



## EVALUATION OF RENAL PROTECTIVE EFFECTS OF *ANOGEISSUS LATIFOLIA* W. IN STREPTOZOTOCIN – INDUCED DIABETIC RATS

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### ABSTRACT

*Anogeissus latifolia* is a small to medium sized tree up to 20-36m tall, belonging to the family Combretaceae. This herb is widely used in Ayurveda for the treatment of various disorders. Animals were allocated into six groups of six animals per each. The treatment for Streptozotocin (65mg/kg) induced diabetic rats with Whole Plant extract of *Anogeissus latifolia* is (100, 200 and 400mg/kg body weight) for 30days showed the decreased levels of Lipid Peroxidation markers such as Malondialdehyde (MDA). The activities of enzymatic antioxidants Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx) and levels of non-enzymatic antioxidants reduced Glutathione (GSH) increased in diabetic treated rats. And Blood Urea Nitrogen (BUN), Serum Creatinine & Urea levels were estimated. The results of this study showed that Ethanolic ALEE has antidiabetic activity along with that of antioxidant activity and renal protective effects.

**Keywords:** Diabetes Mellitus, Oxidative Stress, Streptozotocin, *Anogeissus latifolia*, renal markers.

### INTRODUCTION

Plants play the most important part in the cycle of nature. Without plants there could be no life on earth. These are the primary producers that sustain all other forms of life. Plants often contain substantial amounts of antioxidants including  $\alpha$ -tocopherol, carotenoids, ascorbic acid, flavonoids and tannins, and it has been suggested that antioxidant action may be an important property of plant medicines used in diabetes.<sup>1</sup>

In diabetes mellitus, chronic hyperglycemia produces multiple biochemical sequelae, and diabetes-induced oxidative stress could play a role in the symptoms and progression of the disease. Oxidative stress in cells and tissues results from the increased generation of reactive oxygen species and/or from decreases in antioxidant defense potential. Several hypotheses have been put forth to explain the genesis of free radicals in diabetes.<sup>2</sup> These include auto oxidation processes of glucose, the non-enzymatic and progressive glycation of proteins with the consequently increased formation of glucose-derived advanced glycolysation end products (AGEs), and enhanced glucose flux through the polyol pathway.<sup>3</sup>

Diabetes mellitus (DM) is a syndrome characterized by abnormal insulin secretion, derangement in carbohydrate and lipid metabolism, and is diagnosed by the presence of hyperglycemia. During diabetes or insulin resistance, failure of insulin-stimulated glucose uptake by fat and muscle causes glucose concentrations in blood to remain high. Diabetes is becoming the third "KILLER" of mankind, after cancer and cardiovascular diseases because of its high prevalence, morbidity and mortality<sup>4</sup>.

*Anogeissus latifolia* is a small to medium sized tree up to 20-36 m height, belonging to the family Combretaceae. It is commonly found in deciduous or semi- evergreen forest and is a native of India, Myanmar, Nepal and Srilanka.

### Medicinal uses

Its leaves contain large amounts of tannin<sup>5</sup>, and used for various disorders including stomach and skin diseases. The plant provides important timber with the leaves and bark being used for tanning. The bark was first examined by Reddy *et. al*<sup>6</sup> who isolated ( $\pm$ ) leucocyanidin. Later, ellagic acids and 2 new glycosides of ellagic and flavellagic acids were reported<sup>7</sup>. Etanobotanically, the bark has been reported to be used in the treatment of various skin diseases such as sores, boils and itching<sup>8</sup>, snake and scorpion bites, stomach disease<sup>9</sup>, colic<sup>10</sup>, cough<sup>11</sup>, and diarrhoea.<sup>12</sup>

*Anogeissus latifolia* contains flavonoids, sugars, sterols, essential oils, and resin, and nonglucosidal bitter substance, tannin, large amount of potassium nitrate and other constituents.<sup>13</sup>

As the evidence of earlier studies shows that the whole plant of *Anogeissus latifolia* possesses flavonoids, tannins, glycosides which are the major chemical constituents responsible for exhibiting antioxidant activity, the present study has been undertaken to evaluate the protective effects of ethanolic extract of whole plant of *Anogeissus latifolia* on liver and kidney tissues in streptozotocin induced oxidative stress.



## MATERIALS AND METHODS

### Collection of drug

The plant *Anogeissus latifolia* was collected from the forest area of Chittoor district in AP (India) during the month of October. Botanist of Sri Venkateshwara University, Tirupathi, authenticated the plant.

