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Research Article

In Vitro Antioxidant and *Ex Vivo* Anticataract Activity of Hydroalcoholic Extract of *Cordia obliqua* Willd

Arpit Shrivastava[1*], Harshita Jain[1], Shikha Mishra[1], Basant Khare[2], Prateek Kumar Jain[2]

[1*]*Adina Institute of Pharmaceutical Sciences, NH86A, Lahdara, Sagar, MP, 470001*

[2]*Adina College of Pharmacy, ADINA Campus Rd, Lahdara, Sagar, MP, 470001*

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*Address for Correspondence:

Arpit Shrivastava, Adina Institute of Pharmaceutical Sciences, NH86A, Lahdara, Sagar, MP, 470001

Abstract

Cordia obliqua has a long history of use in the treatment of cataract and other eye-related problems in Indian traditional medicine. High oxidative stress is one of the major underlying causes of cataract which results in the precipitation of natural protein present in the lenses with aging. This research has been carried out to determine the anti-cataract activity of *Cordia obliqua* by performing various antioxidant techniques such as 1,1-diphenyl-2-picrylhydrazyl, hydrogen peroxide, ABTS radical cation and Ferric reducing antioxidant potential studies in oxidative stress–induced ex vivo cataract model. Results of the study conducted in the hydroalcoholic extract of leaves of *Cordia obliqua* revealed the presence of various phytoconstituents such as alkaloids, phenols, flavonoids, etc. Total phenol and total flavonoid content was found to be 53 ± 3.21and 26.12 ± 2.54% respectively, which revealed that the plant contains a good amount of these compounds and hence possesses good antioxidant activity. Furthermore, IC_{50} values obtained from all the methods gave strong evidence regarding the antioxidant potential of this plant. Anti-cataract activity was also investigated using goat eye lenses and promising results were obtained which speak voluminously about its anti-cataract potential and support its well-prescribed use. Results obtained with this study clearly supported the significant antioxidant potential and anticataract activity of this plant. Further, this plant demands great attention for the development of suitable novel dosage forms for the effective treatment of cataract.

Keywords: *Cordia obliqua,* cataract, High oxidative stress

INTRODUCTION

Cataract is the opacification or optical dysfunction of the lens, associated with the breakdown of the lens micro-architecture, which interferes with transmission of light onto the retina. It is the most important cause of blindness worldwide and the total number of persons with cataracts is estimated to rise to 30.1 million by 2020[1]. Numerous biochemical processes such as oxidative stress, higher glycosylated hemoglobin due to diabetes mellitus, altered epithelial metabolism, calcium accumulation, calpain-induced proteolysis, crystalline precipitation, phase transition, and cytoskeletal loss levels are significantly associated with increased the development of cataract. Oxidative stress associated with diabetes may play an important role in the initiation and progression of diabetic cataract. The toxic effects of reactive oxygen species (ROS) or free radicals are neutralized in the lens by antioxidant systems[2,3]. The antioxidant defense system is composed antioxidant enzyme and biological antioxidants former include major enzyme like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and latter are reduced glutathione (GSH) vitamin C, and vitamin E[4]. This antioxidant system collectively prevents lens damage and subsequent development of cataract due to oxidative stress[5]. Although a number of agents have been tried for effective management of cataract butnone have proved useful[6]. The epidemiological studies have demonstrated a reduce risk of developing cataract in person who consume a diet with high content of nutritional antioxidants. These foodstuffs contain essential vitamins, micronutrients, flavonoids and carotenoids which either alone or in combination attributes to their anticataract activity[7]. *Cordia obliqua* (family Boraginaceae) is a medium-sized tree, found scattered throughout the mid Himalayas up to elevations of 1,470 meters South Asia, China and America. It is used as a functional food and dietary component in Indian subcontinent[8]. The *Cordia obliqua* are reported for presence of taxifolin, α-amyrin, lupeolhesperetin 7-rhamnoside, taxifolin-3-rhamnoside, β-sitosterol and 3, 4, 5, 7-tetrahydroxy flavonone-3-O-β-D-xylopyranoside. The *Cordia obliqua* leaves used in Indian traditional medicine for the treatment of ophthalmic and other eye infections, it also has astringent, nephroprotective, hepatoprotective, expectorant[9]. *Cordia obliqua* is well reported for its anti-inflammatory, antinociceptive and cytotoxic activities. Therefore the present study was under taken to investigate the *ex vitro* anticataract potential of *Cordia obliqua* against glucose induced cataract in goat eye lenses along with this an attempt has been also tried to explore it's in vitro antioxidant potential by using several antioxidant assays. In addition the effect of *Cordia obliqua* on various biochemical parameters was also observed to elucidate the mechanism of protection.

