RESEARCH ARTICLE

Inhibition of Indoleamine 2, 3-Dioxygenase (IDO) Activity of *Hemidesmus indicus* Root Extract and its Attenuate Effect on Streptozotocin Induced Diabetic Cataract in Rats

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Abstract

Background: Increased Indoleamine 2, 3-dioxygenase (IDO) activity has been performed in the development of cataract and IDO Inhibition with natural medicine is a therapeutic strategy for the management of diabetic cataract. Our present study assessed the inhibitory effect IDO and anti- cataract properties of *Hemidesmus indicus* R. Br. root extracts in Streptozotocin (STZ) induced diabetic rats.

Material and Methods: *In vitro* enzyme kinetics were carried out using hIDO (purified from cloned *E. coli* cells) with *Hemidesmus indicus* root extracts. To explore in-vivo anti-cataract properties of Ethyl acetate extract (EAHI), diabetes was in SD rats by single intraperitoneal injection of STZ at dose 35 mg/kg body weight. Experimental rats were divided into four groups (Control & diabetic) orally treated with 0, 250, 350 mg/kg bw of the EAHI up to 6 weeks. The evaluation of enzymatic parameters of kynurenine pathway (KP) metabolites, i.e Kynurenine, 3-hydroxy kynurenine, kynurenic acid, tryptophan and IDO expression level were examined in experimental rat lens and histopathological change were evaluated in retina by using Hematoxylin & eosin staining. .

Results: Among all, EAHI exhibited strong inhibition of hIDO activity with IC 50 value of 34.74±0.82 ug/ml in a non-competitive manner. Extract doses treated with EAHI (250 & 350 mg/kg bw) have been showed a significant alternative changes in retinal morphology, expression of IDO and were significant ($p<0.05$) suppresses kynurenine pathway (KP) metabolites, i.e Kynurenine, 3-hydroxy kynurenine, kynurenic acid and tryptophan in experimental rat lens. Beside this, rat fed with EAHI have shown significant decreased level ($p<0.05$) of IDO in lenticular tissues.

Conclusion: Overall results obtained in the study concluded that EAHI possess pharmacologically active constituents attributing its IDO inhibitory potential and delaying the cataract in lenses. Thus the study clearly suggested the traditional medicinal claim of the plant *Hemidesmus indicus* possessing the anti-cataract drug.

Key words: *Hemidesmus indicus*, Kynurenine pathway, Indoleamine 2,3-deoxigenase, Enzyme-inhibitory activity, Diabetic cataract.

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Introduction

Opacification of the crystal clear eye lens (Cataract) is the major cause of blindness throughout the world, more so in emerging countries like India, caused by high predominance of diabetes.[1] Cataract is a late stage obstacle in diabetic patient and one which intensely disturbs the quality of life. Numerous biochemical pathways connected with diabetes have been implicated,[2] among these; the kynurenine pathway has been extensively studied. Indoleamine 2, 3-dioxygenase (IDO) is a heme-containing dioxygenase (EC 1. 13. 11. 42, 42 KDa) that play the initial and rate limiting role in the kynurenine pathway (KP) and catalyze the L-tryptophan (L-Ttrp) into N-formyl kynurenine. N-formyl kynurenine is readily converted to kynurenine and subsequent metabolites like quinolinic acid (QA).[3,4] Imbalance of the KP, mainly linked with the elevation of IDO activity and over QUIN production, have been associated in the pathogenesis of neurodegenerative (Alzheimer's, disease), neuro-inflammatory, HIV encephalitis, depression and age related cataract.[5-7] A developing baby of clinical data has revealed that many tumor cell lines overexpresses IDO and KP metabolites, and thus strongly correlates with meager prognosis for survival.[8,9] Thus, IDO has emerged as a therapeutic target for neurological disorders, diabetes and it's complications and other diseases considered by pathological tryptophan metabolism. Earlier reports revealed that, there is a growing need to look for new IDO inhibitors

keeping in mind of their efficacy and related safety issues to development novel IDO inhibitors. Recently, Tryptophan (Trp) analogues have been used as IDO inhibitors, which displayed lower affinity with IDO. For instance, 1-methyl tryptophan (1-MT) is the most rottenly used competitive inhibitor (Ki of 34 mM) and some effective IDO inhibitors have been discovered there with unique structural skeleton.[10-14] However, some molecules are identified to be IDO inhibitors, most of them either exhibited low potency or was unsuccessful in *in vivo* studies and therefore are considered as negligible drug candidates. Therefore, it is still of this time great interest to find novel potent IDO inhibitors. For many years, we have focused on the development of novel inhibitors of IDO under the direction of *in vitro* enzymatic studies.

