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Anticataract and antioxidant activities of *Coleus forskohlii* against *in-vitro* glucose-induced experimental cataract.

Pranay Soni and Surendra H. Bodakhe*
Department of Pharmacology, Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya (A Central University), Bilaspur (Chhattisgarh)-495009, India
***Corresponding Author:** drbodakhe@gmail.com

ABSTRACT
The aim of this study was to determine in vitro anticataract and antioxidant activities of Coleus forskohlii. C. forskohlii leaves were collected, washed, air dried, powdered and extracted with hydroalcoholic solvent (70% ethanol and 30% water) for 36 h. The extract was subjected to column chromatography using silica gel (60-120 mesh size), and eluted with the following solvents hexane: dichloromethane (DCM), DCM:ethanol and DCM:methanol (MeOH) in different ratios to isolate the active compounds. The fractionation of C. forskohlii extract revealed that compound B2 exhibited highest yield 210 mg. C. forskohlii extract (500 µg/ml) and three different concentrations of B2 i.e. B2a (10 µg/ml), B2b (25 µg/ml) and B2c (50 µg/ml) were used for evaluation of biological activities. Antioxidant activity was determined by measuring levels of oxidative stress markers such as catalase, glutathione, malondialdehyde, total protein and water soluble protein. Cataract reducing activity was evaluated by measuring lens opacity on goat lenses in glucose induced cataract model. Results indicated that both extract and isolated compound exhibited significant antioxidant and anticataract activities. Treatment with C. forskohlii extract significantly increased level of catalase (6.2 kU/L), glutathione (5.2 µmoles/g protein) as compared to control group. In contrast, MDA level was reduced by C. forskohlii extract to 31.2 nmoles/mg protein in comparison to control group. Isolated compounds B2a, B2b and B2c also exhibited effects on oxidative stress markers dose dependently. The opacity of lens was reduced significantly with the treatment of compound B2c, however, effect shown by the C. forskohlii extract was insignificant. Thus, C. forskohlii may be an effective herbal drug for the treatment and management of diabetes induced cataract.
Keywords: *Coleus forskohlii, glucose induced cataract, oxidative stress markers, goat lens, fractionation technique.*

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INTRODUCTION

Cataracts are prime cause of reversible blindness worldwide that affect lens transparency which is one of the most prevalent diseases of the lens. The clarity of the lens is required for refracting light onto the retina to get clear images [1]. Unfortunately, there is no drug available in the market for prevention or cure of cataracts. However, surgery is the only way of correction of the visual impairment as a result of cataract. Several drugs and formulation strategies are now being investigated to prevent cataract formation but these may not be devoid of side effects. Cataract develops due to aging, diabetes, general disease, congenital disorder or injury that leads to a decrease in vision. Symptoms of cataract include cloudy or blurred vision, glare, faded colors, poor night vision and double vision [2, 3]. According to the World Health Organization the number of cataract blind people will reach 40 million in 2025 [4]. Cataracts can be categorized into cortical, nuclear and posterior subcapsular cataracts according to the regions in the lens where the light scattering cataract first originates. Cortical cataract produces in lens cortex generally associated with diabetes; nuclear cataract is associated with ageing, which is further graded by its progression and color; and subcapsular cataract shows the sign of clouding, which is often associated with corticosteroids [5]. Nuclear cataracts are most common type and oxidative stress is known to contribute to the pathogenesis of age related nuclear cataracts (ARNC). It is due to excessive generation of free radicals such as reactive oxygen species (ROS) in old ages [6]. ROS can harm cell structures in several ways such as by causing damage to DNA strands; peroxidation of lipids and oxidation of proteins. Under normal physiological conditions, the lens is able to inactivate ROS by non-

enzymatic (glutathione) and enzymatic (catalase) antioxidant defense systems [7]. The clouding of the lens in cataract is associated with the depletion of antioxidants level and an impaired balance between soluble and insoluble lens proteins [8]. The delivery of antioxidants to the lens might be a solution with the aim of slowing down or preventing the formation of cataract [9]. Therefore, delaying in onset of cataract with medical therapies have been an area of interest for research; with the intention on increasing the antioxidant levels to minimize oxidative damage to the lens nucleus [10, 11].

