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Toxicological evaluation of naringin-loaded nanocapsules *in vitro* and *in vivo*

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ABSTRACT

Naringin is a flavonoid widely known for its pharmacological properties, such as: anti-inflammatory and antioxidant ones, being an ally to avoid oxidative damage. Although naringin is an active easily found in citrus fruits, it has low bioavailability, biodistribution and also undergoes biotransformation in naringenin, limiting the described effects. The use of nanocapsules as drug carriers may increase solubility, improve biodistribution, impede the biotransformation thereof, and thus could improve the performance of naringin for use in treating neurological diseases. Therefore, the objective of this work is to produce a nanocapsule containing naringin, validate an analytical method by RP-HPLC to determination of the drug in nanoparticle and evaluate the toxicity. To that end, the blank nanocapsules (NB, without the drug) or naringin-loaded nanocapsules (NN) at the concentration of 2 mg/mL were prepared by interfacial deposition of the preformed polymer and the quantification of naringin by HPLC. Toxicity of the formulations was evaluated *in vitro* in rat hippocampal slices and *in vivo* models with *C. elegans* and *Danio rerio* (zebrafish). The analytical parameters evaluated (linearity, limit of detection and quantification, specificity, precision, accuracy and robustness) indicated adequate method to assay of naringin in nanocapsules by HPLC. There was no indication of toxicity by the nanocapsules in the evaluated biological assays.

1. Introduction

Naringin is a flavonoid, chemically known as 4′,5,7-trihydroxy flavone 7-rhamno-glucoside, originated from from citrus fruits [1]. Naringin contains the basic structure of a flavonoid, together with two rhamnose units attached to its aglycone moiety [2]. In humans, naringin is metabolized in aglycone naringenin by naringinase found in the liver. This happens in two steps: firstly, naringin is hydrolyzed by α-L-rhamnosidase in rhamnose and pruning; then the formed prunin is hydrolyzed by β-D-glucosidase in naringenin and glucose [3].

Many studies have reported that naringin has several pharmacological activities including anti-inflammatory, anti-tumor, antioxidant and antimicrobial [4–8]. In addition, naringin has been widely studied for its beneficial effects on neurological pathologies such as Parkinson's disease [9,10], Huntington's disease [11,12], epilepsy [13,14], stroke [15], Alzheimer's disease [16,17], anxiety [18], memory dysfunction [19,20] and cognitive dysfunction [21,22] in various *in vitro* and *in vivo* models.

According to the Biopharmaceutical Classification System (BCS), naringin belongs to class IV which are drugs with low solubility and low permeability, that is, drugs with significant limitations for oral administration [70]. The oral bioavailability of naringin is less than 5 %, which can be attributed to its low solubility in water [23]. To overcome this, nanoparticles have been an alternative to improve the solubility of these compounds, improving their physico-chemical characteristics, increasing bioavailability and reducing toxicity [24,25]. In addition, it preserves pharmacological activities and increases the stability of compounds [26].

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In the literature, there are has few reports about the encapsulation of bioactive compounds such as naringin. Previous studies have shown that nanoencapsulated naringin produced fewer toxic effects than free form, and can be explained by its greater bioavailability and efficacy from nanoencapsulation [27]. Nanocapsules suspensions are complex matrices composed of surfactant and polymers. Thus, the analysis and method of drug testing in such systems should be developed and validated with great discretion to demonstrate their suitability [28,29]. The rapid spread of nanotechnology raises serious questions about its impact on health and environment. Thousands of nanoproducts are already available in the market [30,31], raising concerns about exposure of the population through inhalation, ingestion, dermal contact or a combination of these routes. Only a few studies have analyzed its impact on the highly vulnerable nervous system, as seen in several reviews [32–36].

Based on their characteristics and requirements, both *Caenorhabditis elegans* (*C. elegans*) and *Danio rerio* (zebrafish) are suitable animal models for conducting early biological testing of nanomaterials in laboratories. Immediate toxicological characterization *in vivo* of this nanoparticle could be alternative methods that aim to complement cell culture testing and mammalian studies. It is worth noting that the use of *C. elegans* and zebrafish is in accordance with widely accepted ethical principles known for animal experiments, such as reduction of superior animal use and refinement of current techniques [37].

We developed nanocapsules loaded with naringin for further neuroprotection studies. Therefore, the aim of this study is developing and validating a simple and reliable RP-HPLC method to quantify of naringin in nanocapsule suspensions. This study also evaluates the toxicity of naringin-containing nanocapsules in different *in vitro* and *in vivo* models.

2. Materials and methods

Naringin, bromide 3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium (MTT) and sorbitane monostearate (Span® 60) were purchased from Sigma Aldrich®. The Eudragit® L100 surfactant (anionic copolymer based on methacrylic acid and methyl methacrylate) was purchased from Evonik®. Diisopropyl adipate was purchased from Polytechno®. Acetonitrile was purchased from Merck®. Polysorbate 80 was purchased from Synth®. Acetic acid and absolute ethyl alcohol were purchased from J.T.Baker®. Other chemicals and solvents were from pharmaceutical grade and used as received.

2.1. Production of nanocapsule suspensions

The nanocapsules were prepared by the interfacial deposition technique of the preformed polymer [38], through the preparation of two separate phases. We have published a similar nanoformulation with minor modifications [77]. The organic phase was prepared by dissolving the polymer Eudragit® L 100 (250 mg), sorbitane monostearate (96.2 mg) and diisopropyl adipate (395 mg) in ethanol (62.5 mL) at 40 °C, with magnetic stirring. Afterwards, naringin (50 mg) was dissolved at organic phase. The aqueous phase was prepared by dissolving polysorbate 80 (385 mg) in ultrapure water (125 mL). The organic phase was then injected into the aqueous phase at a controlled rate, nanocapsules were formed and kept under stirring for 10 min. The organic solvent and some of the water were evaporated under reduced pressure for the final volume of 25 mL to final concentration of naringin 2 mg/mL of suspension.

2.2. Validation of the analytical method by High-performance liquid chromatography (HPLC) for the quantification of naringin in nanocapsules suspensions

2.2.1. Chromatographic analysis

The concentrations of naringin mentioned below in the nanosamples were determined with the use of the high-performance liquid chromatography system (HPLC) Shimadzu System Controller SCL-10AVP (Kyoto, Japan), equipped with an LC-10ADVP pump, SPD-10AVP D2 detector and DGU-14A controller system. The Purospher® STAR RP-18, endcapped (5 µm) column was used. The mobile phase was acetonitrile-water (60:40 v/v) pH 4.0 (adjusted with acetic acid). The samples were eluted at isocratic flow rate (0.7 mL/min), 25 °C of column temperature and assessed by a UV detector set at 285 nm. The injection volume was 20 µL and initial run was 8.0 min.

2.2.2. Preparation of the samples

The nanocapsule suspensions used for the evaluation of all parameters were prepared at a concentration of 2000 µg/mL. An aliquot of 100 µL of the suspensions was diluted in acetonitrile at a concentration of 100 µg/mL and centrifuged for 20 min at 4305 *g*. Subsequently, an aliquot of this solution (200 µL) was diluted with mobile phase to give a final concentration of 10 µg/mL. The resulting solution was filtered through a 0.45 µm membrane and injected into the HPLC system (n = 3 samples).

2.2.3. Preparation of standard solution

The standard naringin solution (400 µg/mL) was prepared by dissolving naringin (10 mg) in ethyl alcohol (25 mL) with 15 min of ultrasonic shaking. Subsequently, the standard solution was diluted with mobile phase in 5 different concentrations of naringin (2, 5, 10, 15 and 20 µg/mL), which were used for the linearity study. All solutions were filtered (0.45 µm) before being injected into the HPLC system (n = 3 samples).

