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SPECTRAL TRANSMITTANCE OF RAT LENSES TREATED WITH NARINGIN-LOADED NANOPARTICLES

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ABSTRACT

Oxidative stress is one of the main factors in the cataract development. Various antioxidants are currently being investigated to prevent cataract formation. The present study evaluated the effect of Naringin-loaded nanoparticles (NLN) in preventing cataracts using an ex vivo model of cataractogenesis. The NLN was prepared using modified single-step nanoprecipitation technique; particle size, surface charge, and polydispersity index by dynamic light scattering, encapsulation efficiency were determined spectrophotometrically. NLN morphology was investigated by scanning electron microscope (SEM). Lenses of Wistar rats were grown in Dulbecco's modified Eagle's Medium alone or with the addition of 100µM sodium selenite and NLN (Naringin 100µM). Photographs of lenses were taken before and after culture. The lens opacity (transmittance) was determined using a UV-Vis spectrophotometer. The NLN had an average diameter of 208.30 ± 19.94 nm, polydispersity index of 0.27 ± 0.08, zeta potential of -44.50 ± 7.78 mV, and EE% of 72.66 ± 3.18 %. The photographs and transmittance spectra of lenses incubated with NLN showed attenuation in the progression of opacity induced by sodium selenite. The treatment with NLN delayed the opacification of lenses in an ex vivo model of cataractogenesis, suggesting a therapeutic option to prevent cataract formation.

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INTRODUCTION

Cataracts are the pathological opacification of the lens, which, results in the scattering of visible light as it passes through the lens and subsequently degrades visual acuity and become blinding if untreated (Lin *et al.*, 2010; Moreau and King, 2012). Although the incidence of most cataracts is age-related, the etiology of the disease is multifactorial and the only current treatment is cataract surgery. (Hildreth, Burke and Glass, 2009; Thompson and Lakhani, 2015). Pharmacological treatments to delay or prevent cataract development and subsequent blindness would result in increased quality of lifeas well as reduced economic burdens (Moreau and King, 2012).

Efforts were generally directed to the search for natural antioxidants as a drug to cataracts prevention (Doganay *et al.*, 2006; Toh *et al.*, 2007; Rooban *et al.*, 2012; Sunkireddy *et al.*, 2013). Naringin, a glycosylated polyphenol of the flavanones class and its aglycone, naringenin, have been shown to possess many health benefits, such as their vasorelaxant, antioxidant, phosphodiesterase inhibitor, antiteratogenic, hepatoprotective and anti-inflammatory properties, as well as preventing lipid peroxidation and platelet anti-aggregation agent (Shpigelman *et al.*, 2014; Cordenonsi *et al.*, 2016; Feng *et al.*, 2017; Ghosal, Ghosh and Kumar, 2018). However, drug delivery of antioxidants to ocular tissues presents challenges (Pathak, Sutariya and Hirani, 2016; Janagam, Wu and Lowe, 2017), due to the barriers of the eye have to be circumvented to

achieve localized and targeted treatment. Hence, it is suggested the anti-cataract potential of Naringin could be improved by formulation in biodegradable and biocompatible polymeric nanoparticles (Patil and Gacche, 2017). The lens opacification found on cataracts blocks the light to the retina due to the protein precipitation. Therefore, opacification grade could be measured by transmittance spectroscopy may provide an initial platform for broad screening of potential novel therapeutic agents towards pharmacological treatment of cataract (Chemerovski-Glikman *et al.*, 2018). Thus, this study aimed to develop and characterize Naringin-loaded nanoparticles (NLN), evaluate their activity in an *ex vivo* experimental model of sodium selenite-induced cataract and contribute with experimental data on spectral transmission using UV-Vis spectroscopy.