### Drugs and chemicals

All the drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All solvents were of analytical grade and were obtained from Sd. Fine Chemicals, Mumbai, India.

### Preparation of the ALEE

Dried and powdered whole plant material of *Anogeissus latifolia* was purchased from a commercial source (Madhavchetty). The powdered material was soaked with 70% ethanol overnight in Soxhlet thimble. The residue in the R.B flask was transferred into a beaker and was concentrated under reduced vacuum pressure to give an average yield of 70% (w/w). Solutions of the *Anogeissus latifolia* extract (ALEE) were prepared freshly for the pharmacological studies.

### Animals

The male wistar albino rats (150-200gms) were procured from Shadan Animal Husbandary and from Sai Animal Distributors, Musheerabad. The animals were acclimatized for 1 week. They were fed with commercial pelleted rats chow and were given free access to water ad libitum throughout the study. The animals were handled gently to avoid giving them too much stress, which could result in an increased adrenal output.

### Induction of diabetes

Diabetes was induced by administering intraperitoneal injection of a freshly prepared solution of Streptozotocin (65mg/kg b.w) in 0.1M cold citrate buffer (P<sup>H</sup> 4.5) to the overnight fasted rats. Since Streptozotocin is capable of producing fatal hypoglycemia as a result of massive pancreatic release of insulin, the rats were kept on 5% glucose for next 24hrs to prevent hypoglycemia<sup>14-15</sup>

### EXPERIMENTAL DESIGN

Forty rats were used and were classified into 6 groups (6 animals/ group; n=6).

Untreated:

- Group I : Normal STZ treated groups
- Group II: Diabetic (Positive) control
- Group III: ALEE 100mg/kg b.w
- Group IV: ALEE 200mg/kg b.w
- Group V: ALEE 400mg/kg b.w
- Group VI: Glibenclamide treated (600µg/kg b.w)

Out of forty rats, six were retained as normal group and remaining 34 animals were induced diabetes using STZ (65mg/kg b.w) through i.p, Initial blood glucose levels were estimated followed by estimations on 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> day. After a period of one week i.e., on seventh day the rats with blood glucose levels >280mg/dl were considered diabetic and used for research work.

The three different doses of drug were suspended with 2% Gum Acacia and given to the Group III to V. The above treatment was given for thirty days. At the end of the treatment i.e., after 30days the animals were fasted overnight. Blood was taken from the retro-orbital plexus under mild chloroform anesthesia. Blood sugar level was evaluated by the method of Glucose Oxidase Peroxidase method. Serum was then separated from blood and was used for estimation of Plasma renal marker enzyme levels.

The animals were then sacrificed by cervical dislocation. The tissues (kidney) were removed and cleared off blood, rinsed in ice-cold physiological saline. Small portions of tissue were fixed in 10% neutral Formalin solution for histology. The remaining tissue was homogenized by tissue homogenizer with Teflon pestle at 4°C in 0.1M Tris-HCl buffer at P<sup>H</sup> 7.4. The homogenate of kidney were centrifuged in a cooling centrifuge (500 X g) separately to remove the debris and supernatant was kept at -20°C and used for biochemical analysis.

### BIOCHEMICAL ESTIMATION

#### Determination of blood glucose levels

Blood glucose levels were checked at weekly intervals during the duration of the experiment. Blood samples for blood glucose determination were collected from tail tip at intervals of 7, 14, 21, 28 days. The blood glucose levels were detected by Glucose – Oxidase Principle (*Beach and Turner*) and results were reported as mg/dl.<sup>16</sup>

#### Estimation of serum urea, creatinine and BUN

The plasma levels of Urea estimated by using the method of Berthelot using End Point Assay technique<sup>17</sup>. Creatinine was estimated by using Modified Jaffe's reaction by initial Rate Assay Technique<sup>18</sup>. Blood Urea Nitrogen was estimated by method of Berthelot using Endpoint Assay technique<sup>19</sup>.

#### Estimation of antioxidant markers

##### A. Malondialdehyde (mda) levels

The activity of MDA was estimated by Okhawa *et al.*,<sup>20</sup>. With minor modification.

TBARS was estimated by reaction with thiobarbituric acid in the presence of butylated hydroxytoluene and measuring the absorbance at 535 nm of the pink coloured chromogen formed against reagent blank. MDA levels were expressed as nmol of MDA/mg protein or nm/100gm tissue.



