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Putative free radical-scavenging activity of an extract of *Cineraria maritima* in preventing selenite-induced cataractogenesis in Wistar rat pups

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Purpose: To investigate the possible free radical-scavenging activity of an extract of *Cineraria maritima* on selenite-induced cataractous lenses in Wistar rat pups.
Methods: In the present study, Wistar rat pups were divided into three experimental groups. On P10, Group I (control) rat pups received an intraperitoneal injection of 0.89% saline. Rats in groups II (selenite-challenged, untreated) and III (selenite-challenged, *C. maritima* treated) received a subcutaneous injection of sodium selenite (19 μmol/kg bodyweight); Group III rat pups also received an intraperitoneal injection of the extract of *C. maritima* (350 mg/kg bodyweight) once daily P9–14. Both eyes of each pup were examined from P16 until P30. Cytochemical localization of nitroblue tetrazolium salts and generation of superoxide, hydroxyl, and nitric oxide levels were measured. The expression of the inducible nitric oxide synthase gene was evaluated with reverse transcription-PCR. Immunoblot analysis was also performed to confirm the differential expression of the inducible nitric oxide synthase protein.
Results: Subcutaneous injection of sodium selenite led to severe oxidative damage in the lenticular tissues, shown by increased formation of formazan crystals, elevated generation of superoxide, hydroxyl, and nitric oxide radicals, and elevated inducible nitric oxide synthase gene and protein expression that possibly contributed to the opacification of the lens and thus cataract formation. When rat pups were treated with intraperitoneal administration of the extract of *C. maritima*, the generation of free radicals as well as the messenger ribonucleic acid and protein expression of inducible nitric oxide synthase were maintained at near normal levels.
Conclusions: The data generated by this study suggest that an ethanolic extract of *C. maritima* possibly prevents cataractogenesis in a rat model by minimizing free radical generation.

Cataract is the major cause of preventable blindness worldwide, especially in developing countries in Africa and Asia. Currently, the only treatment available for the disease is the surgical extraction of the cataractous lens followed by replacement with a synthetic implant. Although such a surgical replacement of the natural lens with an artificial lens is significantly effective in restoring vision to most patients, this procedure is not free of complications. Thus, attempts to prevent cataract formation, or at least significantly slow the onset of the disease, would be of great value [1].

Although many factors have been implicated, cataract formation is primarily associated with oxidative stress produced by free radicals. Generation of excessive free radicals and reactive oxygen species (ROS), such as superoxide anion (O_2^-), nitric oxide (NO), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·), leads to oxidative damage; this has been identified as one of the major triggering factors for human senile cataract formation. The oxidative hypothesis of cataract formation posits that ROS can damage lenticular proteins and fiber cell membranes [2]. ROS can also perturb the homeostasis of the lens by disrupting the water and electrolyte balance and by causing DNA damage and proteolysis, thus leading to loss of lenticular transparency [3,4].

Hydroxyl anions, which are highly reactive free radicals, have been shown to modify the lenticular crystalline structure [5]. Lenticular crystalline proteins, fibers, and lipids are susceptible to O_2^- damage, which presumably accelerates nuclear cataractogenesis [6]. Excessive production of NO due to inducible nitric oxide synthase (iNOS) causes extensive damage to the structure and composition of the lens, thus leading to formation of cataract [7]. Therefore, preventing ROS production or scavenging of free radicals may be an effective strategy for preventing or delaying cataract formation or progression. This strategy, in particular using natural resources as antioxidants, has been the subject of several investigations [8-12].

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Cineraria maritima, which belongs to the family *Asteraceae*, is an annual exotic medicinal herb. The aerial parts of the plant (leaves and stem) are used in homeopathic preparations for treating ophthalmic conditions such as corneal clouding, opacity, cataract, and conjunctivitis [13]. We have previously shown that an ethanolic extract of *C. maritima* exerts an anticataractogenic effect in in vitro and in vivo rat models [14]. The extract of *C. maritima* has also shown in vitro antioxidant activity (submitted for publication). In the present study, an attempt has been made to determine whether the extract of *C. maritima* exhibits free-radical scavenging potential in vivo in lenticular tissue, thus slowing free-radical generation and maintaining lenticular transparency.

METHODS

Chemicals: Ethylenediaminetetraacetic acid (EDTA), nitroblue tetrazolium (NBT), phenazine methosulphate, ascorbic acid, riboflavin, thiobarbituric acid (TBA), trichoracetic acid (TCA), and selenite were all purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents used were of analytical grade and were obtained from HiMedia (Mumbai, India).

Animals: Nine-day-old rat pups (Wistar strain) were used in this study. The pups were housed with parents in large spacious cages, and the parents were given food and water ad libitum. The animal room was well ventilated and had a regular 12 h:12 h light-dark cycle throughout the experimental period. These animals were used in accordance with institutional guidelines (Reference No. of Institutional Ethical Committee: BDU/IAEC/2012/57/28.03.2012) and with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Research. The rat pups were divided into three experimental groups (six each). In Group I (control), saline (0.89%) was injected intraperitoneally on P9. In groups II and III, sodium selenite (19 mmol/kg bodyweight) was injected subcutaneously on P10. In addition, the pups in Group III received intraperitoneal injections (350 mg/kg bodyweight) of the extract of *C. maritima*; the first dose of extract was administered 1 day before the selenite injection (that is, on P9), and was repeated once daily for five consecutive days thereafter (on P10 through P14). On P10 alone, Group III pups received the extract of *C. maritima* 1 h before selenite was injected.