Hemidesmus indicus R. Br. (*H. indicus*)) which belongs to the Asclepiadaceae family, locally called anantamul (Indian sarsaparilla) is extensively used in traditional medicine like as one of the Rasayana plants of Indian Ayurvedic system. The plant is woody, with thick and brown bark, woody and aromatic roots, slender twining shrub and dispersed throughout India.[15] In traditional medicines and Ayurveda, it has been far used as a blood purifier and to cure different disorders like leprosy, fever, rheumatism, impotency, diuretic and skin infections, bronchitis, leukoderma and leucorrhea.[16] An extraction of the *H. indicus* roots is used for the treatment of fever, cough, blood diseases, loss of taste, dyspepsia, dyspnea, poison, menorrhagia and diarrhea.[17] Previous studies revealed that the alcoholic fractions of its roots have anti-inflammatory, antithrombotic, anti-nociceptive, anti-diarrheal, anti-enterobacterial, hepatoprotective and Reno protective activities.[18-22] Radiation induced strand breaks of DNA were protected by *H. indicus* root extract has been reported[23] and is found to be as effective as insulin in the restoration of blood glucose to normal levels. [24,25] *H. indicus* mainly consists of several essential oils and root oil has been shown to contain over 20 major and 40 minor constituents, these were reported to be chief antioxidant, aromatic and bioactive principles. Hence, in this present study ethyl acetate extract of *H. indicus* root was assessed for its inhibitory effect on Indoleamine 2, 3-dioxygenasee (IDO) and Cataract progression in STZ induced diabetic rats.

Meterials and Methods

Chemicals and reagents

Streptozotocin (STZ), Thiobarbituric acid (TBA), NADPH, β-mercaptoethanol, Coomassie Brilliant Blue, Bradford reagent, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and Quercetin were purchased from Sigma Aldrich (St. Louis, MO). DNA, protein size markers, restriction enzymes, DNA ligation kit, cDNA synthesis kit, PCR master mix and primers were obtained from Invitrogen Pvt Ltd, USA and plasmid isolation kit and PCR product purification kits were purchased from Qiagen Pvt Ltd, USA. Bacterial strains of E. coli, BL 21 (DE 3), DH5α, TOP 10, and plasmid cells were obtained from the Centre for Cellular & Molecular Biology (CCMB), Hyderabad, India. The components of LB media and other reagents, analytical grade chemicals and solvents were obtained from indigenous companies such as SRL, HiMedia, Merck Pvt Ltd (Mumbai, India) and SD Fine Chemicals, India.

Collection and Authentication of plant material

Dried roots of *H. indicus* have been collected from the Nallamala forest, Telangana state, India in the month of August 2018. The prescribed authentication was done by the chief botanist Prof. P. Rama Chandra Reddy, Department of Botany, UCS, Osmania University, Hyderabad and voucher specimen (Bat/OU/0119/HYD) was deposited in the Botany Department herbarium, Osmania University, Hyderabad for future reference.

H. indicus root Extraction

H. indicus roots were meticulously washed, and shade dried then grinded into coarse powder by using mechanical grinder and stored in air tight brown bottles. The extracts of *H. indicus* roots were prepared with four solvents of increasing polarity in order of methanol, ethyl acetate, petroleum ether & water, respectively by using cold maceration method.[26] About 400 g of the dried fine powdered form have been macerated with 1.5 L of solvent at room temperature for 5-8 days with occasional mixing to dissolve the soluble matter. The macerated solvent extracts were sieved trice through filter paper and extracts thus obtained were concentrated under reduced pressure by using a rotary evaporator (Buchi, Germany). The semisolid residue was collected and stored in a refrigerator and all the extracts were subjected to conduct hIDO inhibition and anti-diabetic cataract evaluation.

Expression and purification of recombinant human Indoleamine 2, 3-dioxygenasee (rhIDO)

Luria- Bertani (LB) medium was routinely used for growth of *Escherichia coli* at 37 °C containing 50 mg/mL kanamycin. A single positive colony of the plasmid (pET32a-hIDO) was inoculated to 10 mL LB medium and cultured overnight. About 10 mL overnight growth culture was added to 0.5 L of the fresh LB medium incubated to a density of 0.6 OD at 600 nm at 37 °C. The temperature was reduced to 25 °C before IPTG (0.6 mM) induction then culture was incubated for 3 h at 32 °C. The cell residue were harvested by centrifugation at 6000 rpm using Sorvall Primo R (RA-6 rotor) centrifuge for 20 min at 4 °C and the pellet was either processed for purification or stored at -80 °C until use. The pellet obtained as described above about 0.5 L of bacterial culture was suspended in ice cold lysis buffer (10 mL) with combination of protease inhibitor cocktail, sonicated on ice using a Virtis Virsonic sonifier for15 min at the extreme power.

