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Investigation of Anti-cataract Potential and Irritancy of *Dicliptera paniculata* in Diabetic-Cataract: An *Ex Vivo* and *In Vitro* Study

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Abstract: Cataract is one of the major causes of blindness globally. Diabetes worsens this condition. Surgery is a very effective treatment of cataracts in which an artificial lens replaces an opaque lens due to cataracts, but surgery has many complications during and post-surgery. So, alternative treatment can solve the problem of the cost of treatment and complications of the surgery. Using plant extract as aldose reductase inhibitors prevents sorbitol accumulation that prevents oxidative stress, and thus cataracts can be prevented. Presently, many plant products are being used to treat cataracts. Traditionally *Dicliptera paniculata* herb is used as an analgesic, anti-inflammatory, antipruritic, antibacterial, and to treat eye infections in the cattle. In the present study, we have evaluated the antioxidant, and anticataract potential of *D. paniculata* alcoholic leaves extract using diabetes induced cataract in goat eyes. Antidiabetic and the irritancy of leaves extract was also determined using the HET-CAM test. The present study shows plants have good antioxidant potential, and the plant extract shows promising results in reducing diabetes induced cataracts. It significantly increase protein and potassium lebvel and decreases sodium level. The HET-CAM study shows that plant extract has no irritancy.

Keywords: Cataracts, Aldose reductase, Polyolpathway, Eye irritation, HET-CAM Test, Goat eye

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Introduction

A cataract is one of the significant causes of blindness globally. A cataract is defined as a clouding or protein precipitation of an eye lens. The eye lens is responsible for focusing and producing a clear, sharp image (Bandaru *et al.*, 2016). The lens protein precipitation causes low vision or vision loss (Renju *et al.*, 2018). Cataracts are the second leading cause of visual impairment after myopia, hyperopia, or astigmatism (Coronini -Cronberg *et al.*, 2012). A report suggests that

about 17.8 million people worldwide are affected by cataracts (IMPRI, 2021). A diabetic cataract is the main reason for visual disturbance in diabetic patients; the prevalence of occurring cataracts is higher in diabetic patients, and various studies support this hypothesis (Javadi and Zarei-Ghanavati, 2008). The incidence of diabetic cataracts is steadily increasing worldwide (Klein and Klein, 1997).

Various biochemical pathways are involved in the pathogenesis of diabetic cataracts, such as the formation of advanced glycated end products, polyol pathways, and oxidative stress. (Kyselova *et al*., 2004). Aldose reductase (A.R.) is the first rate-limiting enzyme of the polyol pathway. Hyper-glycemia causes A.R. activation, increasing sorbitol formation in insulin-insensitive tissues such as the lens, nerve, and retina of diabetic patients. Sorbitol accumulation increases osmotic stress and swelling, which may damage the tissues. Excess polyol synthesis due to enhanced A.R. activity is considered one of the significant mechanisms resulting in diabetic cataracts (Pollreisz *et al*., 2010; Vangalapati *et al*., 2020).

Oxidative stress is the second most common reason for cataracts (Vinson, 2006). To counterbalance this effect, plants and animals internally synthesized antioxidants such as catalyzes and superoxide dismutase (Arya *et al*., 2022). Many plants have been traditionally used to treat diabetes and associated symptoms in different cultural areas. Some plants like *Ocimum sanctum*, *Tribulus alatus*, *Curcuma longa*, *Punica granatum*, and *Azadirachta indica* have been reported to possess anti-cataract activity (Halder *et al*., 2003). *Dicliptera paniculate* (*D. paniculate*) an annual herb which belongs to Acanthaceae family. It is widely grown throughout India, Vietnam, tropical Africa, Myanmar, and Afghanistan. *D. paniculate* is traditionally used for treating cattle eye infections (Mekala *et al*., 2023). It is used as an expectorant, analgesic, anti-inflammatory, antipyretic, antibacterial (Renju Krishna *et al*., 2018). The fresh leaves are used to treat conjunctivitis by adding 2-3 drops of the plant for two to three days (Satyanarayana *et al*., 1993). Several reports suggest that it has good antioxidant properties (Renju *et al.,* 2018). This study aimed to evaluate the anticataract potential of *D. paniculate* leaf extract using the goat eye model (*ex vivo*) and the eye irritation capacity using the HET-CAM method.