Medicinal plants have been widely used in ancient traditional system of medicine are now-a-days being explored as the sources of raw materials for important drugs for use in modern medicine, neutraceuticals, food supplements, pharmaceutical formulations and as chemical entities for synthetic drugs [12]. *Coleus forskohlii* Briq. belongs to the mint family, *Lamiaceae* and grown in the subtropical climates in India, Nepal, Sri Lanka and Thailand [13]. It has been used since ancient times in Ayurvedic medicine due to its medicinal and therapeutic properties [14]. It has been used in the treatment of glaucoma, diabetes, hypertension, heart diseases, obesity, angina, cancer, depression, psoriasis, spasms and hypothermia [15]. *C. forkohlii* is rich in various kinds of alkaloids. It reported that the isolated forskohlin is believed to be most effective agent in *Coleus forskohlii* plant responsible for biological activity [16, 17]. Thus, in the present study *C. forkohlii* was evaluated against the experimental cataract using goat lens model.

MATERIAL AND METHODS

Collection of plant materials

Fresh leaves *C. forskohlii* was collected from Amarkantak region, Anuppur district of Madhya Pradesh, India during the month of December 2014. It was identified by the local healers and voucher specimen was deposited in this TCB College of Agriculture, Bilaspur (V.No. Speci./2014/12A).

Extraction of *C. forskohlii* leaves

Leaves of *C. forskohlii* were washed, air dried and powdered before extraction. The extraction was carried out using soxhlet apparatus with hydroalcoholic solvent (70% ethanol and 30% water) for 36 hours. The reddish-brown concentrate was obtained with 12.23% yield. The concentrate was heated over a waterbath to obtain a solvent free extract, which was stored in a refrigerator at 4^0C [18].

2.3 Isolation of compounds from the extract of *C. forskohlii*

The extract was subjected to column chromatography using silica gel (60-120 mesh size), and eluted with the following solvent ratios of Hexane: dichloromethane (DCM) i.e. 100:0, 80:20, 60:40, 40:60, 20:80, 0:100, then with 100:0, 90:10, 80:20, 70:30, 60:40, 50;50, 40:60, 30:70, 20:80, 10:90, 0:100, DCM:Ethanol (Eth). Finally eluted with 100:0, 90:10, 80:20, 70:30, 60:40, 50;50, 40:60, 30:70, 20:80, 10:90, 0:100, DCM:Methanol (MeOH). The fractions (25 ml) of final eluted solvents were collected from the column and monitored by thin layer chromatography for homogeneity in which eight different fractions (FB1-FB8) were collected and dried. The yield of fraction FB5, FB6 and FB8 was higher i.e. 253 mg, 360 mg and 400 mg. FB5, FB6 and FB8 were eluted with the solvent DCM:MeOH in the ratios of 3:2, 9:1 and 9:1 to get the compounds B1 (35 mg), B2 (210 mg) and B3 (150 mg), respectively. Based on high yielding of B2, it was further selected for biological activity. Figure – 1 represents the fractionation procedure of *C. forskohlii* for isolation of compounds.

