Published online in http://ijam.co.in

ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (2), 2024; 570-575

Assessment of polyherbal formulations in managing diabetic retinopathy: An in-vivo experimental model study

Research Article

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Abstract

Background: Chronic hyperglycemia induced oxidative stress may leads to many complications in diabetic patient i.e. nephropathy, neuropathy, retinopathy, stroke, ischemic heart diseases etc. The present study was designed to find the protective effect of polyherbal formulation in rat model of diabetic retinopathy. Material & Methods: Healthy 36 male Wistar rats were randomly divided into four groups namely normal control (n=6), diabetic (n=10), standard metformin (n=10) and test drug (n=10) consisting *Triphala* (*Haritaki, Aamlaki, Bibhitaki), Devdaru, Daruharidra* and *Musta* After induction of diabetes with streptozotocin (45mg/kg), rats were kept without any treatment for period of three weeks for induction of diabetic retinopathy followed by treatment with test drug (540mg/kg) for further 8weeks. HbA1c level was tested. Eye tissue homogenates were subjected to biochemical analysis to evaluate the aldose reductase (AR) activity, oxidative stress parameters namely Superoxide dismutase (SOD), catalase, glutathione (GSH), lipid peroxidation (LPO). Histopathological analysis of retinal tissue was conducted. Results: Test drug showed significant reduction in HBA1C level equivalent to standard metformin. Significant reduction was observed in AR and LPO levels and non-significant reduction in SOD, GSH were observed. Histopathological evaluation indicative of restoration and enhancement in cellular stability of retinal tissues. Conclusion: The herbal formulation showed potent hypoglycemic and antioxidant effect. It can be used as an adjuvant medication in prevention and treatment of diabetic retinopathy.

Keywords: Aldose reductase, Diabetic retinopathy, Hyperglycemia, Oxidative stress, Polyherbal formulation, SOD, Streptozotocin, SDG 3.4.

Introduction

Diabetes mellitus (DM) is a group of chronic metabolic disorders, prognosis of which depends on poorly controlled hyperglycemia.(1)Long term uncontrolled DM creates risk of micro-vascular complication such as retinopathy, nephropathy, neuropathy and macro-vascular complication like stroke, peripheral vascular diseases, ischemic heart disease etc.(2) Diabetic retinopathy (DR) is most commonly reported microvascular complication of diabetes, which has remained a leading cause of vision loss in working adults.(3)

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Chronic hyperglycemia is considered as a key factor for the pathogenesis of DR due to its tissue damaging effects. Its lead to the activation of multiple metabolic pathways for excess glucose metabolism such as polyol pathway, advanced glycation end products (AGEs), protein kinase C pathway (PKC), increased hexosamine flux. The end result of these pathways is the production of cytokines and inflammatory growth factors, leading to micro-vascular occlusion, increased vascular permeability, vascular endothelial dysfunction and neovascularization. (4)

The current line of treatment for DR includes laser photo-coagulation, intravitreal anti-VEGF injection, intravitreal steroid injection and vitreo-retinal surgery etc. (5) These treatment helps only for stabilisation of visual acuity & slow down further vision loss but fails to prevent blindness and associated with adverse effects like pain, peripheral & colour vision loss, reduction in night vision, contrast sensitivity and development of macular oedema etc. (6)The research

thrust trace an urgent need for adjuvant, cost effective line of treatment for diabetes and its complications. The World Health Organisation expert committee on diabetes has recommended that traditional system of medicine should be encouraged for prevention and treatment of diabetic complication. (7)

Present study depicts the protective effect of polyherbal formulation for the treatment of DR established through animal experimentation.

Materials and Methods

Institutional Animal Ethics Clearance

Approval for experimentation was taken from the Animal Ethics Committee of laboratory (No: IAEC/Reg no. 87/1999/CPCSEA) in accordance with the guideline formulated by the Committee for the Purpose of Control and Supervision on Experiments on Animals, India.