MATERIALS AND METHODS

Reagents and solvents: The Naringin 94 % purity and poly ε-caprolactone (PCL, MW14,000) were purchased from Sigma–Aldrich (St.Louis, MO, USA). Tween 80 and Acetone P.A. ACS were supplied by BIOTEC, Brazil; and Sodium Selenite 98% from BioReagent, Brazil. Double de-ionized water was prepared using a Milli-Q™ system (Milli pore Corporation, Bedford, MA, USA). All other chemicals and reagents used were of analytical grade.

Synthesis and Characterization of Naringin-loaded nanoparticles: The NLN were prepared using the nano-precipitation method (Dinesh Kumar, Verma and Singh, 2016; Ghosal, Ghosh and Kumar, 2018). Briefly, PCL (0.2% m/v organic solution) and Naringin (50 mg) were dissolved in acetone at 30°C using a magnetic stirrer. The organic solution was then slowly added to an aqueous phase containing Tween 80 (0.5 % v/v water) under moderate magnetic stirring, using a syringe, until the formation of a milky solution. The acetone and water were then evaporated at 40°C under reduced pressure, using a rotary evaporator. The nano-suspension was then centrifuged at 15,000 RPM using ultra-centrifuge for 20 min at 4°C. The supernatant were used to analyze encapsulating efficiency. The resulting pellets were re-suspended in distilled water, and this suspension was freeze-dried and stored at 4°C until further use for ex vivo cataract studies. The size, zeta potential and polydispersity index were determined by dynamic light scattering (DLS) with ZETASIZER NANO SERIES (MALVERN Instruments) equipment, model NANO ZS90. The morphological and surface evaluations of the liposomes were performed using a MYRA 3 LMH (Tescan) scanning electron microscope with field emission.

Encapsulation efficiency (EE%) was determined by an indirect assay method by using an VARIAN CARY 50 NIR UV-VIS spectrophotometer (Dinesh Kumar, Verma and Singh, 2015; Priyadarshini *et al.*, 2016; Fawzy *et al.*, 2017; Ghosal, Ghosh and Kumar, 2018). A standard solution is prepared by dissolving 100 mg of naringin in 100 mL of acetone. A standard calibration curve was made by plotting absorbance value against concentration. The calibration equation (Absorbance = 0.0339 ∗ concentration − 0.0376) of naringin was generated and it was found to be linear between 5 and 35 µg/mL concentration range, Absorbance values of each concentration were noted at λ_{max} 283 nm. The regression value was found to be 0.9973. EE% was calculated by following formula:

$$EE(\%) = \frac{\text{Weight of Naringin determined (mg)}}{\text{Weight of Naringin added (mg)}} \times 100$$

Lens culture: Wistar rat´s control groups from different experiments with normal lenses were used in this experiment (between 8-12 weeks old). All animals were procured, maintained, and used in accordance with the University Ethical Committee guidelines (State University of Ponta Grossa, Brazil). The lenses were gently removed and deposited in a 24-well plate containing sterile balanced sterile saline (BSS) solution at 25 - 37°C to avoid cold cataract (Artigas, Navea and López-murcia, 2014). Then, each isolated lens was placed in a Falcon plastic culture plate (24-well) containing 2ml of Dulbecco's Modified Eagles Medium (DMEM) supplemented with 20% fetal bovine serum, 100µg/ml of streptomycin, and 100 IU/ ml penicillin (Suresh K. Gupta *et al.*, 2010; Suresh Kumar Gupta *et al.*, 2010; Rooban *et al.*, 2012). The lenses were incubated at 37ºC under 90% moisture, 95% air, and 5% CO2 gas atmosphere for 4 h (Suresh K. Gupta *et al.*, 2010). Morphology of the cataract was visualized by dark background photography to avoid using damaged lenses. Photographs were recorded, and spectroscopic analyzes were performed. The Lenses were re-washed with a sterile BSS in the laminar flow cabinet before culture and cataract induction with sodium selenite (Doganay *et al.*, 2006). Only transparent, undamaged lenses were selected and then divided randomly into four specific groups, the Control group consisted of lenses cultured in DMEM alone, while lenses in the CAT group were cultured in DMEM supplemented with 100 µM sodium selenite. "NLN Treated" group corresponds to lenses treated with the antioxidant, and are cultured in DMEM containing 100 µM sodium selenite and the freeze-dried NLN (100 µM Naringin). The "Nblank Treated" group corresponds to lenses cultured in DMEM medium containing 100 µM sodium selenite and the nanoparticles without Naringin loading (Nblank). Lenses were then incubated in a CO_2 incubator at 5% CO2 and 37 °C. The medium in each well in the plate was discarded and replaced with the respective treatment group every 24 hours (Muralidharan *et al.*, 2015; Heruye *et al.*, 2019). The morphology of the cataract was visualized by dark background photography and the images were captured with a microscope digital camera at 0, 24, and 48 incubation hours.