Isolation of lens: At the end of the experiment, each rat pup was euthanized by pentobarbital injected intraperitoneally (50 mg/kg bodyweight), and the eyes were enucleated. The lens was immediately dissected from each eye, washed in ice-cold saline to remove blood, and frozen at −70 °C. Homogenates of the lenses were prepared using 0.1 M Tris–HCl buffer (pH 7.4), and the supernatants obtained after centrifugation (12,000 ×g, 30 min, 4 °C) were used to analyze NO alone. To measure O_2^- and OH·, the lens was immediately dissected from the eye and washed in saline. The entire lens was used directly for assays.

Cytochemical localization of nitroblue tetrazolium–reducing substances in the ocular lens: Generation of O_2^- in the lenses of Wistar rat pups was detected cytochemically using the nitroblue tetrazolium (NBT) reduction method [15]. Briefly, each entire lens was incubated with 100 µl of 0.3% NBT for 1 h at 22 °C. After incubation, the lenses were washed with Tris–HCl buffer (pH 7.4, 0.1 M Tris) and then examined for blue formazan deposits under bright-field optics (total magnification 5X) using a Carl Zeiss Axiolab (Oberkochan, Germany) microscope.

Measurement of superoxide anion generation in the ocular lens: Generation of O_2^- in the lenses of Wistar rat pups was measured spectrophotometrically using the cytochrome c method [16]. Briefly, undamaged lenses were incubated, each with 500 µl phosphate buffer (pH 7.8, 0.1 M EDTA) and 100 µl of cytochrome c (0.002 mM), for 15 min. At the end of the reaction, the absorbance was read at 550 nm in a UV-160A Spekol (Jena, Germany) spectrophotometer against a suitable blank. The O_2^- generated was expressed as absorbance at 550 nm/15 min.

Determination of hydroxyl radical generation in the ocular lens: Generation of OH· in the lenses of the Wistar rat pups was determined spectrophotometrically [17]. Briefly, lenses were incubated in 700 µl phosphate buffer (pH 7.8, 0.1 M EDTA), 2 mM sodium salicylate, and 40 µl 10 N HCl, to which 0.25 g of NaCl was added. To this mixture, an equal volume of chilled diethyl ether was added and incubated for 30 min at 25 °C. The absorbance was read at 510 nm in a Spekol (UV-160A) spectrophotometer against a suitable reagent blank. The generation of OH· was expressed as absorbance at 510 nm/30 min.

Determination of nitric oxide generation in the ocular lens: Generation of NO in the lenses of Wistar rat pups was determined with Ozbek et al.'s method [18]. For this assay, 100 µl of the supernatant from each homogenized undamaged lens was mixed with 150 µl Tris–HCl buffer (pH 7.4) and incubated with 5 µl of 0.01 U nitrate reductase and 10 µl of 2 mM β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (β-NADH) for 20 min at 22 °C in the dark with constant shaking. Following this, 50 µl of 1% sulfanilamide and 50 µl of 0.1% naphthylethylenediamine dihydrochloride (Griess reagent) were added and incubated for 10 min at room temperature. Following incubation, the samples were centrifuged (17,000 ×g, 15 min, 4 °C) to pellet any precipitate that may have formed, and the absorbance of the clear supernatant was read at 540 nm in a Spekol (UV-160 A) spectrophotometer against a reagent blank consisting of buffer and Griess reagent. The nitrite (=NO) generated in the lens was determined against sodium nitrite in a standard curve, and the amount of nitrite was expressed as µM nitrite.

Reverse transcription-polymerase chain reaction analysis of messenger ribonucleic acid transcripts of inducible nitric oxide synthase in the ocular lens: Total RNA was extracted from each lens using TRIzol (Sigma-Aldrich, St. Louis, MO) reagent (1 ml/100 mg tissue) according to the manufacturer's instructions. The concentration and purity of total RNA were determined by absorbance at 260/280 nm in a UV-spectrophotometer [19]. The purity of the RNA obtained was >1.8.

Reverse transcription-polymerase chain reaction (RT–PCR) was performed to measure the expression of inducible nitric oxide synthase (iNOS) mRNA transcripts relative to the expression of the reference gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used for generating cDNAs were as follows: for rat iNOS (amplicon size=257 bp) sense primer 5′-CCA ACC TGC AGG TCT TCG ATG-3′; antisense primer 5′-GT CGA T GC ACA ACT GGG TGA AC-3′ [20]; for rat GAPDH (amplicon size=207 bp), sense primer 5′-TCA AGA AGG TGG TGA AGC AGG-3′; antisense primer 5′-GGT CCA CCA CCC TGT TGC TGT-3′ [21]. Two micrograms of total RNA were reverse transcribed with Qiagen One-Step RT–PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and further amplified with PCR. The reverse transcription (RT) reaction was performed at 50 °C for 30 min followed by initial PCR activation at 95 °C for 1.5 min. The three-step PCR cycles included (i) denaturation at 94 °C for 1.5 min, (ii) annealing at 58 °C for 1.5 min, and (iii) extension at 72 °C for 3 min. PCR amplification was performed for up to 30 cycles; to ensure that the products were extended completely, a final extension at 72 °C for 10 min was performed.