2.2.4. Linearity, limits of detection, and quantification

Analytical curves (n = 3) were obtained from naringin 2, 5, 10, 15, and 20 µg/mL. The calibration curves were plotted and the linearity assessed by linear regression analysis using the least squares regression method. The limit of detection (LOD) and the limit of quantification (LOQ) were determined according to the equations of the Brazilian Health Regulatory Agency (ANVISA) guidelines and European Medicines Agency (EMEA) [39,76].

2.2.5. Specificity

The specificity is determined by the ability of the method to dose accurately the compound even in the presence of other components [39,76]. In order to evaluate the possibility of interference of the nanocapsule components in naringin quantification, the specificity was evaluated through comparative analysis between naringin samples in standard solution, blank nanocapsules and naringin-loaded nanocapsules.

2.2.6. Precision and accuracy

The precision of the method was determined in naringin-loaded nanocapsules at 10 µg/mL by intermediate precision variating in different days and different analysts, between run reproducibility (n = 6) and within runreproducibility (n = 6), respectively [39,76]. The between run was calculated from the results obtained by analysing naringin at 3 different days. The within run was evaluated by measuring naringin under the same experimental conditions at the same day (12 determinations, n = 6 at triplicate) by two different analysts. Accuracy was assessed using the standard recovery method in naringin-loaded nanocapsules at 10 µg/mL by adding 3 different standard solutions with concentration of 3.75, 7.5 and 11.25 µg/mL to the final concentrations of 13.75, 17.5 and 21.25 µg/mL, respectively. Accuracy was calculated as the percentage of naringin recovered from the formulations and expressed as relative standard deviation (RSD) among the replicates (9 determinations, n = 3 at triplicate).

2.2.7. Robustness

Robustness was evaluated by the analysis of the effect of small

modifications on the chromatographic conditions. The alterations on chromatograph conditions were the flow rate (± 0.1 mL/min), the pH of the water used in the mobile phase (± 0.20) composition of the mobile phase (acetonitrile ± 5.0 %), and wavelength ± 2.0 nm). Samples (n = 3) were evaluated at each variation condition and the results were compared to the recommended conditions.

2.3. Toxicity in vivo in a model of Caenorhabditis elegans

2.3.1. Worm maintenance

C. elegans Bristol N2 (wild-type) was provided by the *Caenorhabditis Genetic Center* (CGC, University of Minnesota). All strains were grown at 20 °C on NGM plates M9 (1.7 % agar, 2.5 mg/mL peptone, 25 mM NaCl, 50 mM KH_2PO_4 pH 6.0, 5 µg/mL cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) with fresh *Escherichia coli* OP50 as food source [40]. For each experiment, synchronized populations were obtained by disruption of gravid adults. Worms were grown to the L4 larval stage on NGM/OP50-seeded plates.

2.3.2. Concentration range

Survival curves were generated for treatment (twenty-four hours) at 0.6, 1.2 and 1.8 mg/mL^{-1}. Worms were considered dead when no movement response was obtained upon gentle touch and/or pharyngeal pumping was unnoted. Three experiments were individually performed in duplicates with approximately one hundred worms per treatment.

2.3.3. Pharyngeal pumping and defecation cycle

After treatment with free naringin and blank or naringin-loaded nanocapsules, animals were washed with M9 and transferred to a fresh NGM plate seeded with E. coli OP50. Pharyngeal pumping was individually verified for ten seconds at three-time points in animals seeded over bacteria at 22 ± 2 °C [41]. The results were expressed as pharyngeal pumping/min. Ten worms were used in each experiment, with three independent replicates. The mean of three defecation cycles from each animal was used as an indirect measurement of intestinal traffic [42]. This experimental procedure was performed at 22 ± 2 °C with ten worms and independently replicated in triplicate worm preparations.

2.3.4. Survival assay over juglone-induced oxidative stress

Worms at the L4 larval stage were treated with 200 µM of each compound as described above (~500 worms per group). After three washes in M9, the worms were picked and transferred to tubes containing 100 µM juglone (5-hydroxy-1,4-naphthoquinone). Afterwards, one hour of exposure under constant shaking, the worms were washed again with M9 and transferred to NGM plates where survival was examined twenty-four hours later following the same criteria described above [43].

2.3.5. Microorganisms

For microbiological experiments four *E. coli* strains (OP450, ATCC 25922, ATCC 35218 and ATCC 8739) were used. The inoculums were prepared by taking three to four colonies of the strains grown in brain heart infusion (BHI) for 24 h. The bacterial strains were subcultured on nutrient agar and diluted in 0.9 % saline to achieve the turbidity (DO_{600} = 0.08 a 0.1) equivalent to 0.5 McFarland scale (1.5×10^8 UFC/mL) [44].

2.3.6. Disk diffusion assay

The susceptibility test was performed by disk diffusion method on Mueller Hinton agar [45]. The inoculums containing the strains were seeded on Petri dishes with Mueller Hinton agar. After, 10 µL of samples (free naringin, blank or naringin-loaded nanocapsules) were added to the disks and then, they were deposited on the agar surface. The plates were incubated for 24 h at 37 °C. Ceftriaxone was used as positive control. The assay was performed with 2 replicates in 3 independent experiments.

2.3.7. Minimal inhibitory concentration

The antimicrobial activity was performed by the microdilution technique. Assays were performed in 96 well plates using Mueller Hinton broth [46]. Free naringin, blank or naringin-loaded nanocapsules were diluted (1 – 0.015 mg/mL) in culture medium, the plates were incubated at 37 °C for 24 h. Tests were performed in triplicate, using the inoculum as a positive control and the liquid medium as a negative control. The developing agent 2,3,5-triphenyltetrazolium chloride was used to determine the minimum inhibitory concentration.

2.4. Toxicity in vivo in a model of Danio rerio

2.4.1. Animals

Wild-type AB adult *Danio rerio* fishes (zebrafish) from the breeding stock held at the Pontificia Universidade Católica do Rio Grande do Sul (Brazil) were maintained in an automated recirculating tank system (Aquatic Blue™ - Tecniplast Group, Buguggiate (VA), Italy), and used for obtaining fertilized eggs. Fertilized eggs were collected and treated with free naringin, and blank or naringin-loaded nanocapsules solutions diluted in the maintenance water (water from reverse osmosis reconstituted with marine salt, 0.4 parts per thousand - Instant Ocean, Crystals™). The eggs were kept individually in 96-well microplates in an incubator (Bio-Oxygen Demand) at 28.5 °C on a 14:10 light/dark cycle.

2.4.2. Treatment

Embryos aged 3 – 5 hours post-fertilization (hpf) were distributed in 96-well plaques and immersed in 100 µL of free naringin, and blank or naringin-loaded nanocapsules solutions diluted in maintenance water to reach the concentrations 0.005, 0.05, 0.5, 1 and 2 mg/L. Solutions were daily prepared and replaced. The pH of treatment solutions was kept at 7. Animals were monitored every 24 h until 5 days post-fertilization (dpf). After the experiments, animals were euthanized with tricaine overdose (500 mg/L; pH7.2). All procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Ethical Council from Pontificia Universidade Católica do Rio Grande do Sul (PUCRS) (Registration number 8080/2017).

2.4.3. Survival, hatching and morphological evaluation

Survival and hatching rates were monitored during each day until 5 dpf. Morphological evaluation was daily performed based on Fish Embryo Test (FET) [47] under a stereomicroscope (3 x) to search for body defects until larvae reached 5 dpf. Photographic registration was performed using the software NIS-Elements D 3.2 for Windows, supplied by Nikon Instruments Inc. (Melville, USA). The morphological parameters were coagulation, tail not detached, no somite formation, no heartbeat, malformation of eyes, heart tail spinal cord, yolk, pigmentation, and general growth retardation.

