Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: http://www.tandfonline.com/loi/iphb20

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To cite this article: Luana Mota Ferreira, Mailine Gehrcke, Verônica Ferrari Cervi, Paula Eliete Rodrigues Bitencourt, Elita Ferreira da Silveira, Juliana Hofstatter Azambuja, Andiara Prates Ramos, Kátia Nascimento, Maria Beatriz Moretto, Elizandra Braganhol, Michele Rorato Sagrillo & Letícia Cruz (2016): Pomegranate seed oil nanoemulsions with selective antiglioma activity: optimization and evaluation of cytotoxicity, genotoxicity and oxidative effects on mononuclear cells, Pharmaceutical Biology, DOI: 10.1080/13880209.2016.1199039

To link to this article: http://dx.doi.org/10.1080/13880209.2016.1199039

Published online: 29 Jun 2016.

PHARMACEUTICAL BIOLOGY, 2016
http://dx.doi.org/10.1080/13880209.2016.1199039

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RESEARCH ARTICLE

Pomegranate seed oil nanoemulsions with selective antiglioma activity: optimization and evaluation of cytotoxicity, genotoxicity and oxidative effects on mononuclear cells

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ABSTRACT

Context: Glioma is a malignant brain tumor with rapid proliferation, infiltrative growth, poor prognosis and it is chemoresistent. Pomegranate seed oil (PSO) has antioxidant, anti-inflammatory and antitumor properties. This study showed the optimization of PSO nanoemulsions (NEs) as an alternative for glioma treatment.

Objective: The study aimed to evaluate PSO NEs cytotoxicity on human blood cells and antiglioma effects against C6 cells.

Materials and methods: NEs were prepared by the spontaneous emulsification method, using PSO at 1.5 and 3.0%, and were evaluated regarding their physical stability and antioxidant activity. Toxicity evaluations in human blood cells were performed in terms of cell viability, genotoxicity, lipid peroxidation, protein carbonylation, catalase activity and hemolysis at 0.1, 0.25 and 0.5 mg/mL PSO, after a 72-h incubation period. *In vitro* antitumor effect was determined against glioma cells after 24 and 48 h, and astrocytes were used as a non-transformed cell model.

Results: Formulations presented droplet size below 250 nm, low polydispersity index, negative zeta potential and pH in the acid range. NEs and PSO had scavenging capacity around 30% and promoted a proliferative effect in mononuclear cells, increasing about 50% cell viability. No genotoxic and oxidative damage was observed in lipid peroxidation, protein carbonylation and catalase activity evaluations for NEs. Hemolysis study showed a hemolytic effect at high concentrations. Moreover, formulations reduced only tumor cell viability to 47%, approximately.

Discussion and conclusion: Formulations are adequate and safe for intravenous administration. Besides, *in vitro* antitumor activity indicates that NEs are promising for glioma treatment.

ARTICLE HISTORY
Received 14 January 2016
Accepted 4 June 2016
Revised 27 April 2016
Published online 28 June 2016

KEYWORDS
Antitumor effect; astrocyte cells; C6 cells; catalase activity; colloidal systems; comet assay; lipid peroxidation; protein carbonylation; punica granatum

Introduction

Pomegranate, an Asia and Mediterranean native fruit, has been studied as a functional and nutraceutical source in recent years. Pomegranate seeds composition have polyunsaturated fatty acids, vitamins, polysaccharides, polyphenols and minerals. When crushed and dried, the seeds produce oil, which can be extracted by mechanical or chemical methods (Syed et al. 2007).

The main constituents of pomegranate seed oil (PSO) are conjugated fatty acids, including oleic, linoleic, palmitic, punic and stearic acids, which have an important role in preventing cardiovascular diseases, by reducing cholesterol levels and promoting an anti-atherogenic effect (Schubert et al. 1999; Mirmiran et al. 2010). The phenolic and flavonoid contents might contribute to the PSO antioxidant activity, by eliminating free radicals and decreasing oxidative stress and lipid peroxidation, which plays an important role in the prevention and treatment of cancer (Poulsen et al. 1998; Schubert et al. 1999). PSO may also inhibit the proliferation of different types of tumor cells, disrupting the cell cycle by inducing apoptosis and thus, reducing the tumor growth (Aslam et al. 2006; Lansky & Newman 2007). For this reason, PSO is a promising alternative for cancer therapy and it has been reported for breast, prostate and skin tumors (Kim et al. 2002; Albrecht et al. 2004; Johanningsmeier & Harris 2011). However, until now, no study has shown the antiproliferative effect of PSO against glioma cells.

Glioma is a brain tumor originated from glial cells. Usually, it presents a poor prognosis for patients, mainly due to its rapid proliferation and infiltrative growth. Furthermore, malignant gliomas are resistant to therapeutic strategies and the poor penetration of many drugs across the blood–brain barrier contributes to the drug resistance (Behin et al. 2003). In this sense, nanostructured carriers have shown promising results in terms of enhancing drug transport across the blood-brain barrier (da Silveira et al. 2013).

Considering the numerous potentialities of PSO, it is relevant to study the incorporation of this oil in an effective and stable system for its administration. In this regard, nanoemulsions are very attractive colloidal systems for therapeutic and nutritional oils (Ostertag et al. 2012). By definition, nanoemulsions are kinetically stable emulsion systems, where oil droplets are dispersed in an external aqueous phase, with a thin layer of emulsifier as

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stabilizing agent (Bouchemal et al. 2004). Due to their small droplet size (100–500 nm), instability phenomena caused by gravitational flocculation, creaming, sedimentation and/or coalescence are prevented. Besides the kinetic stability, the nanoemulsions smaller droplet size turns the formulations less viscous and more translucent or transparent than conventional emulsions (Solans et al. 2005). Moreover, due to their reduced droplet size, nanoemulsions have been studied to increase the bioavailability of drugs, enhance skin penetration, improve water solubility of lipophilic compounds and to ameliorate the stability of labile substances.

Taking all of this into account, the aim of this study was to prepare and characterize PSO nanoemulsions, as well as to evaluate their physical stability and antioxidant activity. Moreover, a study of cytotoxic, genotoxic and oxidative effects on human blood cells was performed and formulations were tested against glioma cells.