Cell debris was separated by centrifuged at 12000 rpm for 20 min at 4 °C and the transparent supernatant (10mL) was then applied to a Nickel ions charged Hi-Trap Chelating column (1mL). Further, column was equilibrate with imidazole (10 mM) in phosphate buffer (20 mM, pH 7.4) and

rotate 180 min at 4 °C. Later washing with 4 times of basal buffer (40 mM imidazole, Tris 25 mM, pH 7.4,) recombinant human Indoleamine 2, 3-dioxygenasee was eluted with the imidazole solution (250 mM) that was prepared in the similar phosphate/NaCl buffer. The protein was collected at the elution step by using Sephadex G 25 column with buffer exchanging (50 mM Tris, pH 7.4) and simultaneously protein concentration checked by Bradford method. [27] The pooled protein were desalted by using agarose desalting column (GE Healthcare, UK) and protein fractions have been stored in aliquots at -80 °C until further analysis.

IDO inhibition assay

Human Indoleamine 2,3-deoxygenase (hIDO) enzyme was used for IDO inhibition assay and purified hIDO were obtained from Physiology and Molecular Biology Lab, Department of Zoology, Osmania University. The effects of plant extracts on IDO activity were carried out by according to the method described by Matin et al. with slight modifications.[28] Different concentrations of 100 µl extract was added to 200 µl of standard reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 20 mM ascorbic acid (neutralized with NaOH), 100 mg/mL catalase, 10 mM methylene blue and 5 µg/mL hIDO solution, 200µM L-tryptophan, and incubated at 37 °C for 30 min. Then 250 µl of Trichloroacetic acid (30% w/v) was added to each reaction test tube, and incubated at 65 °C for 15 min to hydrolyze N-formyl kynurenine into kynurenine. After that, the reaction mixture was centrifuged at 12000 rpm for 10 minutes and 100 µL supernatant was transferred into a fresh test tube and mixed with 100 µL of p-dimethyl amino benzaldehyde in acetic acid (2% w/v). The yellow color change noted and absorbance was read at 480 nm using a spectrophotometer (Hitachi U-2910). The results were expressed as % inhibition calculated using the formula: IDO inhibition activity= $[1-\{A_1 - A_2) /A_0\}] \times 100$.

Where A_1 is the absorbance of the test sample, A_2 is the absorbance of product control (sample without IDO solution) and A_0 is the absorbance of negative control (IDO without extract). The concentration of each extract resulting 50% inhibition (IC_{50}) was determined by non-linear regression analysis of log concentration of extract verses percentage inhibition and Ethyl acetate extract of *H. indicus* (EAHI) which showed higher inhibitory activity was employed for kinetic inhibition.

Kinetic analysis of IDO inhibition

The enzyme kinetic analysis of IDO was performed by using method described.[29] The assay was conducted with 50-300 mM L-tryptophan and a amount of inhibitor solution (EAHI) concentration varied over a three-fold range above and below the concentration yielding approximately 50% inhibition. Otherwise the reaction conditions were exactly the same as those described above. Based on the assay results, a function of rate [V] against substrate amount [S] was plotted and data were analyzed with Lineweaver Burk plots.

Experimental animals

Heathy two months old male SD (Sprague Dawley) rats of average weight of (180±8) g were obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. The animals are housed in the Animal house of Department of Zoology, Osmania University, Hyderabad in polycarbonate cages. The rats were monitored under standard environmental conditions (22±3 °C, relative humidity 50-60%, and 12-h light & dark sequence. They were allowed to a semi synthetic AIN-93 diet (14) and water *ad libitum* throughout the 6 week experimental period. All the protocols and experimental procedures in this study have been executed according to the Institutional Animal Ethical Committee (Register Number: 383/01/CPCSEA) and care of animals was taken as per the guidelines of CPCSEA.

Acute toxicity study

The median lethal dose (LD_{50}) of the EAHI was performed by administrating the extracts to different groups of animals (n=6) at doses of 100, 500, 1000, 3000 and 5000 mg /kg BW respectively according to according OECD 423,2001 guidelines.[30] The fasted rats administered orally with extracts prepared distilled water, while distilled water administered (2 mL/kg BW) to control groups (*n*=3) as a vehicle orally. The animals were observed for any adverse effect and mortality within six hours and then daily up to 10 days. Hence, the effective doses (ED_{50} value) of *H. idicus* root extracts were calculated in the different groups of rats for further animal experiments.

Induction of Diabetes in Animals

Diabetes was induced in rats by a single intraperitoneal injection of freshly prepared Streptozotocin (35 mg/kg BW) in a 0.1 M citrate buffer (pH 4.5). Another set of control rats received an equivalent amount of sham 0.1 M citrate buffer (pH 4.5). After 48 h, the fasting blood glucose levels (tail vein blood) were measured by a portable glucometer and animals with fasting glucose level ≥ 180 mg/dL were considered as diabetic and used in the study. After one week of acclimatization period, the animal groups and dosage schedule are described in Table 1.