UV % transmittance: Spectral transmittance of the lenses was measured using a NIR VARIAN CARY 50 UV/VIS spectrometer, with a spectral range of 200-800 nm. The measurements correspond to the total transmission of the crystalline lens. A suitable lens holder was adapted to place the sample (lens) directly in front and covering the complete entrance hole of the integrating sphere of the spectrophotometer. The air was taken as a reference to measure transmittance. The UV beam is focused on the anterior surface of the lens and irradiated the whole surface of the lens (Artigas *et al.*, 2012; Artigas, Navea and López-murcia, 2014).

Statistical analysis: The data obtained were statistically analyzed and the results were expressed as mean ± standard deviation (SD). The significance of differences between groups was evaluated using one-way ANOVA followed by the Tukey test (Heruye *et al.*, 2019). Differences were considered significant at $p < 0.05$. All the tests were performed using the

Statistical Package for Social Sciences (SPSS) software program (version 20.0 for Windows) and BIOESTAT 5.0 software.

RESULTS

Synthesis and characterization of NLN: The NLN production proved to be easy to perform by the nanoprecipitation method, resulting in a milky homogeneous solution and no precipitation. Tween 80 is widely used in ophthalmic preparations due to its safety (Ammar *et al.*, 2009). The sizes of NLN and NBlank were similar, but the encapsulation of naringin modified the polydispersity index value and increased the zeta potential (Table 1).

NLN was administered to a selenite-induced cataract model of rats. This *ex vivo* model of cataractogenesis technique demonstrated that sodium selenite was able to produce opacification on the rat lens (Figure 2), where opaque areas correspond to refractive regions; thus, preventing the complete passage of light through the lens. All lenses not exposed to sodium selenite (control group) remained transparent, while the incubation with sodium Selenite 100 μM in the CAT and NBlank treated groups generated opacity. The lenses in the NLN treated group, meanwhile, showed lower refractive areas, maintaining transparency for up to 24 hours, with slight opacity formation. Nonetheless, this group is less opaque than CAT and NBlank treated groups, equivalent in sodium selenite concentration.

Table 1. The physicochemical properties of nanoparticles (Mean ± SD, n = 3)

Samples	Particle size (nm)	PDI	Zeta potential (mV)	EE (%)
NLN	208.30 ± 19.94	0.27 ± 0.08	-44.50 ± 7.78	72.66 ± 3.18
NBlank	201.25 ± 2.90	0.14 ± 0.01	-26.05 ± 3.89	---