Materials and methods

Materials

Pomegranate seed oil was purchased from ViaFarma (São Paulo, Brazil). Span 80® (sorbitan monooleate), dimethylsulfoxide (DMSO), 3(4-5-dimethyl)-2-5diphenyl tetrazolium bromide (MTT) and 1-1-diphenyl-2-picrylhydrazyl (DPPH) were acquired from Sigma Aldrich (São Paulo, Brazil). Tween 80® (polysorbate 80) was furnished by Delaware (Porto Alegre, Brazil). Dulbecco's modified Eagle's medium (DMEM), Fungizone, penicillin/streptomycin, 0.25% trypsin/EDTA solution and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA). Histopaque-1077® was purchased from Sigma-Aldrich Co. (St Louis, MO). Thiobarbituric acid (TBA) was purchased from Merck® (Germany). Butylated hydroxy toluene (BHT) was acquired from Alpha Química® (Porto Alegre, Brazil) and trichloroacetic acid (TCA) was obtained from Neon® (São Paulo, Brazil). Blue coloring tripan and hydrogen peroxide was furnished by Nuclear® (São Paulo, Brazil). Culture claw 25 cm^2 was obtained from TPP® (USA) and culture medium RPMI from Vitrocell® (São Paulo, Brazil). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Ultrapure water was obtained from Milli-Q® Plus apparatus. All other solvents and reagents were analytical grade and used as received.

Preparation of nanoemulsions

Nanoemulsions were prepared by spontaneous emulsification according to the method described by Ferreira et al. (2015). In brief, pomegranate seed oil [1.5% (w/v) –NE PSOA or 3.0% (w/v)–NE PSOB] and Span 80® (0.077 g) were solubilized in acetone (50 mL). After 60 min under moderate stirring, the organic phase was added in a Tween 80® (0.077 g) aqueous phase (50 mL). The O/W emulsion was formed instantaneously by diffusion of the organic solvent in the aqueous phase, forming the nanodroplets. The magnetic stirring was kept for 10 min and then, the organic solvent was eliminated by evaporation under reduced pressure to achieve 10 mL final volume. The formulations were prepared in triplicate.

Characterization of nanoemulsions

The mean droplet size and polydispersity index (PDI) ($n = 3$) were measured at 25 °C by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments, UK) after diluting the samples in ultrapure water (1:500). ζ Potential analyzes (ZP) were measured using the same instrument after the dilution of the samples in 10 mM NaCl (1:500). The pH values of nanoemulsions were determined by immersing directly the electrode of a calibrate potentiometer (Model pH 21, Hanna Instruments, Porto Alegre, Brazil) in the formulations. Measures were performed at room temperature (25 ± 2 °C) in triplicate.

Physical stability

Centrifugation test

For the centrifugation test, 10 mL formulations were submitted to three different rotation speeds: 1000, 2500 and 3500 rpm (MTD Plus III) for 15 min at each speed (Gumiero & da Rocha 2012). After this, nanoemulsions were analyzed for macroscopic changes and were classified according to the instability events in normal, slightly modified, modified and intensely modified.

Heating/cooling cycle

The heating/cooling cycle was performed in order to evaluate the nanoemulsions stability at extreme temperature changes. This test consisted in submitting the samples at 40 °C in an air stove (Mecalor, Brazil) for 24 h and then at 4 °C in a refrigerator (Consul, Brazil) for another period of 24 h, thus completing six cycles (12 days) (Gumiero & da Rocha 2012). Physicochemical characteristics (droplet size, polydispersity index, ζ potential and pH) were evaluated before and after heating/cooling cycle ($n = 3$).

Thermal stress

Thermal stress test was carried out according Gumiero and da Rocha (2012), with some modifications. For this, the samples were placed in a thermostatic bath (Cientec, Brazil) and submitted to a range of temperature (from 50 °C up to 80 °C, increasing by 5 °C intervals) for 30 min at each condition. Physicochemical characteristics (droplet size, polydispersity index, ζ potential and pH) were evaluated before and after thermal stress ($n = 3$).

DPPH radical scavenging capacity estimation

To evaluate the antioxidant property of PSO nanoemulsions (NE PSOB), a 0.5 mL aliquot of each sample at 0.1, 0.25 and 0.5 mg/mL was mixed with 1.5 mL DPPH reagent at room temperature, following the method described by Serpen et al. (2007), with minor modifications. After 30 min, the absorbance values were measured at 518 nm and expressed as percentage of scavenging capacity following equation described below (Blois 1958; Brandwilliams et al. 1995) (Equation 1). An ethanolic solution of PSO and nanoemulsions were used as a blank, while DPPH solution was used as a negative control. Moreover, ascorbic acid was used as reference standard for comparison purposes.

$$SC\,\% = 100 - \frac{(Abs - Abb) \times 100}{Abc} \quad (1)$$

Where, SC%: Scavenging capacity in percentage; Abs: sample absorbance; Abb: blank absorbance; Abc: control absorbance.

Cytotoxicity, genotoxicity and oxidative effects on mononuclear cells

Blood collection

Peripheral human blood was used for these studies. The samples were obtained from School Laboratory of Clinical Analyzes of Franciscan University, under approval by the Ethics Committee for Research with Human Beings of the institution (CAAE: 31211214.4.0000.5306) with no identifying data. For this, human blood was collected from healthy voluntaries by venipuncture using a Vacutainer® (BD Diagnostics, Plymouth, UK) and heparin tubes. The Histopaque-1077® density gradient was used to separate mononuclear cells using 4 mL blood samples. After separation, cells at an initial density of 2×10^5 cells were treated with PSO and NE PSOB at concentrations of 0.1, 0.25 and 0.5 mg/mL, and incubated for 72 h at 37 °C in a 5% humidified CO_2 atmosphere.

Cell viability

Cell viability was evaluated after 72 h incubation employing MTT assays. The culture medium was removed and cells were incubated with MTT at 37 °C, protected from light, until the observation of the presence of violet formazan crystals (3 h). Subsequently, the absorbance was measured spectrophotometrically at 540 nm. Results were expressed by cell viability (%) in comparison to the negative control. Hydrogen peroxide was used as positive control.

Comet assay

The comet assay was performed according to Singh et al. (1988), modified by Garcia et al. (2004). After incubation period, on a glass plate previously covered with a layer of 1.5% agarose, samples were deposited already suspended in agarose of low melting point. The material was immersed in lysis solution (89.0 mL lysis solution to 10.0 mL DMSO and 1.0 mL Triton X-100) for the removal of membranes and cytoplasm. Subsequently, the slides were incubated in alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA in distilled water), and subjected to electrophoresis for about 30 min at 25 V and 300 mA. After, the neutralization, fixation and coloring process were conducted in order to analyze the genetic material. The analysis of each slide was performed under a light microscope and the cells were classified according to the format of the image into four damage classes, varying from zero (no damage) to four (maximum injury), but also including cellular apoptosis rating.

