**RESEARCH ARTICLE**

# Effectiveness of *Syzygium aromaticum* Extract in Prevention of Diabetic Retinopathy in Experimental Animals

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## ABSTRACT

**Aim of the study:** *Syzygium aromaticum* has been reported to possess aphrodisiac, stomachic, carminative, antispasmodic and anti-cataract properties in traditional ayurvedic medicine. The current study determined the efficacy of *S. aromaticum* alcoholic extract in the prevention of diabetic retinopathy in rats with type 2 diabetes.

**Materials and Methods:** Type 2 diabetes was induced in neonatal rats with streptozotocin. The effect of *S. aromaticum* alcoholic extract in different doses on glycemic parameters, lenticular changes, vessel diameter, antioxidant enzymes, inflammatory markers and thickness basement membrane of retinal capillaries was assessed with electron microscopy. The results of the treatment groups was compared with that of the diabetes control group.

**Results:** The treatment of diabetic rats with *S. aromaticum* alcoholic extract at different doses significantly reduced blood glucose, glycated hemoglobin, lenticular changes, vessel diameter, inflammatory markers and thickness of basement membrane of retinal capillaries and antioxidant enzymes status.

**Conclusion:** *S. aromaticum* alcoholic extract may be used strategically to prevent diabetic retinopathy.

**Keywords:** Basement membrane thickness, Clove, Markers, *Syzygium aromaticum*, Type 2 Diabetes, Retinopathy.

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**Conflict of interest:** None

## INTRODUCTION

Debilitating diabetic retinopathy, which is the main cause of blindness in western countries among working-age individuals, is caused by diabetic microvascular problems.[1] The prevalence of diabetic retinopathy is increasing with an increase in the number of diabetic patients.[2] The mechanisms of diabetic retinopathy and microvascular complications are not well understood, but some vasoproliferative markers are involved in the pathogenesis of diabetic retinopathy. Animal models of hyperglycemia and human studies have demonstrated increased retinal levels of tumor necrosis factor-alpha (TNF- α), vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF-1α).[3-5] Tissue hypoxia has been hypothesized to be the major component stimulating the development of new vessels through up-regulation of VEGF and HIF-1α in human and animal models of proliferative diabetic retinopathy.[6] The intravitreal anti-VEGF medications, which are currently licensed for the treatment of diabetic retinopathy and macular oedema, have severe side effects, are expensive, and are poorly tolerated by patients. Pharmacological agents for the prevention of diabetic retinopathy are not yet available. Thus, it is the need of the hour to look for more effective antidiabetic agents which can check the progression of microvascular complications with fewer side effects.

Cloves are dried flower buds of *Syzygium aromaticum* (L.) Merr. and Perry. (Family: Myrtaceae) which is an evergreen tree native to India, Indonesia, Zanzibar, Mauritius, and Sri Lanka. It is most significantly used in Indian and Unani medicine. Clove possesses aphrodisiac, stomachic, carminative, and antispasmodic properties.[7] It is reported to be beneficial in cataracts. Clove extract also has anticarcinogenic properties.[8] It inhibits platelet aggregation and arachidonic acid metabolism in human platelets. Phytochemical studies have shown that cloves contain free eugenol, eugenol acetate, caryophyllene, sesquiterpenes, phenyl propanoid,

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β caryophyllene, eugenol and acetyl eugenol.[9] Eugenol, the primary component of cloves, suppresses lipid peroxidation and keeps glutathione peroxidase-6 phosphate dehydrogenase, catalase, and superoxide dismutase active;[10] and also has vasodilator and smooth muscle relaxant properties.[11] *In-vitro* studies with clove extracts have shown that clove causes similar cellular effects as that of insulin.[12] Eugenol treatment has also been used in diabetic rats and has shown beneficial effects on nerve and vascular endothelium function as it possesses antioxidant and anti-inflammatory properties.[13] As there are no systematic studies reported on the treatment of diabetic retinopathy with *S. aromaticum*. All these properties of *S. aromaticum* could be of potential in the treatment of diabetic retinopathy. Therefore, the current study aimed to evaluate the effectiveness of *S. aromaticum* alcoholic extract in the prevention of diabetic retinopathy in type 2 diabetic rats.

## MATERIALS AND METHODS

### Plant Material

Glimepiride USP (Gift Sample). Batch No. PO10743397 Provided by- Panacea Biotec Ltd. Malpur-Baddi,Solan (H.P.)173205.