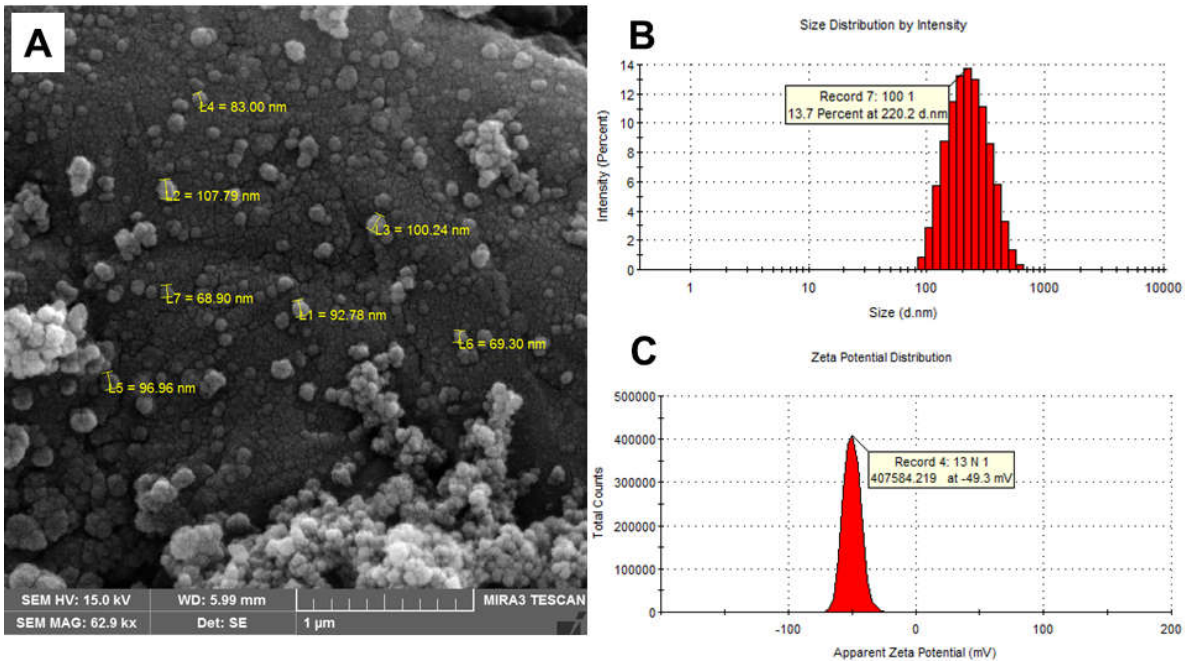

Figure 1. Basic characterization of NLNs. (A) The NLN morphology under SEM, (B) size distribution, and (C) zeta potential determined by DLS

The inclusion of Naringin in microspheres has been related using Polycaprolactone (Ghosal, Ghosh and Kumar, 2018) and revealed viability of cells in different concentrations of drug samples. Moreover, Cordenonsi*et al.* (2016) developed Nanoparticles loaded with Naringin prepared by the method of interfacial deposition with submicrometric particle diameters, although the value of the zeta potential found was -16.60 ± 0.03 mV. In our study, the NLNs were spherical or ellipsoidal in shape and can be seen in Figure 1A.

Ex vivo study analysis of sodium selenite model: Collected lenses were visually inspected against a black grid and cultured to avoid the imperceptible rupture of the capsule. Only lenses that maintained their integrity could continue with the experiment, so the images obtained with a digital microscope corresponds to 0 hours, 24 hours, and 48 hours of incubation in the determined aforementioned groups. To elucidate the effect of antioxidants on cataract prevention,

UV % transmittance: Figure 3 demonstrates the percentage of transmittance by UV-Vis spectroscopy in the wavelength range between 400 and 800 nm. For this experiment, the total transmission of the whole mouse lens was performed to facilitate comparison between transmittance curves of the groups after the incubation period. It is observed that the average spectral transmission factor decreases, and for wavelengths shorter than 400 nm the transmission percentage starts reducing down to very low values. The transmittance cutoff occurs at approximately 320 nm (Boettner and Wolter, 1962). Rat lenses of control group maintained transmittance in the visible spectrum at 24 and 48 hours and displayed a higher optical clarity compared to other groups. A similar observation was made by Artigas *et al.* (2012) and Heruye et al. (2019) about the effect of time on lens transmittance and opacification using the lens culture model (Artigas *et al.*, 2012; Heruye *et al.*, 2019). On the other hand, the transmission spectrum of the rat lens varies when it is incubated in sodium selenite.