### B. Superoxide dismutase levels (SOD)

The activity of Superoxide Dismutase was estimated by the method of Mc Cord and Fridvoich<sup>21</sup>. These are metallo enzymes, the amount of SOD present in cellular and extracellular environments are crucial for the prevention of diseases linked to oxidative stress. Absorbance of the blue color formed was measured again. Percent of inhibition was calculated after comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). The volume of the sample required to scavenge 50% of the generated superoxide cation was considered as 1 unit of enzyme activity and expressed in U/protein.

### C. Estimation of catalase (CAT)

The activity of Catalase was estimated by the method of Aebi. H<sup>22</sup>. Catalase activity was measured by using the rate of decomposition of H<sub>2</sub>O<sub>2</sub> and the values are expressed in nmol H<sub>2</sub>O<sub>2</sub> consumed U/mg protein.

### D. Estimation of reduced glutathione (GSH)

The activity of Reduced Glutathione was estimated by the method of Ellamn GL<sup>23</sup>. Total reduced glutathione was measured by the use of DTNB assay using Elman's method wherein the incubation mixture at 37°C contained 0.08M sodium phosphate (pH 7.0), 0.08M EDTA, 1.0 mM sodium azide, 0.4nM GSH and 0.25mM H<sub>2</sub>O<sub>2</sub>. GSH was determined at 3-min intervals using DTNB and is expressed as n.mol/mg protein.

### E. Estimation of glutathione peroxidase (GPX)

The activity of Glutathione Peroxidase was estimated by the method of Paglia and Valentine<sup>24</sup> GPx, an enzyme with Selenium, works with Glutathione in the decomposition of H<sub>2</sub>O<sub>2</sub> or other organic hydroperoxides to non-toxic products at the expense of GSH. Reduced activities of GPx may result from radical induced inactivation and glycation of enzymes.

One unit of enzyme activity was defined as decrease in GSH 0.001/mm after subtraction of the decrease GSH per

minute for the non-enzymatic reaction and is expressed as units/mg protein.

### Statistical analysis

Values were represented as Mean ± S.E.M. and data was analyzed by ANOVA followed by Dunnet's test using WINKS STAT SOFTWARE.

## RESULTS

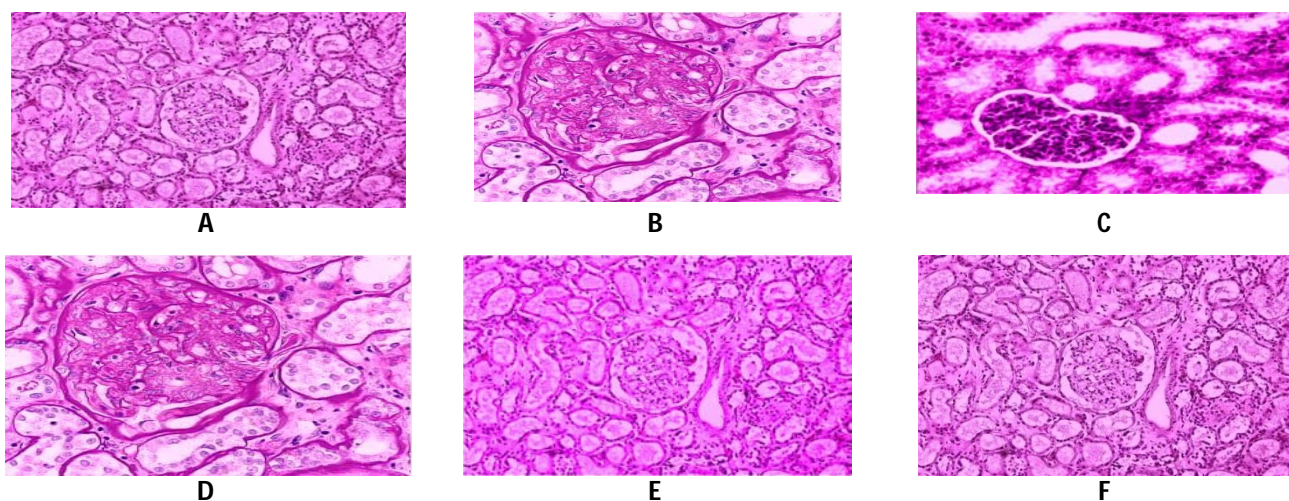
The present study was designed to evaluate the renal protective activity of ethanolic extract of whole plant of "*Anogeissus latifolia* W." (ALEE) in Streptozotocin (at dose of 65mg/kg) induced diabetic rats. In this study the various antioxidant parameters, FBG levels, plasma renal marker levels were used for the assessment of renal protective activity of "*Anogeissus latifolia* W."