Materials and Methods

Plant Collection and authentication:

D. paniculata leaves were collected from Bhimtal (Uttrakhand), India, in August and authenticated by the Botanical Survey of India, Dehradun, with accession number 570.

Preparation of plant extract:

Leaves of the plants were shade-dried and powdered. The powdered leaves were divided into three sets (40 g each), which were then extracted with 200 ml ethanol using the Soxhlet apparatus. The solvent was evaporated using a rotary vacuum evaporator, and the percentage yield (w/w) was calculated. Dried extracts of the plant were stored in an air-tight container and kept in refrigerators for the experimental work (Ali *et al*., 2017).

Phytochemical analysis:

The presence of various phytochemicals such as flavonoids, alkaloids, carbohydrates, Tannins, Proteins, and other constituents were determined in the ethanolic extract of *D. paniculate* by performing the phytochemical analysis (Guglani *et al*., 2022).

Antioxidant activity:

The antioxidant study of *D. paniculata plant* extract was performed using following methods:

Phosphomolybdate assay:

10 mg of ascorbic acid was dissolved in 100 ml of ethanol and from serial dilution 20,40,60,80 and 100 µg/ml were made. 0.1 ml ascorbic acid (Standard) solutions from every dilution were combined with 1 ml of reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM

Table 1: Experimental design

Group I	Normal lens only with lens culture (Normal control)
Group II	lens culture +Glucose 55 mM (Toxic control)
Group III	lens culture + Glucose 55 mM + ethanolic extract of *D. paniculata* (500 µg/ml) (treated group)
Group IV	lens culture +Glucose 55 mM +Enalapril (10 ng/ml) (standard drug)

ammonium molybdate). At the same time, 0.1 ml of *D. paniculata* solution (1 mg/ml) was added to 1 ml reagent, and a blank (mother solvent without extract) was added with 1 ml of reagent. All the tubes were tightly closed and incubated in a water bath at 95 ℃ for 90 min. After cooling all blank and ascorbic acid tube samples were kept at room temperature and the absorbance was measured at 695 nm against a blank in UV/ VIS-spectro-photometer. A graph between percentage inhibition and concentration was plotted. The liner equation obtained from this graph was used for IC_{50} calculation (Kausar *et al*., 2023).

DPPH *Radical Scavenging* assay:

50 ml of *D. paniculata* ethanol extract were taken to form various concentrations of 20,40,60,80 and 100 µg/ml in ethanol. 5 ml of 0.1 mM methanol solution of DPPH was added to each concentration of the plant extract and kept for half an hour at room temperature, and absorbance was taken at 517 nm with the help of a U.V. spectrometer against a blank. Ascorbic acid was taken as standard. The percentage radical scavenging activity of the ascorbic acid and *D. paniculata* plant extract was calculated using the following formula (Guglani *et al*., 2020):

$$(\%\ RSA = A_{control} - A_{sample} / A_{control\ x100})$$

A graph between the percentage of radical scavenging activity (RSA) and concentration was plotted. From this graph, a linear equation was obtained and used for IC_{50} calculation.

$$\%\ RSA = A_{control} - A_{sample} / A_{control\ x100}$$

Preparation of lens culture:

Artificial lens culture was prepared by taking sodium chloride: 140 mM, potassium chloride: 5 mM, magnesium chloride: 2 mM, sodium bicarbonate: 0.5 mM, sodium dihydrogen phosphate: 0.5 mM, calcium chloride: 0.4 mM, glucose: 5.5 mM), pH 7.8. Furthermore, 1 ml of gentamycin (40 mg) prevents microbial growth. (Moghaddam *et al*., 2005; Pooja *et al*., 2014).

Cataract Induction:

Fresh goat eyeballs were collected from the nearby slaughterhouse and immediately transported to the laboratory at 0-4℃. A total of twenty eyeballs was taken for study. The eyeball lenses were removed from each eye and divided into six groups, containing six lenses in each group. All the lenses in each group were placed in artificial lens culture and treated as per the experimental design (Table 1). All lenses in each group were incubated for 48 h at 37℃. (Hajarnavis and Bulakh, 2020; Aware *et al*., 2021).

Photograpic evaluation:

The morpholical changes were detemnined for evalution of cataracts induction by comparing scores obtained by the lens. The lenses were placed on a wired mesh with the posterior surface touching the mesh, and the mesh pattern was observed to measure the lens opacity. The degree of opacity was graded as mentioned in Table 2 (Shreya *et al*., 2021).