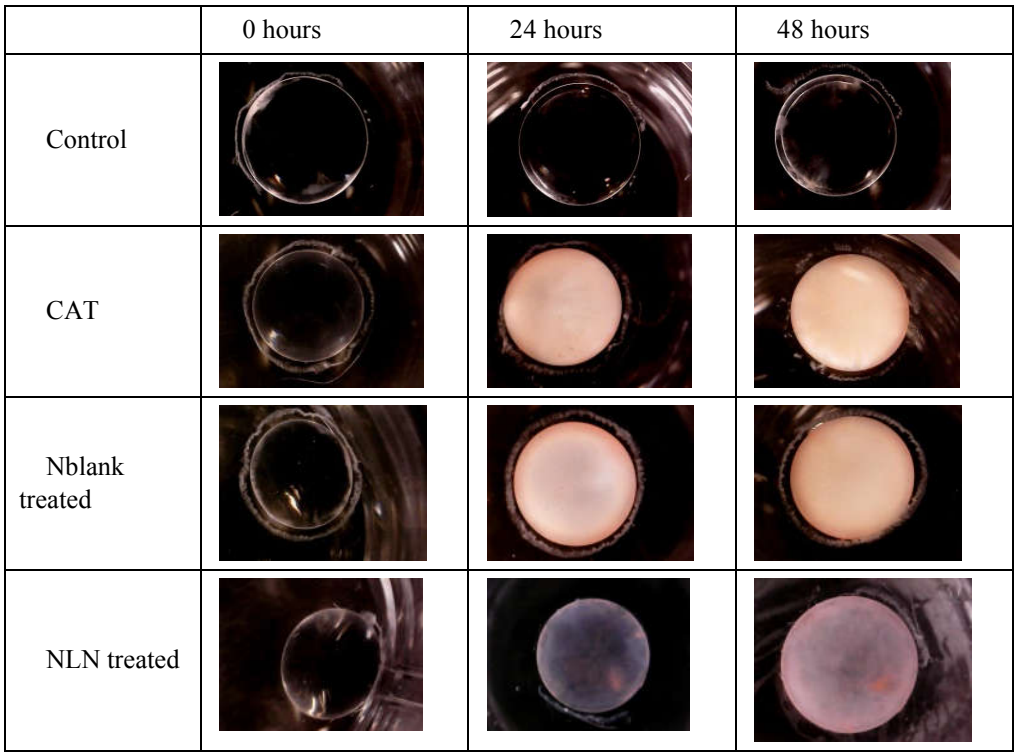

Figure 2. Comparison of response of cultured Wistar rat lenses: Control Group (cultured in DMEM only); CAT Group (cultured in DMEM and 100 μM sodium selenite); NBlank treated Group (cultured in DMEM + 100 μM sodium selenite + NBlank); and NLN Treated group (cultured in DMEM + 100 μM sodium selenite + NLN).