MATERIAL AND METHOD

Chemicals and reagents

2,4,6-tripyridyl-s-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2' azobis(2-amidinopropane) dihydrochloride (AAPH),β-phycoerythrin (β-PE), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox),Triton X-100, bovine serum albumin (BSA) and *Folin- Ciocalteau* reagents were purchased from SigmaSt. Louis, MO, USA). Thiobarbituric acid (TBA), trichloroacetic acid (TCA), potassium chloride, sodium chloride, sodium bi-carbonate, sodium phosphate, and calcium chloride were purchased from Central Drug House, New Delhi India

Extraction and phytochemical screening

The fresh leaves of *Cordia obliqua* were collected from local area of, Jabalpur in October 2012. The material was identified and authenticated by Dr. P.K. Tiwari, Senior scientist and Botanist, Department of Botany, Hari Singh Gour Central University, Sagar, M.P., INDIA and the voucher specimen number Bot/Her/B/3110was deposited. The leaves were air-dried and made into powder with the aid of local mortar and pestle. Extract was prepared by taking 100 g of *C. obliqua* leaf powder and soaking it in solvent consisting of 95% of ethanol and water (1:1) for 72 h. The residue was filtered by whatman filter No. 4, and the extraction solvent was removed under reduced pressure by rotary vacuum evaporator (Super fit, India) at 50°C, which gave a Greenish-brown residue. Then this hydro alcoholic leaves extract of*Cordia obliqua*(HECO) was freeze dried and stored at 6°C until use and subjected to qualitative as we as quantitative phytochemical estimation[10].

Determination of total phenolics

The total phenolic content of extract was determined as per the reported Folin-Ciocalteau method (Singleton and Rossi, 1965). In brief, 200 µl of diluted extract was added to a test tube and then mixed with 500 µl of Folin-Ciocalteau reagent (1:10). Thirty seconds later and just prior to 8 min, 800 µl of Na_2CO_3 (7.5%) was added. The reaction mixture was incubated at 24°C and absorbance of mixtures was traced at 750 nm against blank. The standard curve was prepared by 1, 10, 100 and 200 mg/Solutions of gallic acid in ethanol: water (50:50 v/v) solvent. The values of total phenolic were estimated by comparing the absorbance of each with those of a standard response curve generated with gallic acid and the total phenolic content was expressed as gallic acid equivalents per mg of HECO[11].

Determination of total flavonoids

The concentrated extract was again exhaustively defatted by refluxing with *n*-hexane and benzene (twice for 15 h). HECO (0.5 ml of 1:10 g/ml) in ethanol was separately mixed with 1.5 ml of ethanol, 0.1ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min. A dark yellow color indicated the presence of flavonoids. The absorbance of the reaction mixture was measured at 415 nm[12].

Phytoanalytical studies

HPLC analysis

HPLC analysis was performed by following the previously reported method with slight modification[13]. In brief, for this purpose Shimadzu LC-20A apparatus coupled with a semiautomatic injector (SIL-20A, Shimadzu) and UV/VIS detector (SPD-20A, Shimadzu), and spin-chrome CFR software was used. A Shim-pack ODS column luna C-18 (5 µm, 4.6/250 mm; Shimadzu) was employed coupled to the respective guard-column, using the following elution profile: 0–10 min: 2% B (isocratic), 10–100 min: 2–20% B (linear gradient), 90–120 min: 20–100% B (linear gradient); solvents: A = aqueous acetic acid 2% (v/v); B = Me CN with 2% acetic acid (v/v). Flow rate: 1 ml/min. UV detection between 200 and 750 nm.