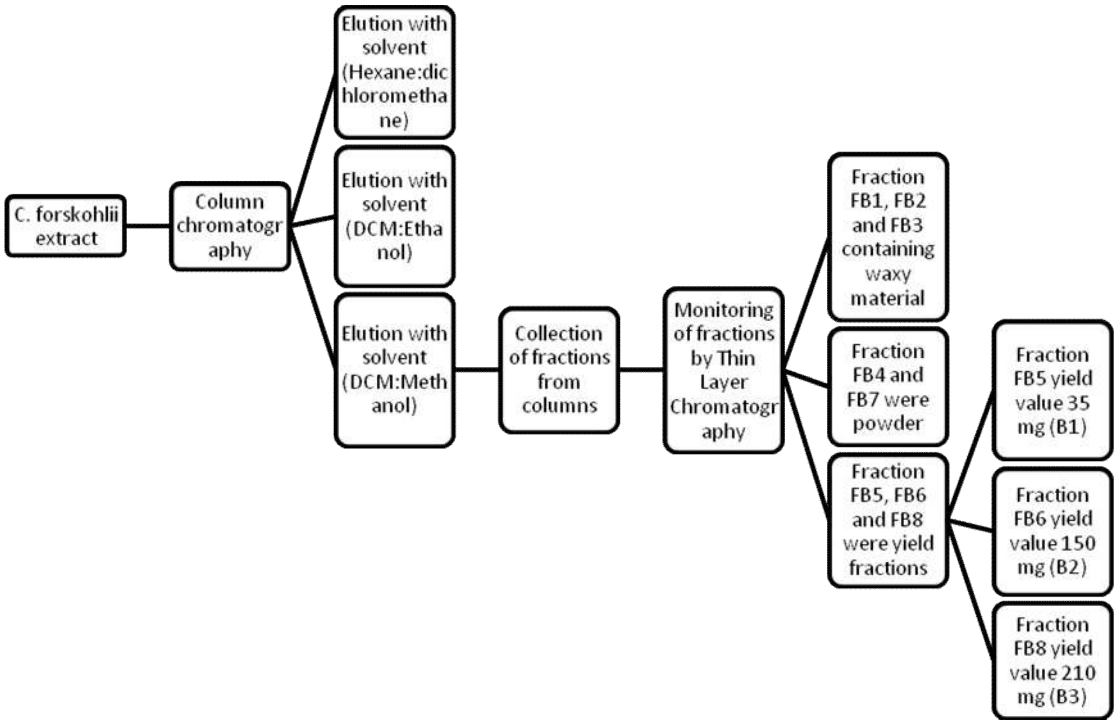

Figure – 1 Schematic representation of isolation of compound from *C. forskohlii* extract

In vitro* antioxidant activity of *C. forskohlii

DPPH reducing assay

The DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) reducing assay was performed by the method of Aadil et al, 2014, for the determination of free radical scavenging activity [19]. In the assay, 1 ml of 0.135 mM DPPH prepared in methanol was mixed with 50 µL of different concentrations (50-250 µg/mL) of extract. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm and scavenging ability of the *C. forskohlii* extract was calculated using the following formula:

$$\text{DPPH Scavenging activity (\%)} = \frac{(\text{Ab control} - \text{Ab sample})}{\text{Ab control}} \times 100$$

Where, Ab control = absorbance of DPPH + methanol; Ab sample = absorbance of DPPH radical + sample

Reducing power assay

This method was performed according to Oyaizu, 1986 [20]. It was based on the principle of measurement of absorbance of the reaction mixtures to determine antioxidant activity. The sample was taken in the fixed concentration of 150 µg/mL in a test tube containing 1 mL of phosphate buffer and 5 mL of 0.2 M phosphate buffer, pH 6.6. To this, 5 mL of 1% potassium ferricyanide solution was added and mixture was incubated at 50^0C for 20 min followed by addition of 5 mL of 10% TCA and the content was centrifuged at 1,000 rpm for 10 min. The upper layer of the supernatant (5 mL) was mixed with distilled water (5 mL) followed by addition of 1 mL of ferric chloride (0.1%) and vortexed then the absorbance of the reaction mixture was read spectrophotometrically at 700 nm using ascorbic acid as standard.

***In vitro* anticataract activity**

Lens culture

Goat eyes were collected from the slaughterhouse of Bilaspur that was immediately carried to the laboratory at 0-4 then lenses were removed by extracapsular extraction and incubated in artificial aqueous humor [NaCl (140 mM), KCl (5 mM), $MgCl_2$ (2 mM), $NaHCO_3$ (0.5 mM), $NaH(PO_4)_2$ (0.5 mM), $CaCl_2$ (0.4 mM) and glucose (5.5 mM)] maintained at pH 7.8 in room temperature for 72 h. The culture media was maintained antiseptic to prevent bacterial contamination by addition of penicillin (32 mg) and streptomycin (250 mg) [21].

Induction of *in vitro* cataract

Cataract was induced by addition of glucose (55 mM) in the culture media for 72 h according to Ganeshpurkar *et al*, [22]. Goat lenses were divided into six groups each containing six lenses. Group I (vehicle control) received 5.5 mM of glucose, Group II (toxic control) received 55 mM of glucose, Group III

(*C. forskohlii* extract, 500 µg/ml), Groups IV to VI received isolated compound B2 in three doses 10, 25 and 50 µg/ml, designated as B2a, B2b and B2c, respectively.

Evaluation of lens opacity

The opacity in lens was evaluated after 72 h of incubation by placing lenses on a graph paper with posterior surface touching the paper and the pattern of graph (number of squares clearly visible through the lens) was observed. The degree of opacity was graded as 0, absence; +, slight degree; ++, diffuse opacity and +++, extensive thick opacity [22].