Ten microliters of each PCR product were analyzed with gel electrophoresis on 2% agarose gel. The molecular size of the amplified products (iNOS and GAPDH) was determined by comparison with molecular weight markers (100 bp DNA ladder, Genei, Bangalore, India) run in parallel with the RT–PCR products. Gels were subjected to densitometric scanning, and the band intensity of the cDNA fragment of the iNOS gene was normalized against the band intensity of the cDNA fragment of the GAPDH gene, using Quantity One Software (Bio-Rad, Hercules, CA).

Immunoblot analysis of inducible nitric oxide synthase in the ocular lens tissue: Each lens was homogenized with 10 times its mass of 20 mM phosphate buffer containing 1 mM ethylene glycol-bis (2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA; pH 7.2), and centrifuged at 14,000 ×*g* at 4 °C for 15 min. This process was repeated twice. The supernatant obtained was used for immunoblot analysis. Proteins subjected to sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-PAGE) were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane using a semidry blotting apparatus (Bio-Rad). Blotting was done at 25 V for 1 h. Blotted membranes were stained with Ponceau S solution to check for the efficiency of transfer; subsequently, blocking was done with 5% non-fat milk powder in Tris buffer saline (pH 7.5) with 0.1% (v/v) Tween-20 for 3 h, following Towbin et al.'s method [22]. Antibody (purchased from Sigma) against iNOS (1:1,000 dilution) was used. Immunoreactivity was visualized with alkaline phosphatase conjugated to antimouse immunoglobulin G secondary antibody and 5-bromo 4-chloro 3-indolyl phosphate/nitroblue tetrazolium chloride (BCIP/NBT; Genei, Bangalore, India). To detect even minor changes in the intensity of the bands, densitometry was performed on scanned images of the membranes. The program Quantity One SW (Bio-Rad) was used to analyze the intensity of the bands in each lane of the membrane

Statistical analysis: Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) software package for Windows (Version 16.0; IBM, Armonk, NY). Differences between all experimental groups were assessed with one-way ANOVA (ANOVA). Post-hoc testing was performed for intergroup comparisons using the least significance difference test. The experiments were performed at least three times with duplicate samples. P values ≤0.05 were considered statistically significant.

RESULTS

Effects of the extract of C. maritima on selenite-induced free radical generation in lenses of Wistar rat pups: Levels of the free radicals O_2^-, NO and OH· were assayed in the lenses of Group I (control), Group II (selenite-challenged, untreated), and Group III (selenite-challenged, *C. maritima* extract-treated) rats.

Cytochemical localization of superoxide generation in rat lenses: Using the NBT salt reduction assay, cytochemical localization of formazan in the lenses of Group II revealed intense blue deposits, which suggested O_2^- had been generated. However, lenses from the Group III rats exhibited negligible formazan deposition; the pattern was similar to that in the Group I (control) rat lenses (Figure 1).

Superoxide anion generation in rat lenses: O_2^- generated in the lens, measured as a cytochrome c reduction reaction,