Preparation of test drug polyherbal formulation

A polyherbal formulation was prepared by using raw material namely, Stem bark of *daruharidra* (*Berberis aristata* DC, Berberidaceae), fruits of *amalaki* (*Phyllanthus emblica* L, Phyllanthaeceae), *haritaki* (*Terminalia chebula* L, Combretaceae) and *bibhitaki* (*Terminalia belerica* L, Combretaceae), rhizome of *musta* (*Cyperus rotundus* L, Cyperaceae) and stem bark of *devadaru* (*Cedrus deodara* Roxb. ex. Lamb., Pinaceae).

The raw material were purchased from market and authenticated in Alarsin pharmacy. The preparation of formulation was carried out in Department of Rasashastra and *Bhaishajyakalpana*, at R. R. A. Podar Ayurved College, Worli, Mumbai.

Chemicals

Streptozotocin was purchased from Sisco Research Laboratory (SRL), India. All the reagents and chemicals used in this study were of analytical grade.

Test Animals

Healthy 36 male wistar rats weighing between 200-250 gm were procured from National Institute of Biosciences, Pune (1091/abc/07/CPCSEA). They were housed in registered house facility at study center, Mumbai. Animals were maintained on a 12h light-12h-dark cycle with temperature 25+2^{0}C and relative humidity 50-70%. They were fed a commercial diet, pellets and water ad libitum. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC).

Induction of experimental diabetes

Diabetes was induced in 4-hour fasted male wistar rats by single intra-peritoneal (*i.p.*) injection of streptozotocin (45 mg/kg) in citrate buffer pH 4.5. For each rat, dose of streptozotocin was weighed in an amber coloured micro-centrifuge tube which was placed on ice until used. The dose was reconstituted with cold citrate buffer (pH 4.5) and immediately injected intra-peritoneally within 10 s. Post six hours of streptozotocin administration, drinking water was replaced by 10% glucose solution, for the next 24 hours to prevent fatal hypoglycaemia due to heavy insulin release from the necrotic b-cells of pancreas. After 72 hours, fasting blood glucose level of rats was determined using ACCUCHECK glucometer by tail vein sampling method. Rats with blood glucose level greater than 250 mg/dL were considered as diabetic and included in the study.

Seven days after induction, fasting blood glucose was determined using commercially available kit (Accurex, Mumbai). Animals with blood glucose level higher than 13.88 mmol/L (>250 mg/dL) were termed diabetic and included in the study.

Experimental procedure

Animal was randomly divided into 4 groups i.e. normal non-diabetic group (n= 6), diabetic group (n=10), standard group (n=10), test drug group (n=10) by cheat method. Animals were kept without any treatment for period of 21 days (3 weeks) for induction of diabetic retinopathy. Treatment was started from 4th week and continued till 8th week. Generally, 10% mortality has been noted in streptozotocin treated diabetic rats; thus, to compensate animals against this mortality, extra rats were included in diabetic control group and treatment group. Test drug (540 mg/kg powder) was uniformly suspended in 1 ml of distilled water and administered orally using stomach tube. The dose conversion was done using tables of Pagets and Barnes.

Experimental animals were grouped were as follows:

- Group 1 = Normal control group received distilled water only. (n=6)
- Group 2 = Diabetic control group, given streptozotocin injection intra-peritonially. (n=10)
- Group 3 = Standard control group received metformin500 mg/kg. (n=10)
- Group 4= Treatment group received poly-herbal formulation orally (540mg/kg). (n=10)

Samples

At end of the test period (8 weeks), rats were anesthetized under light isoflurane anesthesia & blood was withdrawn from retro-orbital plexus following 6-h fasting for various biochemical and histopathological analysis. Blood was centrifuged at 4000g for 10 min, subsequently serum was separated & stored at -20^0 C for further analysis. Serum was used for estimation of HbA1c using standard commercial kit.