In vitro antioxidant activity of *Cordia obliqua* extract

DPPH (2, 2 -Diphenyl-1-picryl-hydrazyl) method

The antioxidant activity of the various extractives of the selected plants was assessed on the basis of the radical scavenging effect of the stable DPPH free radical. Each of the extractives and the standards were dissolved in DMSO to prepare 100 µg/ml solutions. From the above stock solution further dilutions were made to get different dilutions such as 20µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml 100 µg/ml and absorbance was recorded at517 nm[14, 15].

H_2O_2 radical scavenging method

Hydrogen peroxide is one of the least reactive molecules among the reactive oxygen species.Underphysiological pH and temperature it is generally stable in the absence of metal ions.The hydroethanolic extract was dissolved in water and ethanol (1:1) to prepare 100 µg/ml solutions. From the above solution further dilutions was made to get different dilutions such as 20, 40, 60, 80 and 100 µg/ml. Absorbance was recorded by using UV-visible spectrophotometer at 230 nm[14, 15].

ABTS radical cation method

This method involves the scavenging of ABTS [2, 2' azinobis (3- ethylbenz-thiazoline-6-sulfonic acid) di-ammonium salt] radical cation. The principle behind the technique involves thegeneration of ABTS radical cation, a blue green chromogen which is produced by a reaction between ABTS and potassium persulphate. The coloured radical is converted to colourless ABTS in the presence of antioxidant reductant. The absorbance of this colourless ABTS is measured at 734 nm[14, 15].

Ferric reducing antioxidant potential (FRAP)

Transformation of Fe^{3+} to Fe^{2+} in the presence of extractives under study was taken as the parameter to study the measurement of the reductive ability. 10 mg of test (extract) and standard solution (ascorbic acid) were weighed accurately and dissolved in 100 ml DMSO to get 100 µg/ml solutions. These solutions were diluted with DMSO to obtain 20, 40, 60, 80 µg/ml and 100 µg/ml solutions, and have separate aliquots for each solutions[14, 15].

In vitro anti cataract activity

Lens culture

Fresh goat eyeballs were obtained from local slaughter house immediately after slaughter and transported to the laboratory at 0-4°C. The lenses were isolated by extra-capsular 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) at room temperature and pH 7.8 for 72 hr. Penicillin 30 mg % and streptomycin 200 mg % were added in culture media to prevent bacterial contamination[16].

Induction of cataract

Glucose in a concentration of 55 mM was used for the induction of cataract. At high concentrations, glucose in the lens was metabolized through sorbitol pathway and accumulation of polyols (sugar alcohols), causing over-hydration and oxidative stress. This led to cataractogenesis[16].

Experimental design and groups

For the purpose of in vitrocataract study sixteen extra-capsular extracted lenses were divided into four groups of four lenses in each group incubated as follows:

Group I: Glucose 5.5mM (Normal Control)

Group II: Glucose 55mM (Toxic control)

Group III: Glucose 55 mM + extract 200 µg/mL

Group IV: Glucose 55 mM + extract 400 µg/mL

Biochemical estimation of lens after treatment

Protein estimation

Intended for total protein estimation the lens homogenate was prepared in 5% TCA. The precipitated protein was dissolved in sodium hydroxide and used as aliquots for the estimation of total proteins. Soluble and insoluble fractions of the proteins were estimated by preparing lens homogenate in distilled water. The water soluble supernatant was used for estimation of soluble protein and the residue was dissolved in sodium hydroxide and used for the estimation of insoluble protein. The protein content of the samples was determined by the method of using BSA as the standard.

Glutathione estimation

For the estimation of glutathione in treated lens, the lens was taken on filter paper, dried and homogenized in 1 ml of 10% trichloroacetic acid solution (TCA) using tissue homogenizer. The homogenate was centrifuged (5000 rpm) for 15 min and 0.8 ml of supernatant was taken and mixed with 1.79 ml of Tris HCI buffer containing 0.2 M EDTA (pH 8.2) and 30 µl of Ell man's reagent. The final volume was brought to 3 ml with 0.05 M EDTA and the absorbance of the solution was measured immediately at 415 nm[17].