The effect of the extract on fasting blood glucose levels was observed, using STZ at dose of 65 mg/kg bodyweight. After one week, it was observed that there was marked elevation in fasting blood glucose levels. The diabetic rats with FBG>280mg/dl were selected for further studies.

The blood glucose level in diabetic control group significantly increased and was higher than those of the control group. On the other hand, oral administration of ethanolic extract of whole plant of "*Anogeissus latifolia*" for 30 days was found to lower the blood glucose levels significantly in a dose dependent manner in treated diabetic groups when compared with those of the diabetic group.

### LIGHT Microscopy study of KIDNEY

Light microscopy of kidney sections of diabetic rats showed degenerations in proximal tubules epithelial cells in the cortex of kidneys, hemorrhage in the interstitial area and periglomerular lympholytic infiltration and hyalinization of the arterioles. Examination of kidneys of the diabetic rats treated with whole plant of extract ALEE indicated that the kidneys appeared more or less as control. Fig 1: (A-F)



**Figures 1: (A-F)** Kidney histological section of Norma I(A), DC (B), ALEE 100mg/kg (C), ALEE 200mg/kg (D), ALEE 400mg/kg (E), STD (F).



**Table 1:** Effect of oral administration of ethanolic extract of ALEE on FBG levels for 30 days

| Sample    | 1 <sup>st</sup> day | 7 <sup>th</sup> day | 15 <sup>th</sup> day | 21 <sup>st</sup> day | 28 <sup>th</sup> day |
|-----------|---------------------|---------------------|----------------------|----------------------|----------------------|
| Group I   | 79.77±1.72          | 74.23±3.64          | 78.68±5.27           | 75.66±2.46           | 76.12±1.66           |
| Group II  | 295.27±3.44         | 308.91±2.53         | 312.46±3.16          | 313.71±3.11          | 319.34±3.67          |
| Group III | 215.15±2.98         | 198.01±3.44         | 155.66±4.23          | 122.01±2.98          | 99.98±2.11*          |
| Group IV  | 210.15±2.86         | 167.48±2.97         | 127.11±3.09*         | 108.47±2.77*         | 89.67±3.43           |
| Group V   | 210.65±1.43         | 145.76±5.11         | 106.24±3.00*         | 99.45±3.11**         | 79.42±2.06*          |
| Group VI  | 201.03±4.36         | 140.37±4.61         | 99.56±2.76**         | 89.67±2.87**         | 78.34±2.66**         |

Values are expressed as Mean ± SEM values for six rats in each group. Diabetic control rats were compared with normal control rats. Diabetic + "Anogeissus latifolia" and Diabetic + Glibenclamide treated were compared with diabetic controlled rats. P<0.05, \*P<0.01, \*\*\*P<0.001 with respect to the Diabetic Control Group (ANOVA with Dunnet's t-test).

**Table 2:** Effect of ethanolic extract of whole plant of on *Anogeissus latifolia* MDA, GSH, Catalase, SOD, GPx levels in Kidney.

| Parameters       | Group I       | Group II     | Group III      | Group IV        | Group V          | Group VI        |
|------------------|---------------|--------------|----------------|-----------------|------------------|-----------------|
| MDA <sup>a</sup> | 1.33 ± 0.09   | 2.34 ± 1.16  | 1.71 ± 0.97*   | 1.63 ± 0.82*    | 1.47 ± 0.60***   | 1.43 ± 0.71***  |
| GSH <sup>b</sup> | 118.77 ± 2.31 | 46.17 ± 2.47 | 62.32 ± 2.89 * | 71.23 ± 3.14 *  | 91.33 ± 2.89 **  | 95.17 ± 2.55**  |
| CAT <sup>c</sup> | 38.33 ± 1.22  | 20.55 ± 1.75 | 27.37 ± 1.26*  | 31.65 ± 1.20 *  | 35.24 ± 1.81**   | 37.57 ± 1.41 ** |
| SOD <sup>d</sup> | 20.61 ± 1.21  | 8.95 ± 0.18  | 10.22 ± 2.11*  | 16.37 ± 2.40 ** | 18.91 ± 1.21 *** | 19.42 ± 1.46*** |
| GPx <sup>e</sup> | 7.33 ± 0.14   | 4.99 ± 1.01  | 5.22 ± 0.44 *  | 5.54 ± 0.21*    | 5.97 ± 0.76**    | 6.97 ± 0.58 *** |