Measurement of lipid peroxidation

The evaluation of lipid peroxidation induced by PSO or NE PSOB was determined by thiobarbituric acid reactive species (TBARS). For this purpose, mononuclear cells were incubated with the samples for 72 h. Then 1.0 mL of suspension was collected and centrifuged for 10 min at 1000 rpm. The supernatant was discarded and the pellet washed with 0.9% NaCl for three times. The supernatant was discarded one more time and it was added 300 µL saline/phosphate buffer, 100 µL 10 nM BHT and 500 µL 20% TCA. Homogenization and centrifugation for 5 min at 2000 rpm was performed. In a fresh tube, 900 µL of the supernatant was mixed with 140 µL water, 300 µL TBA and 60 µL 10% phosphoric acid. Incubation was performed for 90 min at 95 °C and measurements were made in spectrophotometer at 532 nm, after the tubes reached room temperature. The results were expressed as nmol of MDA/10^6 cells.

Protein carbonyl determination

Protein damage was determined by carbonyl content assays, as previously described by Chagas et al. (2015), with modifications. After 72 h, an aliquot of cell suspension (50 µL) was diluted with Tris/HCl buffer pH 7.4 (1:8). 10 mM DNPH in 2.0 M HCl (200 µL) was mixed with 1000 µL of diluted samples. For blank, only 200 µL of 2.0 M HCl were used. All tubes were kept for 1 h at room temperature in dark and shaken using a vortex mixer every 15 min. Then, 500 µL denaturizing buffer (sodium phosphate buffer, pH 6.8, containing 3% SDS), 2000 µL ethanol and 2000 µL of hexane were placed in all tubes. The tubes were mixed in a vortex for 40 s and subsequently centrifuged for 15 min at 3000 rpm. The supernatant was discarded and the pellet washed two times with 1 mL ethanol:ethyl acetate (1:1, v/v). Then, it was added to the denaturizing buffer (1 mL), and the tubes were kept in a water bath (40 °C) for 20 min until the complete dissolution of the pellet. Absorbance was measured at 370 nm and results were expressed as carbonyl content (nmol carbonyl content/10^6 cells).

Catalase activity

The catalase (CAT) activity was measured spectrophotometrically at 240 nm by the disappearance of hydrogen peroxide in the presence of the cell suspension treated with PSO or NE PSOB (Aebi 1984). An aliquot of cell suspension (200 µL) was withdrawn and placed into a quartz cuvette with 2965 µL 50 mM potassium PBS pH 7.0. Next, it was added to 120 µL hydrogen peroxide. Measurements of absorbance were taken at 0, 30, 60, 90 and 120 s for the kinetic analysis of CAT activity. The results were expressed in U of CAT/10^6 cells.

Hemolytic activity

Hemolysis induced by PSO or NE PSOB was evaluated spectrophotometrically. For this, fresh human blood was collected as described above. Red blood cells (RBC) were separated, washed three times with PBS and subsequently centrifuged for 10 min at 1000 rpm. RBC (400 µL) was dispersed in PBS (1000 µL) and treated with increasing concentrations of PSO or NE PSOB. After incubation (1 h at 37 °C in a humidified atmosphere with 5% CO_2) samples were subjected to a centrifugation for 5 min at 1000 rpm. The supernatant was placed in a 96-well plate and then the spectrophotometric analyzes were performed at 405 nm. Negative control was performed by incubating cells with PBS and for positive control hydrogen peroxide was used. Results were expressed as percentage of hemolysis, following the equation below (Bender et al. 2012; Ferreira et al. 2015).

$$\% = ((Abs - Abnc)/Abpc) \times 100 \quad (2)$$

Where, Abs means sample absorbance; Abnc means negative control absorbance; and Abpc means positive control absorbance.

Cytotoxicity assay in glioma C6 cells and astrocyte cells

The rat C6 glioma cell line was obtained from American Type Culture Collection (ATCC; Rockville, MD). The cells were grown

and maintained in low-glucose DMEM containing 0.1% Fungizone and 100 U/L penicillin/streptomycin and supplemented with 5% fetal bovine serum (FBS). Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2. Primary astrocyte cultures were prepared as described by da Silveira et al. (2013). Briefly, brain cortex of newborn Wistar rats (1–2 days old) were removed and dissociated mechanically in a Ca^{+2} and Mg^{+2} free balanced salt solution, pH 7.4, (137 mM NaCl, 5.36 mM KCl, 0.27 mM Na_2HPO_4, 1.1 mM KH_2PO_4 and 6.1 mM glucose). After centrifugation for 5 min at 1000 *g*, the pellet was suspended in DMEM supplemented with 10% FBS. The cells (5×10^4) were plated in poly-L-lysine-coated 96-well plates. After 4 h plating, they were gently shaken and PBS washed, and medium was changed to remove neuron and microglia contaminants. Cultures were allowed to grow to confluence by 20–25 days. Medium was replaced every four days.

The C6 glioma cells were seeded at 1×10^3 cells/well in DMEM/5% FBS in 96-well plates. Astrocyte cultures were prepared as described above. The cell cultures were treated with PSO or nanoemulsion formulation (NE PSOB) at increasing concentrations [0.87; 1.74; 2.61; 4.35 and 8.7% (v/v)] dissolved in 5% DMEM or 10% FBS, for C6 glioma or astrocyte cultures, respectively. Cells treated with DMSO were used as controls. After 24 and 48 h of treatment, cell viability was determined by MTT reduction, as previously described (da Silveira et al. 2013; Ferreira et al. 2015).

Statistical analyses

Formulations were prepared and analyzed in triplicate and the results were expressed as mean ± standard deviation. GraphPad Prism, version 5 were used for the *t*-test and analyses of variance (ANOVA) one-way followed by *post hoc* Tukey's tests. Values of $p < 0.05$ were considered to be statistically significant.

Results

Preparation and characterization

After preparation, the formulations had milky appearance, characteristic of colloidal systems. Table 1 shows the values of droplets average diameter, PDI, ζ potential and pH. By comparing formulations, it is possible to notice a slight increasing trend in the characteristics for the formulation prepared with PSO at 3.0%; however, there is no significant difference among the parameters evaluated. Mean droplet diameter was below 250 nm, PDI was less than 0.2, ζ potentials were negative and pH values were in the acid range.