Preparation of lens homogenization

After completing the 6 week's experimental period, the animals were sacrificed by cervical dislocation and their eyeballs were collected for biochemical evaluation. The lenses were dissected from eyeballs by posterior approach in normal saline (0.9%) then placed into pre-weighed Eppendorf tubes and stored at −80 °C until further analysis. A lens homogenate (10 %) was prepared in 50 mM phosphate buffer (pH 7.4) and centrifuged at 16000 rpm for 20 min. The supernatant of homogenate was used for enzyme assays while the lens IDO expression study.

Estimation of kynurenine metabolites

The kynurenine metabolites in lenticular tissues have been estimated as per the method of previously described.[31] To

Table 1: Percentage inhibition of rhIDO by using different solvent extracts of *H. indicus* and IC_{50} values have been compared with standard (Quercetin).

Conc. of extract (µg/ml)	*% Inhibition of hIDO with corresponding extract used*				*Conc. of Quercetin (µg/ml)*	*% inhibition of hIDO*
	MEHI	*EAHI*	*PEHI*	*AQHI*		
20	36.78±2.09	37.57±1.48	11.44±1.13	20.10±2.03	10	40.63±1.15
40	63.29±2.27	70.30±2.21	31.45±2.08	55.53±1.48	20	71.74±1.23
60	86.48±1.6	89.11±1.58	41.31±1.41	74.91±0.96	30	89.99±0.82
80	93.10±2.5	95.41±1.36	48.94±2.06	84.64±0.70	40	98.18±0.37
100	97.63±0.84	97.82±0.44	57.53±2.14	92.08±0.91	50	99.69±0.27
IC_{50} value (µg/ml)	36.74±0.76*	34.74±0.82*	81.15±2.28*	47.27±2.93*	**IC_{50} value (µg/ml)**	16.51±0.18

Values were represented as mean±SD (n=3)
Superscript * denotes in column are statistically significant (p≤0.05) compared with standard. Ethyl acetate extract showing better inhibitory property with hIDO.

100 µl of 10 % lens homogenate 0.3 ml of 100 % ethanol was added and incubate at room temperature for 1 h & then centrifuged at 12000 rpm / 15 min. The supernatant was removed, kept at -20 °C, whereas the pellet was re extracted twice with 0.3 ml of 80% ethanol and the supernatant absorbed to be pale yellow color. The supernatants were pooled and concentrated in Eppendorf tubes and kynurenine metabolites have been quantified by reverse phase HPLC (Shimadzu Corp., Japan). The column was equilibrated in 0.1% TFA (v/v), at flow rate of 0.5 ml/min and samples were suspend in 100 µl of 0.1 % TFA, centrifuged for 10 min at 12000 rpm and loaded on to the column. The metabolites (Kynurenine, Kynurenic acid, 3-hydroxy kynurenine and tryptophan) were eluted with the following gradient; 0-5 min 0% buffer B (0.08 % (v/v) TFA, 80 % (v/v) Acetonitrile, water), 5-50 min 0-50% B, 50-60 min 50-0% B. The eluents of Kynurenic acid & tryptophan were monitored using fluorescent detector at 400 nm and 255 nm respectively and UV absorbance for kynurenine and 3-hydroxykynurenine have been set at 360 nm. In similar way, reference kynurenine tryptophan, and 3-hydroxykynurenine have been used to construct standard curves.

Quantify IDO expression in rat lens

The levels of IDO mRNA in lenticular tissue of experimental rats were assessed by the method using semi-quantitative RT-PCR. Briefly, total RNA was purified from lens homogenate using Tri reagent according to manufacturer's instructions. The purity and concentrations of RNA were determined spectrophotometrically at 280 nm (proteins), 260 nm (nucleic acids) and 320 nm (background), respectively. Samples with a ratio of OD_{260}/OD_{280} values greater than 1.8 were used for quantification. Total RNA (~1.2 µg) was reverse transcribed using AMV reverse transcriptase and the cDNA was amplified in the presence of gene specific primers as following, Housekeeping gene GAPDH (F 5′-GCCAAGGTCATCCATGACAAC-3′and R 5′-GTCCACCACCCTGTTGCTGTA-3′) and IDO (F 5′-GACTTCGTGGATCCAGAC-3′ and R 5′-TCTAAGGAGGAGAGGAAG-3′) used with the primary concentration of 20 pmol. The amplification conditions have been initial denaturation was 95 ^{0}C for 5 min for all genes. Denaturation was at 94°C (30 sec-GAPDH and 2 min for IDO); annealing for GAPDH 55°C (30 sec) and for IDO 63.5°C (60 sec); and extension was at 74°C (2 min) for all the genes. The number of cycles for GAPDH and IDO is 30 and respectively the final extension was at 74°C (10 min). The amplicons were electrophoresed on a 2% agarose gel in Tris-acetate EDTA buffer (pH 8.2) and final PCR product hold at 4 °C. Bands were visualized using ethidium bromide, photographed using an UV-trans illuminator (Syngene, USA).[32]

Histopathological Evaluation

For histopathological examinations, eyes have been collected from rats by posterior approach in each experimental group and preserved in 10% formaldehyde. Formalin-fixed eyes were collected and dehydrated in a graded series of ethanol (70–100%), cleared in xylene, and finally embedded in paraffin. Thereafter, 5 mm thin sections were prepared and stained with Hematoxylin and Eosin (H&E) for histopathological examination according to earlier described [33]. The photomicrographs of the respective tissue sections were taken using Olympus BX41 research optical microscope fitted with Olympus DP 25 digital camera.

