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In vitro effect of triphala constituent in sodium selenite-induced cataractogenesis

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Abstract
Globally the burden of blindness is majorly due to cataract. The major risk factor responsible for cataract is the oxidative stress generated by the free radicals. As of now panacea for cataract is the surgical removal of cataractous lens. This is often accompanied with several post-operative complications. Here opacification of crystalline fibres of eye lens is induced by protein oxidation. Antioxidant enzymes like SOD, CAT and GSH does plays significant role by neutralizing free radicals. In the present study we have demonstrated the anti-cataract potential of Triphala and its constituents. Cultured goat lenses were divided into nine groups containing six lenses each. From the experimental, Group I remained at natural transparency, while Group II exhibited dense opacification of lens indicating cataract formation. All the treated lenses were protected from opacification. Methanolic extract (Group VI & VII) that contained *Emblica officinalis* in combination with *Terminalia bellirica* and *Terminalia chebula* showed better anti-cataract efficacy. Whilst, Triphala extract (Group IX) was confirmed and demonstrated to contain highest anti-cataract potential.

Keywords: Cataract, Goat lens, Triphala, *Emblica officinalis*, *Terminalia bellirica*, *Terminalia chebula* anti-cataract, Selenite-induced cataract model

Introduction
Cataract is foremost among the elements leading to blindness globally [1]. Opacification of crystalline fibres in eye lens impedes the passage of light and causes cataract [2]. At present, about fifty million cataract victims are reckoned globally and the number rises up rapidly in proportional to the population explosion. It includes more than 75% cataract sufferers from developing countries [3]. The factors responsible for cataract comprise ageing, congenital defects, accidental injury and inflammation or disease [4, 5, 6].

One such significant risk factor associated with cataract is oxidative stress [7]. It arises as a result of an imbalance between free radical production and antioxidant defences. Oxidative stress is an imbalanced condition between the production of free radicals and the ability of body to defend or detoxify their effects by antioxidant neutralization [8]. The elements of risk factor inducing opacification include dementia, diabetes, prolonged exposure to sunlight, tobacco use and alcohol drinking by liberating free radicals into eye lens leading to cataract [9]. It is also evidenced from epidemiological and experimental reports, that oxidative stress is responsible for stimulation and progression of cataract [10, 11]. As an adaptive response lenses have developed antioxidant systems to defend against the toxic damage that are induced by reactive oxygen species (ROS) or free radicals. It includes antioxidants, such as reduced GSH (glutathione), antioxidant enzymes like SOD (superoxide dismutase), CAT (catalase), GST (glutathione S-transferase) and GR/Gpx (glutathione reductase/peroxidase) [12, 13]. As of now, the only available treatment for cataract is surgical replacement of malfunctioned lens with synthetic clear lens. Whilst, the short comes of the panacea encompasses, (i) post-operational complications, (ii) quality of lens, and (iii) inequity in feasibility. Thus preventive measures through pharmacological approach that could delay the onset of cataract need to be adopted to eradicate the blindness. It is also confirmed that ingestion of antioxidants impedes the progression of nuclear cataracts. Selenite-induced cataract is a common model of senile cataract that is employed frequently in preclinical studies for screening potential anti-cataract agents [14].

In this backdrop, the present study has aimed to harness the pharmacological intervention for cataract with a special reference to scientific validation of Triphala and its constituents(*Emblica officinalis*, *Terminalia bellirica* and *Terminalia chebula*). The anti–cataract efficacy of Triphala constituents (methanolic extract) were assessed in *Selenite-induced cataract* model. In addition the efficiency of antioxidant enzymes such as SOD, CAT, GSH and levels of lipid peroxidation were determined from *in vitro* goat eye lens culture.

Materials and Methods

Drugs and chemicals

All the drugs and chemicals were obtained from the following sources: Chemicals required for the enzyme assays were received from Sigma Chemical Co., (USA); Sodium selenite (an oxidative stress inducing agent) was received from Central Drug House (P) Ltd., (New Delhi); Triphala extract was received from Promed Exports (P) Ltd., (New Delhi).