Catalyse estimation

Lens catalase activities were determined by Goth's colorimetric method, in which serum was incubated in H_2O_2 substrate and the enzymatic reaction stopped by the addition of ammonium molybdate. The intensity of the yellow complex formed by molybdate and H_2O_2 was measured at 405 nm[18].

Estimation of malondialdehyde

The lipid peroxide formed was estimated by measuring thiobarbituric acid reacting substances (TBARS). For this, 0.4 ml of the incubation mixture (as prepared as same under protein estimation) was treated with sodium dodecyl sulphate (8.1%, 0.2 ml), TBA (0.8%,1.5 ml) and acetic acid (20%, 1.5 ml, and pH 3.5). The total volume of the mixture was then made up to 4 mL by adding distilled water and the reaction tube was kept in a water bath at 100 °C for 1 h. After cooling, 1 ml of distilled water and 5mL of a mixture of n-butanol and pyridine (15:1, v/v) were added, and this was shaken vigorously. Subsequent to centrifugation, the absorbance of the organic layer was measured at 532 nm. The % of lipid peroxide-scavenging ability of the extract was calculated.

Lens water content

For the detection of lens water content, the lens was taken out and fresh weight was estimated. Lens was then dried in an oven at 110°C till constant dry weight was obtained. The differences in the weights were used as an index of the percentage water content in that lens[19]. **Morphophotometric evaluation of lenses**

Lenses were placed on a wired mesh with posterior surface touching the mesh, and the pattern of mesh (number of squares clearly visible through the lens) was observed through the lens as a measure of lens opacity.

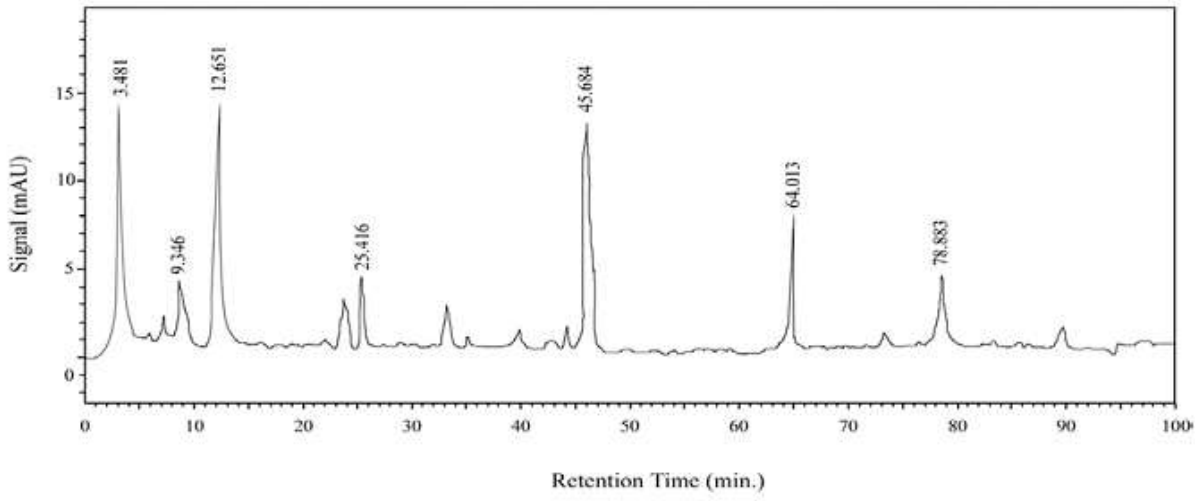
Statistical analysis

Studies were performed in triplicate. Data were expressed as mean ± SEM. Multiple comparisons Test by employing statistical software. Differences between groups were considered significant at $P < 0.05$ levels.

RESULTS

HPLC

Cordia obliqua is extensively used in the traditional system of medicine in India and other countries for treating abdominal pain, diarrhea, wound healing, fever, constipation, blood disorders and acne. The plaster of leaves is applied for curing inflammation locally. Reports of phytochemical investigation of *C. obliqua* have previously demonstrated the presence of phenolic compounds such as catechin, procyanidin B2, epicatechin. Other constituents like tartaric acid, mucilage, pectin, arabinose, xylose, galactose, glucose, uronic acid and triterpenes have also been identified in *C. obliqua*.. An attempt has been made through the membrane stabilizing property of leaves extract to investigate the ability of *C. obliqua* in the treatment of inflammatory disorders and its antinociceptive action.