Preparation of lens homogenate:

After 48 h of incubation, the lenses from each eyeball were removed and washed with saline, and the lens weight was estimated. A 10% homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was then centrifuged in a refrigerated centrifuge at 6000

Table 2: Grade to measure the degree of opacity

Grade	Clarity and transparency of the lens
Grade 0	Totally clear lens, with no opacity
Grade 0.5	Clear lens with mild opacity
Grade 1	Moderate haze and less transparency
Grade 2	Mild haze is seen easily but very little transparency

rpm for 30 min. The calibration curve of lens protein was prepared from the supernatant using U.V spectrometer at 280 nm (Hajarnavis and Bulakh, 2020).

In-vitro aldose reductase inhibition:

The glyceraldehyde converted into glycerol in the presence of aldose reductase enzymes in lens homogenate. The reducing amount of the glyceraldehyde indicates aldose reductase in lens homogenate. For determination of aldose reductase inhibition, 0.2 ml was taken in the cuvette, and in that cuvette, 1.4 ml of phosphate buffer (0.067 M), 0.2 ml of NADPH ($25x10^{-5}$ M) and substrate (0.2 ml DL-glyceraldehyde ($5x10^{-4}$ M) was added and volume was made up to 2 ml, and after adding substrate, the absorbance (O.D.) was recorded at 340 nm for 3 min at 30 sec interval (Suryanarayana *et al.,* 2004).

Biochemical evaluation:

Each group (n=6) of goat lens and a fresh group (n=6) lens were homogenated separately in mortar with 190 mM phosphate buffer (pH 7.3). All insoluble aggregates of lens cells and other insoluble materials were removed by centri-fugation of homogenate at 6000 rpm for 30 min and supernatant obtained by vacuum filtration were taken flame-photometry.

Estimation of Lens protein:

To determine lens protein, six fresh goat eye lenses were taken and homogenated in demineralized water with the help of a mortar pestle and centrifuged for 30 min at 6000 RPM. The obtained supernatant was filtered to get a clear solution. The protein was estimated using the Lowery method (Hajarnavis and Bulakh, 2020).

Estimation of sodium and potassium:

For the estimation of sodium and potassium, a calibration curve of sodium and potassium was prepared by using aliquots of sodium and potassium calibration (20,40,60,80,100 µg/ml**).** With the help of the calibration curve, sodium and potassium in goat eye lens (fresh and other groups A, B, and C) were estimated. Sodium and potassium were calculated from lens homogenate using flame photometry. After 48 h, all lenses from different groups were homogenated similarly to fresh lenses to obtain clear supernatant and protein content was calculated using an equation obtained in a standard calibration curve. Then, using the sodium and potassium calibration curve, the remaining clear supernatant was taken for flame photometry to estimate sodium and potassium concentration in the lens after 48 h (Kumari and Singh, 2021).

HET-CAM Test for Eye Irritancy of The Plant Extract:

Twenty eggs were purchased from a poultry farm and examined for shell breakage. The eggs were upright in an egg tray in such a way that the direction of the narrow end was downward. The tray was kept in an incubator at 38^0C and water in a beaker for eight days. All eggs were rotated four to five times a day during that period. After eight days, all eggs were examined under a flashlight for embryo formation. Nine fertile eggs were selected. Then, the eggs were inverted so that the broad end was upward. Again, all these eggs were incubated for two days at the same incubation conditions mentioned above but without rotations. The eggs were divided into positive, negative, and test groups containing three eggs. All eggs from each

Table 3: Study design

Groups	Name of groups	0.3 ml of solutions
Group I	Positive control	0.9 % w/v NaCl
Group II	Negative control	1% w/ v Sodium laurate sulphate
Group III	Test control	*Dicliptera paniculeta* (500 µg/ml)

Table 4: Score for Irritancy Study

Score	Reaction
0	No reaction
1	slight reaction
2	moderate reaction
3	severe reaction

group were now opened carefully from the broad portion of the eggs. All groups were treated with 0.3 ml of the respective solution per the study design (Table 3). All eggs were carefully examined for reactions on the CAM for 5 min, and the hemorrhage (Bleedings), vascular lysis (Blood vessel disintegration), and coagulation (protein denaturation intra- and extravascular) was monitored, and scoring was done as per Table 4, and irritation score (IS) was calculated using equation (Batista-Duharte *et al.,* 2016; de Araujo *et al.,* 2022).

$$IS = \frac{(301-H)}{300} X5 + \frac{(301-L)}{300} X7 + \frac{(301-C)}{300} X9$$

Where H: Haemorrhage, L: Vessel lysis, and C: Coagulation, all readings of H, L, and C were taken in seconds.