Plant material collection: The fruits of *E. officinalis*, *T. bellirica* and *T. chebula* were collected from Jaipur (Rajasthan), India. The plant specimens were identified and confirmed from the State Forest Research Institute, Jabalpur, India.

Plant extract preparation: The fruits of the *E. officinalis*, *T. bellirica* and *T. chebula* were shade dried and crushed mechanically to thin powder. Methanolic plant extracts were prepared using the method mentioned elsewhere [15, 16].

Lens culture

The study was conducted with goat eye lens collected from local slaughter house, then transported immediately and stored in the laboratory at 0–4 °C. The specimen was isolated by extra capsular extraction technique and preserved in artificial aqueous humour (NaCl–140 mM, KCl–5mM, $MgCl_2$–2mM, $NaHCO_3$–0.5 mM, NaH $(PO_4)_2$–0.5mM, $CaCl_2$–0.4mM and Glucose–5.5mM) at room temperature and pH 7.8 for 72 hrs. Penicillin (32*mg*) and streptomycin (250*mg*) were added to the culture media to prevent further bacterial contamination [17]. Lenses that developed damage with artificial opacities were discarded, and only those transparent were further used for *in–vitro* experiments [18].

Experimental groups

About 54 goat eye lenses were used in the experimental study. The lenses were then divided into nine groups and each group contained six lenses. Latter the lenses were incubated in artificial aqueous humour with methanolic plant extract of triphala and its constituents for 24 hours. The doses were selected on the basis of stoichiometric experiments conducted with three different concentrations of Triphala (400, 800, and 1200µg/ml). The groups were categorised as follows: Group I: Control (C); Group II: Sodium selenite treated (SS); Group III: SS + *E. officinalis*; Group IV: SS + *T. bellirica*; Group V: SS + *T. chebula*; Group VI: SS + *E. officinalis* + *T. bellirica*; Group VII: SS + *E. officinalis* + *T. chebula*; Group VIII: SS+ *T. bellirica* + *T. chebula*; Group IX: SS + Triphala. Group I lenses were incubated in DMEM (Dulbecco's modified Eagle's medium), while Group II lenses were incubated in DMEM supplemented with 100 µM sodium selenite. Post-incubational lenses were examined visually by placing them on a graph sheet for its opacity and photo documentation was recorded. Subsequently, all lenses underwent cleaning, weighing and were processed to carry over biochemical assays. Prior to the initiation of the experimental, all the protocols were approved by the institutional animal ethical committee.

Biochemical assays

Lenses were processed for biochemical assay after 24 hours of incubation. 10% of homogenate (*W/V*) were prepared using York's homogenizer (dispersed in 1*mM* phosphate buffer), fitted with Teflon plunger. The homogenate was centrifuged at 4000 *RPM* for 10 minutes in refrigerated centrifuge and the supernatant was used for determining the biochemical parameters.

Protein and protein carbonyl content: The protein content was determined [19] using bovine serum albumin (BSA) as standard. It was represented as *mg/gm lens*n. Protein carbonyl content was estimated in the samples by measuring the DNPH adducts at 375 nm. Carbonyl contents were calculated by using a molar extinction coefficient (e) of 22,000 M^{-1} cm^{-1}. Data were expressed as n moles carbonyl/mg protein.

Lipid peroxide levels (LPO): The lipid peroxide (LPO) levels were calculated by the standard method [20]. The thiobarbituric acid reacting substances (TBARS) of the sample were recorded using spectrophotometer at the wavelength of 532 nm. The determined lipid peroxidation was expressed as n mole of MDA/g of tissue.