Physical stability

After centrifugation test, the formulation NE PSOA remained the initial characteristics, and no instability phenomena were observed. On the other hand, the formulation NE PSOB showed a moderate creaming above 2500 rpm. PSO nanoemulsions were also subjected to thermal stress and heating/cooling cycle tests (Figure 1). Over the studies, no macroscopic modifications were observed, suggesting absence of instability phenomena. However, among the physicochemical parameters analyzed, only ζ potential was affected by higher temperatures. Regarding heating/cooling cycle study, no significant differences in the characteristics of the formulations were observed. The droplet size, PDI, ζ potential and pH values after thermal stress and heating/cooling cycle tests remained in the ranges 185–250 nm, 0.096–0.163, −17.39 to −22.47 mV and 4.39–4.89, respectively.

Table 1. Characteristics of pomegranate seed oil nanoemulsions.

Analysis	NE POSA	NE PSOB
Mean diameter (nm)	219 ± 29	250 ± 43
PDI	0.10 ± 0.03	0.16 ± 0.08
ζ potential (mV)	−23.20 ± 3.74	−20.10 ± 4.16
pH	4.5 ± 0.03	4.7 ± 0.17

DPPH radical scavenging capacity estimation

Considering that NE PSOB showed adequate physicochemical characteristics and has the higher PSO concentration, this formulation was selected for the *in vitro* evaluations. The capacity of PSO and NE to scavenge DPPH, a stable free radical, was evaluated in comparison to ascorbic acid, a standard antioxidant that has a scavenge capacity around 98%. Radical scavenge activity of PSO was 27.51 ± 2.05, 35.76 ± 3.37 and 37.89 ± 1.47%, for concentrations of 0.1, 0.25 and 0.5 mg/mL, respectively, and no significant differences were observed ($p > 0.05$). For NE, values were similar: 27.80 ± 2.58, 28.47 ± 4.50 and 32.59 ± 9.87% for concentrations of 0.1, 0.25 and 0.5 mg/mL, respectively ($p > 0.05$). The comparison among PSO and NE presented no statistical difference between the radical scavenging activity of the oil and the nanoformulation, indicating that the nanoemulsification process did not affect the antioxidant activity of PSO.

Cytotoxicity, genotoxicity and oxidative effects on mononuclear cells

Cytotoxicity was evaluated by MTT reduction assay after 72 h incubation. As can be seen in Figure 2, all tested concentrations of PSO and NE PSOB promoted proliferative effects on mononuclear cells, increasing the cell viability. PSO presents cell viability of 143.5 ± 2.73, 151.1 ± 6.5 and 158.8 ± 4.63% for concentrations of 0.1, 0.25 and 0.5 mg/mL, respectively. By ANOVA one-way analysis, no significant difference was observed between PSO concentrations ($p > 0.05$). For nanoformulation, results were 138.5 ± 12.50, 142.8 ± 3.3 and 139.2 ± 6.9% for concentrations of 0.1, 0.25 and 0.5 mg/mL, respectively. *p* Values were higher than 0.05, indicating no significant difference. Comet assays showed no DNA damage for all tested concentrations, as can be seen in Figure 3 were below 1, indicating that formulations are not genotoxic to cells.

Figure 4 shows results of TBARS (A), protein carbonyl determination (B) and CAT activity (C). TBARS levels were expressed in terms of nmol MDA/10^6 cells and all tested concentrations presented values below the control values, indicating that PSO and NE PSOB did not cause any oxidative damage to lipids in cell membranes. By ANOVA one-way analysis, no significant difference was observed between PSO and NE PSOB, for all concentrations ($p > 0.05$). In terms of protein damage, ANOVA one-way analysis shows that only PSO concentration of 0.5 mg/mL caused carbonylation of cell proteins (0.0539 ± 0.0036 nmol carbonyl content/10^6 cells) ($p > 0.05$). Other concentrations presented values close to the negative control, below 0.0084 nmol carbonyl content/10^6 cells. In relation to CAT activity, it was observed a significant difference for all concentrations ($p < 0.05$) by ANOVA one-way analysis. For nanoemulsions, at 0.1 and 0.25 mg/mL there was a reduction in the enzyme activity ($p < 0.05$) and at 0.5 mg/mL concentration values were similar to the negative control ($p > 0.05$). PSO at 0.1 mg/mL showed no changes in the enzyme activity