Values are expressed as Mean ± SEM values for Six rats in each group. Diabetic control rats were compared with normal control rats. Diabetic + *Anogeissus latifolia* and Diabetic + Glibenclamide treated were compared with diabetic controlled rats. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 with respect to the Diabetic Control Group (ANOVA with Dunnet's t-test). <sup>a</sup>MDA= nM/100g tissue; <sup>b</sup>GSH= nM of DTNB conjugated/mg protein; <sup>c</sup>CAT= U/mg protein; <sup>d</sup>SOD= U/mg protein; <sup>e</sup>GPx= U/min/mg protein

**Table 3:** Effect of ethanolic extract of whole plant of *Anogeissus latifolia* on Serum Urea and Serum Creatinine levels

| Parameters               | Group I      | Group II     | Group III    | Group IV       | Group V        | Group VI       |
|--------------------------|--------------|--------------|--------------|----------------|----------------|----------------|
| Serum Urea (mg/dl)       | 29.49 ± 1.59 | 48.27 ± 2.78 | 42.36 ± 3.12 | 39.27 ± 2.72*  | 33.18 ± 2.10** | 31.31 ± 2.65** |
| Serum Creatinine (mg/dl) | 0.73 ± 0.04  | 1.98 ± 0.05  | 1.90 ± 0.21  | 1.68 ± 0.07 *  | 1.37 ± 0.06**  | 1.01 ± 0.03*** |
| BUN (mg/dl)              | 17.98 ± 2.20 | 31.78 ± 0.97 | 27.65 ± 0.56 | 20.68 ± 0.59** | 20.68 ± 0.59** | 26.57 ± 2.65** |

Values are expressed as Mean ± SEM values for Six rats in each group. Diabetic control rats were compared with normal control rats. Diabetic + *Anogeissus latifolia* and Diabetic + Glibenclamide treated were compared with diabetic controlled rats. P<0.05, \*P<0.01, \*\*\*P<0.001 with respect to the Diabetic Control Group (ANOVA with Dunnet's t-test).

## DISCUSSION

DM is probably the fastest growing metabolic disease in the world and as the knowledge of multifactorial or heterogeneous nature of the disease increases so does the need for more challenging and appropriate therapies<sup>25</sup>. Traditional plant remedies have been used for centuries in the treatment of diabetes but only a few have been scientifically evaluated. Therefore the present study was done to investigate the effect of ethanolic extract of whole plant of *Anogeissus latifolia* on biomarkers of oxidative stress, and renal markers in Diabetic rats<sup>20</sup>.

Oxidative Stress in diabetes co-exists with the decrease in antioxidant status which can increase the deleterious effects of free radicals. Evidence has accumulated indicating that the generation of reactive oxygen species (Oxidative stress) may play an important role in the etiology of diabetic complications<sup>21</sup>.

Glibenclamide is a standard antidiabetic drug used. It has been involved in stimulating insulin secretion from pancreatic β-cells principally by inhibiting ATP sensitive K<sub>ATP</sub> channels in the plasma membrane.

Antioxidant enzymes, Super oxide dismutase, Catalase, Reduced Glutathione and Glutathione peroxidase form the first line of defense against ROS and decreased activities were observed in the STZ diabetic rats.

In this study, we have found that poor glycemic control in diabetic patients was also associated with decreased free radical scavenging activity. In hyperglycemia, glucose undergoes auto-oxidation and Produces free radicals that in turn lead to peroxidation of lipids in lipoproteins. Elevated Levels of lipid peroxidation, as seen in diabetic patients are clear manifestation of excessive formation of free radicals resulting in tissue damage<sup>22, 23</sup>. The activity of super oxide dismutase was found to be lower in diabetic patients when compared to normal. This decrease in activity could result from activation of the enzyme by H<sub>2</sub>O<sub>2</sub> or by glycation of the enzyme, which are known to occur during diabetes. Super oxide dismutase scavenges superoxide anion to form H<sub>2</sub>O<sub>2</sub> and diminishes the toxic effects derived from secondary reaction. The activity of Super oxide dismutase was found to be lowered in diabetic controlled rats.