Rats were perfused with normal saline and excision of both eyes were performed. With ice-cold saline solution eyes were rinsed and, blot dried. Right eye of each rat was fixed in 10% formalin solution for histopathological evaluation. Each left eye was cut by scalpel into 2 equal parts; weighed individually and stored at 80^0 C for further biochemical analysis. One half was used for determination of aldose reductase (AR).

Published online in http://ijam.co.in ISSN No: 0976-5921
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Biochemical assessment

HbA1c

For estimation of HbA1c whole blood was used using standard commercial kit from Accurex (Mumbai).

Aldose reductase activity in eye tissue homogenate

Aldose reductase (AR) activity in eye tissue homogenate was measured by as per the procedure of Hayman and Kinoshita with slight modifications. (8) One half of each right eye was homogenized in 3 volume of cold 5mm Tris – HCL buffer followed by the centrifugation at -4 C (10,000 rpm: R-248M of CPR24 plus instrument, Remi, India) for 20 min. Enzyme activity was measured spectrophotometrically at 340 nm with d-xylose as substrate.

Histopathology of retina

Right eye of each rats was fixed in 10% formalin in potassium phosphate buffer (0.1 M pH 7.4). The samples were subjected to dehydration, through graded alcohol series and embedded in paraffin. Then obtained paraffin blocks were cut into 4 mm sections and stained with Hematoxylin & Eosin. The slides were examined at 400X magnification under light microscopy.

Statistical Analysis

The results were analysed for statistical significance using 'One Way ANOVA' followed by Dunnett's test. Data was expressed as mean ± SEM, n=6rats in control group & n=10 rats in other group. $P<0.05$ value has been considered as the level of significance.

Results

Glycated haemoglobin (HbA1c)

HbA1c level was significantly ($p<0.001$) increased in diabetic group (HbA1c: 7.13 ± 0.25) as compared to normal group (HbA1c: 4.72 ± 0.46). However, with test drug treatment, HbA1c level (HbA1c: 4.20 ± 0.26) was significantly ($p<0.001$) decreased as compared to all other groups. The results obtained in TD and STD are statistically significant in lowering HBA1C. The data has been depicted in table 1.

Aldose reductase activity in eye tissue homogenate

Aldose reductase activity was significantly ($p<0.001$) increased in diabetic group (AR activity: 5.28 ± 3.13 Units/ mg protein) as compared to normal group (AR activity: 3.126 ± 8.1654 Units/ mg protein). While with test drug (AR activity: 4.449 ± 8.667 Units/ mg protein) aldose reductase activity was significantly decreased as compared to diabetic groups. (Table 1)

Table 1: Results of HbA1c and aldose reductase levels activity in Wistar rats

Animal groups	HBA1C	Aldose reductase (activity U/mg protein)
Normal Control	4.72 ± 0.46	3.12635E-07 ± 8.16543E-08
Diabetic Control	7.13 ± 0.25	5.28E-07 ± 3.13E-07
STD- Metformin	4.30 ± 0.78	3.48746E-07 ± 1.3152E-07
Test drug (Polyherbal formulation)	4.20 ± 0.26	4.4496E-07 ± 8.66734E-08
Value reflects Mean+ SDM One Way ANOVA' followed by Dunnett's test.		

Oxidative stress parameters in eye tissue homogenate

Oxidative stress was increased in diabetic control group as compared to normal control group evidenced by decreased in levels of endogenous antioxidant enzymes and increase in level of lipid peroxidation (LPO). Level of superoxide dismutase (SOD) and catalase was significantly ($p<0.001$) increased in diabetic control group (SOD: 0.243 ± 0.072 Units/ mg protein, Catalase: 0.272 ± 0.045 Units/ mg protein) as compared to normal control group(SOD: 0.478 ± 0.108 Units/ mg protein, Catalase: 0.539 ± 0.152 Units/ mg protein).

Treatment with test drug increased in levels of superoxide dismutase and catalase (SOD: 0.337 ± 0.059Units/ mg protein, Catalase: 0.433 ±0.078Units/ mg protein) however, it was noted non-significant statistically.