Statistical analysis:

Results were expressed as mean± standard error of the mean. The statistical significance of the difference between groups for the various treatments was determined by a one-way analysis of variance followed by Bonferroni test. $P<0.05$ was considered statistically significant.

Results and Discussion

In the present study, we successfully prepared the alcoholic leaves extracts of *D. paniculata* using soxhlet apparatus, the yield was found to be 5.76%, and then the extract was subjected to phytochemical analysis, antioxidant assay, *ex-vivo*aldose reductase study, and *in-vitro* irritation study.

Photochemical analysis:

The phytochemical analysis showed the presence of various phytochemicals in the ethanolic leaf extract of *D. paniculata*, including flavonoids, alkaloids, carbohydrates, tannins, proteins, and other constituents, and results are given in Table 5.

Antioxidant study:

The plant extract possesses good antioxidant properties (Renju Krishna *et al*., 2018), which is confirmed by our study. The extract showed an antioxidant effect in a dose-dependent manner in both phosphomolybdate and DPPH radical scavenging assays. In phosphomolybdate assay, the IC_{50} of *D. paniculata* was found to be even better than ascorbic acid, which was found to be 54.25 µg/ml and 58.42 µg/ml, respectively (Table 6).

From DPPH radical scavenging assay, the radical scavenging activity of using ascorbic acid (as standard) and *D. paniculata* estimated, IC_{50} of were found to be 34.24 µg/ml and 72.92 µg/ml, respectively (Table 7), from the antioxidant study we found that *D. paniculata* has good antioxidant properties that can help to reduce cataract generation by oxidative stress.

Table 5: Showing phytochemicals constituent present in extract

Phytoconstituents	Ethanol extract
Alkaloids	+
Tannins	+
Carbohydrates	+
Anthraquinone glycosides	-
Cyanogenetic glycosides	-
Cardiac glycosides	-
Steroids / Triterpenoids	+
Saponins	+
Coumarins	+
Flavonoids	+
Proteins	-
Amino acids	-
Fixed oils	+

Table 6: Showing the percentage inhibition of ascorbic acid and *D. paniculata in the* phosphomolybdate assay

Concentration (µg/ml)	Ascorbic acid		*D. paniculata*	
	% Inhibition	IC_{50}	% Inhibition	IC_{50}
20	45.7	58.42 µg/ml	21.5	54.25 µg/ml
40	51.9		31.5	
60	61.7		51.7	
80	76.8		64.9	
100	86.7		80.5	

Table 7: Showing % RAS of ascorbic acid and *D. paniculata in DPPH* radical scavenging assay

S. No.	Concentration (µg/ml)	Ascorbic acid		*D. paniculata*	
		% RSA	IC_{50} (µg/ml)	% RSA	IC_{50} (µg/ml)
1	20	44.69	34.24	16.04	72.92
2	40	51.97		27.53	
3	60	59.62		39.75	
4	80	71.11		55.43	
5	100	78.27		68.40	
6	Absorbance of control = 0.810				

Biochemical study:

Total lens protein was estimated to verify the precipitations of eye proteins (crystallins). The protein content of different groups was estimated after 72 h. The study showed that protein level was low in groups II (negative) as compared to groups I and it was significantly high in group III (extract treated) as compared to the groups I and group IV. The study showed the leaf extract was effective in increseasing the protein level effectively (Table 8; Fig. 1).

