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Evaluation of Anti-Cataractogenic Activity of *Spinacia Oleracea* On Glucose - Induced Cataract in Isolated Goat Lens.

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ABSTRACT

Cataract, a leading cause of poor vision and blindness worldwide is recognized as a major complication of diabetes mellitus and hyperglycemia. Increasing evidence that oxidative stress plays a role in cataractogenesis has led to the development of antioxidant nutrients from plant and synthetic origins that could be effective in preventing cataract formation. The present study evaluated the *in vitro* anticataract potential of *Spinacia oleracea* leaf extract against glucose-induced cataractogenesis in goat lenses. Freshly isolated goat lens was divided into the following five experimental groups and incubated for 72 h: 55 mM glucose alone (Group I), 100 µg/ml SE+55 mM glucose (Group II), 300 µg/ml SE+55mM glucose (Group III), 500 µg/ml SE+55mM glucose (Group IV) and 12 ng/ml Enalapril+55mM glucose. On photographic evaluation of lenses, a reduction in the opacity of the lens incubated with the plant extract was observed. After 72 h incubation, various biochemical parameters such as total protein, H_2O_2, malondialdehyde (MDA), reduced glutathione (GSH) and Cu^{2+}-induced lipoprotein dienes; enzymatic antioxidants like glutathione reductase (GR) and peroxidase (POX) were measured in the lens homogenate. Thus, the leaf extract of spinach (100 µg/ml SE) protected the lens against glucose-induced oxidative damage which might help delay the progression of cataract.

Keywords: antioxidant, cataract, hyperglycemia, goat lenses, spinach.

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INTRODUCTION

Various factors including sedentary lifestyle, population growth, urbanization, ageing and increase in obesity may cause an estimated 4.4% rise in the number of patients of diabetes mellitus by 2030 [1]. The major secondary complication of diabetes is cataract with 20% cataract surgeries performed for diabetics alone [2]. In India, about 65.2 million people lose vision due to cataract [3,4]. Even short episodes of hyperglycemia may cause tissue damage via mechanisms causing acute changes in cellular metabolism. Increased intracellular glucose rapidly enters the polyol pathway wherein it is converted to sorbitol via an associated decrease in NADPH. Sorbitol accumulation in the intra lenticular space triggers hyper osmotic effects, lenticular increase in sodium and decrease in the levels of potassium, glutathione, ATP and free amino acids [5,6]. Membrane permeability is thus altered resulting in increased sensitivity to oxidative stress.

Cataract treatment commonly use aldose reductase inhibitors and non-steroidal anti-inflammatory drugs (NSAIDs) [7]. Side-effects of surgery such as progression of retinopathy, vitreous haemorrhage, iris neovascularization and a decrease or complete loss of vision have been reported [8]. According to the World Health Organization (WHO), traditional medicine incorporates health practices, approaches and knowledge of plants, applied either singularly or in combination to treat and prevent illnesses or maintain general well-being. Many indigenous plants have been explored as potential promising sources of antioxidants to reduce the development or even prevent cataract formation, thereby reducing the need for surgery [9].

Spinacia oleracea, belonging to the family of Amaranthaceae is an edible flowering plant extremely rich in antioxidants and carotenoids like lutein, β-carotene, violaxanthin and 9'-(Z)-neoxanthin [10]. In herbal medicine, fresh or dried spinach leaves are used to cure aliments of the gastrointestinal tract, as a blood-generating remedy, treat fatigue, appetite stimulator and accelerating convalescence [11]. Notably, high amounts of carotene and lutein found in spinach prevents age-related macular regeneration and cataract [12]. The present study was done to specifically test the local anti-oxidant and anti-cataract effects of methanolic extract of *S. oleracea* leaves on glucose-induced cataractogenesis using goat lenses. Lens morphology, lipid peroxidation, and specific antioxidant activity were the areas of interest.

MATERIAL AND METHODS

Preparation of spinach leaf extract: The spinach plants were purchased from a local market in Bengaluru, India. The leaves were washed thrice with tap water to remove any debris, rinsed with distilled water and then sun-dried. The dried leaves were pulverized using an electric grinder and then extracted in methanol to a final concentration of 10% (w/v). The resultant extract was centrifuged at 6000 rpm for 15 min at 4 °C and the centrifugate obtained was stored at 4 °C in dark bottles.