HPLC-UV analysis of the hydroethanolic extract of *C. Obliqua* leaves

Flavonoids and total phenolic contents by HECO

The HECO showed the presence of flavonoids and polyphenols (Table 1 and 2). The results were in accordance with the previously reported data.

Table1 Phytochemical composition of HECO

Sr. No.	Constituents	Hydro alcoholic extract
1.	Flavonoid	+
2.	glycosides	+
3.	alkaloids	+
4.	Carbohydrate	+
5.	protein and free amino acids	+

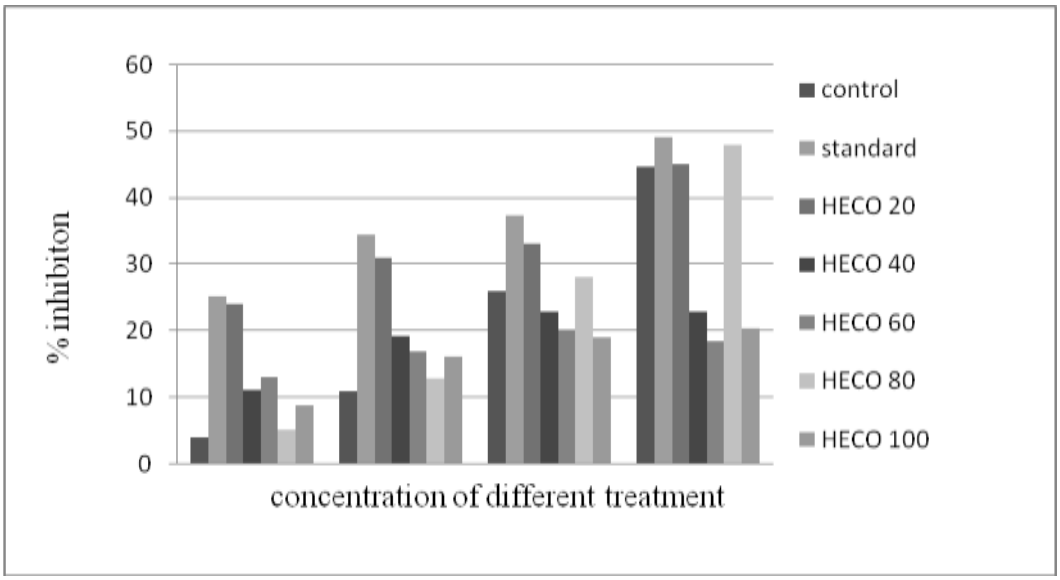
Table 2 Flavonoids and total phenolic contents HECO

Extract	Flavonoids (mg/g)	Polyphenols (mg/g)
Hydro ethanolic extract of *Cordia obliqua* (HECO)	26.12 ± 2.54	53 ± 3.21