2.4.4. Locomotor activity

In the locomotor activity assessment, the animals were individually placed in 24-well microplates, filled with 10 mL of maintenance water. Locomotor activity was recorded for 5 min after 1 min of adaptation. Three parameters were evaluated: the distance traveled (m), the average speed (m/s), and the time in the inner or outer zone of the well. All data were registered through the computerized system Ethovision XT (Noldus, Wageningen, NL).

2.5. Toxicity in hippocampal slices

Slices were prepared as described by Boeck et al. (2004) [71]. Adult rats, 90–180 days, ± 300 g, regardless of sex, were used. All procedures described were approved by the Ethics Committee on Animal Use - CEUA of the Franciscan University (protocol 02/2017). The animals

were killed rapidly by decapitation, and the hippocampi removed and maintained in PBS buffer supplemented with 0.6 % glucose (pH 7.4). Slices of 0.4 mm thick hippocampus were obtained using a tissue slicer (McIlwain Tissue Chopper) and preincubated in PBS-glucose buffer (NaCl, Na_2HPO_4, NaH_2PO_4 and glucose) for 15 min at 37 °C, for metabolic recovery of the tissue. Free naringin, blank or naringin-loaded nanocapsules were diluted in the incubation medium (PBS-glucose) at concentrations of 0.1 μg/mL, 1 μg/mL and 10 μg/mL [75]. Free naringin was solubilized in Milli-Q water, with a temperature of approximately 40 °C at the same time as magnetic stirring, at the concentration of 2000 μg/mL (stock solution) and then diluted at concentrations of 0.1 μg/mL, 1 μg/mL and 10 μg/mL in incubation medium (PBS-glucose). Afterwards, the toxicity of the nanocapsules was evaluated.

2.5.1. Treatment

The hippocampal slices were treated in triplicate [69]. After recovery of the tissue for a period of 15 min, the medium was replaced by a new medium containing the free naringin, blank or naringin-loaded nanocapsules, the control group was incubated only with incubation medium, the 100 mM H_2O_2 was used as a death inducer (death control). The hippocampal slices were housed at 37 °C containing 95 % O_2 and 5 % CO_2 for 1 h.

2.6. Statistical analysis

For the evaluation of the parameter robustness in the quantification of naringin by HPLC, parametric *t*-test was used. The results of the biological tests were initially evaluated for the normal distribution by the Shapiro-Wilk test followed by one-way or two-way analysis of variance (ANOVA) followed by Tukey or Bonferroni post-tests, respectively. Otherwise, Kaplan-Meier method estimated the survival of zebrafish, and Kruskal-Wallis analyzed locomotor activity. The differences between the groups were considered significant for results of $p < 0.05$. The GraphPad Prism® (version 6.0) program was used.

3. Results

For analytical validation different conditions were tested during the development of the method. The pH was also tested using different solutions (acetic acid and hydrochloric acid), then, choosing pH 4.0 acidified with acetic acid due to its chromatographic profile. Acetonitrile and water in different proportions were tested and an isocratic method composed of 60 % acetonitrile and 40 % water was chosen because of the better chromatographic profile obtained. The retention time of naringin was around 3.5 min.

3.1. Linearity, limit of detection and limit of quantification

In order to determine linearity in HPLC, five different concentrations in the range 2–20 μg/mL of the standard sample were evaluated in triplicate. RSD was below 5 %. A linear equation ($y = 51766x - 19951$) was obtained by linear regression, between naringin concentration and the obtained areas. A correlation coefficient of 0.998 was obtained within the standards determined by ANVISA. The limits of detection and quantification were 0.26 μg/mL and 0.79 μg/mL, respectively.

3.2. Specificity

The specificity of the analytical method was evaluated by comparative analysis between free naringin, blank or naringin-loaded nanocapsules at a concentration of 10 μg/mL. The assessment of specificity allows the detection of impurities and the determination of whether the method is suitable for the product to be quantified. Chromatograms were obtained (Fig. 1). It is observed that the naringin-loaded nanocapsules (red line) showed the same retention time as the free naringin (blue line), demonstrating the presence of the active in the formulation and efficiency of the naringin detection method in these nanocapsules. As expected, the blank nanocapsule (green line) did not show any chromatographic signal, demonstrating that the method is specific for drug detection, and there is no interference for the constituents of the nanocapsule formulation.

3.3. Precision

The values of the precision tests revealed RSD of 1.41 %, results lower than the acceptance criterion which requires a maximum RSD of 5 % [39], indicating that the method presents adequate within run and between run reproducibility, confirming the intermediate precision of the proposed method (Table 1).

3.4. Accuracy

Regarding the evaluation of accuracy, adequate recoveries were obtained (99–100 %) (Table 2). The percentage of recovery indicated appropriate accuracy and, consequently, an agreement between theoretical concentrations and actual concentration.

3.5. Robustness

The results of the robustness evaluation, due to the deliberate variations in the conditions of the method, did not show any significant effect on the quantification of naringin nor on the chromatographic performance, indicating the robustness of the method. Regarding the composition of the mobile phase, there was no significant influence on the naringin content found when the composition of the mobile phase was changed to 55:45 (acetonitrile-water) or 65:35. Also, the content did not change when, the flow rate was changed to 0.6 mL/min or 0.8 mL/min; nor when the detection wavelength was changed to 283 nm or 287 nm; nor when the pH of the mobile phase water modified to 3.8 or 4.2 (Table 3).

3.6. Toxicity assessment

In order to evaluate the safety of the use of blank or naringin-filled nanocapsules, toxicity tests were performed in different alternative models for use in nanotoxicology. *In vivo* models (*C. elegans* and *Danio rerio*) and *in vitro* models (hippocampal slices) were used for these evaluations.

3.6.1. Toxicity in vivo in a model of Caenorhabditis elegans

Young adult nematodes were exposed to free naringin, blank or naringin-loaded nanocapsules at concentrations of 0.6 mg/mL, 1.2 mg/mL and 1.8 mg/mL for 2 h at 20 °C. Control animals were exposed only to M9 buffer. It was found that treatment with the nanocapsules and free naringin did not interfere in the survival of the worms (data not shown).

3.6.2. Behavioral analysis

In the behavioral analysis it was observed that the treatment with the free naringin, blank or naringin-loaded nanocapsules did not interfere in the pharyngeal pumping, which was analyzed 2 h and 24 h after the treatment (data not shown). However, treatment with nanocapsules (blank and naringin) significantly decreased the defecation cycle when compared to untreated worms in the first two hours of exposure. However, in the analysis after 24 h of exposure, the defecation cycle depicted no significant difference when compared to untreated worms (Fig. 2).