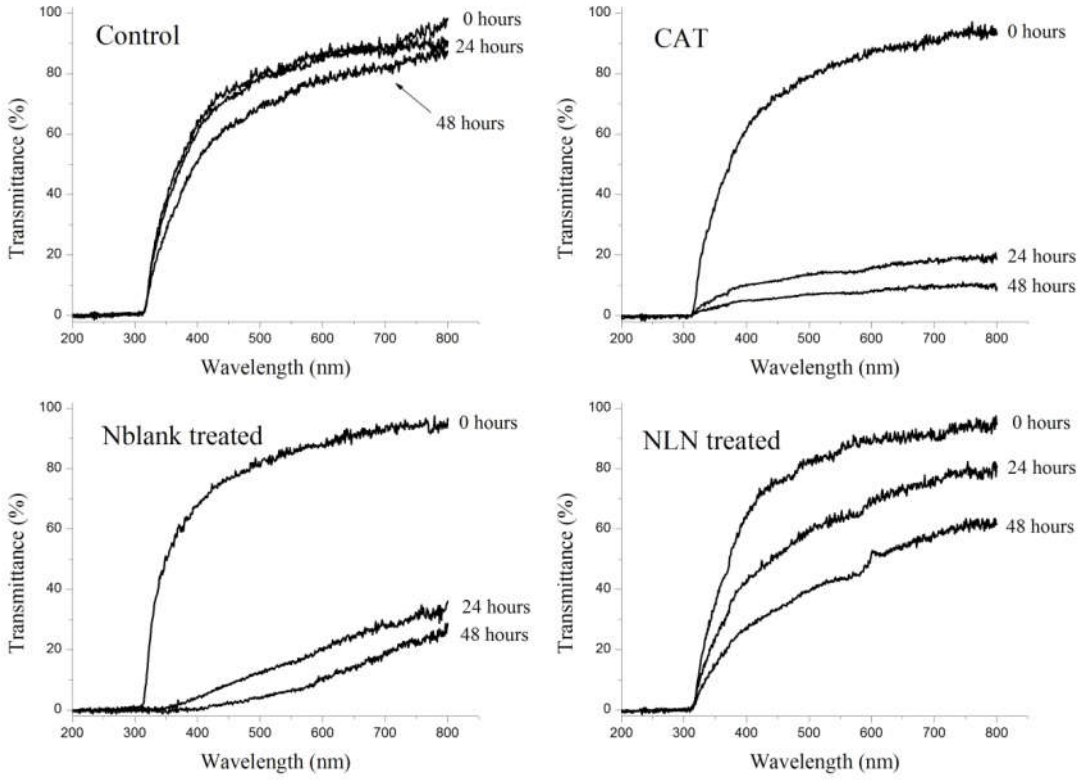

Figure 3. Spectral transmission of Wistar rat lenses in Control, CAT, Nblank treated, and NLN treated groups at three different times: before incubation (0 hours), 24 hours, and 48 hours, n=3

In the visible region, it is observed a decrease in light transmittance of CAT and Nblank treated groups of lenses after 24 and 48 hours of incubation, compared to the same lenses before incubation (0 hours). These lower transmittances is due to a presence of opacities that block the transmission of light, particularly in the blue and ultraviolet areas, which indicates the cataract development. In contrast to the CAT and Nblank group, lenses in the NLN treated group, showed a not severe decline in transmittance percentage. This indicates that the loading of Naringin in a nanoparticle delayed the opacity

formation. The highest reduction in transmittance was consistently observed on areas of the visible spectrum, mainly between 400 nm and 450 nm. Changes in 420 nm transmittance are considered as an acceptable indicator for the development of opacification (Heruye *et al.*, 2019). Thus, considering 420 nm as a point of wavelength comparison (Figure 4), no significant difference ($p>0.05$) in spectral transmission between 0, 24, and 48 hours of incubation in the control group was observed, but the light transmittance of lenses cultured in CAT reduced sharply by 83,80 % ± 4,82 and 92,16 % ± 0,47 after 24 and 48 hours of incubation respectively.