Measurement of endogenous enzymes

The lens homogenates were used for enzymatic antioxidant assays. SOD (Superoxide dismutase, EC 1: 15.1.1) activity was determined from its inhibition ability to the reduction of NBT in presence of PMS [21]. The reaction was monitored using spectrophotometer at 560 nm. The SOD activity was represented as unit/mg protein (1 unit is the amount of enzyme inhibiting the reduction of NBT into one half above reaction mixture). CAT (Catalase, EC 1.11.1.6) activity was determined using hydrogen peroxide (H_2O_2) as substrate and the decomposition of H_2O_2 was monitored at 240 nm using the spectrophotometer. The CAT activity was denoted as unit/mg protein [22]. The reduced GSH (glutathione) was estimation using Ellman reagent (5, 5'dithiobis (2-nitro benzoic acid). The appearance of pale colour was measured by taking optical density on the spectrophotometer at 412 nm. An appropriate standard (pure GSH) was run simultaneously. The level of GSH was expressed as unit/mg protein [23].

Statistical analysis

Experimental data were summarized as Mean ± SD (n=6). Groups were compared each other through one way variance analysis followed by Student Newman-Keuls post hoc test. The acceptance level of significance was $p<0.05$. InStat (version 3) was used for analysis of data.

Results

Lens morphology *in vitro*

Figure 1 reveals that all the lenses of Group I found to be transparent while lenses incubated with SS (group II) exhibited dense opacification indicates the formation of cataract. The majority of lenses incubated with methanolic extract of triphala constituents, such as Group III, Group IV and Group V showed significant results with constrained opacification of lens. *E. officinalis* in combination with *T. bellirica* (Group VI) and *T. chebula* (Group VII) exhibited enhanced anti-cataract potential than the combinations thereof. The methanolic fruit extract of triphala (Group IX) revealed the presence of transparent lens, among the studied group. It might have resulted due to the presence of higher concentration of *E. officinalis* constitution in Triphala, which keeps the loss of lens transparency in check (Figure 1). In addition, presence of phenolics as well as flavonoids in *E. officinalis* could have acted as potential antioxidant [15] enhancing the anti-cataract efficacy.

Biochemical assays: The total protein content was found to be least in Group II among the conducted experiments. Significant recovery was documented in all the treated groups. The most significant recovery was observed in Group IX (49%), Group VII (45%), Group VI (40%) and Group III

(31%). The protein carbonylation was found to be significantly higher in Group II with 106% than the experiments thereof. Positive trend was record in all the treated groups. The most significant recovery was observed in Group IX (50%), Group VII (49.5%), Group VI (49%) and Group III (48%) as compared to experimental control (Group II). The lipid peroxidation was estimated to be 135% in Group II than that of Control (Group I). Here significant recovery was evidenced in all the treated groups. The rate of recovery was observed to be higher in Group IX (53%), followed by Group VII (49%), Group VI (47%), and Group III (24%) respectively as compared to experimental control (Group II). The antioxidants enzymes SOD level was found to be least in Group II (101%) as compared to the control Group I. The most significant recovery was observed in Group IX (253%), Group VII (128%), Group VI (24%) and Group III (24%) as compared to experimental control (Group II).

The CAT level was found to be significantly decreased in group II (54%) as compared to the control group I. The significant recovery was observed in all the treated groups. The most significant recovery was observed in Group IX (100%), Group VII (74%), Group VI (61%) and Group III (52%). The antioxidants enzymes GSH level was found to be significantly decreased in group II (61%) as compared to the control group I. The significant recovery was observed in all the treated Groups. The most significant recovery was observed in Group IX (185%), Group VII (159%), Group VI (137%) and Group III (124%).

