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Effects of *Laurus Nobilis* Eye Drop on Selenite-Induced Cataract Formation and Oxidative Stress-Related Parameters in Rabbits: An Experimental Study

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ABSTRACT

Purpose: To evaluate the protective role of *Laurus Nobilis* eye drops on selenite-induced cataracts in suckling rabbits.

Methods: Fifteen male albino suckling rabbits with no signs of ocular inflammation were randomly assigned to three groups: controls (Group A), sodium-selenite group (Group B) and sodium-selenite plus *Laurus Nobilis* group (Group C). By selenite treatment, cataract formation was experimentally induced and then graded. The grade of oxidative stress was defined in the lens, measuring the concentration of malondialdehyde, alpha-tocopherol, oxidized glutathione, ascorbic acid and hydrogen peroxide, and in blood samples as levels of alpha-tocopherol and malondialdehyde.

Results: Mean lens concentrations of GSSG, H2O2, and MDA levels in group B were significantly higher than in both group C and control. Ascorbic acid and alpha-tocopherol concentrations were lower in group B than in both group C and A. As plasma oxidative status markers, the level of MDA was higher in group B respected group C and A. The mean alpha-tocopherol levels in group B were significantly lower than in both group A and group C.

Conclusions: In animals treated with *Laurus Nobilis*-based eye drops, inflammation was inhibited, and lipid peroxidation was significantly reduced. *Laurus nobilis* leaves extract represents a good source of antioxidant components that may contrast sodium selenite-induced cataractogenesis in suckling rabbits.

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Cataract; selenite; *Laurus nobilis*; oxidative stress

Introduction

Cataract is the most common treatable worldwide cause of bilateral blindness in aged patients. Over the years, due to the normal aging process, there is an aggregation and oxidation of the lens proteins which causes the gradual opacification of the lens. Age-related changes are the key factor in the formation of cataracts; however, there may also be other causes that contribute to this degeneration: familiarity, smoking, UV radiation exposure, metabolic and inherited defects.[1] According to data from the World Health Organization, cataracts are responsible for over 75% of cases of visual impairment in the world, mainly concentrated in developing countries.[2] The costs of blindness and severe visual impairment are very large in economic, health, and psychosocial terms. Therefore, researchers started to look for and find out alternative strategy for medical treatment of cataract or to delay its surgery time.

In the field of medical treatment for cataracts, according to the key role of oxidative stress in cataract's development, many molecules and natural compounds have been considered as protective factors, yet not effective for preventing it: daily protein intake of 100–150 µg, vitamin C intake of approximately 135 g/day, increased consumption of vegetables with carotenoids, vitamin A or B, vitamin E, or antioxidant supplements.[3,4]

For the first time, Ostadalova et al. demonstrated that a subcutaneous overdose injection of Na-selenite caused cataracts in suckling rats.[5] Subsequently, the model was widely used for medical research.[6] The mechanism of selenite-induced cataract encompasses many stages including alteration of epithelial metabolism, calcium accumulation, calpain-induced proteolysis, crystalline precipitation, and cytoskeletal loss, all contributing to lens opacification.[7] Although the pathogenesis of cataract is a multifactorial event, oxidative stress plays a fundamental role in the lens opacification.[8,9]

The lens, as well as other organs, has a well-developed defence system against oxidation, based on non-enzymatic antioxidants such as glutathione, vitamin E, vitamin C, and carotenoids.[10–12] When this defence system is not able to counteract the formation of free radicals, lens opacity occurs.[13,14] Research has turned to find natural antioxidant compounds capable to delay the onset and the progression of cataract.[15,16]

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Laurus nobilis (L. nobilis) is an ever-green plant belonging to the *Lauraceae* family in the genus *Laurus*, native to the Mediterranean countries,[17,18] which shows high levels of nutritional contribution, related to high concentration of proteins, free sugars, organic acids, PUFA and tocopherols with antioxidant activity. More specifically, *L. nobilis* is a great source of flavones, flavonols and hydroxycinnamic acids, which are effective to attenuate oxidative cells' stress (in relation to scavenging activity, reducing power and lipid peroxidation inhibition).[19]

The purpose of this study has been to evaluate the possible effect of *Laurus nobilis* extract in preventing selenite-induced cataract and the possible correlation between blood and lens antioxidant parameters and lipid peroxidation, in suckling rabbits.