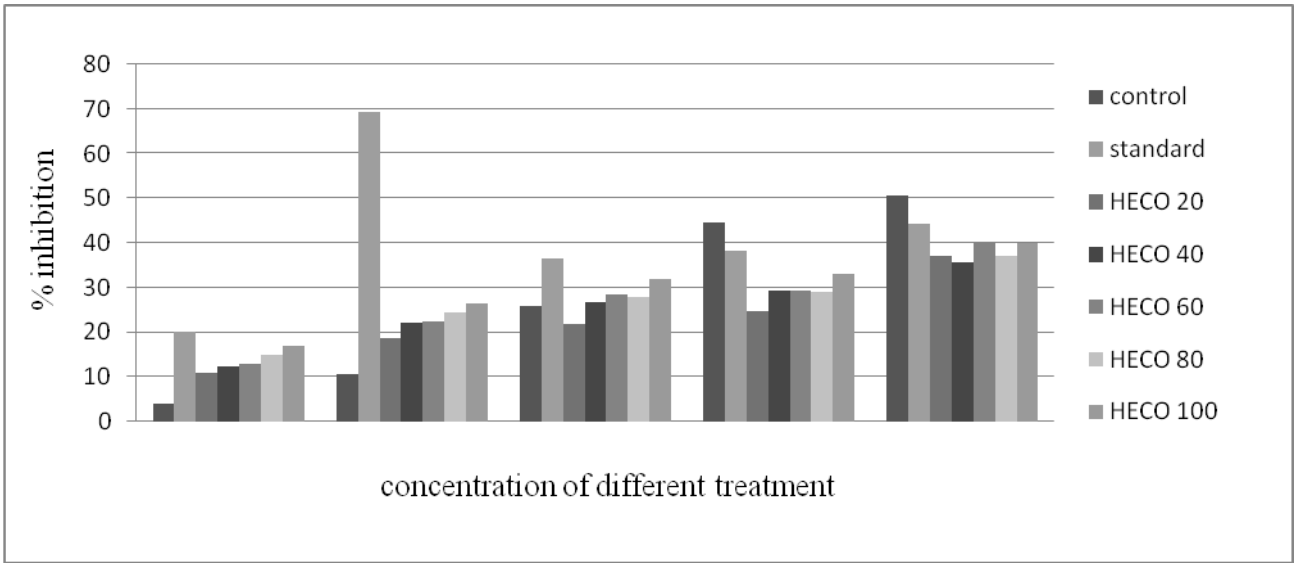
Values Were Expressed As Mean ± S.E.M. (n=3).