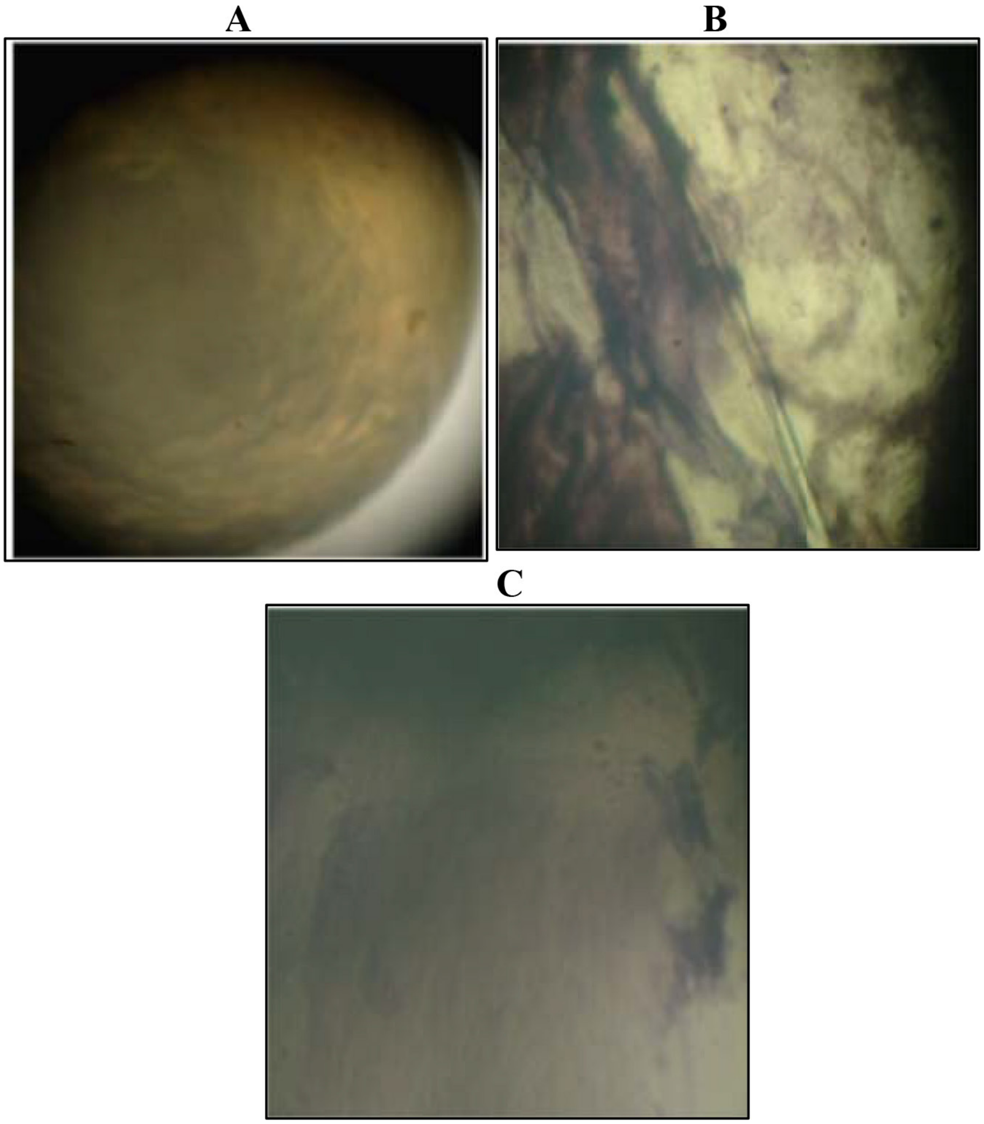

Figure 1. Results of nitroblue tetrazolium (NBT) salt reduction assay in the ocular lens of Wistar rat pups. **A**: No blue color formation was observed upon incubation with NBT for 45 min in Group I rat lenses. **B**: Formation of intensely blue-colored formazan deposits, suggestive of superoxide (O_2 −) generation was observed in Group II rat lenses. **C**: Negligible blue formazan deposition was observed in Group III rat lenses.

was significantly ($p<0.05$) higher in the selenite-challenged, untreated rat lenses compared to that in the control and selenite-challenged, *C. maritima* extract-treated rat lenses. However, the mean quantum of the superoxide anion generated in the lenses of the extract-treated rats was significantly ($p<0.05$) higher than that in the control rat lenses (Figure 2).

Hydroxyl radical generation in rat lenses: The OH· generated in the lenses of the Group II (selenite-challenged, untreated) rats was significantly ($p<0.05$) higher than that in the lenses of the Group I (control) and Group III (selenite-challenged, *C. maritima* extract-treated) rats. Interestingly, the amount of OH· radicals generated was significantly ($p<0.05$) lower in the Group III rat lenses than in the Group I rat lenses (Figure 3).

Nitric oxide levels in rat lenses: Similar to O_2^- and OH·, NO levels were significantly ($p<0.05$) higher in the lenses of the Group II (selenite-challenged, untreated) rats than in the lenses in the Group I (control) and Group III (selenite-challenged, *C. maritima* extract-treated) rats. The levels of NO in the Group III rat lenses approximated those in the Group I rat lenses (Figure 4).

Reverse transcription–polymerase chain reaction analysis of messenger ribonucleic messenger ribonucleic acid transcript levels of inducible nitric oxide synthase in Wistar rat lenses: iNOS mRNA was generated with RT–PCR, and the transcript levels attained in the lenses of the different groups of rats were compared. The mean relative expression of the gene encoding iNOS was significantly ($p<0.05$) higher in the Group II (selenite-challenged, untreated) rats than that

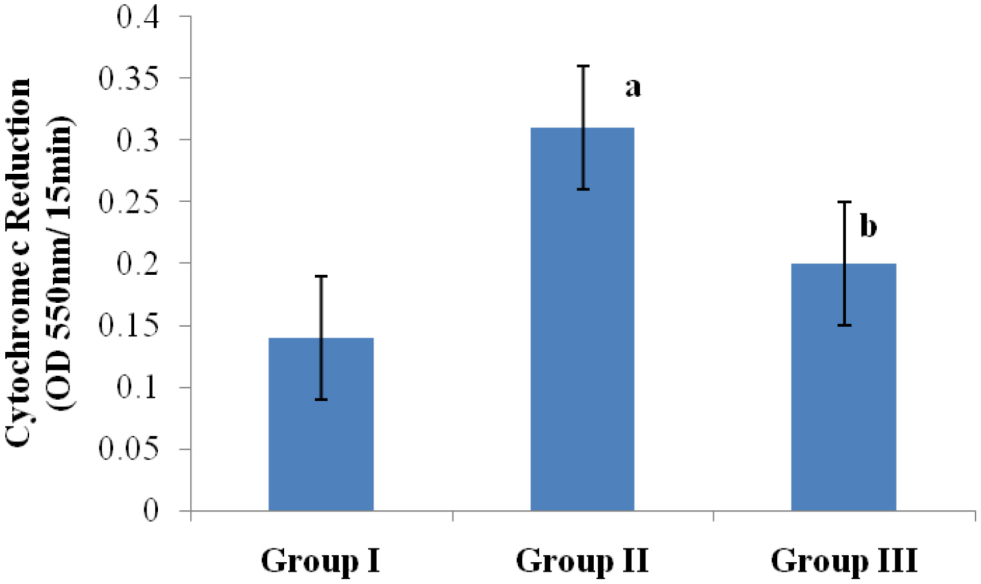

Figure 2. Superoxide anion radical generation in lenticular tissues of Wistar rat pups. Group I=Control rat lenses; Group II=Selenite-challenged, untreated rat lenses; Group III=Selenite-challenged, *C. maritima* extract-treated rat lenses. [a]Group II versus Group I ($p<0.05$); [b]Group III versus Group I ($p<0.05$).