Statistical analysis

All data have been analyzed and results are expressed as standard deviation of mean (mean ± SD) and graphical

Table 2: Animal groups and their dosage schedule

Groups	*Type*	*Treatments*	*Dosage schedule*
Group - I	Normal Control	Normal saline	0.5 ml/kg bw
Group - II	Diabetic Control	Normal saline +STZ	0.5 ml/kg bw + 32 mg/kg bw
Group- III	DC+ EAHI low	STZ + EAHI 250 mg/kg BW/day	250 mg/kg bw
Group- IV	DC+ EAHI high	STZ + EAHI 350 mg/kg BW/day	350mg/kg bw

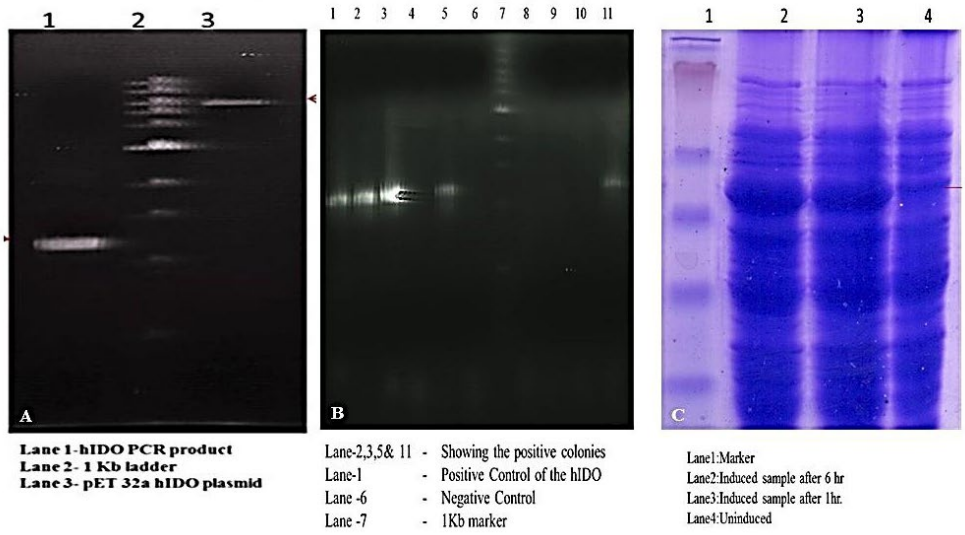

Figure 1: Separation of IDO product (1.2 Kb) and clone (pET 32a-hIDO) on 0. 8 % agarose gel along with 10 Kb DNA marker (A). Colony PCR of hIDO gene (B). SDS-PAGE analysis of overexpression of hIDO protein pre and post induction of IPTG have been shown along with molecular weight markers (C).

data were prepared by OriginPro 2021. Statistical significant difference between groups was performed by ANOVA followed by Tukey's test and $P \leq 0.05$, was considered to be statistically significant by using Statistical Package for Social Science (IBM SPSS 20).

Results

Purification of the human IDO

The 1209 bps amplified fragment was digested with EcoRI and XhoI and this resulting fragment was cloned into TOP10 vector to generate plasmid TOP10 -hIDO. The plasmid was then used to transform *E. coli* Top10 cells. The presence of 1209 bp insert was tested by double digestion with XhoI and EcoRI of the plasmid DNA isolated from transformed cells and colony PCR was done by using gene specific primers which were used to amplify hIDO cDNA was represented in Figure 1A. This 1209 bp EcoRI – XhoI fragment was cloned into pET 32a' expression vector and the coding sequence of hIDO would be in frame with the Histidine coding sequence of this vector. This would permit the expression of a hIDO fusion protein from the pTac promoter (Lac promoter and T7) of the vector. This plasmid, termed pET 32a-rhIDO, was used to transform *E. coli* BL-21(DE3) cells (Figure 1B). The restriction fragment cloned into the pET32a' vector was sequenced using forward and reverse primers. The sequence of both strands was identical to the coding region and it confirmed that the insert in plasmid pET 32a -hIDO is the coding sequence of the hIDO gene. *E. coli* BL-21 (DE3) cells transformed with pET 32a'-rhIDO have been grown in the existence of IPTG to induce the expression of the hIDO fusion protein. A protein seemed to be induced in grown cultures in the present of IPTG and the purified rhIDO protein resolved as a single band on SDS-PAGE as shown in figure 1C.