Level of reduced glutathione (GSH) was significantly increased in diabetic group (GSH: 0.060 ± 0.006 g/ mg protein) as compared to normal control group (GSH: 0.078 ± 0.003 g/ mg protein). While, in treatment group reduced glutathione level was non-significantly increased (GSH: 0.087 ± 0.013 g/ mg protein).

Table 2: Oxidative parameters studied in wistar rats

Groups	Catalase (Units/mg protein)	Superoxide dismutase (Units/mg protein)	Reduced glutathione (µg/mg protein)	Lipid peroxidation (nmols MDA/mg protein)
Normal Control	0.539 ± 0.152787	0.478 ± 0.108	0.078 ± 0.003	0.0120 ± 0.0014
Diabetic Control	0.272 ± 0.045768	0.243 ± 0.072	0.060 ± 0.006	0.0142 ± 0.0029
Standard Control	0.510 ± 0.058608	0.482 ± 0.096	0.089 ± 0.007	0.0021 ± 0.0005
Test drug	0.433 ± 0.078036	0.337 ± 0.059	0.087 ± 0.013	0.0000 ± 0.0000

Lipid peroxidation, a marker of cellular injury was increased significantly in diabetic group (LPO: 0.0142 ± 0.0029nmol MDA/ mg protein)as compared to normal control group (LPO: 0.0120 ± 0.0014nmol MDA/ mg protein).While in treatment group lipid peroxidation level was significantly decreased in (LPO: 0.000 ± 0.000 nmol MDA/ mg protein) as compared to all other groups. The data has been mentioned in table 2.

Histopathological analysis by hematoxylin & eosin staining

Histopathological analysis by hematoxylin and eosin staining demonstrated that, the retinal tissue cellularity in inner and outer nuclear layer was decreased in diabetic control group which can be attributed to increase in lipid peroxidation of cellular membranes along with decrease in levels of antioxidant enzymes. However, treatment with TD restored the cellularity which was better than the diabetic group and comparable to standard group. TD restored the cellularity to normal condition by ameliorating oxidative stress. (Figure 2) The outer nuclear loss of cells and total cellularity score is depicted in table 3.

Table 3: Outer nuclear layer and total cellularity loss score

Group Name	Outer nuclear layer loss of cells (Yellow arrow)	Total cellularity loss score
Normal Control	0	0
Diabetic Control	+++	3
STD-Metformin	+	1
Test drug	+	1

Note: += Mild Change; ++= Moderate change/Damage; +++= Severe change /Damage

Discussion

Diabetic retinopathy is a major micro-vascular complication of diabetes which often leads to decrease in vision or blindness. Currently available treatment for diabetes and retinopathy includes insulin, oral hypoglycemic drugs, laser photocoagulation, intra-vitreal steroid &anti-VEGF injections, vitero-retinal surgery etc. Although these treatments are quite effective but have some limitations such as its highly expensive, required frequent administrations of intraocular injections, acts for short durations & may causes side effects like macular edema, triggering retinal detachment in patients. Therefore, it's necessary to find out some cost effective, safer natural herbal remedies which can easily available to all class of world populations.

Many herbal compounds contain different phytochemical constituents that have ability to acts on metabolic pathways responsible for pathogenesis of diabetes and its complications.

In present study, a test drug containing poly-herbal formulation including *Haritaki, Amalaki, Bibhitaki, Devdaru, Daruharidra* and *Musta*, which are mentioned for the management of *Prameha* (a group of diabetic disorders).(9) These herbs are reported to possess anti-diabetic, antioxidant, anti-inflammatory and immune-modulator thus performs protective role in diabetes and its complications. (10,11 ,12)

The study explore in-vivo efficacy of these herbs in powder dosage form in prevention of diabetic retinopathy using rodent model of DM/RAT.