Antioxidant activity

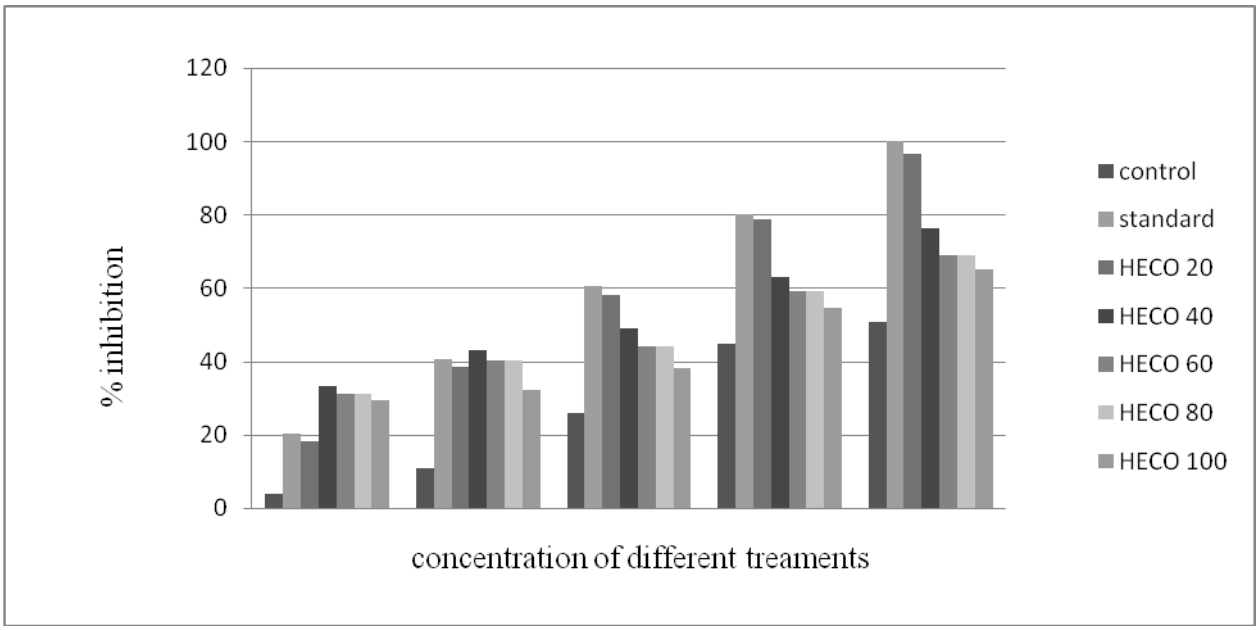
Ferric reducing activity



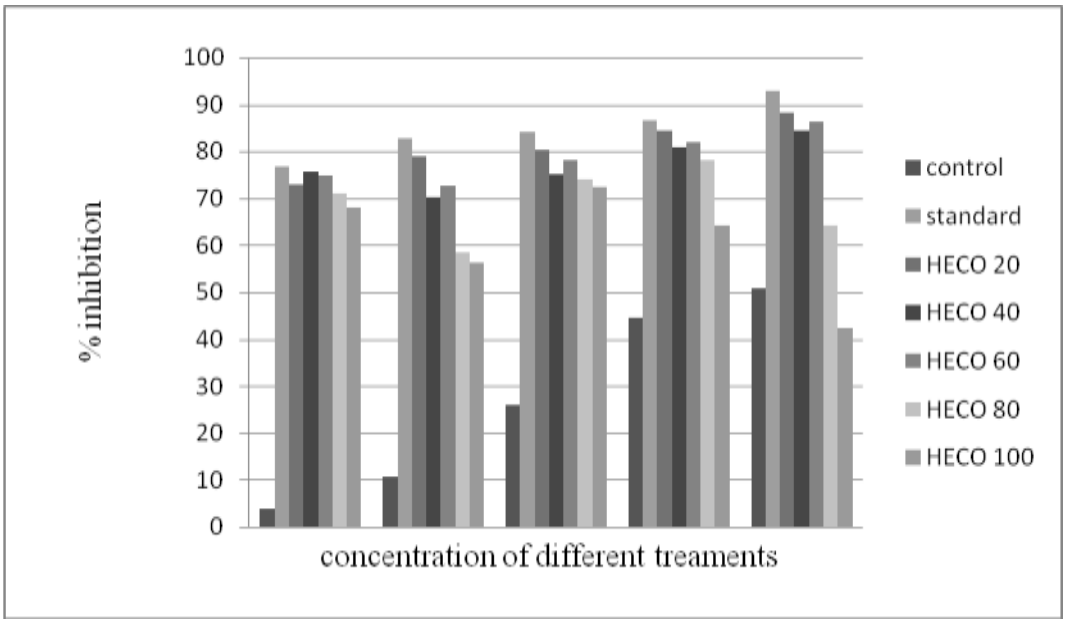
DPPH activity



H_2O_2 Activity



ABTS Activity



In vitro anti cataract activity

Protein content

The lens protein level of glucose (55µM) treated lens (Group II) showed significant decrease($P<0.005$) as compared to normal control group(Group I).HECO extract at the concentration of 250µg/ml and 500 µg/ml showed significant increase ($P<0.05$) in lens protein as compared to toxic control (Group II).

Lens glutathione level

The lens glutathione level of glucose (55µM) treated lens (Group II) showed significant decrease ($P<0.005$) as compared to normal control group (Group I) HECO extract at the concentration of 250µg/ml and 500 µg/ml showed significant increase ($P<0.05$) in lens glutathione as compared to toxic control (Group II). Table only treated group (Group IV) showed almost the same levels of glutathione as that of control.

MDA Level

MDA Levels were found to be very high in glucose 55mM treated lens compared with normal lens (61.77 vs. 2.78).Lens treated with HECO extract had significantly reduced MDA content ($P<0.05$) at both concentrations compared with high glucose group.

Table 3: Glutathione and MDA Content

Group	Catalase	Glutathion	MDA	Total protein
I	8.450±0.156***	7.982 ±0.128**	3.072±9.964*	195.568±0.289**
II	3.908±0.175	5.768±0.259	60.774±17.473	160.248±7.721
III	6.370±0.121***	6.310±0.121**	47.114±8.323	171.878±5.077*
IV	7.712±0.230***	7.158±0.129**	29.782±6.944*	187.168±2.663*

Water content

To determine lens water content, lenses were dried to constant weight in an oven at 105 °C. After culture for 3 days we found no significant difference between cultured and fresh lenses in water content, indicating that loss of water could not account for the lack of weight increase in the cultured lenses.