Effects of EAHI on IDO inhibitory activity

Different solvent extracts (Methanol, Ethyl acetate, Petroleum Ether and Aqueous) of *H. indicus* were tested for their inhibitory activity against hIDO. In this assay, dose dependent increase in the percent inhibition activity against hIDO enzyme was noted. Inhibitory concentration 50 (IC_{50}) values were compared with that of Quercetin, (a known IDO inhibitor) is 16.51 ± 0.18 µg and among other extracts, EAHI was established stronger hIDO inhibitory activity with lower

Table 3: Effect of EAHI treatment on tryptophan levels and kynurenine pathway metabolites in lens homogenate of streptozotocin induced diabetic rats.

Group	*KYN/IDO activity (nM/100mg)*	*KYNA (pM/g)*	*3HKYN (nM/g)*	*TRP (µM/g)*
Group-I	0.67 ± 0.051	0.14 ± 0.035	5.14 ± 0.16	42.39 ± 0 .87
Group-II	2.04 ± 0.111*	0.97 ± 0.088*	13.81 ± 0.44*	55.20 ± 1.10*
Group-III	1.49 ± 0.093$^{*\#}$	0.32 ± 0.068$^{*\#}$	8.64 ± 0.68$^{*\#}$	49.20 ± 1.66$^{*\#}$
Group-IV	0.94 ± 0.049$^{\#}$	0.22 ± 0.46$^{*\#}$	6.76 ± 0.56$^{\#}$	45.21 ± 0.88$^{\#}$

Data ware expressed as mean ± SD ($n=3$). Data ware analyzed by one way ANOVA.* *vs* Group-I rats (Narmal control). (; $p \leq 0.05$). $^{\#}$ *vs* Group-II rats (Diabetic control) at $p \leq 0.05$.

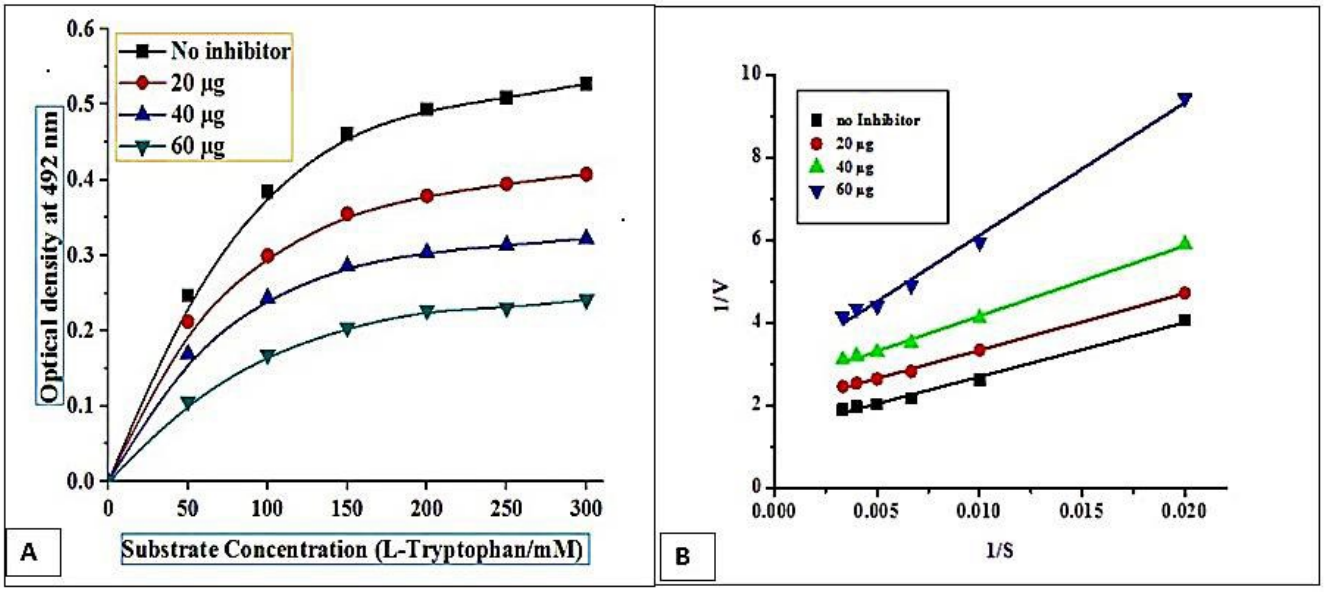

Figure 2: Effect of H. indicus on IDO inhibitory activity. A) Represents IDO inhibitory ability of Ethyl acetate extract of H. indicus with different doses and graph expressed as substrate concentration vs enzyme velocity. B) Effects of Ethyl acetate extract of H. indicus (EAHI) on the Lineweaver- Burk plot of hIDO activity. Graph expressed as reciprocal values of enzyme velocity of IDO vs reciprocal values of substrate (L-tryptophan, mM) by non-competitive manner. Values were presented as Mean ± SD (n=3).