Chronic hyperglycemia activates multiple metabolic pathways, leading to oxidative stress in retina by producing Reactive Oxygen Species (ROS), superoxide, catalase. In present study, with TD treatment HbA1c was significantly decreased as compared to diabetic group. This indicates that, TD acts on pancreas as an insulin stimulator as well as insulin sensitiser to increase peripheral glucose utilisation. However, it also helps in correcting the metabolic imbalance caused due to DM.

Hyperglycemia induced oxidative stress has been considered as a crucial cause in pathogenesis of DR. It produces mitochondrial dysfunction, inflammation and cell death by apoptosis and neuro-degeneration that leads to vascular, neural and retinal tissue damage. (13)The imbalance between reactive oxygen species (ROS) production and the antioxidant defense system induces several oxidative stress related mechanism that endorses the pathogenesis of DR. Oxidative stress in eye tissue homogenate was measured by calculating the level of intracellular antioxidant enzymes namely, SOD, catalase, GSH and LPO. Treatment with TD has showed decrease level of SOD, catalase and reduction in GSH; however it was found statistically significant.

Significant reductions in LPO level were observed in eye tissue homogenate as compared to diabetic group. Malondialdehyde (MDA) is a highly reactive three-carbon dialdehyde, produced as an end product of lipid peroxidation. It is a highly toxic and reactive with DNA to form adducts with deoxyguanosine and deoxyadenosine. This is suggestive of TD acts by reduction of LPO levels and achieves anti-oxidant action.

The TD also decreases AR levels significantly as compared to diabetes control but in compare to standard the results were non-significant. AR is an enzyme implicated in development of diabetic cataract as well as retinopathy. In normal glucose conditions, only trace amount of glucose (approximately 3%) enters the polyol pathway, however, under hyperglycemic conditions about 30 % of glucose is metabolized through this pathway to form sorbitol.

The rate limiting step of the polyol pathway is the reduction of glucose to sorbitol catalyzed by aldose reductase (AR), at the expense of reduced nicotinamide adenosine dinucleotide phosphate (NADPH). Sorbitol is, in turn, converted to fructose by sorbitol dehydrogenase (SDH) with the oxidized form of nicotinamide adenine dinucleotide (NAD+) as a co-factor.There are three potential mechanisms by which the polyol pathway contributes to oxidative stress. First, under hyperglycemic condition, 30% of the glucose is channeled into AR-dependent polyol pathway, which depletes NADPH and consequently reduces GSH level. Second, oxidative stress is generated during the conversion of sorbitol into fructose by SDH (i.e., the second step of polyol pathway). In this step, the co-

factor NAD+ is converted to NADH by SDH. NADH is a substrate for NADH oxidase leading to production of superoxide anions. Third, the polyol pathway converts glucose to fructose, and fructose can be further metabolized into fructose-3-phosphate and 3-deoxyglucosone, which is more potent non-enzymatic glycation agent than glucose. Thus, the flux of glucose through the polyol pathway would increase AGEs formation, ultimately leading to ROS generation. Considering these facts, inhibition of aldose reductase is an important target for prevention of diabetic retinopathy.

Histological analysis of the retinal tissue demonstrated that, the cellularity in nuclear layer of retinal tissue was restored with TD.

Test drug contains *Triphala* has been frequently mentioned by Ayurveda for treatment of diabetes and its complications, inflammation, immunomodulatory etc. The bioactive compounds of *Triphala* are tannins, saponins, flavonoids, amino acids, ellagic acids are potent antioxidant. (14) In addition, polyphenols derived from *Triphala* namely chebulinic acid, tannins, ellagi tannins, gallotannins also effectively helps to increase insulin responsiveness and lowers the blood glucose level by inhibition of protein glycation in vitro. Clinical researches validate blood sugar lowering and immunomodulatory effect of *Triphala.* (15)

In-vivo experiments states, administration of *Triphala* effectively reduces the lysosomal enzymatic activity in the arthritis induced rats, which supports its anti-inflammatory claim. (16)