in the lenses of the Group I (control) and Group III (selenite-challenged, *C. maritima* extract-treated) rats (Figure 5A). The mean relative expression of this gene in the Group III rat lenses approximated that noted in the Group I (control) rat lenses (Figure 5B).

Immunoblot analysis of inducible nitric oxide synthase in Wistar rat lenses: To further validate the data on the mRNA transcript levels of iNOS, immunoblot analysis was performed with specific antibodies against iNOS. Lenses of the selenite-challenged, untreated (Group II) rats revealed a significantly ($p<0.05$) higher band intensity than those in the control (Group I) rat lenses. Interestingly, the lenses of the selenite-challenged, *C. maritima* extract-treated (Group III) rat lenses exhibited a band intensity that was almost similar to those in the control lenses. The protein loading control β-actin was confirmed with the specific antibody (Figure 6).

DISCUSSION

Oxidative free-radical damage is an initiating or early event in the overall sequence leading to cataract formation [23]. In the cells, ROS may initiate a surge of toxic biochemical reactions, such as peroxidation of membrane lipids and

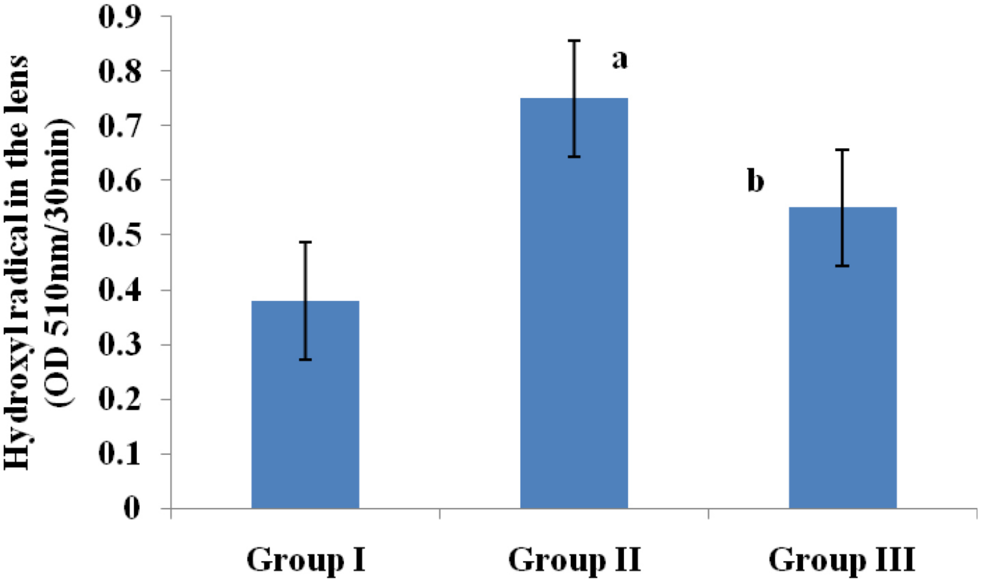

Figure 3. Hydroxyl radical generation in lenticular tissues of Wistar rat pups. Group I=Control rat lenses; Group II=Selenite-challenged, untreated rat lenses; Group III=Selenite-challenged, *C. maritima* extract-treated rat lenses. [a]Group II versus Group I ($p<0.05$); [b]Group III versus Group I ($p<0.05$).