IC_{50} values 34.74±0.82 µg (Table 1). Figure 2A represent kinetic parameters such as Km & Vmax to understand the mechanism of inhibition of IDO by EAHI. Vmax was found to decrease in the presence of different concentration of EAHI. However, there was no change in Km with L-Tryptophan being substrate as evaluated by Line weaver–Burk plots in a non-competitive manner as described in Figure 2B.

Acute toxicity and effective dose calculation

Acute toxicity studies have been conducted according to a fixed dose method as per the OECD 423 test guidelines.

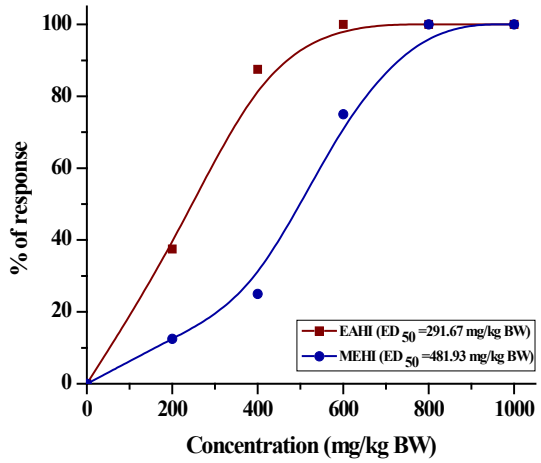

Figure 3: The effective dose (ED 50) of H. indicus extracts have been carried out on different groups of rats. Each group contain 8 rats and experimental time is seven days. EA: ethyl acetate, ME: methanol, HI: Hemidesmus indicus, BW: body weight.

In this study revealed that the administration of EAHI extract showed no sign of behavioral pattern, no mortality and weight loss at the concentration of 2000 mg/kg BW. According to toxicology guidelines, $1/10^{th}$ (200 mg/kg) & $1/5^{th}$ (400 mg/kg) were taken as effective doses according to respective ED_{50} values calculated by Figure 3 and for all further experiments with rodent model.

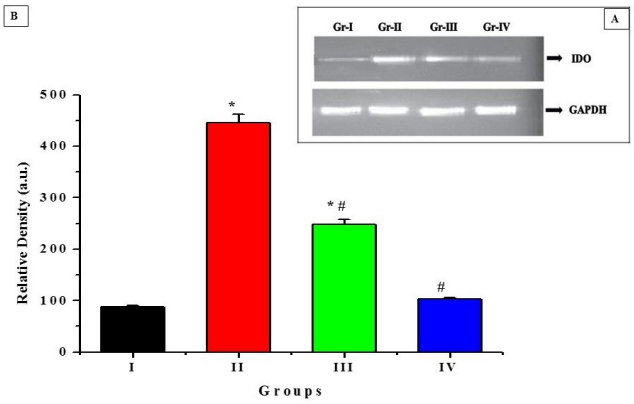

Figure 4: The effect of EAHI on the expression and quantification of IDO gene and housekeeping gene (GAPDH) in the experimental rat lens. Representative Agarose gel analysis showing the expression of IDO in groups of experimental rats along with GAPDH (A). Quantification of data presented as the relative density of IDO gene expression compared with that of GAPDH (B).
The data are the average of three independent experiments (Mean±SD, n=3).
Statistically significant from Group-I (analyzed by ANOVA; p≤0. 05).
#Statistically significant from Group-II (analyzed by ANOVA; p≤0. 05).

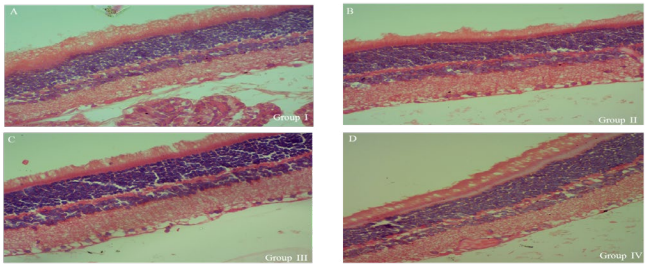

Figure 5: The end of the experiment eyes were collect and dissected from different groups of rats (G-I to G-IV), histopathological studies of retina were performed by Hematoxylin and Eosin (H&E) stain and examine under microscope (Olympus C X 21)for histological changes.