Oxidative stress markers

The oxidative markers molecules are modified by interactions with reactive oxygen species (ROS). It can be studied by measuring cellular antioxidants enzymes such as catalase, reduced glutathione, lipid peroxidation, and protein estimation [23].

Reduced glutathione (GSH)

Total reduced glutathione content was measured by the method given by Ellman, 1959 [24]. Lens homogenate (0.5 ml) was deproteinized with 5 % trichloro acetic acid (3.5 ml) and centrifuged. The supernatant (0.5 mL) was mixed with Ellman's reagent and 3.0 ml phosphate buffer. It lead to development of yellow color that was read at 412 nm. A series of standards (10-60 pg) were treated in a similar manner with a blank and values were expressed as µmoles/g of lens protein.

Lipid Peroxidation

It was determined by assaying malondialdehyde (MDA) formation according to the method of Ohkawa et al, [25]. In this assay, 4 ml of tert-butyl alcohol and 2 ml of trichloro acetic acid were added to 1 ml of 10 % lens homogenate then heated in a water bath for 30 min followed by cooling and centrifugation. The absorbance of the supernatant was read at 535 nm. The extent of lipid peroxidation was expressed in nmoles of MDA formed/l0 mg lens using a molar extinction co-efficient of MDA as $1.56 \times 10s\ M^{-1}CM^{-1}$.

Protein estimation (Total and Soluble proteins)

The protein estimation assay was performed according to the method of Lowry, [26]. In this assay, 10% lens homogenate was centrifuged (16,000 rpm) with 50 mM potassium phosphate buffer at pH 6.2 and the supernatant was used to determine soluble protein while whole homogenate was used for determination of total protein in which 10 µl of 10% lens homogenate was made upto 1ml with distilled water then 5 ml of alkaline solution was added, mixed thoroughly and allowed to stand at room temperature for l0 min. For protein estimation, 0.5ml of Folin-Ciocalteau reagent was added that lead to development of color after 30 min and read at 610 nm. A series of standards (40-200 µg) were also treated in a similar manner along with a blank. Protein content of lens was expressed as mg/g lens.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 software (GraphPad Prism Software, Inc., USA). The results were expressed as mean ± SEM.

RESULT AND DISCUSSION

In vitro* antioxidant activity of *C. forskohlii

C. forskohlii extract strongly scavenged DPPH radical in a dose dependent manner, highest activity was exhibited at concentration of 250 µg/mL i.e. 85.26% (Table – 1). However, the radical scavenging activity of standard drug ascorbic acid (50 µg/mL) was found to be 92.14%. Plans possess enzymatic antioxidant activity as an intrinsic defense tool to resist oxidative damage [27]. Similar result was found in antioxidant activity of C*. forskholii* reported by Khatun *et al,* [28]. Free radical-scavenging is one of the known mechanisms through which antioxidants inhibit lipid oxidation [29]. Likewise, the extract exhibited reducing property as antioxidant activity (26.43%), read at absorbance 700 nm (Table – 2). The presence of reducers (the antioxidants) causes the conversion of the Fe^{3+} /ferricyanide complex to the ferrous form. Researchers reported that reductones are the agents possess reducing properties by breaking the free radical chain by donating a hydrogen atom [30]. Accordingly, the leaves of *C. forskohlii* might contain higher amount of reductone that could react with free radicals to stabilize and block radical chain reactions. It has been reported by many researchers that plant extracts rich in polyphenols and essential phytoconstituents have been shown to exert potent antioxidant and free radical scavenging activities in various antioxidant models [31-33].

Table – 1 Free radical scavenging capacity of *C. forskohlii* extract

Concentration (µg/mL)	DPPH Scavenging %	
	Ascorbic Acid	*C. forskohlii* extract
50	92.14±0.63	20.17±1.02
100	-	36.51±1.23
150	-	59.62±1.05
200	-	68.45±0.45
250	-	85.26±0.63

Data are represented as mean ± SEM (n=6)

Table – 2 Reducing activity of *C. forskohlii* extract

Particulars	Concentration (µg/mL)	Absorbance at 700 nm	Antioxidant activity (%)
Ascorbic acid	50	0.749±0.03	93.54
C. forskohlii extract	150	0.198±0.04	26.43