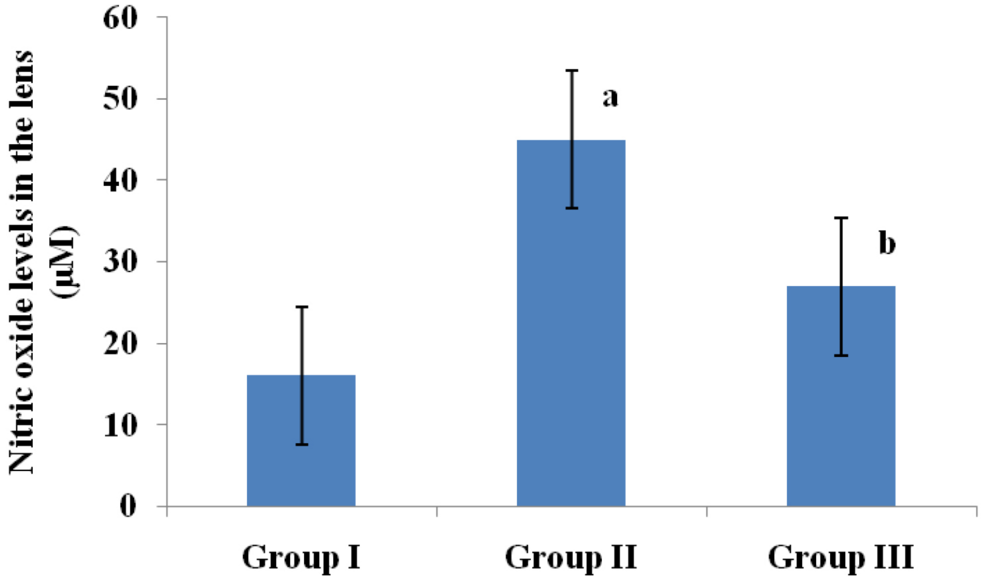

Figure 4. Nitric oxide generation in lenticular tissues of Wistar rat pups. Group I=Control rat lenses; Group II=Selenite-challenged, untreated rat lenses; Group III=Selenite-challenged, *C. maritima* extract-treated rat lenses. [a]Group II versus Group I ($p<0.05$); [b]Group III versus Group I ($p<0.05$).

extensive damage to proteins, causing intracellular protein aggregation and precipitation and eventually leading to opacification of the lens [2]. Since the extract of *C. maritima* exhibits scavenging of free radicals, and reducing power and chelating effects on ferrous irons (unpublished observations), an attempt was made to test its potential to prevent putative free radical-mediated cataractogenesis in the lenses of Wistar rat pups in the present study.

NBT is a useful marker for indicating decreases in a blue insoluble formazan by free radical intermediates. NBT has been widely used to demonstrate radical production by activated monocytes and macrophages [24]. In this study, NBT staining was used for localizing superoxide (O_2^-) generation in the ocular lens. Selenite administration led to a drastic increase in SOD-inhibitable O_2^- generation in the lens, suggesting selenium-induced free radical generation that could have contributed to enhanced oxidative stress and cataractogenesis. The mechanism of oxidative damage to the lens through intraocular photogeneration of O_2^- and its derivatives has already been studied [25]. In addition, O_2^- may lead to other ROS through metal-catalyzed nonenzymatic reactants such as free radicals or lipid hydroperoxides. When NBT reacts with superoxide, a dark-blue, insoluble formazan compound is produced [26]. Superoxide is believed to be the major oxidant species responsible for reducing NBT to formazan [27]. The lenses of the Group II (selenite-challenged, untreated) rats showed intense NBT staining at the central core (Figure 1B) while the lenses of the Group I (control) rats did not show any visible staining (Figure 1A). The lenses of the Group III (selenite-challenged, *C. maritima* extract-treated) rats showed less intense NBT staining than that of the Group II rat lenses (Figure 1C). Thus, cytochemical localization of formazan suggested that free radical intermediates were much lower in the lenses of the selenite-challenged, extract-treated rats than in the lenses of the selenite-challenged, untreated rats.

Superoxide radical O_2^- is harmful to cellular components, since it is a precursor of more reactive oxygen species [28]. The mechanism of oxidative damage to the lens through intraocular photogeneration of O_2^- and its derivatives has already been studied in an in vivo model [25]. In addition, O_2^- may lead to other ROS through metal-catalyzed nonenzymatic reactants, such as free radicals or lipid hydroperoxides. In the present study, the mean quantum of generation of superoxide anion radicals was significantly ($p<0.05$) higher in the lenses of the Group II (selenite-challenged, untreated) rat lenses than that in the Group I (control) and Group III (selenite-challenged, *C. maritima* extract-treated) rat lenses. This suggests that the extract of *C. maritima* prevented to some extent the increase in generation of superoxide anion radicals that followed exposure to selenite alone (Figure 2)

The generation of highly reactive cytotoxic hydroxyl radicals (OH·) is facilitated by iron, which catalyzes the interaction of O_2^- and H_2O_2 by Fenton or Haber-Weiss reactions [29]. Generation of OH· can promote oxidative stress, cytotoxicity, and tissue injury [30]. One of the earliest changes in nuclear cataractogenesis is the loss of protein sulfhydryl groups. It was postulated that the reaction of the lenticular proteins with H_2O_2 derived from superoxide may be responsible for the changes associated with nuclear cataract formation [31]. H_2O_2 may be derived from the aqueous humor