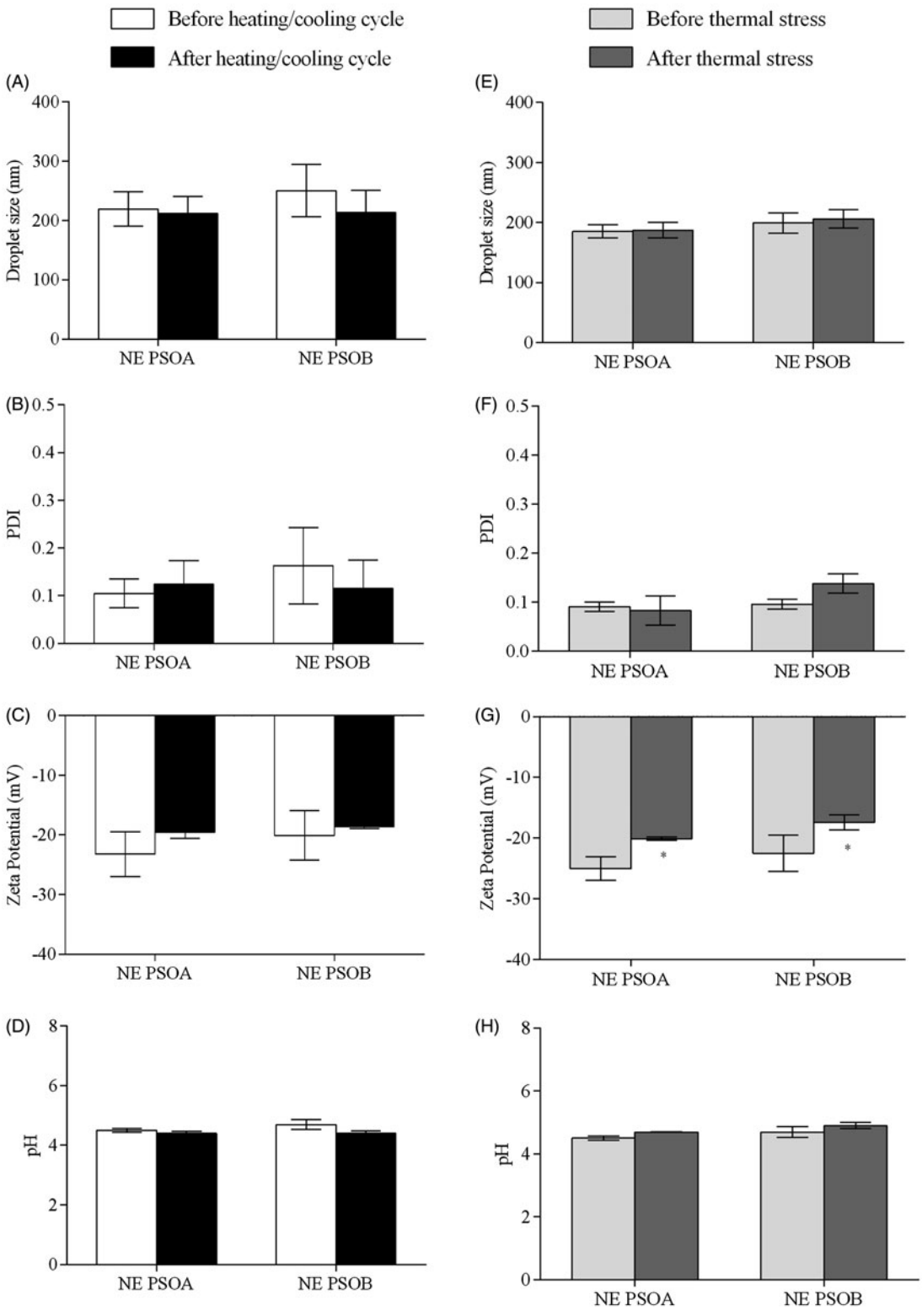

Figure 1. Physical stability: heating-cooling cycle (A) Droplet size (nm); (B) PDI values; (C) ζ potential (mV); (D) pH values. Thermal stress (E) Droplet size (nm); (F) PDI values; (G) ζ potential (mV); (H) pH values. Each column represents the mean with S.E.M. of triplicate. Asterisks denote the significance levels (*) $p < 0.05$ by two-way ANOVA, followed by Tukey's test. NE PSOA means: nanoemulsion containing 1.5% of PSO; NE PSOB means: nanoemulsion containing 3.0% of PSO.

compared to the negative control ($p > 0.05$). On the other hand, concentrations of 0.25 and 0.5 mg/mL caused an increase in CAT activity ($p < 0.05$). Comparing PSO and NE PSOB by ANOVA one-way, it was observed significant difference ($p < 0.05$), since PSO concentrations caused an augmentation in values of U CAT/10^6 cells.

Hemolytic activity

PSO and NE PSOB were evaluated for their ability to cause hemolysis in human erythrocytes (Figure 5). PSO and NE PSOB at 0.5 mg/mL caused significant hemolysis. Moreover, PSO induced 89.57 ± 0.25% of hemolysis, while nanoemulsions caused only

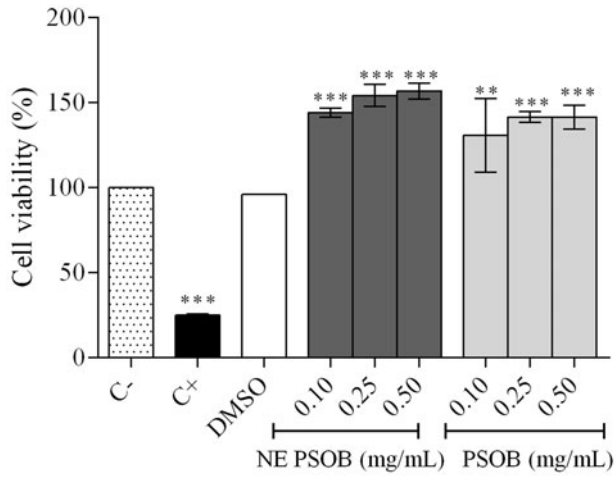

Figure 2. Cell viability of mononuclear cells after 72 h incubation by MTT reduction assay. Each column represents the mean with S.E.M. of triplicate. Asterisks denote the significance levels compared to negative control (ANOVA one-way, Tukey's *post hoc* test) (*) $p < 0.05$. NE PSOB means: nanoemulsion containing 3.0% of PSO and PSO means: pomegranate seed oil.

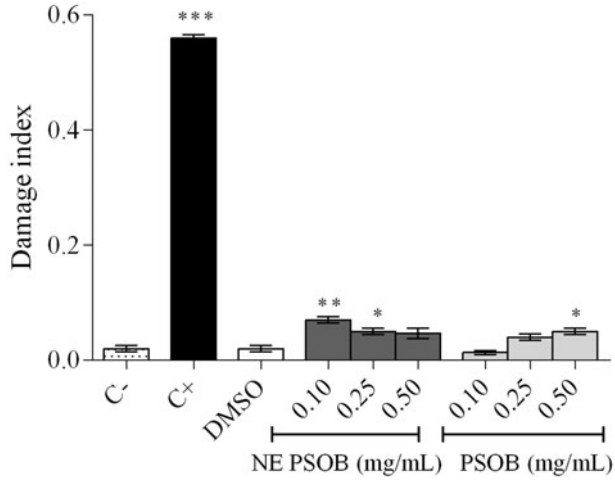

Figure 3. Determination of DNA cells damage by comet assay. Each column represents the mean with S.E.M. of triplicate. Asterisks denote the significance levels compared to negative control (ANOVA one-way, Tukey's *post hoc* test) (*) $p < 0.05$. NE PSOB means: nanoemulsion containing 3.0% of PSO and PSO means: pomegranate seed oil.

28.33 ± 0.73% of hemolysis ($p < 0.05$). For the other concentrations, there was no hemolytic effect ($p > 0.05$) compared to the negative control. However, PSO at 0.1 and 0.25 mg/mL presented lower hemolysis when compared to the same concentration of NE PSOB ($p < 0.05$).

Cytotoxicity assay in glioma rat cells (C6) and astrocyte cells

PSO and NE PSOB were assayed *in vitro* for their cell growth inhibitory activity in rat glioma C6 cells and astrocyte cells by the MTT assay. Figures 6 (A and B) show that PSO reduced the viability of C6 cells, regardless of the dose tested. Moreover, no significant difference was observed among 24 and 48 h of experiment. In relation to the nanoemulsified formulation, NE PSOB also presented cytotoxic action, decreasing C6 cell viability. After 24-h culture, significant suppression of 42 and 65% of growth occurred for the higher concentration (8.7%) of NE PSOB and PSO, respectively. On the other hand, after 48-h culture, NE PSOB presented suppression of 53% of growth, whereas PSO kept the same cell viability. PSO and its nanoemulsion showed excellent antiglioma potential with values higher than 40% inhibition. In this way, their selectivity was evaluated in astrocyte cultures as a non-transformed cell model. Figure 6(C) shows that NE PSOB did not promote significant alterations on astrocyte cell growth. Moreover, PSO presented significant reduction of astrocyte viability. These results suggest that PSO nanoemulsions are nontoxic to healthy cells, being selective for malignant cells.