Materials and methods

The study was conformed to ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law No. 86/609).

Animals

Cataract was experimentally induced by selenite treatment. Fifteen male albino suckling rabbits (15 days old pups, body weight in a range of 300–400 g), with no signs of ocular inflammation, were randomly assigned to three groups of five animals each: controls (Group A), sodium-selenite group (Group B) and sodium-selenite plus *Laurus nobilis* group (Group C).

Rabbits were kept at a temperature of 20 ± 2 °C in single cage with ad libitum access to food and water. A natural light/dark cycle was guaranteed throughout the experimental period. A mixture of selenite sodium (Merk, Germany) and 0.9% sterile normal saline solution (0.1% density) was prepared; 1 mg/kg of body weight (1 ml) was subcutaneously injected to induce cataract. During the 7 days following the injection date, rabbits' eyes were carefully examined every day, using ophthalmoscope, and slit lamp, to ascertain the development of cataract.[20] Finally, euthanasia was performed by injection of an overdose of sodium pentobarbital, preceded by anesthesia with Xylazine and Ketamine HCl. Blood and lens samples were collected after euthanasia.

Ophthalmic preparation

The ophthalmic eye drops were obtained by extraction of dry leaves of *Laurus nobilis*. In particular, the dried parts of bay leaf were powdered in a mill. For water extraction, fifty grams sample was boiled with 50 mL of sterile distilled water for two hours. The extract was first filtered through double layer muslin cloth then centrifuged at 3500 rpm for 30 min. The supernatant was filtered through Whatman No. 1 filters paper and sterilized in an autoclave at 121 °C for 15 min. The extracts were preserved aseptically at 5 °C for further use. For the protocol procedure, rabbit eyes were topically treated every day from the same day of subcutaneous selenite injected, with one drop (approximately 0.05 ml) of an ophthalmic formulation containing 1% of aqueous extract, trice daily. The treatment was carried on for 30 days after the initiation of cataract. The eyes of the animals were observed for the progression/disappearance of the cataract.

Cataract classification

Morphological examination of rabbit eyes was performed according to the procedure described by Carey et al.[21] One hour prior to examination, a drop of both 2.5% phenylephrine hydrochloride and 1% tropicamide ophthalmic solutions were instilled in each eye to induce mydriasis. Cataracts were graded as clear lens, grade 0; lens with slight opacity, grade 1; lens with partial nuclear opacity, grade 2; lens with dense nuclear opacity, grade 3; total opacity of lens, grade 4 (Table 1).[22]

Antioxidant lens profile/markers

For each sacrificed animal, lenses were carefully removed under microscopy visualization, immediately washed with normal physiological saline and processed. To define the grade of oxidative stress, the concentration of malondialdehyde (MDA), alpha-tocopherol, oxidized glutathione (GSSG), ascorbic acid (AA) and hydrogen peroxide (H2O2) were quantified.

Malondialdehyde (MDA) was determined according to the method of Ohkawa et al.[23] Rabbit lens samples were homogenized in a solution of 1.15% of KCl, to form 10% homogenates, and 1500 rev/min, for 1 min on ice. This homogenate was used for the MDA analysis. Prepared solutions were added to test tubes, vortexed, then, the tubes were kept in boiling water for 1 h. The tap water-cooled tubes were centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm, and the results were expressed as μmol of MDA per g of wet tissue.

Alpha-tocopherol was dosed using the modified procedure of Zhao et al.[24] Samples were analyzed by an HPLC system (Kontron Instruments, Milan, Italy) consisting of an autosampler (HPLC autosampler 360, Kontron Instruments, Milan, Italy) with a loop of 20 μL, a high-pressure pump and a C18 column 5 μm, 250 x 4.60 mm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile and methanol (75:25 v/v), and a flow rate of 1 mL min-1 was used. Alpha-tocopherol was identified using a fluorimeter

Table 1. Morphological examination of isolated pup lenses.