In group II (negative control), the sodium level increased compared to group I (Normal control). The study shows the extract decreases the sodium level, a significant decrease in the sodium level was observed in group III (extract treated) compared to group I and Group II, the extract was found even better than standard, having a significant decreased sodium level than group IV (*P < 0.05) (Fig. 1b). In the present study group II (negative control) showed a decrease in potassium level compared to the group I (Normal control). In group II (extract treated) a significant

Table 8: Showing Biochemical parameters of lense

Groups of lenses	Lens protein (mg/ml)	Sodium(µg/ml)	Potassium(µg/ml)
Group I	0.5768±0.0016	23.29324±0.0201	0.08330±0.0021
Group II	0.4467±0.0021	51.086.45±0.0322	0.06965±0.00103
Group III	0.6473±0.012	15.701.21±0.0182	0.09725±0.00134
Group IV	0.5378±0.023	19.079.21±0.0203	0.09445±0.001.24

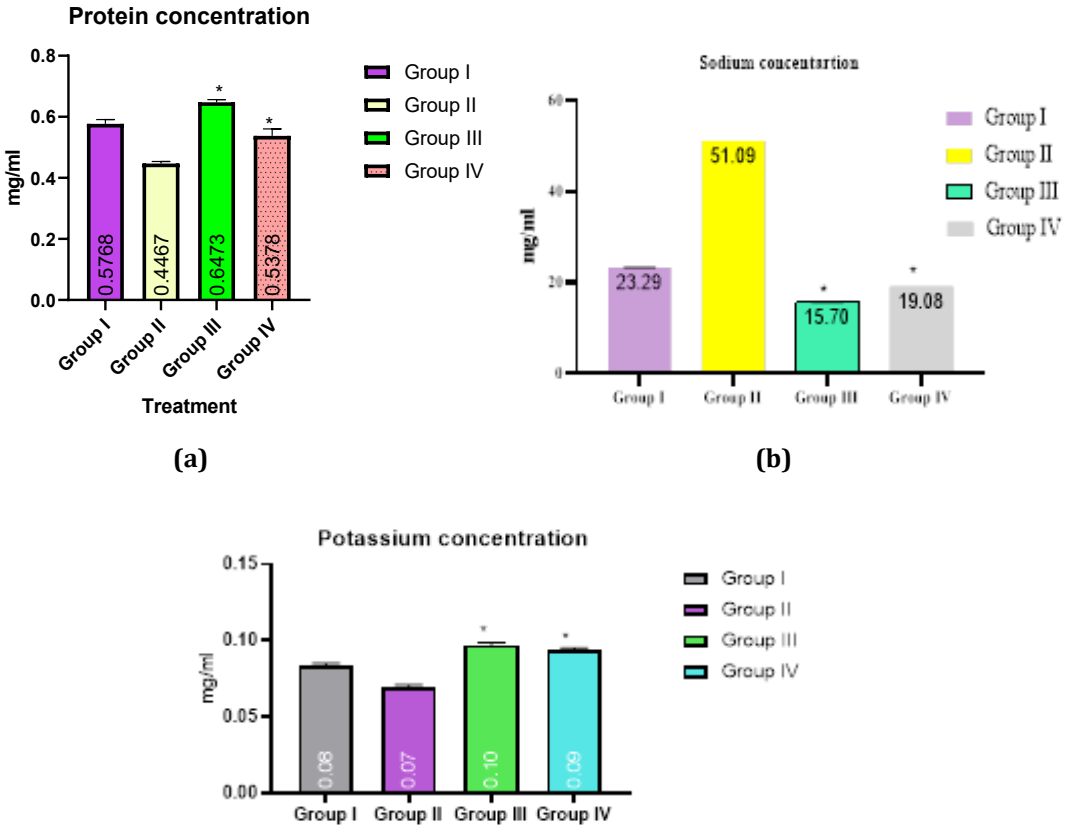

Fig. 1a, 1b, and 1C, showing the effects of *D. paniculata* extract on total protein level, sodium and potassium level; all values are mean ± SD, n=6; Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni test (*P < 0.05).

increase in potassium level was observed than group II (P < 0.001), the extract treated group showed a significantly higher potassium level in group III compared to the IV (P < 0.001 (Standard).

Photographic evaluation:

In photographic evaluation, the clarity of the lens was determined. The plant extract showed good efficacy, effectively reducing lenses' opacity. Group I (positive control) showed the highest lens clarity. However, group II (toxic control) showed the highest opacity than the normal control. Group IV (standard), which was treated with the standard drug, showed no opacity or clear Lense, while group III (treated) showed good Lense clarity, which confirmed the efficacy of extract in reducing the opacity in glucose-induced (diabetic) cataract (Shreya *et al.,* 2021) (Fig. 2).