Discussion

In this work, it was demonstrated the pomegranate seed oil nanoemulsions preparation using an easy and low cost method, and their effects on the mononuclear cells, as well as a high antiglioma activity. There are few reports showing PSO nanoemulsions preparation, employing different methods as sonication (Mizrahi et al. 2014), self-nanoemulsifying (Lu et al. 2015), ultrasonic emulsification/solvent evaporation (Baccarin & Lemos-Senna 2014; Baccarin et al. 2015a, 2015b). Recently, our group developed PSO nanoemulsion by nanoemulsification, employing pullulan as stabilizer (Ferreira et al. 2015). In this study, PSO nanoemulsions were prepared by spontaneous emulsification method, using two different concentrations of PSO, 1.5 and 3.0% (w/v), in order to evaluate the influence of oil concentration in the final characteristics of the formulations. Solans and Sole (2012) reported that for obtaining nanodroplets with this method is necessary a very high solvent/oil ratio. In this study, it was used equal volumes of water and acetone, 50 mL for each phase. This was performed in order to facilitate PSO solubilization and the formation of the droplets. Furthermore, according to the literature, the spontaneity of the emulsification can be affected by interfacial tension, bulk viscosity of oil phase, water miscible solvents and surfactant structure and/or concentration (Saberi et al. 2013). Droplet sizes were below 250 nm and PDI values were less than 0.2, which is compatible with other studies using the same method of preparation (Martini et al. 2007; Ferreira et al. 2015). According to the literature, a narrow size distribution prevents Ostwald ripening and, for this reason, is related to high nanoemulsions stability (Yilmaz & Borchert 2005). ζ potential values were negative and pHs were in the acid range, which is probably influenced by the fatty acids of PSO (Baccarin et al. 2015a) and the presence of Tween 80® at droplet surface (Fontana et al. 2009).

Accelerated stability studies were performed in order to determine possible instability phenomena in a minor period of time. After preparation, nanoemulsions were submitted to the centrifugation test, which allows fast information about phenomena like creaming, sedimentation and phase separation. The moderate creaming observed for NE PSOB can be reversed by stirring, which do not avoid the use of the formulation (Badolato et al. 2008). Temperature modifications can promote changes in the viscosity of the phases and can modify the interfacial tension inducing phase separation, creaming or coalescence. Gumiero and da Rocha (2012) described the physical and chemical stability of babassu nanoemulsions and reported that these systems also showed no difference in their characteristics when the formulations were submitted to changes of storage temperature. The results found in our study showed that after the formulations were subjected to temperature changes, characteristics remained appropriate.

Since several studies have proven that the antiproliferative activity of many natural compounds is related to their antioxidant ability, it was considered relevant to investigate whether nanoemulsification could interfere in PSO activity. Results showed that

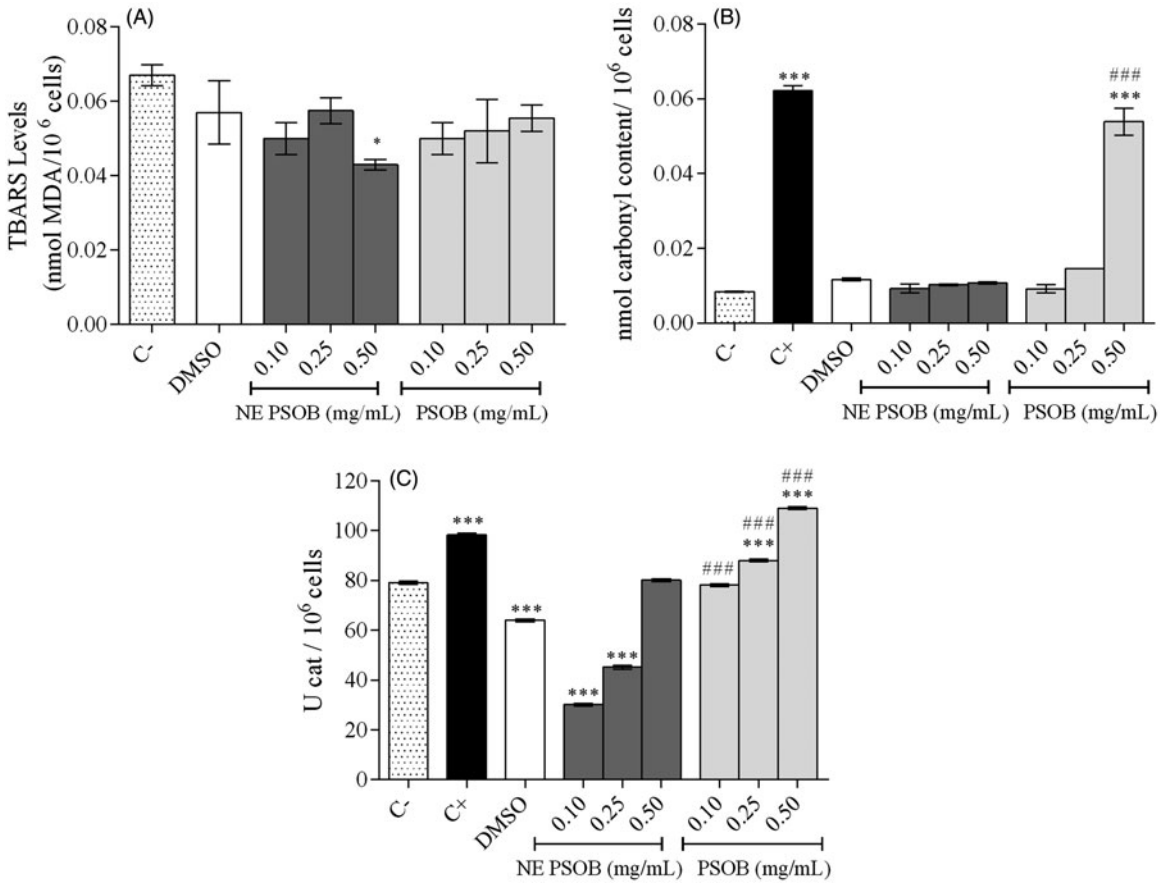

Figure 4. Oxidative stress in mononuclear cells: (A) TBARS levels; (B) carbonyl protein determination; and (C) catalase activity. Each column represents the mean with S.E.M. of triplicate. By ANOVA one-way, followed Tukey's test, asterisks denote the significance levels compared to negative control (*) $p < 0.05$ and sharp denote the significance levels of the concentrations between samples (#) $p < 0.05$ (0.1×0.1 mg/mL; 0.25×0.25 mg/mL and 0.5×0.5 mg/mL, PSO and NE PSOB, respectively). NE PSOB means: nanoemulsion containing 3.0% of PSO and PSO means: pomegranate seed oil.