	Number of lenses with different degrees of lenticular opacification			
Experimental groups	*0*	+	++	+++
Group A ($n = 10$)	10	–	–	–
Group B ($n = 10$)	–	–	3	7
Group C ($n = 10$)	8	2	–	–

Figure legend
0 absence of opacification, + slight degree of opacification, ++ moderate degree of opacification, +++ extensive thick opacification involving the entire lens
Group A = control group; Group B = sodium-selenite group, Group C = sodium-selenite plus *Laurus nobilis* group

detector and comparing the samples retention time with the pure standards (97%) purchased from Sigma Aldrich (St. Louis, USA). The quantification was carried out using the Geminyx system (version 1.91) comparing the area sample peak with that of the reference standards curve. Results were expressed as µmol/mg of eye wet weight. The levels of oxidized glutathione (GSSG) were recorded spectrophotometrically with NADPH 2 and glutathione reductase.[25] The reaction went to completion and the amount of GSSG was calculated from the change in extinction at 340 nm. NADPH 2 was prepared with the method reported by Pirie. The concentrations of ascorbic acid (AA) and hydrogen peroxide (H2O2) in lens were measured simultaneously on the same sample by the spectrophotometric method.[26] The decrease in the absorbance at 610 nm resulting from the addition of 0.05 ml of lens homogenate to 3 ml of 50 mM-phosphate buffer, pH 6-6, containing 0.04 mM-2,6 dichlorophenolindophenol was first recorded. Five microliters of 5 mg/ml horseradish peroxidase solution (Sigma) were then added and the increase in absorbance at the same wavelength due to reoxidation of the dye by H202 was measured. The initial decrease in absorbance is proportional to the concentration of the AA, while the increase in absorbance in the second step is equivalent to the concentration of H202. Concentrations of AA and H202 in the lens were calculated using a molecular extinction coefficient at 610 nm for the oxidized dye.

Alpha-tocopherol and MDA in blood

Blood samples from rabbits were withdrawn into heparinized polypropylene tubes from the external ear vein using a vacutainer method (Venoject, Terumo Europe N.V., Leuven, Belgium) at the end of the study. Collected blood was centrifuged for 20 min at 3000 rpm; alfa-tocopherol and MDA were determined on plasma.

Spectrophotometer was used to determinate the values of thiobarbituric acid reactive substances (TBARS) according to the method of Esterbauer and Zollner and to a standard curve with the 1,1,3,3 tetramethoxypropane (Sigma Aldrich, St. Louis) .[27]

The results were expressed as µmol of malondialdehyde (MDA) per l of plasma. Alfa-tocopherol have been extracted from plasma samples with chloroform, according to the method of Zhao et al. and results were expressed as µg/ml of plasma.[24]

Statistical analysis

SPSS software (version 22.0, IBM Corporation, Armonk, NY, USA) was used for statistical analysis. Values are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare data among the three groups. A p value less than 0.05 was considered statistically significant. The information about the magnitude of correlation, as well as the direction of the relationship were obtained by Pearson's correlation coefficient.

Results

In Table 1 are reported the results of morphological assessment of cataract formation, in group A, all the eyes were clear. In group B, 100% of the eyes developed moderate to severe cataract, indicating success in establishing the selenite-induced cataract model in our experiment, whereas in group C treatment with *Laurus nobilis* reduced the incidence of cataracts.

In Table 2 are summarized the effect of topically treated of ophthalmic antioxidant formulation on lenses of exposed animals.

The mean lens concentrations of GSSG, H2O2 and MDA levels in group B (GSSG: 3.450 ± 0.226 mmol/ml, H2O2: 0.910 ± 0.025 mmol/ml and MDA: 9.030 ± 0.141 µmol/g) were significantly higher than in both group C (GSSG: 0.735 ± 0.028 mmol/ml, H2O2: 0.182 ± 0.012 mmol/ml and MDA: 2.525 ± 0.061 µmol/g) and control (GSSG: 0.713 ± 0.038 mmol/ml, H2O2: 0.183 ± 0.025 mmol/ml and MDA: 2.203 ± 0.133 µmol/g), with a statistical significance of $p < 0.001$. While ascorbic acid and alpha-tocopherol concentrations were significantly lower in sodium-selenite group (0.085 ± 0.008 mmol/ml and 0.033 ± 0.003 µmol/mg, respectively) than in both group C (2.512 ± 0.220 mmol/ml and 0.092 ± 0.007 µmol/mg) and A (2.913 ± 0.202 mmol/ml and 0.108 ± 0.013 µmol/mg).