Data are represented as mean ± SEM (n=6)

3.2 *In vitro* anticataract activity of *C. forskohlii*

3.2.1 Effect of *C. forskohlii* on lenticular opacity and photographic evaluation

Lenses incubated in glucose 5.5 µM remained transparent (normal group), whereas, the lens incubated in 55 µM glucose developed dense opacities (toxic control group). The opacity increased towards center with complete opacification at the end of 72 h. Treatment with *C. forskohlii* extract did not show significant results (Table – 3). However, treatment with isolated compound B2 caused retardation of development of opacity as shown in Figure – 2, which was significant with B2c (50 µg/mL). Phytochemicals are bioactive principles in plants that have been widely implicated in ameliorating vast array of diseases whose pathogenesis are remotely or directly connected with oxidative stress [34, 35]. Glycemic control through varieties of mechanisms such as modulating the activity of enzymes related to antioxidant and anticataract activities [36, 37]. It has reported that accumulation of high levels of fructose in the lens fibers and epithelium leading to osmotic swelling of the tissue and reduction in lens opacity [38].

Table – 3 Effect of *C. forskohlii* on glucose induced cataractogenesis in goat lens

S.No.	Groups	Treatment	Degree of opacity
1.	Normal	5.5 µM glucose	0
2.	Toxic Control	55 µM glucose	+++
3.	*C. forskohlii*	500 µg/ml extract	**+++**
4.	B2a	10 µg/ml fraction	**+++**
5.	B2b	25µg/ml fraction	**++**
6.	B2c	50 µg/ml fraction	**++**

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

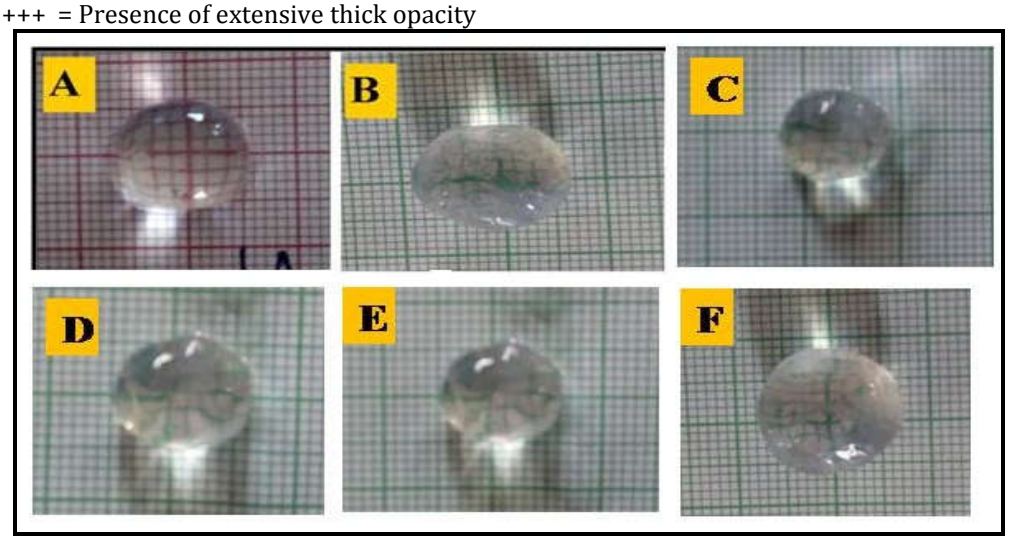

Figure – 2 Effect of *C. forskohlii* on glucose induced cataract in goat lens

A-Normal, B-Toxic control, C- *C. forskohlii* extract treated group (500 µg/ml), D- B2a treated group (10 µg/ml), E- B2b treated group (25 µg/ml), F- B2c treated group (50 µg/ml)