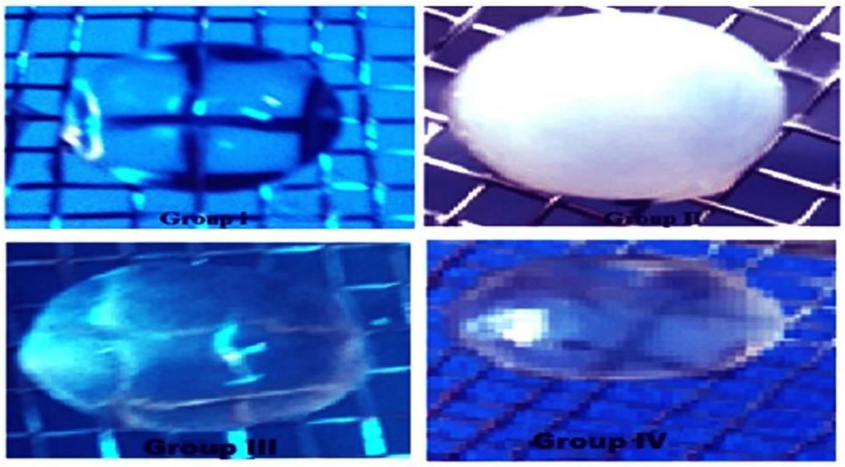

Fig. 2: Comparative opacity of goats' eyes lenses in groups I, II, III, and IV, respectively after 48 h at 37 ℃ in incubation.

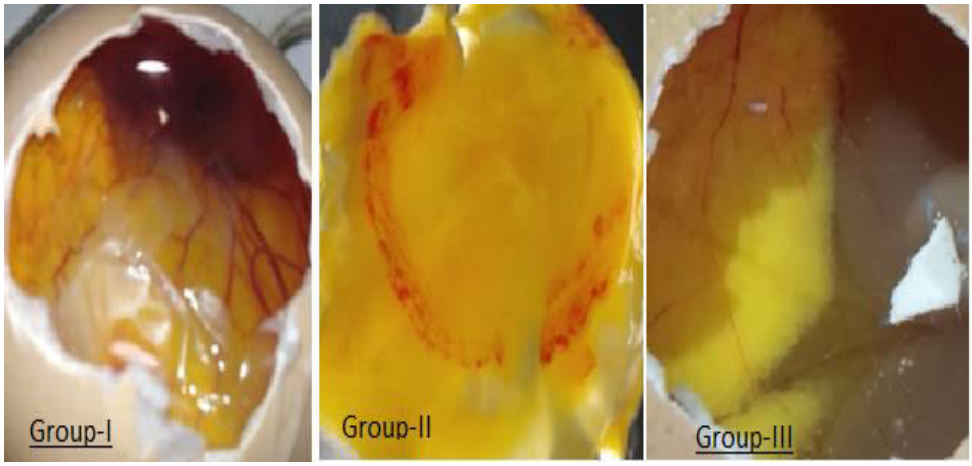

Fig. 3: Results of the HET CAM test.

Group I has an average score of 0.58, the negative control has an average score of 3.67, the group III (extract treated) has the lowest average score of 0.33; the study showed that extract has higher anticataract potential than the standard drug. However, group IV, treated with the standard drug Enalapril, showed a higher average score value than the extract tread group III.

Ocular irritation:

HET-CAM test (Hen's egg-chorioallantois membrane test) was performed to analyze the irritancy level of the extract (Fig. 3)(de Araujo *et al*., 2022). In the ocular irritation study, positive control (Group I) showed an average IS value of 1.45±0.02. The lower IS value indicated low irritancy. However, the negative group (Group II) showed an average I.S. value of 17.73±0.31, indicating high irritancy level and ethanolic leaves' extract of *D. paniculata* (Group III) has low IS value of 0.47±0.07 indicating no or very low irritancy level (Batista-Duharte *et al*., 2016).

Conclusion

We have successfully extracted the alcoholic leaves extract of *D. paniculata*. The alcoholic leaf extract possesses good antioxidant potential. Our study revealed that the *D. paniculata* ethanolic plant extract arrest the diabetes-induced cataract, and the HET-CAM study showed that the *D.*

paniculata ethanolic plant extract has no ocular irritancy. From the study, we concluded that the ethanolic plant extract can be used for treating cataracts; however, further study is required to confirm the potential of *D. paniculata*, and new delivery systems are required to treat cataracts using such harmless extract.

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