Discussion

The opacification was observed in experimental group while significant recovery was observed in experimental groups when treated with methanolic extract of triphala and their constituents. Several previous records confirmed that oxidative stress and radical oxygen species causing opacification of lens and are responsible for cataract [24[. Free radicals perturb the homeostasis of lens and leads to loss of transparency. The protein concentration was found to be lower in experimental group than the untreated control group (Figure 2). These findings are in line with the increased protein carbonization (PC) in group II. The deteriorating effect was decreased with the treatment with triphala and its constituents. The composition of lens poses transparent crystalline protein. Free radicals and reactive oxygen species induces oxidation of lens proteins there by leading to lens opacification [25]. The destructive progression of cataract in goat eye lens might have resulted due to the generation of free radicals by sodium selenite induction. Earlier researchers had documented that accumulation of peroxidation product damages the important fragile membranous structures [26]. It is evident from the present study that increased rate of lipid peroxidation was observed in sodium selenite induced group. Significant recovery was found in groups treated with methanolic extract of triphala and its constituents. The evaluated antioxidant profiles (*i.e.* SOD, CAT and GSH) of goat eye lenses were presented in Figure 3. The SOD and CAT activity were found to increase significantly in methanolic extract treated study group. The maximum reduction was observed in the Group II. The mode of action of sodium selenite induced cataractogenesis and protective effect of methanolic extract of triphala and their constituents has not yet been completely defined. However, the present study reports that the main biochemical action of oxidation is increased by reactive oxygen species present in aqueous humour in combination with decreased antioxidant enzyme activity. Catalase an innate enzymatic defence system present in the lens detoxifies H_2O_2. Reduction in the activity of these enzymes builds-up highly reactive free radicals in the tissues and causes injurious effects [27]. The catalase level was found to be less toxic in Group I than in Group II. Reduction in glutathione content was recorded in all types of cataract [28]. It plays an important role in preservation of transparency of lens and protein oxidation [29]. It is suggested that protein aggregation occurs under stressful circumstance and its precipitation leads to protein denaturation resulting in lens opalescence [30].

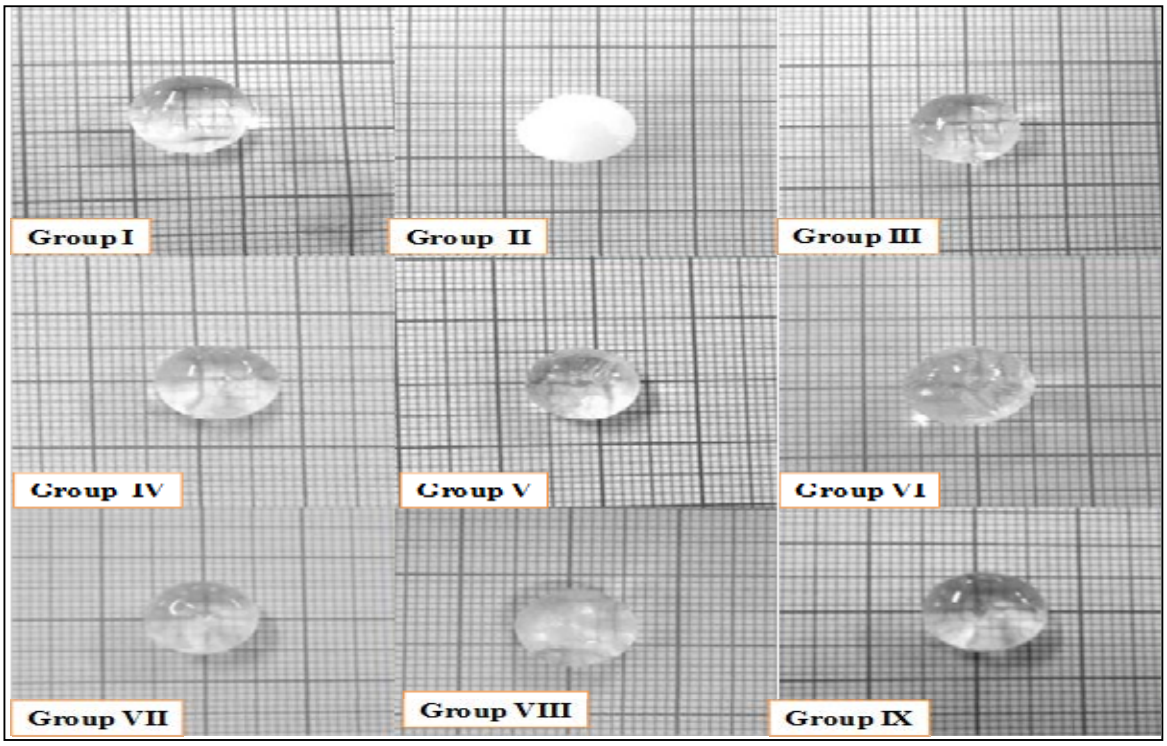

Figure 1: Visual observation of study groups examined for anti-cataract efficacy.