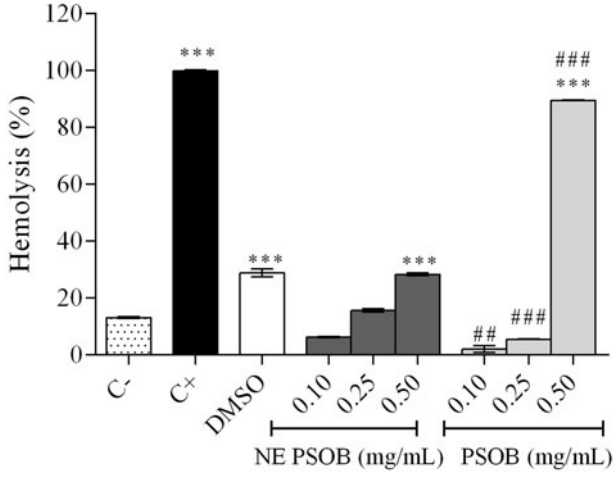

Figure 5. Hemolytic activity of formulations on human red cells blood. Each column represents the mean with S.E.M. of triplicate. By ANOVA one-way, asterisks denote the significance levels compared to negative control (*) $p < 0.05$ and sharp denote the significance levels of the concentrations between samples (#) $p < 0.05$ (0.1×0.1 mg/mL; 0.25×0.25 mg/mL and 0.5×0.5 mg/mL, PSO and NE PSOB, respectively). NE PSOB means: nanoemulsion containing 3.0% of PSO and PSO means: pomegranate seed oil.

PSO maintained scavenging activity. Liu et al. (2012) reported radical scavenging activity higher than 50% for PSO, however, the lowest concentration tested in that study was 0.8 mg/mL, being 1.6 times higher than the maximum concentration evaluated in our study. Schubert et al. (1999) demonstrated that PSO has antioxidant activity higher than red wine, green tea and butylated hydroxyanisole. Shaban et al. (2013) showed that PSO presents low phenolic and flavonoid content, indicating that the antioxidant property is due, in addition to these compounds, to the presence of other substances as triterpenoids, γ-tocopherol, 17-estradiol, estrogens "estrone and estriol", testosterone, β-sitosterol, coumesterol, campesterol, stigmasterol, punicic acid and flavonoids "genistein and daidzein" (Lansky & Newman 2007; Kaufman & Wiesman 2007).

In vitro toxicity evaluations have been accepted by the scientific community to determine the safety of formulations. Human peripheral blood cells are the first site of toxicity after intravenous administration; therefore, some tests were performed in order to elucidate the possible toxic effects of the proposed nanoemulsion. Cell viability was determined by MTT assays, a colorimetric method used for the cytotoxicity evaluations by measuring the living cells capacity to reduce the MTT salt, forming insoluble crystals violet staining of formazan. After an incubation period, a significant increase of cell viability was observed. This effect may be attributed to the stimulation of immune system cells caused by PSO and nanoformulation, probably due to the antioxidant and anti-inflammatory activity related for PSO. According to the literature, some antioxidants compounds might present immunostimulatory effects by modulation of gene expression (Ramiro-Puig & Castell 2009). Darwis et al. (2014) found that *Hydnophytum formicarum* ethanolic extract promoted an increase in lymphocyte proliferation, and they attributed this to the presence of the