*S. aromaticum* flower buds were purchased from the local market and authenticated by Ms. E. R. Nayar from National Bureau of Plant Genetic Resources (ICAR), PUSA, New Delhi. A voucher specimen No. NHCP/NBPGR/2007/104 was deposited at NBPGR, New Delhi.

### Method of Extraction

Dried flower buds of *S. aromaticum* were powdered using a domestic electric grinder. The ground material was passed through sieve no. 22 to obtain moderately coarse powder. The coarse powder was blended and extracted in soxhlet apparatus with rectified spirit until exhaustion at room temperature. Following cotton wool filtration, the filtrate was concentrated using distillation to recover the solvent, and the concentrate was then further concentrated at 40°C using a rotavapor until the solvent had completely evaporated to get the extract. The extract of *S. aromaticum* showed a percentage yield of 22.85%.

### Induction of Experimental Diabetes

Streptozotocin was used to induce diabetes in a neonatal rat model for the study.[14] Streptozotocin in a dose of 90 mg/kg (i.p) in citrate buffer (pH-4.5) was used to induce diabetes in two-day-old rat pups; the rat pups in the normal control animals were injected with buffer solution. Rats were fed a 10% dextrose solution for the first 12 hours. After seven weeks of induction of diabetic blood glucose was measured and rats showing fasting blood glucose levels above 140 mg/dl were selected for the study. By puncturing the distal end of the rat tail with an Accu-Chek Softclix Lancing device and utilizing glucose test strips (Accucheck Active, Roche Diagnostics India Pvt. Ltd.), fasting blood glucose was measured using a commercially available kit that uses the glucose oxidase-peroxidase (GOD-POD) method. (Roche, Germany). All the treatment groups were fed standard laboratory chow and water was provided *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical committee of DIPSAR, New Delhi (Protocol No. IAEC/2007-II protocol No. 9).

### Experimental Groups and Treatments

From the aforementioned streptozotocin-induced 24 *Wistar* albino rats that had type II diabetes, five treatment groups with a total of six animals each were created. Equal numbers of males and females were kept in each group, and they were housed individually as shown in Table 1. Group 1 was designated as normal control and group 2 as diabetic control and received only 2% gum acacia. Glimepiride is used as a positive control. Each treatment lasted for 12 weeks; during the treatment period rats were fed food and water *ad libitum*. Blood glucose and body weight were measured every week for the changes. The percentage change in the fasting blood glucose is represented here.

### Anterior and Posterior Segment Photography

Animals were screened using a slit lamp and ophthalmoscope for inclusion in the study and for measuring blood vessel diameter. The animals with any abnormality in the anterior and posterior chambers of eye were excluded from the study. Anterior segment and fundus photographs were taken using slit lamp biomicroscope using a 90D lens to quantitate the lenticular (cataractous) and retinal changes (vessel diameter). To dilate the pupil tropicamide (1%) was used topically. Moisol eye drops (0.7% hydroxypropyl methyl cellulose) was administered periodically to avoid drying of the cornea. Slit lamp biomicroscope was attached with a digital camera (Nikon, Japan) to obtain the photographs. Rats eye was placed on the focus of the slit lamp lens to take anterior chamber photographs and with the help of 90D lens the posterior chamber photographs were taken. These photographs were saved for further evaluation and measurement of vessel diameter. The methodology of Vucetic *et al.* (2004) was utilized to estimate the diameter of retinal vessels.[15]

Anterior segment photographs were taken to quantitate visible lenticular changes in the rat eye of different study groups. The lens photograph was divided into 12 hours cycle and the number of hours affected were counted to quantitate the lenticular changes. Three different observers were given randomized photographs for the evaluation of the lenticular changes and the averages of the three readings are represented.

Fundus photographs were taken with the help of slit lamp biomicroscope and 90D lens. The magnification of the slit lamp biomicroscope was fixed to 16X. A dimension of 3000x2000 pixels was applied to all the pictures taken to assume it as a standard size. All the fundus photographs were randomized before the measurement of vessel diameter to avoid any bias by the observer. Three vessels were chosen (each in different quadrant) and vessel diameters were recorded for these three vessels. Fundus photographs were evaluated by three independent observers and average of the three readings are represented. The red free fundus photographs were evaluated using Adobe Photoshop CS3 version software.