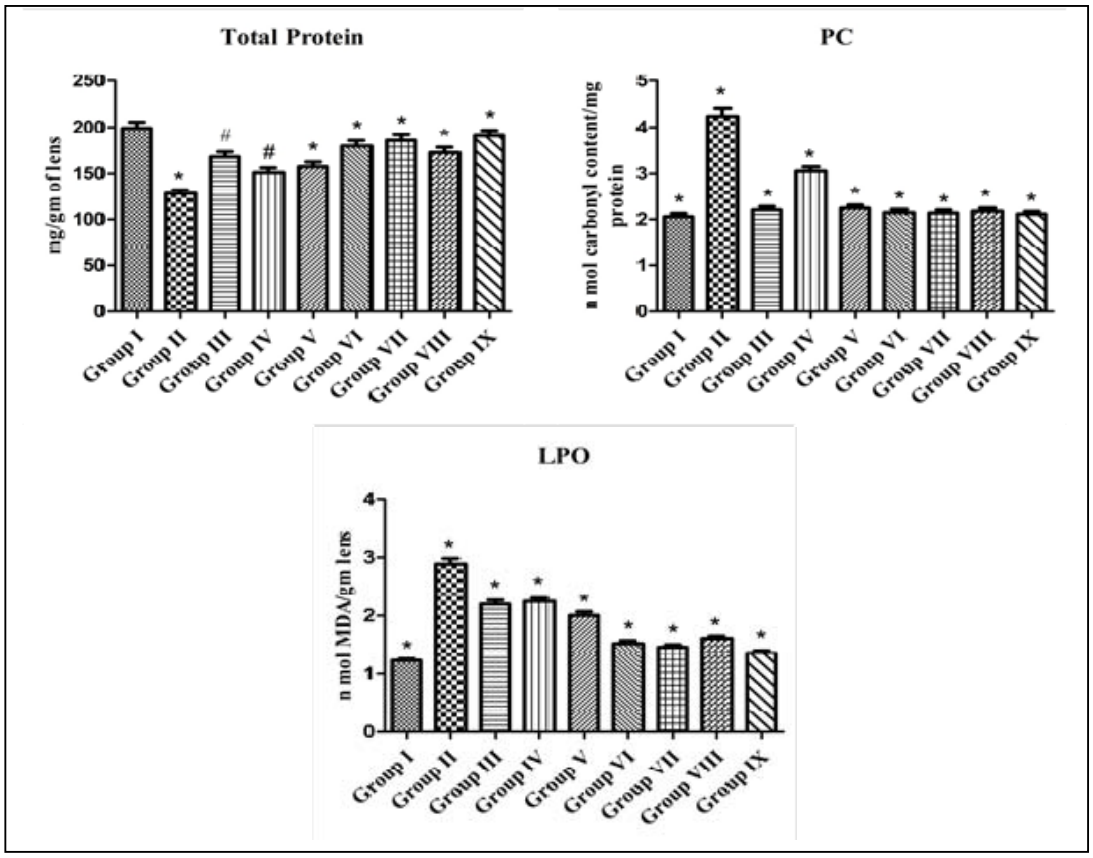

Fig 2: Levels of Total Protein (TP), Protein carbonyl content (PC) and Lipid peroxide level (LPO) in control and experimental groups. The results are expressed as Mean ± SD in six goat eye lens of each group. (*=p<0.001; #=p<0.01; †=p<.05; ns = non significant)