Daruharidra (*Berberis aristata* DC) is projected as anti-inflammatory, wound healing drug in Ayurveda and has been indicated for eye diseases. (17) Berberine prime alkaloid of B. aristata helps for restoration of hepatic glycogen content and blood glucose level. It is reported that, root extract of B. aristata has strong potential to regulate glucose homeostasis through decreased gluconeogenesis and oxidative stress. (18)

Musta (*Cyperus rotundus* L.) is a useful to treat obese diabetes as per Ayurveda. Researchers report its anti-oxidant action by inhibiting and scavenging free radicals. (19)

Devadaru (*Cedrus deodara* L.) root and root-bark is one of the anti-diabetic plant described in ayurveda, have showed promising anti-hyperglycemic action attributed to its constituents namely alkaloids, glycosides, tannins & phenolic compounds, tri-terpenoids, fixed oils, fats, and flavonoids.(20)

Conclusion

The polyherbal formulation containing *Triphala* (*Haritaki, Aamlaki, Bibhitaki), Devdaru, Daruharidra* and *Musta* experimentally proves its protective role in in-vivo model of diabetic retinopathy by effectively lowering hyperglycemia, significantly decreasing HBA_1C levels and reversing the lipid peroxidation. The herbal formulation thus showed potent hypoglycemic and antioxidant effect. It can be used as an adjuvant medication in prevention and treatment of diabetic retinopathy.

Future scope

Although, present study showing protective effect on amelioration of DR, further studies are required for studying impact of formulation on different parameters such as inflammatory cytokines (e.g. tumor necrosis factor -α). In addition fundus and lenticular examination of rat pupils can be studied to know the severity of retinal damage.

Acknowledgment

Authors acknowledges to management Dr. D. Y. Patil College of Ayurved & Research Center, D.Y. Patil Vidyapeeth (Deemed to be University), Maharashtra, Pimpri, Pune for constant support and encouragement.

Figure 1: Levels of oxidative parameters SOD, Catalase, GSH, Lipid peroxidation

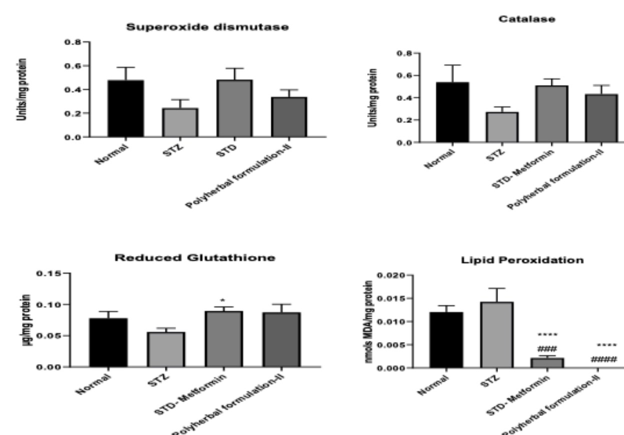
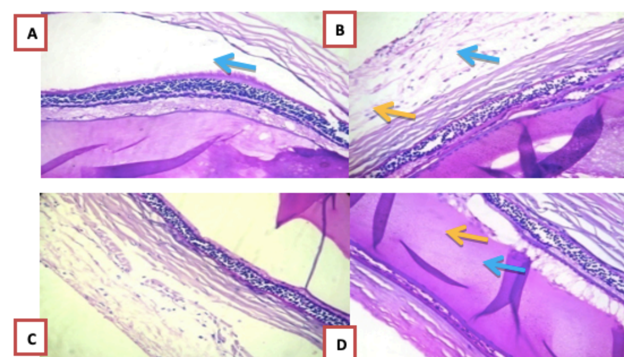


Figure 2: Histopathological examination of retinal tissue



Legends: A: Normal Control, B: Diabetic Control, C: STD-Metformin, D-Test drug.

Yellow arrow- Outer nuclear layer, Blue arrow- Inner nuclear layer

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