**Table 1:** Groups and treatment of experimental animals

| *Treatments (Code)* | *Type of animals* | *n* | *Treated with* |
|---|---|---|---|
| Normal Control (N) | Normal rats | 6 | 2% gum acacia |
| Diabetic control (D) | Diabetic rats | 6 | 2% gum acacia |
| Glimepiride 0.5 mg/kg (G) | Diabetic rats | 6 | Glimepiride in 2% gum acacia |
| *S. aromaticum* 100 mg/kg (S) | Diabetic rats | 6 | *S. aromaticum* in 2% gum acacia according to dose |
| *S. aromaticum* 200 mg/kg (Sa) | Diabetic rats | 6 | *S. aromaticum* in 2% gum acacia according to dose |

**Sample Collection**

Animals in all groups were anesthetized mildly after the 12 week study period was over in order to collect blood from the retro-orbital plexus for the estimation of glycated hemoglobin; this blood was then collected in citrated vacutainers, and the animals were then killed by administering deep anesthesia and their eyes enucleated. The retina is attached at two places in the eye: at the ora serrata and at the optic nerve. With the help of a sharp razor, the eye was cut half transversely so that the anterior chamber (cornea and lens) was separated from the posterior chamber (with retina). The sclera was held with forceps, and a very thin spatula was gently pushed under the retina (between retina and choroid). The retina is not firmly attached to the underlying structures and tissues separate with relative ease. A very thin and delicate scissors was slid under retina to cut the optic nerve. The retina then was totally free, and could float free into the water. The isolated retina was held gently with the help of blunt forceps without damaging the tissue. The retinal tissue was then weighed and transferred in an eppendorf tube containing chilled 50 mM phosphate buffer and was then homogenized with the help of hand-held homogenizer (tubes were dipped in ice till the homogenization was complete to prevent denaturation of proteins from heat generated during homogenization process). The remaining 50 mM phosphate buffer was added in order to remove the tissue from the homogenizer pestle and create a 10% homogenate which was then centrifuged at 4500 rpm at 4˚C temperature for 15 minutes. The supernatant so formed was utilized for further analysis.

**Method of Estimation of Glycated Hemoglobin**

A cationic exchange resin holds hemoglobins after the labile fraction is eliminated from the hemolysate made from the whole blood. The kit from Biosystems (Barcelona, Spain) precisely elutes hemoglobin A1C (HBA1C) after washing away the hemoglobin A1a+b fraction (HBA1a+b), and quantifies it by direct photometric measurement at 415 nm using a spectrophotometer (Hitachi 2900, Japan).

**Preparation of Retinal Homogenate**

The retinal tissue was then weighed and transferred in eppendorf tube containing chilled 50 mM phosphate buffer and was then homogenized with the help of hand held homogenizer (tubes were dipped in ice till the homogenization was complete to prevent denaturation of proteins from heat generated during homogenization process). The remaining 50 mM phosphate buffer was added to wash the tissue out from the homogenizer pestle and prepare a 10% homogenate. The homogenate was then centrifuged at 4500 rpm at 4˚C temperature for 15 minutes. The supernatant so formed was then used for further analysis.

**Method of Assay in Retinal Homogenate**

The estimation of glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) was performed in the aforementioned supernatant using specific kits from Cayman chemical company (USA), and measurements of the vasoproliferative markers TNF-alpha, (Diaclone, France), vascular endothelial growth factor (VEGF), and HIF-1α, (R & D Systems, USA) were measured using enzyme-linked immunosorbent assay (ELISA) reader Model 680 (BioRad, India).

**Histopathological Study**

Transmission electron microscopy (TEM) was done to see the changes in basement membrane thickness and capillary integrity. The retina was fixed for 6 hours at 4˚C in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). It was then separated and cut into 2 x 2 mm pieces in an optic disc to temporal peripheral direction. The fixed retinal tissue was transported to the electron microscopy facility (AIIMS, New Delhi) for further processing. The retinal tissue underwent osmication, dehydration, and then embedded in Araldite CY 212. (TAAB, UK). Cut thin slices of retina (70–80 nm) were contrasted with lead citrate and uranyl acetate. Retinal sections were viewed under Morgagni 268 D transmission electron microscope equipped with digital imaging (Fei Company, Netherlands). Two eyes from each group were subjected for the study. The basement membrane thickness of six vessels was recorded using software (soft imaging system, Munster, Germany) and observed for any changes in integrity. Basement membrane thickness was measured from six different capillaries viewed and their mean thickness was calculated and plotted for different groups.