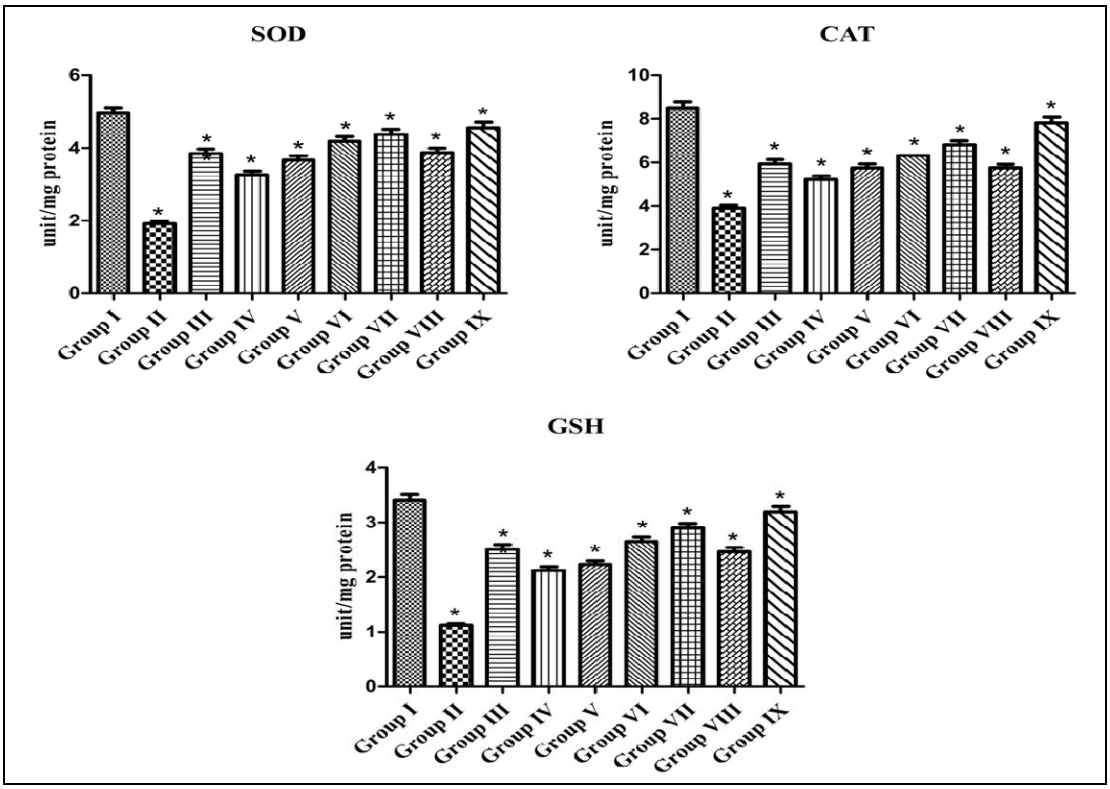

Fig 3: Activity of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione (GSH) in control and experimental groups. The results are expressed as Mean ± SD in six goat eye lens of each group. (*=p<0.001; #=p<0.01; †=p<.05; ns = non significant)

Conclusion

The major cause of cataractogenesis is the excessive free radicals generated due to ageing, diabetes mellitus, alcohol consumption or smoking. The generation of free radicals could be delayed by supplementation of methanolic extract of *E. officinalis*, *T. bellirica*, *T. chebula* and their combination

thereof. In present study, it is observed that *T. bellirica* and *T. chebula* in combination with *E. officinalis* showed maximum anti-cataract efficacy. This might be due to the fact that *E. officinalis* enhances the antioxidant potential of *T. bellirica* and *T. chebula*. The presence of phenolics as well as flavonoids in *E. officinalis* could have acted as potential antioxidant. Thus supplementation of Triphala and its constituents in diet could prevent or delay the free radicals induced cataract formation.

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