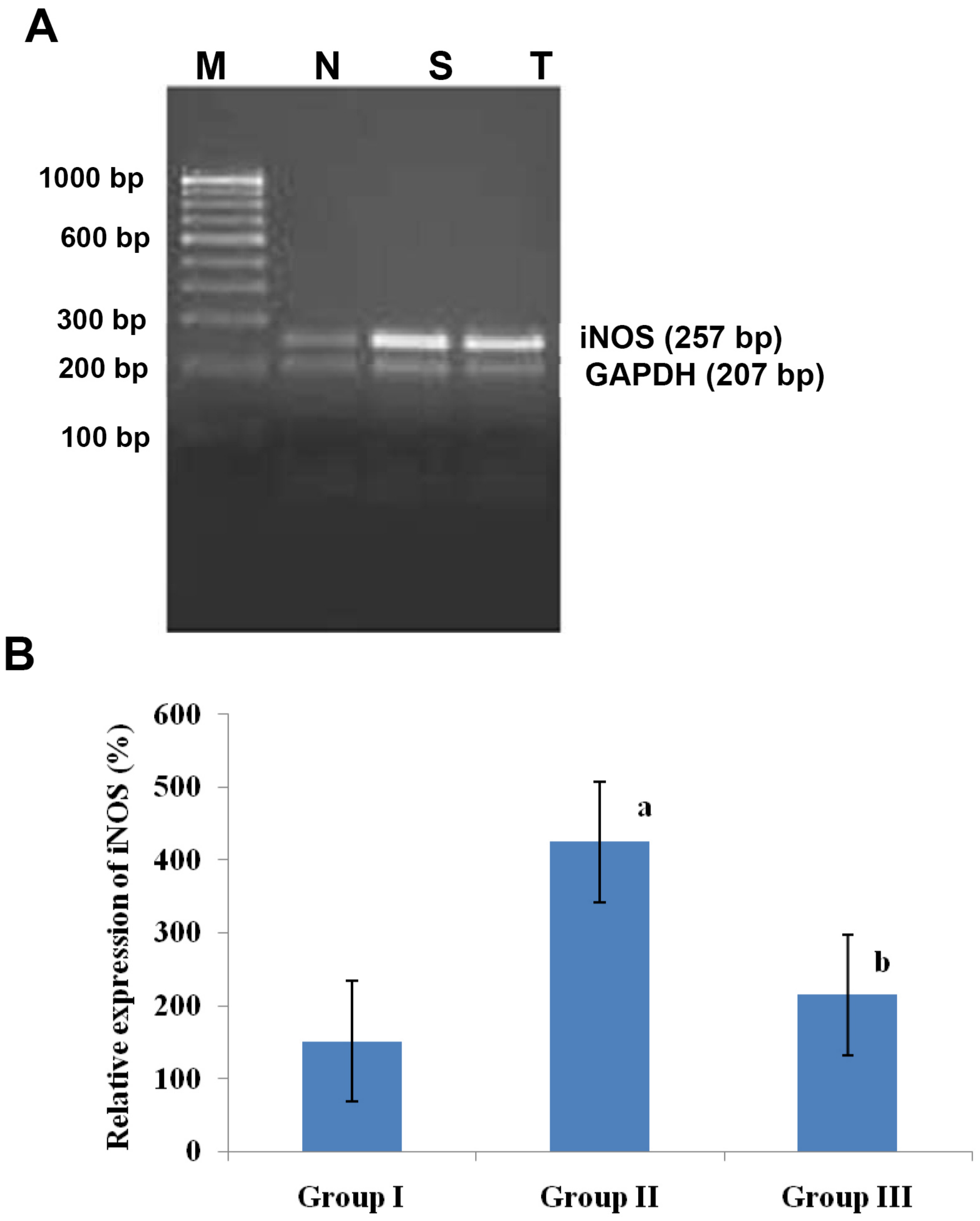

Figure 5. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of inducible nitric oxide synthase messenger RNA (mRNA) in rat lenses visualized on an ethidium bromide-stained agarose gel alongside messenger ribonucleic acid of glyceraldehydes-3-phosphate dehydrogenase. **A**: M=100 bp DNA ladder; N=Group I (control); S=Group II (selenite-challenged, cataract-untreated); T=Group III (selenite-challenged, C. maritima extract-treated). **B**: The results depicted are normalized to levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Data are mean value (experiments run in triplicate) of ratios of intensity for gene of interest divided by that for GAPDH. [a]Group I versus Group II and III values ($p<0.05$); [b]Group II versus Group III values ($p<0.05$).

that bathes the anterior segment of the lens [32]. In addition, protein modifications linked with cataract could be the result of a reaction of lenticular crystallins with oxidizing agents such as the hydroxyl radical [33], which might also partly derive from H_2O_2 through the transition-metal ion catalyzed Fenton reactions [34].

In the present study, a significant increase in OH· generation was observed in the Group II (selenite-challenged, untreated) rat lenses when compared with the Group I (control) rat lenses. This enhanced generation of OH· was not observed in the lenses of the selenite-challenged, extract-treated (Group III) rats; in these rats, the generation of OH· was similar to that in the Group I rat lenses, suggesting that the *C. maritima* extract can scavenge OH· (Figure 3). These observations are consistent with the known antioxidant activities of the extract of *C. maritima*. Other plant extracts, such as *Abutilon indicum* [35] and *Aerva lanata* [36], have been shown to have similar properties.

NO, a short-lived lipophilic chemical transmitter, can diffuse freely across membranes and has been shown to play a crucial role in regulating local blood flow and aqueous outflow in blood vessels [37]. NO levels were enhanced in the selenium-administered group, and such a selenium-induced effect has been recently reported [38]. Inducing iNOS results