**Statistical Analysis**

With the aid of Sigma Stat 3.5 Software, one-way analysis of variance and post hoc multiple comparisons across groups using the Student-Newman-Keuls technique were used to conduct statistical analyses on the data for all the parameters under investigation. $p < 0.05$ was considered statistically significant. The values are represented as average mean and standard error of mean. Bar charts are drawn using Excel 2003 software.

## RESULTS

### Effect of *S. aromaticum* on Body Weight and Blood Glucose

The diabetes control group demonstrated a significantly ($p < 0.05$) lower increase in body weight than the normal control group, although the diabetic control group's fasting blood glucose at the conclusion of the 12 week study period was significantly greater than that of the normal control group. A significant increase in body weight was observed with glimepiride and *S. aromaticum* a higher dose. The increase in body weight in the *S. aromaticum* lower dose was not significant compared to a diabetic control group. Compared to the diabetic control group, all treatments significantly reduced the fasting blood glucose, however, it was significantly elevated in diabetic control group as compared to normal control group. The maximum decrease in blood glucose was observed with *S. aromaticum* higher dose (Table 2 and 3).

### Effect of *S. aromaticum* on glycated hemoglobin

Levels of glycated hemoglobin increased significantly ($p < 0.05$) in the diabetic control group as compared to normal control group. All the treatment groups significantly ($p < 0.05$) decreased glycated hemoglobin levels compared to the diabetic control group (Figure 1). Significant difference among the *S. aromaticum* treatment groups was not observed in glycated hemoglobin levels.

### Effect of *S. aromaticum* on Lenticular Changes and Vessel Diameter

Diabetic control group animals exhibited a significant increase in cataract development and vessel diameter ($p < 0.05$) compared to the normal control group. All treatment groups significantly ($p < 0.05$) alleviated the cataract-like changes and reduced vessel diameter compared to with diabetic control group (Table 4, Figure 2 and 3). These parameters did not observe significant differences among the *S. aromaticum* treatment groups.

### Effect of *S. aromaticum* on Antioxidant Enzymes

Considerable ($p < 0.05$) reduction in antioxidant enzyme (glutathione reductase, superoxide dismutase and catalase) levels was observed in the diabetic control group compared to

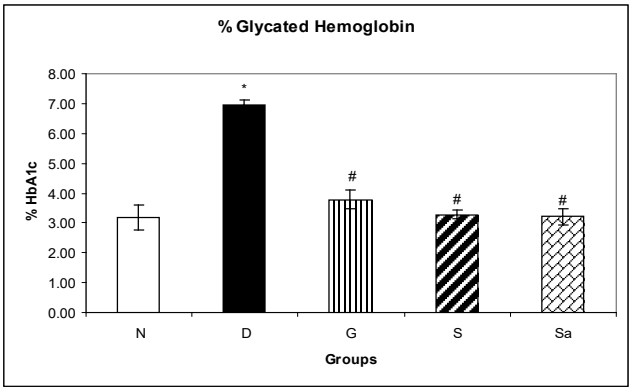


**Figure 1:** Effect of different treatments on Glycated Hemoglobin after 12 weeks of treatment.

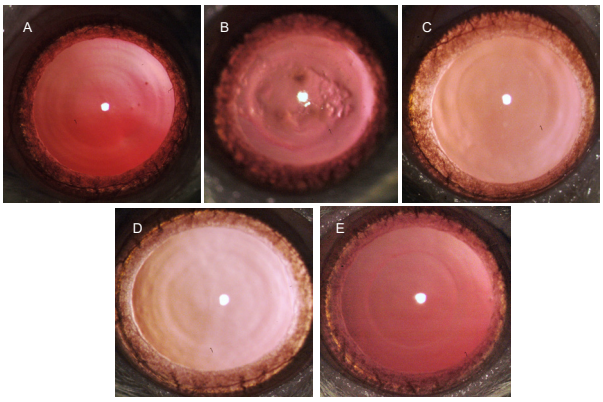


**Figure 2:** Anterior Segment Photographs of the groups after 12 weeks of treatment. Photographs from A-E showing lenticular changes in the groups (Normal Control, Diabetic Control, Glimepiride treated, *Syzygium aromaticum* 100 mg/kg treated, *Syzygium aromaticum* 200 mg/kg treated respectively) ↑ Arrows in the Diabetic control group B showing cataract like changes in the Lens.