Plasma oxidative status markers were reported in table 3. The level of MDA was found to be higher ($p < 0.01$) in group B (6.690 ± 0.113 µmol/l) respected group C (4.746 ± 0.165 µmol/l) and A (4.486 ± 0.276 mmol/l). The mean alpha-tocopherol levels in group B (0.087 ± 0.079 µg/ml) were significantly lower than in both group A (0.184 ± 0.013 µg/ml) and group C (0.187 ± 0.010 µg/ml).

In Table 4 correlation between blood and lens parameters are reported. Coefficient correlation analysis between the antioxidant parameters and the oxidative markers provided

Table 3. Blood oxidative status markers in rabbits. Results are expressed as mean values ± SE.

Parameters		Group A	Group B	Group C
MDA	µmol/l	4.233 ± 0.349	6.695 ± 0.138*	5.653 ± 0.176
Alpha-tocopherol	µg/ml	0.187 ± 0.018	0.087 ± 0.009**	0.101 ± 0.006

*$p < 0.05$;
**$p < 0.001$, A and B versus C

Table 2. *Lenses* oxidative status markers in rabbits. Results are expressed as mean values ± SE.

Parameters		Group A	Group B	Group C
GSSG	µmol/ml	0.713 ± 0.038	3.450 ± 0.226*	0.735 ± 0.028
Ascorbic acid	mmol/ml	2.913 ± 0.202	0.085 ± 0.008*	2.512 ± 0.220
H2O2	mmol/ml	0.183 ± 0.025	0.910 ± 0.025*	0.182 ± 0.012
MDA	µmol/g	2.203 ± 0.133	9.030 ± 0.141*	2.525 ± 0.061
Alpha-tocopherol	µmol/mg	0.108 ± 0.013	0.033 ± 003*	0.092 ± 0.007

*$p < 0.001$, A and C versus B.

Table 4. Correlation between blood and lens oxidative status markers in rabbits.

	Blood parameters		Lens parameters				
Items	Alpha-tocopherol	MDA	Ascorbic acid	H2O2	Alpha-tocopherol	MDA	GSSG
Alpha-tocopherol	1	−0.901*	0.596*	−0.524	0.564*	−0.531	−0.537
MDA		1	−0.776**	0.741**	−0.695**	0.767**	0.734**
Ascorbic acid			1	−0.960**	0.956**	−0.968**	−0.949**
H2O2				1	−0.896**	0.991**	0.993**
Alpha-tocopherol					1	−0.899**	−0.885**
MDA						1	0.976**
GSSG							1

*Pearson correlation: *$p < 0.05$; **$p < 0.001$

different results in blood and in lens. In particular, a positive and significant ($p < 0.05$) correlation was found between the antioxidant lens parameters (ascorbic acid and alpha-tocopherol) and the alfa-tocopherol blood content. Blood MDA levels were positively correlated ($p < 0.001$) with lens oxidant markers tested (H2O2, MDA and GSSG), while negatively correlated ($p < 0.001$) with antioxidant markers (ascorbic acid and alpha tocopherol). Oxidative lens parameters (H2O2, MDA and GSSG) were negatively correlated ($p < 0.001$) with lens ascorbic acid and tocopherol values, whereas lens antioxidant markers (ascorbic acid and tocopherol) were positively correlated ($p < 0.001$) between them.

Discussion

Oxidative stress plays an important role in the opacification of the lens. Like other organs, the lens has a well-designed system of defence against oxidation. Various pharmacological antioxidants have been shown to protect against selenite induced cataractogenesis in experimental animal models.[10,28–30] The purpose of our study was to examine *in vivo* the potential effects of *Laurus nobilis* eye drop in selenite-induced cataract. Preview research demonstrated the efficacy of *Laurus nobilis* intake in preventing or treating cataracts in rabbits.[17] Cataract was produced in sucking rabbits by an overdose of the essential trace mineral selenium, when injected before completion of the critical maturation period of the lens (approximately 18 days of age).