3.6.3. Antioxidant activity assay

Treatment with Juglone significantly decreases the survival rate when compared to control worms. In the treatment with naringin-loaded nanocapsules there was no protection against damage caused by

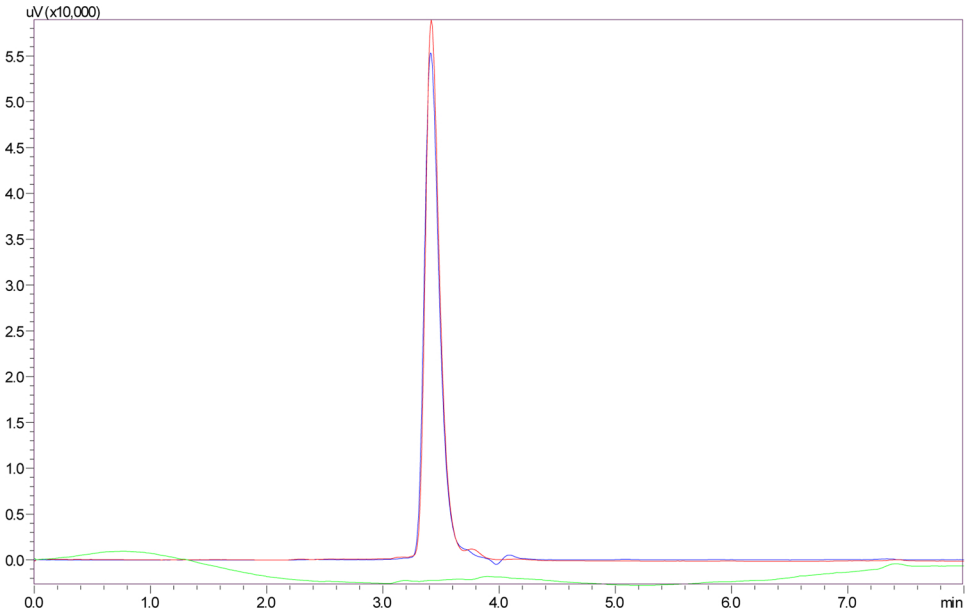

Fig. 1. Chromatograms obtained from solutions (10 μg.mL^{-1}) (Blue line) free naringin, (Red line) naringin-loaded nanocapsule suspensions, and (Green line) blank-nanocapsule (placebo formulation) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Within run and between run reproducibility of the method used to assay naringin content of nanocapsule suspensions (sample solutions at a theoretical concentration of 10 μg mL^{-1}).

Parameter	*n*	RSD (%)
Within run	6	1.78
Between run		
Day 1	3	1,74
Day 2	3	1,20
Day 3	3	1,31
Day 1 + 2 + 3	9	1,4

Abbreviations: RSD, relative standard deviation.

Table 2
Determination of the accuracy of the method (sample solutions at a theoretical concentration of 10 μg.mL-1).

Reference sample (μg·mL^{-1})	Added (μg·mL^{-1})	Found (μg·mL^{-1})	Recovery (%)	RSD (%)
9.96 ± 0.76	3.75	13.69 ± 0.81	99.85 ± 0.53	0.53
	7.50	17.52 ± 0.65	100.62 ± 1.06	1.05
	11.25	21.11 ± 4.57	99.02 ± 3.37	3.41
		Mean	99.83	

Abbreviations: RSD, relative standard deviation.

Juglone (data not shown). In the treatments with free naringin there was protection against the damage caused by Juglone and the treatment with the blank nanocapsules showed a decrease in the mortality in the highest concentration, as shown in Fig. 3.

3.6.4. Microbiological assays

To verify if the nanocapsules did not interfere in the feed of the worms inhibiting the bacterial growth, two antimicrobial tests, disc-diffusion and MIC were carried out. In both assays, the nanoformulations tested or naringin did not cause significant antimicrobial effect on the growth of the analyzed strains including E. coli OP50 serving as a nutrient for *C. elegans* (data not shown).

Table 3
Determination of the robustness of the method. The recommended chromatographic conditions were: *Purospher®* STAR RP-18, endcapped (5 μm) with acetonitrile–water pH 4.0 60:40 (v/v) as mobile phase at a flow rate of 0.7 mL/min and UV detection at 285 nm.

Conditions	%	RSD (%)
Recommended conditions	100.04	0.33
Mobile phase (acetonitrile-water)		
55-45	99.57	1.02
65-35	99.37	0.65
Flow rate (mL/min)		
0.6	100.43	0.69
0.8	99.68	0.36
Wavelength (nm)		
283	99.62	0.58
287	99.37	1.11
pH water		
3.8	99.17	1.55
4.2	99.98	0.60

Abbreviations: RSD, relative standard deviation.

3.7. Toxicity in vivo in a model of Danio rerio

Intact embryos with 3 − 5 hours post-fertilization (hpf) were distributed in 96-well plates and immersed in 100 μL of free naringin, blank or naringin-loaded nanocapsules solutions diluted in maintenance water to reach concentrations of 0.005, 0, 05, 0.5, 1 and 2 mg/L. The animals were monitored every 24 h up to 5 days after fertilization (dpf). It was found that treatment with the nanocapsules and naringin in their free form did not interfere in the survival of the embryos. None of the treatments altered the hatching parameter (Figs. 4 and 5).

3.7.1. Morphological evaluation and locomotor activity

No changes were observed in the morphology of the embryos and the parameters of evaluation of locomotor activity such as time in the peripheral zone, distance travelled and mean velocity were also not affected by the treatment with the nanocapsules suspensions and with the free naringin (data not shown).

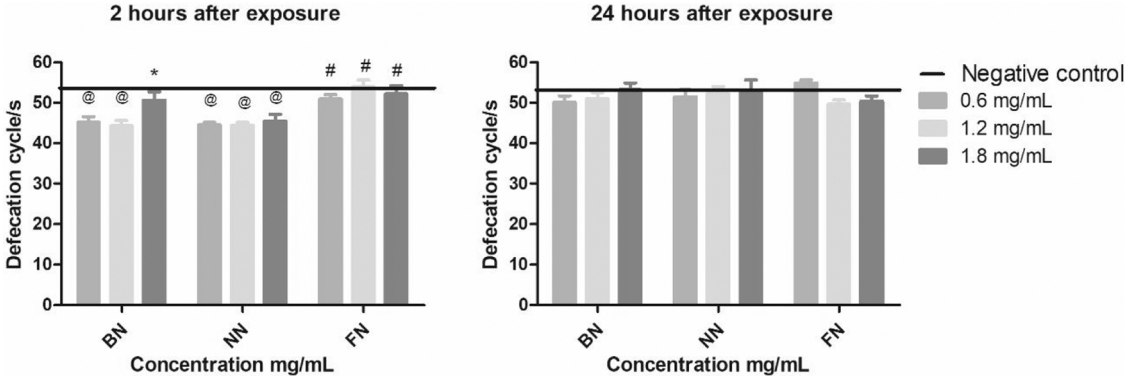

Fig. 2. Defecation cycle of worms treated with blank-nanocapsule (BN), naringin-loaded nanocapsule suspensions (NN) and free naringin (FN) after 2 h and 24 h respectively of exposure, n = 15 animals per group. Control group was free of chemicals (negative control). Comparison among groups and control was performed by one-way ANOVA followed by Tukey post test; $^{@}p < 0{,}001$. Two-way analysis of variance - ANOVA, followed by Bonferroni post test; $^{*}p < 0.0001$ *vs.* BN, $^{\#}p < 0.0001$ *vs.* NN.

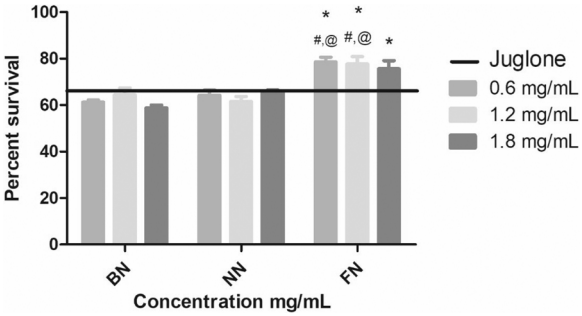

Fig. 3. Juglone resistance of worms exposed to blank-nanocapsule (BN) and free-naringin (FN) previously exposed in liquid medium for 2 h, after being exposed to Juglone for 1 h. The experiments were performed in five independent assays. Comparison among groups and Juglone was performed by one-way ANOVA followed by Tukey post test; $^{@}p < 0{,}001$. Two-way analysis of variance - ANOVA, followed by Bonferroni post test; $^{*}p < 0.0001$ *vs.* BN, $^{\#}p < 0.0001$ *vs.* NN.