**Table 2:** Percentage change in body weight after 12 weeks of treatment

| *Groups (n= 6/ group)* | *Before Treatment* | *After Treatment* | *Percentage Change* |
|---|---|---|---|
| Normal Control (N) | 109.67 ± 5.38 | 240.00 ± 6.71 | 54.31 |
| Diabetic Control (D) | 116.83 ± 3.18 | 201.67 ± 6.41* | 42.07 |
| Glimepiride (G) | 134.67 ± 5.05 | 279.17 ± 12.27$^{\#}$ | 51.76 |
| *S. aromaticum* 100 mg/kg (S) | 99.17 ± 5.83 | 176.67 ± 9.46 | 43.87 |
| *S. aromaticum* 200 mg/kg (Sa) | 85.83 ± 3.27 | 205.83 ± 7.35# | 58.30 |

**Table 3:** Percentage Change in Blood Glucose after 12 weeks of treatment

| *Groups (n= 6/ group)* | *Before Treatment* | *After Treatment* | *Percentage Change* |
|---|---|---|---|
| Normal Control (N) | 104 ± 3.87 | 87.33 ± 2.6 | ↓16.03 |
| Diabetic Control (D) | 151.17 ± 6.36 | 189 ± 13.56$^{*}$ | ↑25.03 |
| Glimepiride (G) | 156.67 ± 4.27 | 114.67 ± 6.5$^{\#,b}$ | ↓26.81 |
| *S. aromaticum* 100 mg/kg (S) | 165 ± 4.88 | 106.17 ± 3.57$^{\#,b}$ | ↓35.66 |
| *S. aromaticum* 200 mg/kg (Sa) | 159.67 ± 5.11 | 93.67 ± 1.67$^{\#}$ | ↓41.34 |

Values are Mean ± SEM. *p < 0.05 vs Normal, $^{\#}$p<0.05 vs Diabetic control, $^{b}$ p < 0.05 vs *S. aromaticum* 200 mg/kg

**Table 4:** Effect of different treatments on Lenticular Changes and Vessel diameter after 12 weeks of treatment

| *Groups (n= 12/ group)* | *Lenticular Changes (Nos affected out of 144)* | *Vessel Diameter (pixels)* |
|---|---|---|
| Normal Control (N) | 5.00 ± 1.0 | 46.49 ± 0.80 |
| Diabetic Control (D) | 90.33 ± 7.13* | 61.99 ± 0.79* |
| Glimepiride (G) | 12.33 ± 4.34# | 50.91 ± 0.52# |
| *S. aromaticum* 100 mg/kg (S) | 9.33 ± 1.45# | 51.34 ± 0.58# |
| *S. aromaticum* 200 mg/kg (Sa) | 8.0 ± 1.16# | 50.65 ± 0.55# |

Values are Mean ± SEM. *p <0.05 vs Normal, #p <0.05 vs Diabetic

**Table 5:** Effect on antioxidant enzymes in retinal homogenate after 12 weeks of treatment

| *Groups (n= 6/ group)* | *Glutathione Reductase (nmol/min/mL)* | *Superoxide Dismutase (U/mL)* | *Catalase (nmol/min/mL )* |
|---|---|---|---|
| Normal Control (N) | 8.62 ± 0.33 | 7.05 ± 0.33 | 14.33 ± 2.47 |
| Diabetic Control (D) | 2.51 ± 0.16* | 4.73 ± 0.23* | 7.46 ± 0.33* |
| Glimepiride (G) | 5.09 ± 0.43# | 6.59 ± 0.33# | 12.12 ± 2.02# |
| *S. aromaticum* 100 mg/kg (S) | 6.11 ± 0.38# | 5.06 ± 0.09 | 8.99 ± 0.31 |
| *S. aromaticum* 200 mg/kg (Sa) | 7.24 ± 0.38# | 5.62 ± 0.31# | 10.16 ± 0.75# |

Values are Mean ± SEM. *p <0.05 vs Normal, #p <0.05 vs Diabetic control

**Table 6:** Effect of treatments on different markers in retinal homogenate after 12 weeks of treatment

| *Groups (n= 6/ group)* | *TNF- α (pg/mL )* | *VEGF (pg/mL )* | *HIF- 1 α (pg/mL )* |
|---|---|---|---|
| Normal control (N) | 25.74 ± 0.80 | 6.81 ± 0.75 | 16.97 ± 6.0 |
| Diabetic control (D) | 58.12 ± 4.57* | 149.90 ± 13.18* | 180.91 ± 24.72* |
| Glimepiride (G) | 27.00 ± 0.7#, a, b | 8.34 ± 1.5 #, a, b | 37.46 ± 10.44#, a, b |
| *S. aromaticum* 100 mg/kg (S) | 44.75 ± 5.9# | 46.95 ± 2.79# | 98.03 ± 9.4# |
| *S. aromaticum* 200 mg/kg (Sa) | 37.59 ± 4.51# | 45.62 ± 5.36# | 88.64 ± 9.12# |