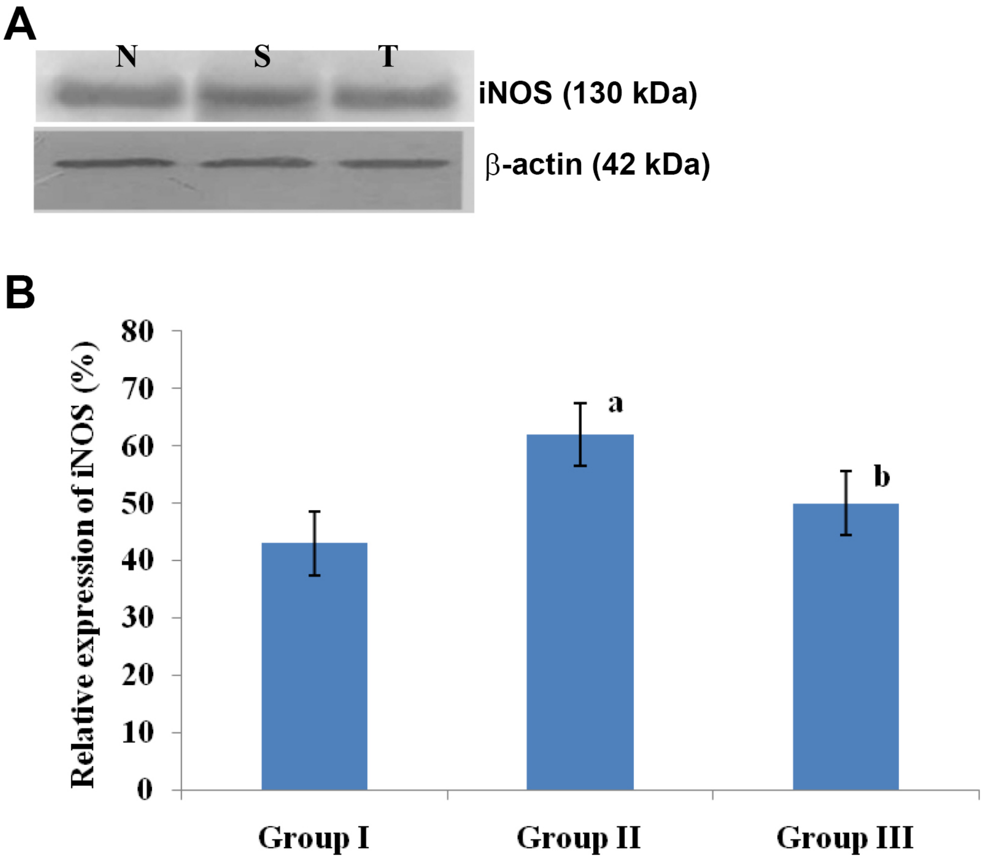

Figure 6. Immunoblot analysis of inducible nitric oxide synthase (iNOS) in lysate of whole lens. **A**: Representative western blot for iNOS (with β-actin as loading control) in lenses of 16-day-old Wistar rat pups. N-Group I (control); S-Group II (selenitechallenged,cataract untreated); T-Group III (selenite-challenged, C.maritima extract-treated). **B**: Bar graphs of mean normalized densitometry readings. N-Group I (control); S- Group II (selenite-challenged, cataract-untreated); T-Group III (selenite-challenged, C.maritima extract-treated). ^{a}Group I vs Group II & III values ($p<0.05$); ^{b}Group II vs Group III values ($p<0.05$).

in a sustained and upregulated release of excessive amounts of NO, which is cytotoxic to neighboring cells. In the present study, NO levels were enhanced in the lenses of the selenite-challenged, untreated (Group II) rats; this observation is consistent with those of other studies [39,40]. Ito et al. [41] demonstrated that NO is involved in selenite-induced cataracts and cataracts can be prevented by treating with NO synthase inhibitors.

Time-course experiments have demonstrated that NO is involved at an early phase of cataractogenesis, but the exact mechanism involved in NO-mediated cataractogenesis remains to be elucidated. However, existing data provide evidence that NO can affect the levels of free protein thiols and total glutathione in cells [42], which appear to correlate with loss of antioxidants and increased oxidative stress. Dallak et al. [43] attempted to determine the generation of $ONOO^-$ as well as OH·, which are more potent oxidants in selenite induced O_2^- and NO generation in ocular lenses. In the present study, the levels of mRNA transcripts and protein expression of iNOS were elevated in the Group II (selenite-challenged, untreated) rat lenses when compared to the levels in the Group I (control) rat lenses. This elevation possibly reflects the increased intracellular calcium pool following the loss of epithelial barrier function [44]. However, in the lenses of the selenite-challenged, *C. maritima* treated (Group III) rats, the upregulation of iNOS was prevented and was almost similar to that in the control (Group I) lenses (Figure 5 and Figure 6). Interestingly, these findings are consistent with the observations made in the present investigation on the generation of nitric oxide in all three experimental groups (Figure 4). These original findings suggest that treatment with the extract of *C. maritima* regulates NO activity at near normal levels as evident by iNOS expression.

In summary, the results of the present study suggest that an ethanolic extract of the plant *Cineraria maritima* possibly prevents or slows selenite-induced cataractogenesis by preventing or dampening selenite-induced free radical formation in lenticular cells. These effects may be attributed to the plant's ability to reduce the formation of formazan crystals. In addition, the generation of hydroxyl, superoxide, and nitric oxide levels were also reduced. Alterations in the mRNA and protein expression levels of iNOS were prevented in the selenite-challenged, *C. maritima* extract-treated rat lenses. These observations strongly suggest that the *C. maritima* extract prevents cataractogenesis by minimizing free radical generation in an in vivo rat model.

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