3.8. Toxicity in vitro in hippocampal slices

The hippocampal slices were incubated with blank or naringin-loaded nanocapsules, or naringin-free at concentrations of 0.1 µg/mL, 1.0 µg/mL and 10 µg/mL for 1 h. Hydrogen peroxide was used as the death control and the viability control was composed only by the incubation medium in the slices for the evaluation of the MTT reduction for the toxicity test. It was verified that only the group H_2O_2 used as a control induced cellular damage in relation to the control group (data not shown).

4. Discussion

Naringin is a natural compound found in citrus fruits mainly in grapefruit (grapefruit hybrid of the orange crossing with grapefruit), which confers its bitter taste. This flavonoid exerts numerous biological and pharmacological benefits due to its antioxidant, anti-inflammatory and anti-apoptotic properties [17]. It has also been considered as a neuroprotective agent not only because it can activate anti-apoptotic pathways, but also because of the induction of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF). In addition to these, the neuroprotective effect of naringin is also due to its ability to eliminate free radicals by its antioxidant action and anti-inflammatory properties [48]. However, this compound is metabolised to the naringenin in the organism, its solubility is limited in water and has low oral bioavailability of around 5 % [49]. As these factors may compromise its biological

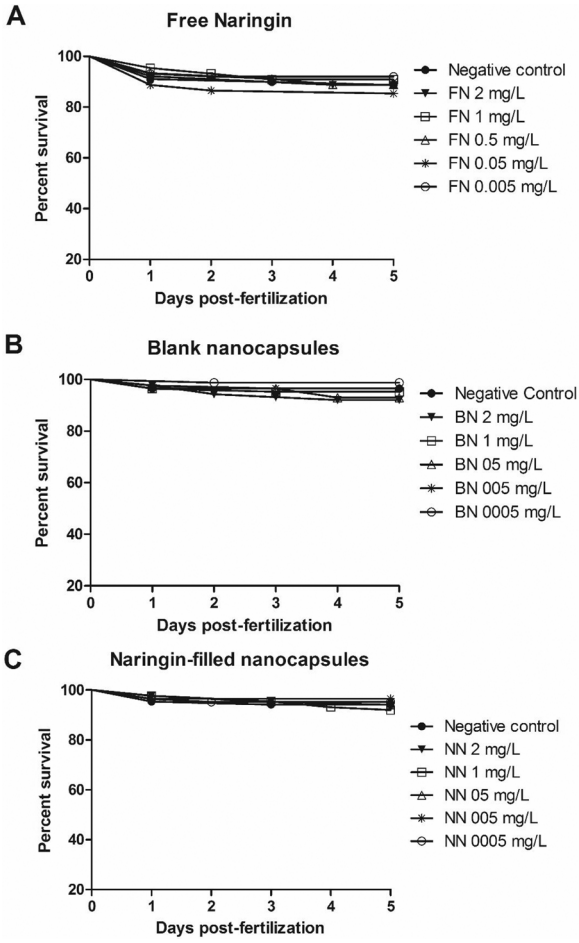

Fig. 4. Kaplan-Meier survival representation of zebrafish larvae after exposure to (A) Free Naringin, (B) Blank Nanocapsules and (C) Nanocapsules with Naringin. The solutions were freshly prepared and renovate daily. Eggs were exposed by immersion from 3 − 5 hours-post fertilization at the concentrations 2, 1, 0.5, 0.05 and 0.005 mg/L. Control group was free of chemicals and Positive control was treated with 4 mg/L of 3,4 dichloroaniline. Data are presented as fraction of survival from at least 70 animals for each group.

applicability, the encapsulation of natural extracts is a promising alteration to improve physicochemical characteristics, increase bioavailability, solubility and avoid metabolites [24,25].

In this work, a nanocapsule containing naringin was produced with the aim of improving characteristics such as solubility, bioavailability

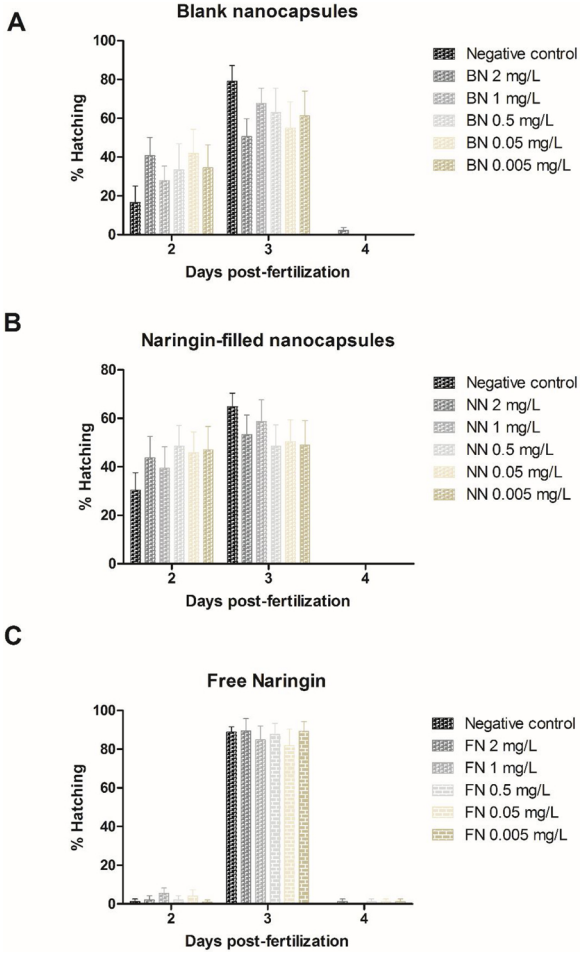

Fig. 5. Percentage of hatchings of zebrafish embryos up to 4 days post-fertilization after exposure to (A) Free Naringin, (B) Blank Nanocapsules and (C) Nanocapsules with Naringin. The solutions were freshly prepared and renovate daily. Eggs were exposed by immersion from 3 – 5 hours-post fertilization at the concentrations 2, 1, 0.5, 0.05 and 0.005 mg/L. Control group was free of chemicals (negative control). Data are presented as mean ± standard error of at least 8 sets of 10 animals for each group.

and biotransformation of naringin, in order to evaluate its effect in neuroprotection models. Among the methods available in the literature it was necessary to select a methodology based on the materials of interest. Therefore, the method described by Fessi et al. (1989) [38] was chosen, which seemed efficient and easy to reproduce. This method is based on the interfacial deposition of a polymer after displacement of a water-miscible solvent from a lipophilic solution. The rapid diffusion of the solvent reduces the interfacial tension between the organic and aqueous phase, leading to the formation of small drops of organic solvent [50].

The HPLC is a useful and fast technique and has been widely used in the validation of analytical methods for the quantification of drugs [51], from which the ANVISA standards are usually used for validation. To do such, a series of analyzes must be carried out to quantify the asset. In this work, a simple and fast method was developed, using a D2 photodiode arrangement detector for naringin nanocapsules. Initially, the maximum wavelength of absorption of the reference standard solution was evaluated. The absorbance at 285 nm was chosen, considering the maximum absorption intensity. This result is in agreement with the literature [52,53].

Linearity, limit of detection and limit of quantification, specificity, intermediate precision (within run and between run reproducibility), accuracy and robustness were evaluated in naringin-loaded nanocapsules. Linearity is used to demonstrate that the results obtained are directly proportional to the drug concentration in the sample, within a specified range [39]. The curve plotted from five concentrations of naringin fitted, validating the data, the correlation coefficient (r) of the curve equal to 0.99 [39]. According to ANVISA standards, the method is specific when there is no detection of interferences with the analyte. The criteria of linearity and specificity were complied as demonstrated in the results.