Effect of *C. forskohlii* on oxidative stress markers

The lens catalase and glutathione level in glucose (55 µM) treated lens were significantly decreased as compared to normal control group (Table – 4). In contrast, MDA level was found to be very high in glucose treated lens compared with normal lens. Treatment with *C. forskohlii* (500 µg/mL) exhibited significantly increased level of catalase (6.2 kU/L) as compared to control group. However, isolated compounds raised the level of lens catalase B2a (3.4 kU/L), B2b (4.7 kU/L) and B2c (5.9 kU/L) dose dependently. Likewise, glutathione level was raised with treatment of *C. forskohlii* extract (5.2 µmoles/g) and isolated compound B2a (3.2 µmoles/g), B2b (4.4 µmoles/g) and B2c (5.6 µmoles/g) dose dependently. However, MDA level was reduced by *C. forskohlii* extract to 31.2 nmoles/mg, which was significant as compared to toxic control group (61.0 nmoles/mg). Similarly, B2a (47.1 nmoles/mg), B2b (39.0 nmoles/mg) and B2c (28.1 nmoles/mg) reduced MDA level dose dependently (Table – 4). Herbal molecules have been tried as ocular aliments in the pathogenesis of many major eye diseases, including glaucoma, and cataracts [39]. Lipid peroxidation is the process in which free radicals steal electrons from the lipids in cell membranes that may cause loss of membrane fluidity, increase in membrane permeability and decrease in physiological performance, thus endangering cell viability [40]. The lens protein level (total and water soluble) in glucose treated groups was reduced as compared to normal control group. Treatment with extract and isolated compound exhibited rise in the levels of total protein and water soluble protein as shown in Figure – 3. The total protein level and water soluble protein was significantly restored by B2c i.e. 174.1 mg and 70.1 mg, respectively (Figure – 3).

Table – 4 Effect of *C. forskohlii* on oxidative stress markers using isolated goat lens model

Groups	Catalase (kU/l)	Glutathione (µmoles/g)	Malondialdehyde (nmoles/mg)
Normal	8.2±0.10***	7.7±0.21***	3.3±0.17***
Toxic control	3.5±0.13	2.6±0.13	61.0±0.62
C. forskohlii	6.2±0.12***	5.2±0.10***	31.2±2.40***
B2a	3.4±0.15	3.2±0.42	47.1±1.40***
B2b	4.7±0.22***	4.4±0.21***	39.0±2.10***
B2c	5.9±0.21***	5.6±0.33***	28.1±2.60***

Values are expressed mean±SEM, n=6, significantly different at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ as compared with their corresponding value in control group

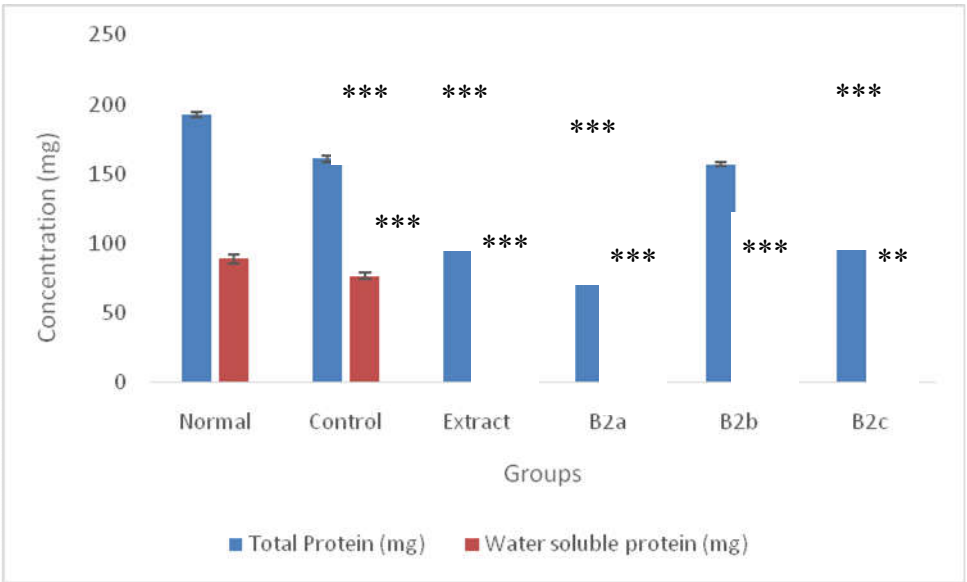

Figure – 3 Protein estimation of *C. forskohlii* on goat lens

Values are expressed mean±SEM, n=6, significantly different at $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ as compared with their corresponding value in control group

CONCLUSION

It can be concluded that hydroalcoholic extract of *C. forskohlii* and isolated compound produced significant antioxidant activity and could alleviate the oxidative stress induced cataract. It can be used as a nutrition supplement to reduce the risk of cataractogenesis.

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