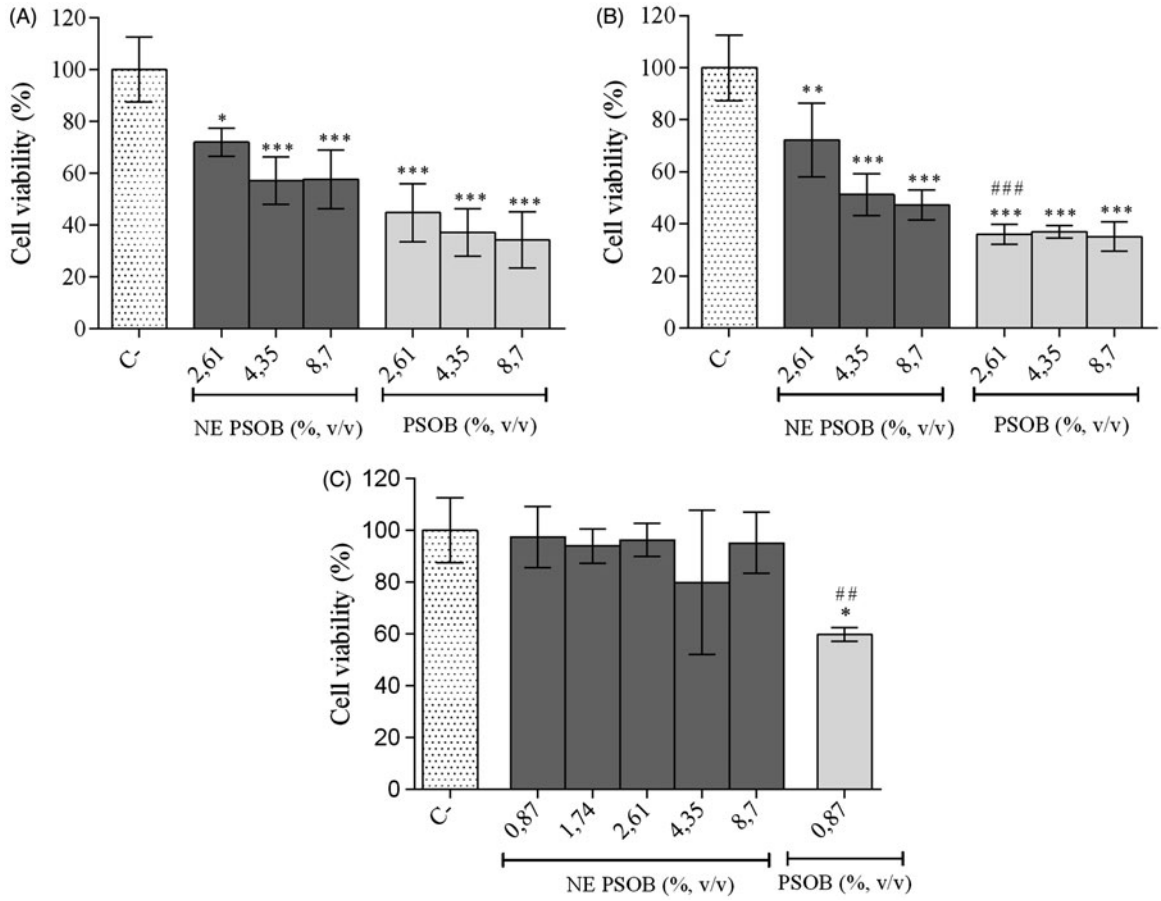

Figure 6. Results of citotoxicity assay in glioblastoma cells (C6): (A) Glioblastoma cells viability at 24 h; (B) Glioblastoma cells viability at 48 h; (C) Primary astrocyte cells viability. By one-way ANOVA, asterisks denote the significance levels compared to negative control (*) $p < 0.05$ and sharp denote the significance levels of the concentrations between samples (#) $p < 0.05$ (0.1×0.1 mg/mL; 0.25×0.25 mg/mL and 0.5×0.5 mg/mL, PSO and NE PSOB, respectively). Each column represents the mean with S.E.M. of triplicate. NE PSOB means: nanoemulsion containing 3.0% of PSO and PSO means: pomegranate seed oil.

antioxidant compounds. Capeleto et al. (2015), who evaluated the effects of resveratrol on human peripheral blood mononuclear cells, found a significant increase of lymphocyte viability at $\geq 5\,\mu M$ by MTT assays.

Genotoxic effects were determined by comet test in order to evaluate DNA cell damage. This assay has high sensitivity and enables to quantify the levels of single strands of DNA breakages. Our results demonstrated no damage caused by PSO and nanoemulsions, since values below 1 indicate no damage (Garcia et al. 2004).

Some diseases, as cancer and neurodegenerative conditions, can involve lipid peroxidation and protein carbonylation (Chagas et al. 2015). Cell membrane integrity was evaluated by the TBA reaction with malondialdehyde (MDA), a product formed by lipid peroxidation. Results showed that PSO and nanosystem did not cause oxidative damage to lipids in cell membranes. Regarding literature reports, PSO at a dose of 0.8 mg/kg has already been reported to reverse Wistar rat's oxidative kidney damage after gentamicin administration, and this effect was attributed to its antioxidant properties (Boroushaki et al. 2014). Protein carbonylation was assayed by reaction of protein carbonyls with dinitrophenyl hydrazine forming dinitrophenyl hydrazone (Chagas et al. 2015). Analysis showed damage only at high PSO concentration (0.5 mg/mL). NE PSOB presented levels close to the control group, probably due to the nanometric size which protects cells from damage.

Free radicals are commonly produced by human organism and may oxidize biomolecules initializing cancer (Poulsen et al. 1998; Cadenas & Davies 2000; Xing et al. 2005). CAT is an enzymatic defense that prevents the oxygen peroxide formation, and consequently, cell damage (Zaib & Khan 2014). Our findings demonstrated that high concentrations of PSO promote an increase of CAT activity, which could indicate an oxidative stress condition that free PSO could not reverse. In this way, nanoformulations protected cells from this damage. It is possible to establish a relationship between these results with carbonylation determination, because damage was observed at the same PSO concentration.

Hemolysis evaluation is considered a rapid and adequate *in vitro* test to determine cytotoxicity and hemocompatibility of nanosystems (Bender et al. 2012; Ferreira et al. 2015). Literature has demonstrated that values between 1 and 5% are the maximum limits indicating the hemolytic effect (Bender et al. 2012; Das et al. 2013). Hemolytic percentage found for NE PSOB may be explained by the nanometric size of the system, which increases the contact area with RBC and promotes lysis. Other possible explanation is the presence of surfactant in the formulation, which can solubilize the lipids and cause membrane destabilization (Kuntworbe & Al-Kassas 2012; Bender et al. 2012).

C6 cells were used in order to evaluate antitumor activity of nanoemulsions and astrocytes as a non-transformed cell model. In our previous study, we tested antiglioma activity of nanoemulsions-loaded ketoprofen containing 1.5% PSO stabilized by pullulan. However, blank formulations did not present cytotoxic effect, so, this action was attributed to ketoprofen (Ferreira et al. 2015). In this study, it was demonstrated that the higher PSO concentration in the nanosystem was capable of reducing tumor cell

viability, without damage to astrocyte cells. PSO antitumor activity has been reported against other kinds of cells such as MCF breast cancer (Kim et al. 2002), colon, skin (Johanningsmeier & Harris 2011) and DU 145 human prostate tumor (Albrecht et al. 2004). The mechanisms involved in the reduction of tumors growth are associated to the presence of campesterol, stigmasterol, γ-tocopherol and conjugated 18 carbon fatty acid, mainly punicic acid. They inhibit lipid peroxidation and/or cyclooxygenase and lipoxygenase activity, hence, oxidative stress and inflammatory process, respectively, which can be involved in the apoptotic cascade (Kim et al. 2002; Johanningsmeier & Harris 2011). In addition, these compounds can inhibit the nuclear factor kappa B (NF-κB) and can promote an antiangiogenic effect, events that are related to the proliferation of tumor cells (Johanningsmeier & Harris 2011). Nanoemulsions represent a promising alternative for PSO administration for glioma prevention and treatment. Due to their colloidal size and aqueous continuous phase, PSO nanoemulsions can be administered by many routes, including intravenous.

Conclusions

The pomegranate seed oil nanoemulsions developed in this study combined adequate physicochemical characteristics with physical stability and antioxidant activity. Formulations presented low toxicity against human blood cells, after analysis of mitochondria, DNA, lipids, protein and erythrocytes. The high antiglioma activity of PSO and its nanoemulsion suggests the potential use of the proposed formulation in the treatment of malignant glioma.

Acknowledgements

We thank Cristiane de Bona da Silva for zetasizer access. Luana Mota Ferreira thanks CAPES/Brazil for master fellowship.

Disclosure statement

The authors report no declarations of interest.

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