Morpho-photographic evaluation

The entire lens incubated in Glucose 5.5µM remained transparent. However, the lens incubated in 55µM Glucose developed dense opacities. The opacity progressively increased towards center with complete opacification at the end of 72 hours. Incorporation of HECO extract (250µg/mL and 500µg/mL) retarded the development of opacity values are mean±SD, n=5 for each group *P< 0.05, **P<0.005 and ***P<0.001 as compared with their corresponding value in glucose 55mM group.

Lens catalase levels

In this study, incubation by sugars resulted in a time dependent inactivation of the enzymes. Lens catalase activities were also significantly lower in the lens of Group II as compared to extract treated groups (Group III and IV).

DISCUSSION

This work supports the possibility that *Cordia obliqua* reduce the oxidative stress and couldprotect diabetes mellitus patients against hyperglycemia-mediated lens damage. Furtherresearch is necessary to determine if the ingestion or administration of this plant extracts could partially abrogate diabetes mellitus chronic complications via anti-diabetic and/or antioxidant effects Cataract is one of the universal processes of ageing and results due to the cumulative effect of various insults to the lens. The oxidation of lens proteins by free radicals and reactive oxygen species play an important role in the multifactorial process leading to lens opacification. Biochemical evidence suggests that oxidation of the lens proteins is involved in the genesis of senile as well as diabetic cataracts. Sugars are one of the well-known cataractogenic agents. Several reports suggest that the cataractogenic effect of the sugars in diabetes as well as in normal aging is initiated by the glycation of the proteins including the enzymes and subsequent formation of more complex and biologically inactive or harmful structures. In this study the level of catalase was found to be less in Group II when compared with normal (Group I).The lens treated with *Cordia oblique* showed significant rise in enzyme level suggesting maintenance of antioxidant enzyme integrity.The amount of Reduced Glutathione in the lens decreases almost in any type of cataract. The role of GSH in the maintenance of lens clarity is of considerable interest; it serves as the major anti-oxidant in the lens and keeps proteins in reduced form[20]. Phytoconstituents from herbal drugs may indirectly inhibit consumption of GSH through oxidation leaving the –SH groups intact. Alternatively, they may directly stimulate Reduced Glutathione synthesis which may be due to a modulating effect on Reduced Glutathionerelated enzymes in the lens [21]. The preventive role of these drugs has also been substantiated by the estimates of lens water content. In this study the levels of MDA were more in Group II when compared with Group I, III, and IV which suggest preventive role against cataract. Flavonoids are most commonly known for their antioxidant activity. Flavonoids have been shown to possess many properties; they inhibit a number of enzymes such as aldose, reductase, xanthine oxidase, phosphodiesterase, Ca^{2+}, ATPase, lipoxygenase, cycloxygenase. They also have a regulatory role on different hormones like estrogens, androgens and thyroid hormone. In vitro anti-cataract effect of *Cordia oblique* on isolated goat lenses incubated in a high glucose medium was dose-dependent. This effect was manifested as amelioration of glucose-induced lens opacity. This study shows that antioxidant enzymes like catalase protect the various tissues of the body against oxidative damage. Hence we can conclude that oxidative stress is an important factor in the development of diabetic cataracts and the use of antioxidants[22] may be advocated in patients to delay or prevent formation of cataract. In conclusion, *Cordia oblique* showed in vitro activity against glucose induced cataract in isolated goat lens model. This effect may be attributed to maintaining higher levels of GSH as well as inhibiting the accumulation of polyols in the lens. This preliminary study is encouraging, but further studies are required to extrapolate the use of this agent in humans.

CONCLUSION

The obtained results of the study clearly show the high antioxidant potential of the plant *Cordia obliqua* and support its well-prescribed and extensive use in the treatment of cataract. The findings support a protective role of *Cordia obliqua* in pathologies involving oxidative stress, namely cataract. Our findings further corroborate the substantial presence of phenolic and flavonoid content signifying the plant to be a promising contender in treating cataract. However, further studies to identify and isolate the main constituents responsible for its anticataract effect from phenolic and flavonoid class to comprehend the proper dosage form for maximum benefits of this plant needs to be carried out in future.

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