Effects of EAHI on kynurenine metabolites

The present study has been attempted to evaluate the concentrations of kynurenine pathway metabolites in experimental rat lens and effect of EAHI in Table 3. The study demonstrated a significant increase in kynurenine metabolites (Kyn, 3-HKYN, KYNA & TRP) in diabetic control rats (Group-II) with respect to control rats (Group-I). The diabetic rats which were fed with EAHI (Group III & IV) in dose dependent manner i.e. 200 mg /Kg body weight & 400 mg/ Kg body weight respectively were found to have decreased kynurenine metabolite levels along with deterioration of TRP levels (Table 3).

Effects of EAHI on IDO Expression by RT-PCR analysis

Biochemical results indicated an increase level of IDO in STZ induced diabetic rats, we have made interest to determine the elevated transcription levels of IDO gene by using semi quantitative RT-PCR in lenticular tissue from experimental rats. For that primers were synthesized corresponding to IDO and one house keeping gene GAPDH. Figure 4A reveals the IDO transcript was detectable very low in control rats, the relative expression of IDO mRNA was significantly increased in diabetic control rats (Group II). The expression of mRNA encoding IDO was remarkably reduced in diabetic rats treated with EAHI (Group III & IV) in dose dependent manner and expression levels of IDO gene relative density were represented in Figure 4B.

Histopathological Examination

Histopathological examinations in retina tissues of all rats have been observed using hematoxylin and eosin stain (H&E). The control group rats showed normal morphological changes observed in inner and outer nuclear layer along with pigmented layers of retina found to have disturbed architecture. Interestingly, the supplementation of EAHI rat retina (Group III & IV) showed prevention of pigmentation of layers in retina as control. These observations suggesting that, delaying cataract progression and ameliorative effect of EAHI confirmed with dose-dependent(Figure 5). Histopathological study have been suggesting an effective potential of EAHI in dose dependent against diabetic induced alternations.

Discussion

Diabetes and hyperglycemia are primary causal factors, play a key role in the onset of diabetic complications such as cataract which contribute to blindness worldwide.[34,35] Recent studies reported that diabetes could become a major threat to public health and the management of cataract blindness.[36] Cataract is a long-term complication of diabetes, the only remedy for the treatment of such kind disease is surgery with undesirable defects. Studies on the pathophysiology of diabetes and its associated complications have revealed, hyperglycemia, oxidative stress, polyol and kynurenine metabolites have been and have been suggested as common underlying mechanisms of cataractogenesis. [37,38] Several mechanisms for the pathogenesis of diabetic complications have been reported from time to time and among them, the IDO-related kynurenine pathway and their metabolites which are act as a pro-oxidants are major contributor to oxidative stress in some tissues, particularly in eye lens.[39-42] IDO is expressed in by many human tumors and results in suppression of T-cell based immune response. IDO has also been implication in depression and in the formation of saline nuclear cataracts.[43,44] Treatment with IDO inhibitors have shown to prevent (or) delay cataract progression and management of crystalline structures observed in the lens, a characteristic features of diabetic cataract. [45]

In this light IDO inhibition would be of immense interest in the prevention or delaying of diabetic cataract and in this context has been made to look into extracts isolated from *H. indicus* and their inhibitory role on IDO activity *in vitro*, *in vivo*. In the present work, a bacterial expression system for production of recombinant human IDO has been constructed, and the purified enzyme has been used to activity assay of rhIDO inhibition that has enabled assay with extracts prepared from a medicinal plant *H. indicus*. The results of the study demonstrated a noticeable inhibition of IDO activity in the lenticular tissue *in virto* and the extent of inhibition is comparable with that of known inhibitor, i.e. Quercetin. Among the four extracts, EAHI showed significant inhibitory potential towards rhIDO, and kinetic studies have suggested that EAHI can interact and inhibit the IDO in a non-competitive manner. Further, the studies have also been extended to know the efficacy of EAHI in STZ induced diabetic rats.

Streptozocin (STZ) is a chemical agent used to induce hyperglycemia in experimental animals mainly due to the rampant generation of reactive oxygen species (ROS), selective pancreatic islet b-cell cytotoxicity and the cytotoxic events subsequently cause b-cell necrosis. However, we observed higher levels of IDO mRNA, as well as enzymatic activity in lens of STZ induced diabetic rats and interestingly, EAHI treated rat's shows the IDO activity and significantly decreased levels of kynurenine metabolites (Kyn, 3-HKYN, KYNA) as well as TRP compared to diabetic control lens. Finally, the results of the RT-PCR, mRNA transcripts have been expressed very low in the EAHI treated groups of rat lens in

a dose dependently like as near to control rats.

Conclusion

Overall results confirmed the antioxidant activities of EAHI root extract led to inhibition of IDO activity and as well as effects on physiological, molecular and histopathological alterations in diabetic rats. Nevertheless, further studies need to be investigated to isolate and characterize the bioactive compounds responsible for these activities and its constituents as promising therapeutic agents treatment for diabetes and it's complications.

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Conflict of Interest

Arthurs declare no conflict of interest.

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