The results of the evaluation of the robustness, in face of the variations of the chromatographic conditions of the method applied in the present work, did not show any significant effect on the quantification of naringin nor on the chromatographic performance. As demonstrated, the validated method to quantify naringin in nanocapsule suspensions complied all the parameters required by ANVISA [39] and EMEA [76] for the production of new drugs. The drug extraction method was considered efficient, since it was possible to recover, in all the initial time analyses, the amount of drug compatible to the naringin added in the preparation. Thus, the isocratic method validated in this work to quantify naringin through HPLC has many advantages over other methods that use elution gradient, longer run time and buffer solution [54].

Nanotechnology has grown and become a very promising tool in many industries. As exposure to nanomaterials increases, an important research area emerges, nanotoxicology [72]. Thus, *in vitro* and *in vivo* toxicological studies have become very relevant and indispensable, since this study is basic for a new formulation developed indicating its safe use. We have used the nanoformulations characterized and validated to evaluate the potential toxicology in C. elegans, zebrafish and slices from hippocampus of rats.

C. elegans is a widely used organism for in vivo assays, but less than a decade ago we began to use it for the screening of new drugs [55]. The first part of the screening of new drugs in *C. elegans* consists in adding the compounds to the culture medium and then evaluating the morphology and locomotion observed under the microscope [56].

In this work we evaluated the survival of the worms when exposed to different concentrations of the nanocapsules in the culture medium, in which it was observed that there was no significant reduction in the percentage of live worms when compared to the control group, indicating the low toxicity of the nanocapsules. In the behavioral assays performed in *C. elegans*, it was possible to verify that the nanocapsules did not interfere in the pharyngeal beats, a parameter governed by the dopaminergic system [73]. However, treatment with nanocapsules, with the exception of free naringin, significantly decreased the defecation cycle in the first two hours when compared to untreated worms. Each defecation cycle begins with a contraction of the posterior body that directs the intestinal contents to the anterior part of the worm and is followed by an anterior muscle contraction that pushes the contents of the intestine into the animal, followed by a contraction of the enteric muscle that expels the intestinal contents. The main pathway governing the defecation cycle of *C. elegans* is the cholinergic system. Our results corroborate the results found by Stefanello et al. (2015) [74], in which organic selenium compounds were tested and obtained similar results, indicating a possible modulation of the cholinergic system. In the test performed 24 h after exposure, the defecation cycle of the worms was already normalized.

Acetylcholinesterase (AchE) is the enzyme responsible for the breakdown of acetylcholine and, in preclinical studies of neurocognitive disorders, levels of AchE have been elevated in different parts of the brain, and any agent that may decrease AchE activity could restore acetylcholine levels, and thus, improve cognitive functions [20,57,58]. The levels of this enzyme are very important in maintaining the normal functions of the brain. Data reported in the literature showed that enzymatic activity was significantly restored after treatment with naringin [20,58–60].

Juglone is a naphthoquinone that generates reactive oxygen species and is widely used as an inducer of damage in tests of possible

antioxidant activity of new compounds in *C. elegans* [43,61]. Juglone can induce oxidative stress through several mechanisms, such as the formation of superoxide ($O2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2), triggering toxic effects due to protein modifications [62]. Nanocapsules containing naringin were not able to prevent oxidative damage caused by juglone. One hypothesis for such an outcome is that the preincubation time has not been sufficient to release the required amount of the active and activates the antioxidant mechanisms necessary to protect the organism against subsequent damage. Naringin-loaded nanocapsules has slow release profile up to approximately 2 h [77]. However, in this case, there is a limitation of the incubation method of worms with juglone, since they have a very fast life cycle, which interferes with the exposure time [63]. In order to verify a possible antimicrobial effect of the nanocapsules, which could also interfere in the metabolism of *C. elegans*, the disc-diffusion test and minimum inhibitory concentration (MIC) in four strains of *E. coli*, including the OP50 used as source nutritional status for the worms, were used to verify if they could cause a decrease of the bacterial viability and, thus, and thus interfering in the useful life of the worms. This data is important because, once again by the limitation of the possible periods of evaluations in the worms, a microbial change could indicate significant changes in the worms in periods after the time used in the present work. Our results established that the nanocapsules at the tested concentrations did not decrease the quality of *E. coli* strains, which provides greater safety for the use of nanocapsules in the *C. elegans* model.

Zebrafish has been recognized as an animal model due to the exceptional set of characteristics. Zebrafish exhibits high similarity to the human genome [64], small size, high reproducibility, rapid development and transparency of embryos and larvae, which allow observation of cells and organs from early stages of development [65]. The zebrafish embryo has been widely used in the field of nanotoxicology to evaluate the effects of nanomaterials such as nanoparticles, carbon nanotubes and fullerene in the nervous system [66–68]. To investigate the potential toxic effects of nanocapsules at different concentrations during initial development, we evaluated *in vivo* parameters as an initial toxicology screen. No statistical difference was found in embryo mortality, morphology or hatching rate. In the behavioral parameters evaluated in zebrafish, there was no change in the residence time in the peripheral zone, distance traveled or average speed, again demonstrating that the nanocapsules were shown to be safe through the tests performed.

In the present study, the hippocampal slices were submitted to different concentrations of the blank nanocapsules or containing naringin. The viability of the tissues was evaluated by the MTT reduction method, which suggests their safety [69]. As there was no reduction of cell viability in the *in vitro* assay for the tested concentrations, the formulations were considered safe by this method. There are still doubts about the period of treatment and the dose to be used, as well as the mechanism of action involving nanoparticles containing naringin.

5. Conclusions

A rapid, specific and reliable HPLC method to quantify naringin in nanocapsule suspensions was developed and validated. The proposed analytical methodology is simple and has fulfilled all ANVISA requirements. The method developed is innovative and presents advantages over previously described methods in the literature, such as the use of a lower proportion of organic solvent, shorter run time and isocratic elution. The toxicological analysis methods used here have demonstrated the safety of the nanocapsules for application at in vitro and in vivo studies. However, it has not yet been possible to clarify how the nanocapsules behave against damage induced by oxidative stress (Juglone) in C. elegans, therefore, more tests are necessary to understand its action.

CRediT authorship contribution statement

Renata Gancine Budel: Conceptualization, Methodology, Validation, Investigation, Writing - original draft. **Denise Ajala da Silva:** Validation, Investigation. **Michele Pereira Moreira:** Validation, Investigation. **Ana Júlia Figueiró Dalcin:** Validation, Investigation. **Aline Franzen da Silva:** Validation, Investigation. **Luiza Reali Nazario:** Validation, Investigation. **Julia Huppes Majolo:** Validation, Investigation. **Leonardo Quintana Soares Lopes:** Validation, Investigation. **Roberto Christ Vianna Santos:** Writing - review & editing. **Felix Alexandre Antunes Soares:** Writing - review & editing. **Rosane Souza da Silva:** Writing - review & editing. **Patrícia Gomes:** Writing - review & editing. **Carina Rodrigues Boeck:** Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The author reports no conflicts of interest in this work.