Preparation of lens culture: The anti-cataract potential of the spinach extract (SE) was analysed using glucose-induced cataractogenesis in goat lenses. The goat eye balls were purchased from an abattoir at Shivajinagar, Bengaluru within 2 h of killing the animals and transported to laboratory at 0-4˚C. Upon extra-capsular extraction, the lenses were incubated in artificial vitreous humor (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM $NaHCO_3$, 0.5 mM $NaH(PO_4)_2$, 0.4 mM $CaCl_2$, and 5.5 mM glucose) at 37 ºC and pH 7.8 for 72 h. To prevent contamination of the lens, Penicillin-32 mg% and Streptomycin-250 mg% was added to the culture media. To induce cataract, 55 mM glucose was added to the media [13].

Experimental design of glucose-induced cataractogenesis: A total of 44 goat lenses were used for the experiment and divided into five experimental groups:

Group I: Glucose 55 mM alone (toxic control)
Group II: *S. oleracea* (100 µg/ml) + Glucose 55 mM
Group III: *S. oleracea* (300 µg/ml) + Glucose 55 mM
Group IV: *S. oleracea* (500 µg/ml) + Glucose 55 mM
Group V: Standard drug Enalapril (12 ng/ml) + Glucose 55 Mm

Photographic evaluation of lens opacity: The analysis of lens opacity was done after 72 h of incubation in the respective solutions. The lenses from the experimental groups and the control were placed on a wire mesh with the posterior surface of the lens on the mesh [14]. The degree of opacity was graded as follows:

Absence of opacity: 0
Slight degree of opacity: +
Presence of diffuse opacity: ++
Presence of extensive thick opacity: +++

Preparation of lens homogenate: After 72 h of incubation, the lenses were homogenised in 10 volumes of 0.1M potassium phosphate buffer, pH 7.0 under cold conditions. Centrifugation was carried out at 10,000 rpm for 15 min at 4°C. The supernatant obtained after centrifugation was analysed for various biochemical parameters [15].

Study of anticataract potential of Spinach extract

Total protein: 4.0 ml of alkaline copper solution was added to 0.02 ml of lens homogenate and incubated for 10 min at room temperature. Then, 0.4 ml of Folin-Ciocalteau reagent was added to the test-tubes, mixed well and incubated for 30 min at room temperature. The absorbance was taken at 610 nm using UV-Visible spectrophotometer against a suitable blank. The protein content was calculated from the standard curve using bovine serum albumin and expressed as µg/g lens tissue [16].

Malondialdehyde (MDA): The analysis of lipid peroxidation was done using the modified protocol [17]. To the lens homogenate, 2 volumes of extraction buffer (0.1% trichloroacetic acid, 0.5% butylated hydroxytoluene and 1.0% polyvinylpyrrolidone) was added. The mixture was boiled for 30 min, chilled on ice for 5 min, and then centrifuged at 10,000 rpm for 10 min at 4 °C. The absorbance of the supernatant was measured at 532nm and subtracted from the non-specific absorbance at 600 nm. The MDA content was calculated from the specific coefficient of 155 $mM^{-1}cm^{-1}$. The values were expressed in µmoles of MDA/g lens protein.

Reduced glutathione (GSH): Lenses were homogenized in 3% metaphosphoric acid and centrifuged at 10,000 rpm for 10 min at 4°C. To 0.02 ml of the supernatant obtained, 0.1M sodium phosphate buffer (pH 7.0) was added followed by 150 µM DTNB [5, 5-dithiobis-(-2-nitrobenzoic acid)] and 20 mM EDTA solution. The tubes were incubated for 30 min at room temperature and the absorbance read at 412 nm. Pure commercial GSH was used as standard for establishing the calibration curve conducted [18].

Hydrogen peroxide (H_2O_2): Homogenisation of the lenses was carried out with 0.1% (w/v) TCA. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C. The homogenate was centrifuged at 10,000 rpm for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM sodium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The tubes were incubated at room temperature for 30 min. The absorbance was observed at 390 nm and calculated from standard curve [19].