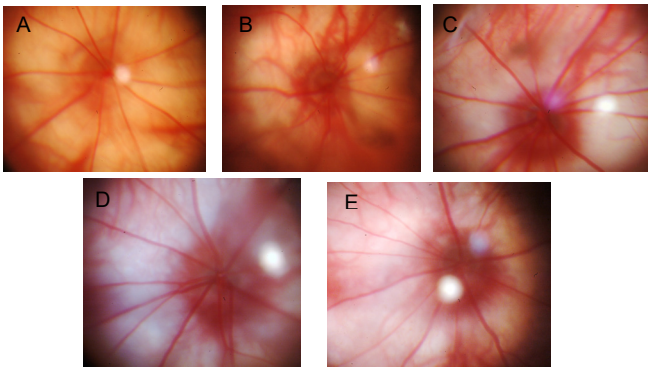


**Figure 3:** Fundus Photographs of the groups after 12 weeks of treatment.Photographs from A-E showing posterior segment changes in the retina in groups (Normal Control, Diabetic Control, Glimepiride treated, *Syzygium aromaticum* 100 mg/kg treated, *Syzygium aromaticum* 200 mg/kg treated respectively). Diabetic control group (B) showing changes in the vessel integrity, vessel tortuosity and leakiness. Other groups showed normal vessel integrity.

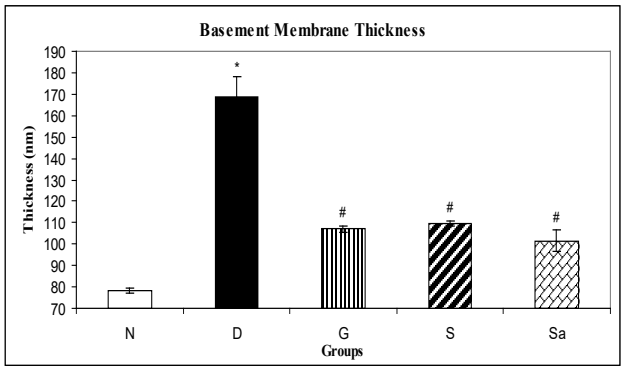


**Figure 4:** Effect of treatments on basement membrane thickness of retinal capillaries after 12 weeks of treatment. Values are Mean ± SEM. Normal Control(N), Diabetic control(D), Glimepiride (G), *S. aromaticum* 100 mg/kg treated (S), *S. aromaticum* 200 mg/kg treated (Sa) *p<0.05 vs Normal, #p<0.05 vs Diabetic control

normal control group. *S. aromaticum* treatment elevated the antioxidant enzyme levels in a dose-dependent manner. The higher dose of *S. aromaticum* significantly ($p < 0.05$) improved antioxidant enzyme status as compared with diabetic control group (Table 5).

### Effect of *S. aromaticum* on Vasoproliferative Markers on Retinal Homogenate

Significantly ($p < 0.05$) higher levels of the vasoproliferative factors (TNF-α, VEGF and HIF-1α) were present in diabetic control group as compared to normal control group. No discernible difference between the lower and higher dosages of *S. aromaticum* treatment was found, however, treatment with the extract significantly ($p < 0.05$) decreased the levels of these variables compared to the diabetic control group. (Table 6).

### Effect of *S. aromaticum* on Basement Membrane Thickness of Retinal Capillaries

Basement membrane thickness of capillary in diabetic control rat retina rose significantly compared to normal control group.

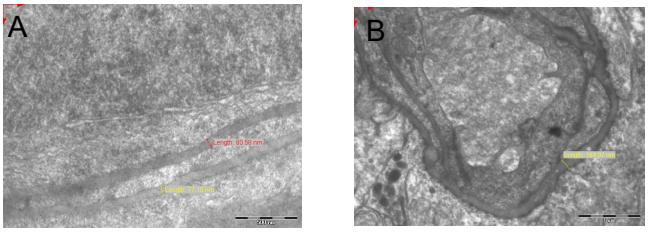


**Figure 5:** Electron micrograph of Normal and Diabetic control rat retina after 12 weeks. A. Normal control: Basement membrane (b) is thin (80.58 nm), endothelial cell cytoplasm (e) is distinct with cell organelles, pericyte cytoplasm (pc), pericyte basement (pb) in the (l) lumen of the capillary. Muller cell cytoplasm (MC) surrounds the capillary. B. Diabetic control: Thickened and Irregular basement membrane (b) of endothelium (184.97 nm), (e) Endothelial cytoplasm is indistinct, (l) Lumen of capillary