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References

[1] G.C. Jagetia, T.K. Reddy, The grapefruit flavanone naringin protects against the radiation-induced genomic instability in the mice bone marrow: a micronucleus study, Mutation Research - Genetic Toxicology and Environmental Mutagenesis 519 (1–2) (2002) 37–48.
[2] M.A. Alam, et al., Effect of Citrus Flavonoids, Naringin and Naringenin, on Metabolic Syndrome and Their, Adv. Nutr. (July) (2018) 404–417.
[3] M.H. Ribeiro, Naringinases: Occurrence, Characteristics, and Applications, (2011), pp. 1883–1895.
[4] H.R. Frydoonfar, D.R. Mcgrath, A.D. Spigelman, The variable effect on proliferation of a colon cancer cell line by the citrus fruit flavonoid Naringenin, *Colorectal Disease* 5 (2) (2003) 149–152.
[5] A.W. Harmon, Y.M. Patel, Naringenin inhibits glucose uptake in MCF-7 breast cancer cells: a mechanism for impaired cellular proliferation, Breast Cancer Res. Treat. 85 (2) (2004) 103–110.
[6] H.J. Heo, et al., Effect of antioxidant flavanone, naringenin, from Citrus junos on neuroprotection, J. Agric. Food Chem. 52 (6) (2004) 1520–1525.
[7] M. Cavia-saiz, et al., Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study, J. Sci. Food Agric. 90 (7) (2010) 1238–1244.
[8] G. Céliz, M. Daz, M.C. Audisio, Antibacterial activity of naringin derivatives against pathogenic strains, J. Appl. Microbiol. 111 (3) (2011) 731–738.
[9] U.J. Jung, S.R. Kim, Effects of naringin, A flavanone glycoside in grapefruits and citrus fruits, on the nigrostriatal dopaminergic projection in the adult brain, Neural Regen. Res. 9 (16) (2014) 1514–1517.
[10] H.D. Kim, et al., Naringin treatment induces neuroprotective effects in a mouse model of Parkinson's disease in vivo, but not enough to restore the lesioned dopaminergic system, J. Nutr. Biochem. 28 (2016) 140–146.
[11] P. Kumar, A. Kumar, Protective effect of hesperidin and naringin against 3-nitropropionic acid induced Huntington's like symptoms in rats: possible role of nitric oxide, Behav. Brain Res. 206 (1) (2010) 38–46.
[12] K. Gopinath, G. Sudhandiran, Naringin modulates oxidative stress and inflammation in 3-nitropropionic acid-induced neurodegeneration through the activation of nuclear factor-erythroid 2-related factor-2 signalling pathway, *Neuroscience* 227 (2012) 134–143.
[13] M. Golechha, et al., Naringin ameliorates pentylenetetrazol-induced seizures and associated oxidative stress, inflammation, and cognitive impairment in rats: possible mechanisms of neuroprotection, Epilepsy Behav. 41 (2014) 98–102.
[14] K.H. Jeong, U.J. Jung, S.R. Kim, Naringin attenuates autophagic stress and neuroinflammation in kainic acid-treated Hippocampus In vivo, Evid. Based