Inhibition of Cu^{2+} induced lipoprotein diene formation: The lens homogenate was made and diluted to 0.67% in phosphate buffered saline. To the mixture, copper sulphate was added as $CuSO_4.5H_2O$ which resulted in the formation of Cu^{+2} due to oxidation. After an incubation of 120 min at 37 °C, absorbance was taken at 234 nm using UV-Visible spectrophotometer. The measurement of lipoprotein diene formation was studied to determine the level of tissue protection against any oxidative stress and free radicals [20].

Glutathione Reductase (GR): Oxidation of NADPH was determined by glutathione reductase activity according to Carlberg and Mannervik [21] with minor modifications. Lens was homogenized using 50 mM tris HCl buffer, pH 7.5 and centrifuged at 10,000 rpm for 10 min at 4 °C to obtain the supernatant. The reaction mixture given below was followed to determine GR activity at 340 nm, for every 20 s time interval, for 3 min to create a time graph. Molar extinction coefficient ε_{NADPH} = 6.22 $mM^{-1}cm^{-1}$ was used for calculation

Guaiacol peroxidase (POX): Decomposition of hydrogen peroxide by peroxidase enzyme was analyzed where, guaiacol acted as the hydrogen donor. Lenses was homogenized using 0.1 M sodium phosphate buffer, pH 7.0. The colour obtained was determined at 470 nm, with a time gap of 20 s for 3 min. The oxidized tetraguaiacol obtained was further calculated using the molar extinction coefficient $\varepsilon_{tetraguaiacol}$ = 26.6 mM^1 cm^{-1} [22].

RESULTS AND DISCUSSION

Diabetes mellitus, a chronic systemic disease has increased in prevalence over time. It affects all ocular structures with cataract being the most common ocular complication and the leading cause of blindness in

individuals above 50 years of age [23]. Oxidative stress is said to play a key role in the molecular mechanism of cataract formation [24]. Cataract surgery is performed when there is severe loss in vision due to the opacity of lenses [25]. At present, the management of cataract involves the removal of the clouded lenses and replacing them with intraocular lenses. However, surgical intervention is often associated with endophthalmitis leading to permanent blindness. Therefore, pharmaceutical and nutraceutical research should be given more thrust so that the isolated potential drug compounds may be used to treat and reverse cataract. This paper makes an attempt to analyse the antioxidative and anti-cataract effect of the methanolic extract of *Spinacia oleracea.*

Photographic evaluation of lens opacities: The goat lenses in normal and experimental groups were photographed (Fig 1). Complete opacity was observed in lenses incubated in 55 mM glucose i.e. Group I (Toxic control) after 72 h of incubation. Opacification was observed from the periphery towards the centre of the lens. The lens from Group II (100 µg/ml of SE + 55 mM glucose) had shown better clarity when compared to Group III (300 µg/ml SE + 55 mM glucose) and Group IV (500 µg/ml SE + 55 mM glucose). The clarity observed in the positive control, Group V (55 mM glucose + 12 ng/ml Enalapril) was comparable to Group II (Table 1).

Table 1: Effect of methanolic extract of *S. oleracea* on glucose-induced cataractogenesis

S.NO	GROUPS	TREATMENT	DEGREE OF OPACITY
1	Group I	55mM Glucose	+++
2	Group II	55mM Glucose + 100 µg/ml SE	0
3	Group III	55mM Glucose + 300 µg/ml SE	+
4	Group IV	55mM Glucose + 500 µg/ml SE	++
5	Group V	55mM Glucose + Enalapril	0

The degree of opacity was graded as follows: 0 = Absence, + = Slight degree, ++ = Presence of diffuse opacity, +++ = Presence of extensive thick opacity