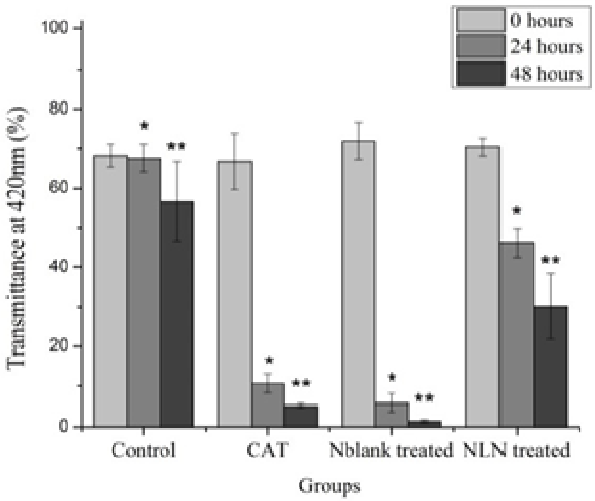

Figure 4. Mean spectral transmission at wavelength of 420 nm before and after incubation period. *, ** Differences between groups are significant at wavelengths of 420 nm ($P < 0.05$). Means differences between CAT and Nblank treated groups are not significant at the 0.05 level (post-hoc Tukey's test)

It is also observed that the transmittance percentage decrease in lenses incubated in Nblank treated group also reduced up to 91,79 % ± 3,47 after 24 hours and 97,99 % ± 0,57 after 48 hours of incubation period. These results contrast with the NLN group, where the transmittance reduction were 34,56 % ± 3,23 and 57,47 % ± 10,45 after 24 and 48 hours of incubation respectively.

DISCUSSION

It has been reported that cataract formation is related to the increase of apoptosis in the lens epithelium (Harocopos *et al.*, 1998). Thereby, the sodium selenite-induced cataracts model was chosen for having several similarities to human senile cataracts (Doganay *et al.*, 2006). 24 hours incubation was needed to observe changes in lens, this because selenite treatment affects the contributions of protein organization, cellular organization, and the occurrence of low-molecular-weight metabolites as participants in maintaining lens transparency (Hess, Mitton and Bunce, 1996). Decreased levels of Glutathione (GSH) in the lens can lead to free radical accumulation, resulting in lipid peroxidation and decreased antioxidant enzyme activity, all of which lead to cataract development (Maddirala *et al.*, 2017). The administration of antioxidants could reduce the cell damage produced by free radicals both *in vitro* and *in vivo* (Ghinelli *et al.*, 2003). Furthermore, the oxidation of amino acids in crystallins that occurs in the lens tissue may affect crystalline functions like the decrease of α-crystallin chaperone activity (Sacharz *et al.*, 2016). The administration of natural antioxidants also was reported to increase the chaperone activity and mitigated cataracts that had formed owing to selenite treatment. (Baskar *et al.*, 2012; Brimson *et al.*, 2012; Yin *et al.*, 2018; Jena *et al.*, 2019; Hussein *et al.*, 2020; Zhang *et al.*, 2020). The effect of selected flavonoids, including Naringin, was observed on glycation induced lens opacity, demonstrating the efficacy of flavonoids as promising leads for inhibition of glycation reaction and amelioration of sugar induced cataractogenesis (Patil and Gacche, 2017). This effect could explain the protective role of Naringin in decreasing lens opacity in this *ex vivo* model of selenite-induced cataract. In the present study, NLN was applied in sodium selenite induced rat lens organ culture studies due to its efficacy as an antioxidant agent. Furthermore, NLN exhibited promising activity.

The CAT and Nblank treated groups, except NLN treatment, had severe opacities and higher stage cataracts than control groups. These results suggested that the treatment with Naringin could delay cataract formation by the maintenance of the lens transparency. This study confirms that the inclusion of Naringin into the nanoparticle system delayed cataract formation. This may suggest that the nanoparticle formulation is suitable for carrying and release others antioxidants in an *in vitro* model of cataract in rat lenses.

CONCLUSION

NLN were successfully obtained, the treatment with this nanoparticles allowed the prevention of cataract advance induced by sodium selenite by the *ex vivo* model of cataractogenesis, and through spectroscopic studies of the percentage of transmittance, it was possible to observe the degree of transparency of the lens after 48 hours. Hence, Naringin may be a promising alternative lead toward the nonsurgical treatment and prevention of cataracts. This study can contribute to the use of spectroscopic techniques for drug discovery in the treatment of cataracts.

Conflict of interest statement: The authors have declared that there is no conflict of interest.

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