Our clinical observations of the morphological examination of the lenses showed that cataract formation caused by selenite administration was attenuated by the administration of *Laurus*, suggesting its anticataract potential. It could be hypothesized that the molecular mechanism of the *Laurus nobilis* effect may be related to the protection against oxidative stress induced by selenite. The mode of action of sodium selenite in cataractogenesis has not been completely defined. Probably, the main biochemical events induced by selenite overdose are triggered by oxidative stress and the generation of reactive oxygen species (ROS) in the aqueous humor in combination with a decrease in the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), and glutathione reductase (GR), as well as glutathione (GSH) content in the lens.[31,32]

According to our results, the mean activities of ascorbic acid and alpha-tocopherol were significantly lower in group B (exposed to selenite alone) than in normal and sodium-selenite plus Laurus nobilis group lenses, A and C respectively. Indeed, we recorded a decline in antioxidant markers activities in animals, daily treated with *Laurus nobilis* eye drops. This observation suggests that treatment of animals/lenses exposed to selenite with an antioxidant helps to maintain antioxidant markers at near-normal activities. Our study shows that *Laurus nobilis* eye drops can not only significantly delay lens opacification induced by sodium selenite, but also significantly enhance the activity of ascorbic acid and alpha-tocopherol ($p < 0.001$) and reduce GSSG, H2O2 and MDA content ($p < 0.001$)

TBARS groups (MDA) are the major indicators of lipid peroxidation in biological membranes and have been found in numerous human diseases. A rise in TBARS con-centrations suggests heightened lipid peroxidation, tissue injury, and the failure of an-tioxidant defence to prevent the development of excessive free radicals.[33] Therefore, TBARS concentrations are essentially the most typical biomarkers of lipid peroxidation. In the present study, the MDA content of the lenses significantly increased in the sodium selenite-treated group; however, the *Laurus nobilis*-treated group had significantly decreased MDA level.

Various antioxidant substances, present in *Laurus nobilis* extract, such as flavones (Cynaroside = 430.3 ± 9.62; Vitexine = 231.9 ± 4.08), flavonols (Rutin= 303.4 ± 17.25), hy-droxycinnamic acids (Trans sinapic acid = 120.0 ± 2.37; Neochlorogenic acid = 109.2 ± 4.49; etc…) show to scavenge free radicals.[17] The antioxidant property of *Laurus nobilis* was confirmed by an increase in lens glutathione content, a decrease in malondialdehyde concentration and lowest MDA levels in the lens of treated animals. These findings agree with those reported by Nair et al. who investigated the anti-cataract effects of aqueous extract of *Embelica officinalis* on selenite-induced cataract in rats. He found a significant decrease in malondialdehyde and simultaneous increase in lenses glutathione levels.[34] Dailami et al. showed the protective effect of *Origanum vulgare* extract on lenses against the selenite-induced nuclear opacity.[9] These authors suggested two mechanisms: a direct influence on lens free radical oxidation and an indirect effect through activation of a system that increases the antioxidant potential in the lenses. Instead, Doganay et al. investigated the effect of resveratrol on selenite-induced cataract formation in rats, which induced an increase of GSH concentration and decrease of MDA levels.[35] Our results also agree with the study reported by Javadzadeh et al. who showed that the instillation of fresh juice of crude

onion into rat eyes could prevent selenite-induced cataract formation in 75% of cases.[36] This effect was associated with higher mean total antioxidant levels and mean activities of GSH and GPX in the lenses of rats that received fresh juice of crude onion and subcutaneous injection of sodium-selenite, compared to those rats that received only sodium-selenite injection. Tsai et al. investigated the effects of Rosmarinic acid against selenite-induced cataract.[37] The treated group presented significantly more GSH content and lower TBARS concentration in their lenses than the untreated group.

Our findings confirmed that the antioxidant activity of *Laurus nobilis* may exert a protective role on the risk of cataract development, secondary to a Na-selenite injection. We assumed that *Laurus nobilis* may have a protective role against selenite-induced cataracts, probably based on direct or indirect antioxidant mechanisms.

According to our data, *Laurus* flavones, flavonols and Hydroxycinnamic acids induce the inhibition of lipid peroxidation, inflammation, and the increase of the activity of the antioxidant defence system.

In conclusion, our results suggest that *Laurus nobilis* leaf extract represents a good source of antioxidant components that may counteract sodium selenite-induced cataractogenesis in suckling rabbits.

Author's contributions

MP, LA: conceptualization, statistical analysis, writing. MC: review and editing. MR: original draft. FT: validation. CC: conceptualization, methodology, validation. All authors contributed to the article and approved the submitted version.

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Data availability statement

The data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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