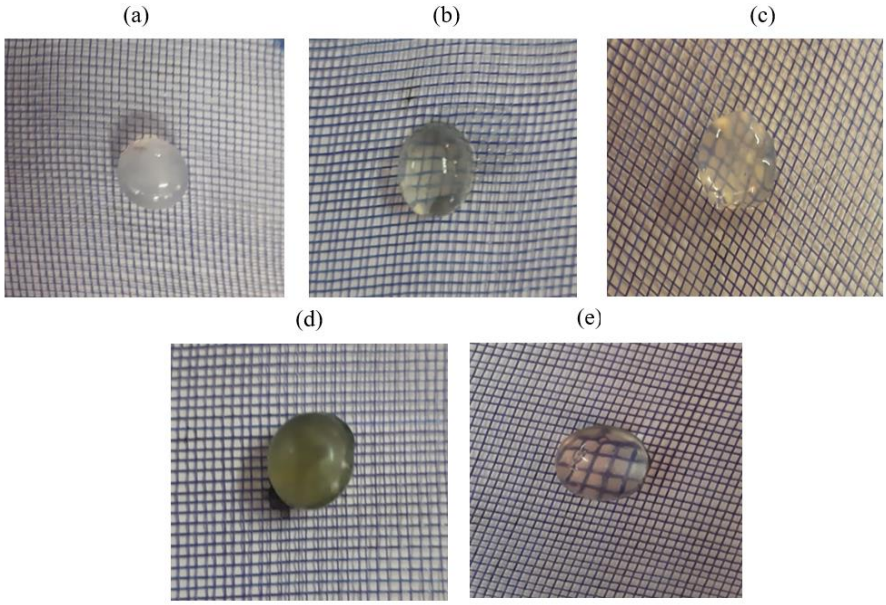

Figure 1: A representative illustration of lens opacity after 72 h of incubation with methanolic extract of *S. oleracea* (SE); only 55mM glucose (a), 100 µg/ml SE + 55 mM glucose (b), 300 µg/ml SE + 55 mM glucose (c), 500 µg/ml SE + 55 mM glucose (d), and 55mM glucose + 12 ng Enalapril (e).

Effect of spinach extract on oxidative stress markers: Oxidative stress has been observed as one of the most common factors influencing the formation of cataract. Due to the high glucose concentration in the lens culture media, glucose enters the lens and is utilized through the sorbitol pathway forming polyols. These polyols result in overhydration and oxidative stress leading [26]. Additionally, hyperglycemia also induces oxidative stress through various pathways [27].

Total soluble lens proteins: Oxidation of the lens proteins particularly crystallins as well as membrane proteins results in the formation of insoluble protein aggregates. This loss of soluble proteins from the lens by

precipitation due to protein oxidation is observed as a decrease in total lens protein levels as evidenced in Table 2. The total soluble lens protein content in the Toxic control decreased by 19.4% as compared to the SE treated sample. The result correlates with the opacity seen in photographic illustration (Fig 2).

H_2O_2: High levels of H_2O_2, an expression of oxidative stress has been linked to cataract formation. A significant increase in H_2O_2 is observed in Group I (7.41 ± 0.79 µmole/g) while comparatively low levels of H_2O_2 is seen in the lenses of Group II (2.89 ±0.01 µmole/g) (Table 2) (Fig 2).

Malondialdehyde: Lipid peroxidation occurs when free radicals sequester electrons from the lipids in cell membranes resulting in loss of membrane fluidity, increase in membrane permeability and decrease in physiological performance. All these autocatalytic changes culminate in cataractogenesis, mainly due to its cross-linking ability [28]. Lipid peroxidation measured in terms of malondialdehyde (MDA) levels showed a significant decrease by 92.1% (Table 2) with control exhibiting 6.04 ± 0.453 µmole/g in comparison to 0.475 ± 0.028 µmole/g of SE incubated lenses.

Reduced glutathione: Formation of any type of cataract results in reduction in the levels of reduced glutathione (GSH). It plays an important role as an antioxidant, stabilizes proteins and preserves lens clarity [29,30,31]. Many herbal extracts used as treatment for cataract work by inhibiting expenditure of GSH through oxidation thereby leaving the -SH groups intact. A decrease is levels of GSH is related to cataract formation. In the present investigation, the level of GSH in the lens homogenate of the toxic group (Group I) was found to be lower (21.04 ± 2.37 µg/g) in comparison to Group II (33.95 ± 2.42 µg/g) which was incubated in 100 µg/ml of SE (Table 2). This justifies the role of GSH in prevention of cataract formation and preservation of lens clarity (Fig 1).

Copper induced lipoprotein diene formation: This assay was performed to determine the antioxidant potential [32]. The decrease in values noted in Group II treated lenses is indicative of its effectiveness in reducing the amount of diene formed and protection of ocular tissue against oxidation (Table 2).