Diabetic control retinae showed irregular basement membrane and increased basement membrane thickness showing signs of microangiopathic lesions of diabetic retinopathy. Basement membrane thickness of retinal capillaries reduced significantly ($p < 0.05$) in a dose-dependent manner when treated with *S. aromaticum* alcoholic extract, albeit the difference among the groups was statistically insignificant (Figures 4-6).

## DISCUSSION

To the best of our knowledge, the current study is the first to use an alcoholic extract of *S. aromaticum* to stop diabetic retinopathy. In T2D, partial or total insulin deficiency causing derangement of carbohydrate metabolism, including continuous glucose excretion, decreased peripheral uptake of glucose and glycogen synthesis, affecting body weight.[16] Streptozotocin-induced diabetic control animals gained less weight than normal control and treatment groups. Failure of diabetic animals to gain weight during the study is due to this derangement of metabolism. Glimepiride, an insulin secretagogue, causes weight gain and corrects the derangement of T2D. The treatment with *S. aromaticum* alcoholic extract boosted body weight and normalized blood sugar levels in the treated animals in a dose-dependent manner. Our study showed beneficial effects of *S. aromaticum* in controlling blood glucose which can be due to its insulin-mimetic activity as *in-vitro* studies with *S. aromaticum* extracts have shown that *S. aromaticum* causes many similar cellular effects as that of insulin.[12]

Diabetes can be treated aggressively to lower the morbidity and mortality associated with it and its long-term complications. Lowering blood glucose levels has been proven to have the same beneficial effects on T2D microvascular complications as it does on type 1 diabetes.[17,18] Presently glycated hemoglobin is regarded as gold standard for quantifying blood glucose control. Direct determination of blood glucose indicates current blood glucose levels whereas glycated hemoglobin reflects the blood glucose levels quarterly.[19] In diabetic individuals, ideal target glycated hemoglobin values should be less than 6.5%.[17] In the current study diabetic control group showed elevated levels of glycated hemoglobin above the recommended target

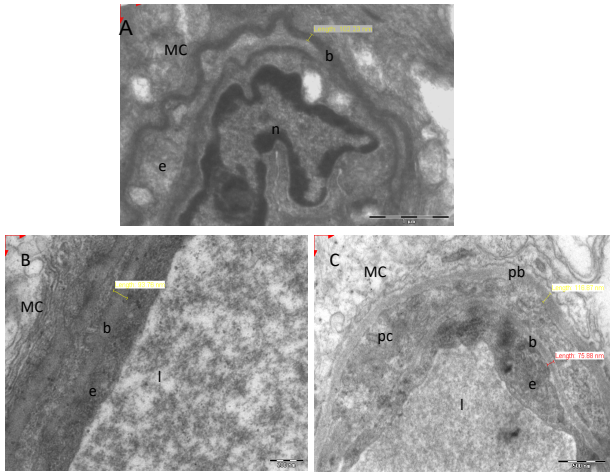


**Figure 6:** Electron micrograph of rat retina after 12 weeks of different treatments. A. Glimepiride treated: Showing a thinner Basement membrane (b) (102.23 nm) than that of diabetic control. Endothelial cell cytoplasm (e) is distinct with cell organelles, n, nucleus of a leucocyte. Muller cell cytoplasm (MC) surrounds the capillary. B. *Syzygium aromaticum* 100 mg/ kg and C. *Syzygium aromaticum* 200 mg/ kg treated showing thinner basement membrane (b) (93.76 nm and 75.88 nm) than diabetic control. Endothelial cell cytoplasm (e) is distinct with cell organelles. A red blood cell (rbc) in the lumen (l) of the capillary, Muller cell cytoplasm (MC) surrounds the capillary, pericyte basement membrane (pb), pericyte cytoplasm (pc) is also distinct.

levels thus showing changes associated with retinopathy. Compared to diabetic control group, *S. aromaticum* treatment in current study considerably lowered blood glucose levels. The HbA1c values in the animals treated with *S. aromaticum* were found to comply with DCCT guidelines for glycemic management.[17]

It is evident that quantitatively measuring the retinal vascular diameter can predict the early development of diabetic microvascular complications, such as retinopathy.[20-23] The retinal vessel diameter significantly increased between the diabetic control group and the normal control group, demonstrating the presence of early diabetic retinopathy in rats. The treatment with *S. aromaticum* extracts showed significant improvement in the vessel diameter, indicating prevention of diabetic retinopathy.