Complement. Altern. Med. 2015 (2015).
[15] V. Gaur, A. Aggarwal, A. Kumar, Protective effect of naringin against ischemic reperfusion cerebral injury: Possible neurobehavioral, biochemical and cellular alterations in rat brain, Eur. J. Pharmacol. 616 (1–3) (2009) 147–154.
[16] D. Wang, et al., Long-term naringin consumption reverses a glucose uptake defect and improves cognitive deficits in a mouse model of Alzheimer's disease, Pharmacol. Biochem. Behav. 102 (1) (2012) 13–20.
[17] S. Qin, et al., Effects of naringin on learning and memory dysfunction induced by gp120 in rats, Brain Res. Bull. 124 (2016) 164–171.
[18] S.P. Fernandez, et al., The flavonoid glycosides, myricitrin, gossypin and naringin exert anxiolytic action in mice, Neurochem. Res. 34 (10) (2009) 1867–1875.
[19] S.R. Maratha, N. Mahadevan, Memory enhancing activity of naringin in unstressed and stressed mice: possible cholinergic and nitriergic modulation, Neurochem. Res. 37 (10) (2012) 2206–2212.
[20] A. Prakash, B. Shur, A. Kumar, Naringin protects memory impairment and mitochondrial oxidative damage against aluminum-induced neurotoxicity in rats, Int. J. Neurosci. 123 (9) (2013) 636–645.
[21] A.K. Sachdeva, A. Kuhad, K. Chopra, Naringin ameliorates memory deficits in experimental paradigm of Alzheimer's disease by attenuating mitochondrial dysfunction, Pharmacol. Biochem. Behav. 127 (2014) 101–110.
[22] A.K. Sachdeva, K. Chopra, Naringin mitigate okadaic acid-induced cognitive impairment in an experimental paradigm of Alzheimer's disease, J. Funct. Foods 19 (2015) 110–125.
[23] S.L. Hsiu, et al., Comparison of metabolic pharmacokinetics of naringin and naringenin in rabbits, Life Sci. 70 (13) (2002) 1481–1489.
[24] S.S. Ajazuddin, Applications of novel drug delivery system for herbal formulations, *Fitoterapia* 81 (7) (2010) 680–689.
[25] V. Dinesh kumar, P.R.P. Verma, S.K. Singh, Development and evaluation of biodegradable polymeric nanoparticles for the effective delivery of quercetin using a quality by design approach, Lwt - Food Sci. Technol. 61 (2) (2015) 330–338.
[26] D.J. Mcclements, Design of nano-laminated coatings to control bioavailability of lipophilic food components, J. Food Sci. 75 (1) (2010).
[27] F.L. Yen, et al., Naringenin-loaded nanoparticles improve the physicochemical properties and the hepatoprotective effects of naringenin in orally-administered rats with CCl4-induced acute liver failure, Pharm. Res. 26 (4) (2009) 893–902.
[28] M.C. Fontana, M.O. Bastos, R.C.R. Beck, Development and validation of a fast RP-HPLC method for the determination of clobetasol propionate in topical nanocapsule suspensions, J. Chromatogr. Sci. 48 (2010) 637–640.
[29] A.J.F. Dalcin, Desenvolvimento, caracterização e avaliação da atividade antimicrobiana e antibiofilme de nanocápsulas de dihidromiricetina em cateteres urinários, Dissertao (2015).
[30] M.E. Vance, et al., Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory, Beilstein J. Nanotechnol. 6 (1) (2015) 1769–1780.
[31] Tulve, et al., Exposures. 218 (3) (2015) 345–357 2015.
[32] W.H. Suh, et al., Nanotechnology, nanotoxicology, and neuroscience, Prog. Neurobiol. 87 (3) (2009) 133–170.
[33] R.A. Yokel, R.C. Macphail, Engineered nanomaterials: exposures, hazards, and risk prevention, J. Occup. Med. Toxicol. 6 (1) (2011) 7.
[34] F.A. Cupaioli, et al., Engineered nanoparticles. How brain friendly is this new guest? Prog. Neurobiol. 119–120 (2014) 20–38.
[35] S. Lung, et al., Brain suppression of AP-1 by inhaled diesel exhaust and reversal by cerium oxide nanoparticles, Inhal. Toxicol. 26 (10) (2014) 636–641.
[36] D. Van berlo, et al., Investigation of the effects of short-term inhalation of carbon nanoparticles on brains and lungs of c57bl/6j and p47phox-/-mice, *NeuroToxicology* 43 (2014) 65–72.
[37] L. Gonzalez-moragas, A. Roig, A. Laromaine, C. Elegans as a tool for in vivo nanoparticle assessment, Adv. Colloid Interface Sci. 219 (2015) 10–26.
[38] H. Fessi, et al., Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 55 (1) (1989) 1–4.
[39] ANVISA, RDC Nº 166, de 24 de julho de 2017, Diário Oficial da República Federativa do Brasil, 2017 v. 2017, p. 22.
[40] S. Brenner, Caenorhabdztzs elegans, *Methods* 77 (1) (1974) 71–94.
[41] C. Huang, C. Xiong, K. Kornfeld, Measurements of age-related changes of physiological processes that predict lifespan of caenorhabditis elegans, Proc. Natl. Acad. Sci. 101 (21) (2004) 8084–8089.
[42] M.L. Migliori, et al., Circadian rhythms in metabolic variables in Caenorhabditis elegans, Physiol. Behav. 103 (3–4) (2011) 315–320.
[43] E. De Castro, S.H. De Castro, T.E. Johnson, Isolation of long-lived mutants in Caenorhabditis elegans using selection for resistance to juglone, Free Radic. Biol. Med. 37 (2) (2004) 139–145.
[44] M.A. Wikler, F.R. Cockerill, K. Bush, M.N. Dudley, G.M. Eliopoulos, D.J. Hardy, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, eighth edition, Approved Standard d, 2009.
[45] A.W. Bauer, et al., Antibiotic susceptibility testing by standadized single disk method, Am. J. Clin. Pathol. 45 (4) (1966) 493–496.
[46] CLSI, Metodologia dos Testes de Sensibilidade a Agentes Antimicrobianos por Diluição para Bactéria de Crescimento Aeróbico, [s.l: s.n.]. v. 23 (2008).
[47] S.E. Belanger, J.M. Rawlings, G.J. Carr, An update to the Fish embryo toxicity-acute Fish toxicity relationship and prospects for support of the use of the FET as an animal alternative document developed for the February, 2012 Meeting of the OECD Ad Hoc Expert Group on the Fish Embryo Test Plea. N. February (2012) 1–156.
[48] M. Golechha, et al., Hydroalcoholic extract of *Emblica officinalis* protects against kainic acid-induced status epilepticus in rats: evidence for an antioxidant, anti-inflammatory, and neuroprotective intervention, Pharm. Biol. 49 (11) (2011) 1128–1136.
[49] D.V. Ratnam, et al., Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective, J. Control. Release 113 (3) (2006) 189–207.
[50] B. Mishra, B.B. Patel, S. Tiwari, Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery, Nanomed. Nanotechnol. Biol. Med. 6 (1) (2010) 9–24.
[51] F.M. Lanças, Cromatografia Líquida Moderna: HPLC/CLAE, editora Átomo, Campinas, SP, 2009.
[52] Y. Zhang, Q. Zhang, L. Li, B. Wang, Y. Zhao, D. Guo, Simultaneous determination and pharmacokinetic studies of dihydromyricetin and myricetin in rat plasma by HPLC-DAD after oral administration of *Ampelopsis grossedentata* decoction, J. Chromatogr. B 860 (2007) 4–9.
[53] L. Ye, H. Wang, S.E. Duncan, W.N. Eigel, S.F. O'keefe, Antioxidant activities of Vine Tea (Ampelopsis grossedentata) extract and its major component dihydromyricetin in soybean oil and cooked ground beef, Food Chem. 172 (2015) 416–422.
[54] L.M. Cordenonsi, et al., Simultaneous separation and sensitive detection of naringin and naringenin in nanoparticles by chromatographic method indicating stability and photodegradation kinetics, Biomed. Chromatogr. 30 (2) (2016) 155–162.
[55] L.P.O. Reilly, et al., NIH Public Access, National Institutes of Health, 2015, pp. 247–253.
[56] Y. Cho, C.L. Zhao, H. Lu, Trends in high-throughput and functional neuroimaging in Caenorhabditis elegans, Wiley Interdiscip. Rev. Syst. Biol. Med. 9 (3) (2017) 1–12.
[57] Y. Chtourou, et al., Naringin abrogates cisplatin-induced cognitive deficits and cholinergic dysfunction through the down-regulation of AChE expression and iNOS signaling pathways in Hippocampus of aged rats, J. Mol. Neurosci. 56 (2) (2015) 349–362.
[58] Y. Chtourou, et al., Protective role of naringin against cisplatin induced oxidative stress, inflammatory response and apoptosis in rat striatum via suppressing ROS-mediated NF-κB and P53 signaling pathways, Chem. Biol. Interact. 239 (2015) 76–86.
[59] A. Kumar, S. Dogra, A. Prakash, Protective effect of naringin, a Citrus flavonoid, against colchicine-induced cognitive dysfunction and oxidative damage in rats, J. Med. Food 13 (4) (2010) 976–984.
[60] A. Kumar, A. Prakash, S. Dogra, Naringin alleviates cognitive impairment, mitochondrial dysfunction and oxidative stress induced by d-galactose in mice, Food Chem. Toxicol. 48 (2) (2010) 626–632.
[61] A. Strayer, et al., Expression of the small heat-shock protein Hsp16-2 in Caenorhabditis elegans is suppressed by Ginkgo biloba extract EGb 761, FASEB J. 17 (15) (2003) 2305–2307.
[62] J.J. Inbaraj, C.F. Chignell, Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes, Chem. Res. Toxicol. 17 (1) (2004) 55–62.
[63] L. Gonzalez-Moragas, A. Roig, A. Laromaine, C. Elegans as a tool for in vivo nanoparticle assessment, Adv. Colloid Interface Sci. 219 (2015) 10–26.
[64] J. Legradi, et al., Comparability of behavioural assays using zebrafish larvae to assess neurotoxicity, Environ. Sci. Pollut. Res. - Int. 22 (21) (2014) 16277–16289.
[65] A. Chakraborty, E. Agoramoorthy, Why Zebrafish? n. January (2015).
[66] L. Truong, et al., Persistent adult zebrafish behavioral deficits results from acute embryonic exposure to gold nanoparticles, Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 155 (2) (2012) 269–274.
[67] A.M. Da Rocha, et al., Gene expression and biochemical responses in brain of zebrafish Danio rerio exposed to organic nanomaterials: carbon nanotubes (SWCNT) and fullerenol (C60(OH)18-22(OK4)), *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 165 (4) (2013) 460–467.
[68] Q. Xin, et al., Silver nanoparticles affect the neural development of zebrafish embryos, J. Appl. Toxicol. 35 (12) (2015) 1481–1492.
[69] J.P. Kamdem, et al., Trichilia catigua (Catuaba) bark extract exerts neuroprotection against oxidative stress induced by different neurotoxic agents in rat hippocampal slices, Ind. Crops Prod. 50 (2013) 625–632.
[70] D. Bonamici, Sistema de classificação biofarmacêutica e bioisenções. Dissertação, Universidade de São Paulo, São Paulo, 2009.
[71] C.R. Boeck, et al., NMDA preconditioning protects against seizures and hippocampal neurotoxicity induced by quinolinic acid in mice, *Epilepsia* 45 (7) (2004) 745–750.
[72] A. Shvedova, A. Pietroiusti, V. Kagan, Nanotoxicology ten years later: lights and shadows, Toxicol. Appl. Pharmacol. 299 (2016) 1–2.
[73] T. Nagashima, S. Ishiura, S. Suo, Regulation of body size in Caenorhabditis elegans: effects of environmental factors and the nervous system, Int. J. Dev. Biol. 61 (6-7) (2017) 367–374.
[74] S.T. Stefanello, et al., Protective effects of novel organic selenium compounds against oxidative stress in the nematode Caenorhabditis elegans, Toxicol. Rep. 2 (2015) 961–967.
[75] Q. Wu, et al., Evaluation of Environmental Safety Concentrations of DMSA Coated Fe 2 O 3 -NPs Using Different Assay Systems in Nematode Caenorhabditis elegans, v. 7, n. 8 (2012).
[76] EMEA, European Medicines Agency gives favourable ruling on NSAIDs, BMJ 333 (7574) (2006) 873.
[77] C.F. Ferreira, et al., Development, characterisation and evaluation of cytotoxitity of nanocapsuled naringin and naringenin, Disciplinarum Scientia. Série: Naturais e Tecnológicas 16 (2) (2015) 285–299 Disponível em: https://periodicos.ufn.edu.br/index.php/disciplinarumNT/article/view/1382/1313.