Table 2: Effect of *S. oleracea* on oxidative stress markers using isolated goat lens model

Parameter analysed	Toxic control	*S. oleracea*
Total protein (µg/g)	79.48 ± 4.072	98.61 ± 1.203
Malondialdehyde (µmole/g)	6.04 ± 0.4535	0.475 ± 0.028
Reduced glutathione (µg/g)	21.04 ± 2.379	33.95 ± 2.425
Hydrogen peroxide (µmole/g)	7.416 ± 0.79	2.893± 0.011
Copper induced lipoprotein diene formation	1.00 ± 0.00	0.295 ± 0.01
Glutathione reductase (IU/g)	0.27 ± 0.002	0.37 ± 0.04
Peroxidase (IU/g)	0.2414 ± 0.00	0.2921 ± 0.01

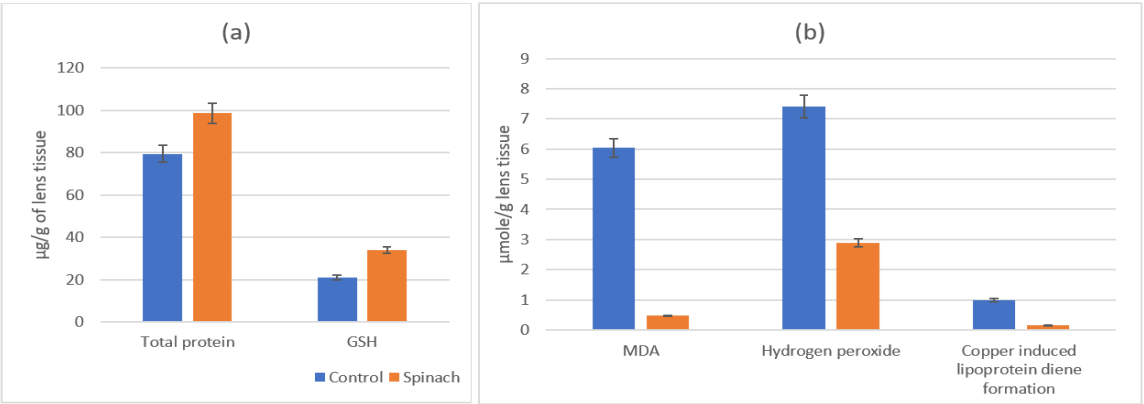

Figure 2: Effects of methanolic extract of *S. oleracea* leaves on total protein and reduced glutathione (A); malondialdehyde, hydrogen peroxide and copper induced lipoprotein diene formation on isolated goat lens. All values are mean ± SD, n =3.

Glutathione reductase (GR) and Peroxidase (POX): The H_2O_2 produced as a result of dismutation undergoes quenching via the CAT and POX system. CAT removes H_2O_2 through the formation of water and oxygen; whereas POX removes H_2O_2 by using it to oxidize GSH [33,34]. The disulphide bond of oxidized glutathione is reduced to GSH using POX enzyme [35]. The high activity of GR in eye lenses provides a robust antioxidant mechanism for survival under high concentrations of H_2O_2 [36]. Additionally, a GSH-dependent thioltransferase system such as GSH-S-transferase (GST) enzyme repairs lens protein oxidation by cleaving protein–thiol groups to maintain a reduced state [37]. All these mechanisms together, preserve protein–protein disulfide bonds, preventing crystallin aggregation and the consequent development of cataracts [35,38]. A statistically significant increase in glutathione reductase by 27.1% was recorded with *S. oleracea* group (Table 2) indicating that GR plays an important role is prevention of cataract. Similarly, a higher activity was noticed in SE treated cataract lenses (Table 2) indicating protection from damage by inhibiting lipid peroxidation of membrane lipids, and thereby reducing oxidative damage to structural proteins. This can be correlated to the reduction in MDA levels seen in SE treated lenses.

CONCLUSION

Oxidative stress aids in the development of glucose-induced cataract. The use of certain plant extracts is advocated to patients to delay or prevent formation of cataract. In isolated goat lenses with experimental glucose induced diabetic cataract, *S. oleracea* leaf extract showed significant antioxidant and anticataract properties. Further studies to access the used of spinach extracts in the prevention of diabetic cataract needs to be worked on even though the laboratory results indicate in a positive direction.

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