Catalase is a haeme protein, which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals. This decrease in Catalase activity could result from inactivation by glycation of the enzyme<sup>24</sup>. The increase in SOD activity may indirectly play an important protective role in preserving the activity of Catalase. The reduced activities of SOD and CAT in kidney have been observed during diabetes.



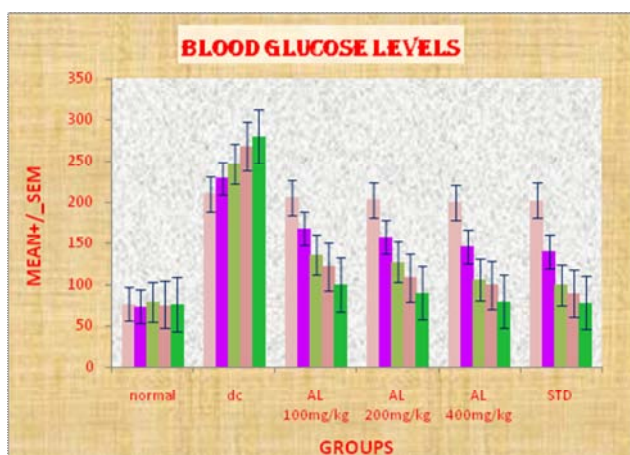
Glutathione peroxidase, an enzyme with selenium, works with Glutathione in the decomposition of  $H_2O_2$  or other organic hydroperoxides to non-toxic products at the expense of GSH. Reduced activities of Glutathione peroxidase may result from radical induced inactivation and glycation of enzymes. Further, insufficient availability of GSH may also reduce the activity of GPx. Reduced activities of GPx in kidney have been observed during diabetes and this may result in a number of deleterious effects due to accumulation of toxic products.

Increased activities of SOD, CAT and GPx after treatment with *Anogeissus latifolia* extract may be due to the presence of flavonoids and isoflavonoids, an antioxidant.

Glutathione is a tripeptide normally present at high concentrations intracellularly, and constitutes the major reducing capacity of cytoplasm. Decreased level of GSH in kidney during diabetes represents its increased utilization due to oxidative stress<sup>25</sup>. GSH plays a pivotal role in the protection of cells against free radicals. Decreased GSH in hyperglycemia is due to decreased formation, GSH formation requires NADPH and Glutathione Reductase (GR). Reduced availability of NADPH may be due to decrease in the activity of glucose 6-phosphate dehydrogenase and hence, decreased level of GSH. Administration of *Anogeissus latifolia* caused an increase in the activity of glucose-6-phosphate dehydrogenase; thereby increasing NADPH levels and, in turn, the GR activity. Thus GSH is replenished by the administration of *Anogeissus latifolia*, which may, in turn, maintain the antioxidant status in the tissues of diabetic rats. This indicates that the extract can reduce the oxidative stress leading to less degradation of GSH, or have both effects.

The increases of serum Creatinine, Urea levels are considered as obvious indicators for kidney damage and dysfunction. The diabetic hyperglycemia induces elevations of blood levels of creatinine urea which are considered as significant markers of renal dysfunction.

**Graph 1: Blood glucose levels**



In our study, it was observed that the levels of antioxidant enzymes (SOD, CAT, GPx, and GSH) and the lipid peroxidation levels were decreased in kidney of diabetic rats. The plasma renal markers also showed changes in

levels. In diabetic rats treated with ethanolic extract of *Anogeissus latifolia* (ALEE), a significant increase in activity of these enzymes was observed, that is the levels were brought back to normal by ALEE, indicate oxidative stress elicited by STZ had been nullified due to the effect of the extract. This might reflect the antioxidant potency of ethanolic extract, which by reducing blood glucose levels prevented glycation and inactivation of enzymes.

## CONCLUSION

Present study was conducted to evaluate the renal protective activity of *Anogeissus latifolia* whole plant and glibenclamide was used as the standard drug. The chemical constituents screened during the phytochemical screening revealed the presence of tannins, flavonoids, saponins, terpenes, glycosides which probably showed the antioxidant potential of the plant. The results showed significant increase in SOD, CAT, GSH, GPX and decrease in TBARS, SGOT, SGPT, ALP, Serum urea, Serum creatinine, BUN which shows antioxidant effect and may be responsible for the reduction of diabetic complications. *Anogeissus latifolia* possesses Renal protective effects which may be due to the number of active principles present, and also may be due to the elevation of lowered enzyme levels like SOD, CAT, GSH etc, and decrease of elevated levels like TBARS.

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