Significant vascular tortuosity has been documented in fundus photographs of spontaneously diabetic rats as a risk factor for diabetic retinopathy.[24] Vascular tortuosity was seen in the diabetic control group in the current investigation, albeit it was not quantified. *S. aromaticum* treated diabetic groups showed absence of vessel tortuosity.

Imbalance between the formation of reactive oxygen species (ROS) and antioxidant defences which results in oxidative stress. This sets off a chain of events that destabilises cellular functioning.[25] SOD, GR, glutathione peroxidase (GPx), and CAT are antioxidant defence enzymes that scavenge free radicals and maintain redox equilibrium. In diabetes, activities of these antioxidant enzymes are lowered in the retina.[26,27] The diabetic control rats in our study displayed subnormal antioxidant enzyme activity (GR, SOD and CAT). Changes

observed in the levels of antioxidant enzymes in our study are consistent with previous studies.[28,29] Treatment with *S. aromaticum* appears to restore the retinal antioxidant defence mechanism to normal, delaying the onset and progression of diabetic retinopathy in rats.

The present study showed increased TNF-α level in diabetic control group as compared to normal control group. Similarly several investigations have revealed that diabetic rats had much higher retinal TNF-α level than healthy rats.[30] In the current study reduced TNF-α levels in *S. aromaticum* treated rats demonstrate its anti-inflammatory potential in dose dependent manner.

Proinflammatory marker VEGF has been linked to neovascularization and enhanced vascular permeability. VEGF increases retinal vascular permeability causing breakdown of the blood-retinal barrier resulting in retinal oedema.[31,32] Two obvious candidates for retinopathy include hypoxia and hyperglycemia, both of which are known to have an impact on angiogenic growth factors. Hypoxia inducible factor type 1 (HIF-1) which induces expression of VEGF[33] which is raised by both hypoxia and hyperglycemia, diabetic animals have increased retinal HIF-1 expression that matches the pattern and timing of VEGF expression.[34] The current study's increased retinal VEGF and HIF-1 levels in the diabetic control group are in line with these investigations. Treatment with *S. aromaticum* in dose dependent manner decreased levels of TNF-α, VEGF and HIF-1α indicating its potential in preventing vasoproliferative changes.

Increased thickness of the basement membrane is the primary structural microangiopathic lesion of the tiny blood vessels in diabetic rats and humans.[35,36] Studies on diabetic animals have revealed that extracellular matrix protein expression increases the basement membrane thickness of capillaries in the kidney, retina, and heart of streptozotocin-induced diabetic rats.[37,38] Early hyperglycaemia in diabetes has been linked to increased synthesis of basement membrane components like fibronectin and collagen types I, III, IV (1, 2), and V are found to be upregulated in the retinal basement membrane in diabetic retinopathy.[39] Our results are consistent with the previous studies as we observed increased retinal capillary basement membrane thickness in diabetic control group compared to normal control group. Treatment with *S. aromaticum* has ameliorated the changes in basement membrane thickness of retinal capillaries.

Eugenol and isoeugenol found in the alcoholic extract of *S. aromaticum* can be attributed for positive benefits. Both eugenol and isoeugenol have been shown to possess antioxidant,[10,40] and anti-inflammatory,[41,42] properties and our results are accordant with these studies.

The favourable effects associated with *S. aromaticum* in the current study were attained by reducing the severity of hyperglycaemia and owing to its anti-inflammatory and antioxidant activities in *S. aromaticum* treated animals compared to diabetic control animals.

## CONCLUSION

In compendium results of our study provide evidence that *S. aromaticum* alcoholic extract in both the doses studied can alleviate hyperglycaemia and be useful for prevention of diabetic retinopathy in streptozotocin induced diabetic rats. It is evident from the study that the extract is capable in preventing the onset and progression of diabetic retinopathy possibly through its hypoglycaemic and antioxidant effects and inhibiting vasoproliferative markers like TNF- α, VEGF and HIF-1α in retinal homogenate. Reduced basement membrane thickness of the retinal capillaries also points towards its beneficial effects. Therefore, *S. aromaticum* can effectively halt the development of diabetic microvascular complications if it is included to the daily diet of T2D patients.

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## CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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