ANTIOXIDANT STATUS IN STREPTOZOTOCIN INDUCED DIABETIC RATS AND EFFECT OF AQUEOUS EXTRACT OF *MURRAYAKOENIGII* LEAVES ON IT

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Abstract:

**Objectives:** This study was done to investigate the antioxidant status in streptozotocin induced diabetic rats and effect of aqueous extract of *Murrayakoenigii* leaves on it.

**Materials and Methods:** Albino Wistar rats weighing 200-250 g were induced diabetes with intraperitoneal streptozotocin and fed with aqueous extract of MK leaves for 30 days. Their blood glucose levels were measured and compared with controls. Erythrocyte malondialdehyde, catalase activity and total antioxidant capacity was measured after sacrificing the rats and compared with control.

**Results:** It was noted that blood glucose levels in diabetic control rats were 2 fold than the control and MK leaves fed group. Erythrocyte malondialdehyde levels, catalase activities were significantly higher in diabetic group in comparison with normal and MK groups. Total antioxidant capacity was significantly higher in MK leaves fed group in comparison with diabetic group.

**Interpretation and conclusion:** Diabetes induced in rats by streptozotocin is indeed a high oxidative stress state. Aqueous extract of MK leaves has resulted in lower blood glucose levels. Also MK leaves are beneficial in diabetes as it has antioxidant effects as shown by the parameters in the present study.

**Key words:** *Murrayakoenigii*, Streptozotocin, malondialdehyde, catalase activity, total antioxidant capacity.

**Introduction:**

Diabetes is a worldwide phenomenon. In 2011 there are 366 million people with diabetes, and this is expected to rise to 552 million by 2030 and the global diabetes epidemic continues to grow.1 The disease will result in microvascular and macrovascular changes in the tissues, largely due to free radical injury. The oxidative stress in diabetes is the major culprit in the pathogenesis of the final metabolic and structural alterations of the organs. The
mechanisms of generation of free radicals are auto-oxidation of glucose, progressive and non-enzymatic glycation of proteins and subsequent formation of advanced glycosylation end products (AGEs). Under normal physiological conditions the free radical injury is kept under vigilance by an active widespread antioxidant mechanism. Increased free radical load may lead to enhanced utilization of antioxidant defence mechanism and lead to disrupted cellular functions and increased chances of lipid peroxidation.

In modern medicine, many hypoglycemic agents are used for glycemic control and have been proved to be beneficial. Of late many herbal medicines are also gaining importance in the treatment of diabetes but often lack the experimental evidence for the beneficial effects. More than 1000 plant extracts are presumed to be beneficial in treating diabetes and many of these are with minimal side effects. Therefore in the present study *Murraya koenigii* leaves are chosen as it is a common ingredient in south Indian diet. MK leaves were popularly known as curry leaves. MK leaves are used as flavouring, condiment and folk medicine and it belongs to family Rutaceae (citrus family). The MK leaves, barks and roots are used in traditional medicine for the treatment of piles, headache, stomach ache, influenza, rheumatism, traumatic injury, insect, snake bites, antivomiting, curing dysentery and diarrhea. MK leaves have carbazole alkaloids, triterpenoids, phenolic compounds and mineral contents such as iron, calcium, zinc and vanadium etc. Carbazole alkaloids present in MK leaves were reported to have antioxidant activities.

As the oxidative stress is the prime mode of cellular alterations in diabetes and MK leaves are reported to have antioxidant activities, the present study was designed to determine the antioxidant status of streptozotocin induced diabetic rats and to investigate the beneficial effects of MK leaves in diabetes, in animal model. Determination of malondialdehyde by thiobarbituric acid is used as an index of the extent of lipid peroxidation. Other parameters used in present study include catalase activity in erythrocytes and total antioxidant capacity of plasma.

**Materials and methods:**

**Preparation of plant extract:** The MK leaves were washed and air dried at room temperature. The aqueous extract was prepared in soxhlet extractor using 30g of dried plant tissue mixed with 150 ml of the solvent (100% v/v) for 24 h. The aqueous extract was lyophilized. 25 mg dry weight of crude extract was further reconstituted in 2.5 ml of distilled water and 1:20 dilution of the extract was used for further studies.

**Animals:** Inbred Albino Wistar rats weighing between 200-250 g were used for the study. They were housed in clean transparent poly propylene cages at room temperature at the natural light – dark cycle with food and water ad
libitum. All the animal experiments were conducted according to the ethical norms approved by institutional ethical committee.

**Induction of diabetes:** Diabetes was induced in rats with a single intraperitoneal injection of a freshly prepared solution of streptozotocin (50mg/kg) in 0.1M cold citrate buffer of pH 4.5. Rats were allowed a week for development and aggravation of diabetes. The rats with moderate diabetes having persistent glycosuria and hyperglycemia (fasting blood glucose range of above 150mg/dl) were used for the experiment. The treatment with aqueous extract of MK leaves was started on 8th day after STZ injection and was considered the 1st day of treatment.

**Experimental protocol:** Fasting blood samples were collected on 8th day and 10th day morning and tested for blood glucose. After confirmation of diabetes the animals were grouped into 3 groups comprising of 10 rats each as follows.

Group I – control rats receiving 0.1M cold citrate buffer.

Group II – diabetic control rats.

Group III – diabetic rats receiving aqueous MK leaves extract orally for 30 days.

At the end of 30 days all animals were dissected under ether anaesthesia. 5 ml blood is collected from inferior vena cava using heparinised syringes. Erythrocytes were obtained after centrifugation at 600g for 10 min. Erythrocytes were washed twice with 0.9% sodium chloride and were centrifuged under the same condition. The 5% erythrocyte suspension in 0.15 M NaCl – 10 mM sodium phosphate buffer, pH 7.4 - was lysed through freezing (-20°C) for 24 h and was used for the measurement of malondialdehyde.

**Determination of malondialdehyde:** Concentration of malondialdehyde was measured by the thiobarbituric acid test. Briefly 1 mM EDTA was added to a 0.5 ml haemolysate and was mixed with 1 ml cold 15 % (w/v) trichloroacetic acid to precipitate proteins. The supernatant was treated with 1 ml 0.5 % (w/v) thiobarbituric acid in a boiling water bath for 15 minutes. After cooling, the absorbance was read at 535 nm and the concentration of thiobarbituric acid reactive substance was calculated by using malondialdehyde as a standard. Results were expressed as mmol thiobarbituric acid reactive substances/mg of protein.

**Determination of Catalase:** Hydrogen peroxide catalysed by catalase to give water and nascent oxygen. The activity of catalase was determined by previously reported method. 5% erythrocyte suspension in 0.15 M NaCl – 10 mM sodium phosphate buffer, pH 7.4 was lysed by freezing (- 20°C) for 24 h and was used for catalase
measurement. The decomposition of \( \text{H}_2\text{O}_2 \) can be documented directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of catalase activity. The results obtained for the sample containing 500 µL haemolysate dilution and 500µL substrate solution (10 mM \( \text{H}_2\text{O}_2 \) prepared in 50 mM phosphate buffer, pH 7.0) were compared with those of a blank containing 1 ml phosphate buffer, instead of substrate solution and 500µL of hemolysate dilution. The reaction was initiated by the addition of the substrate solution and incubated at 20 °C for about 1 min. Catalase activity was expressed as mmol \( \text{H}_2\text{O}_2 \)/min/ mg protein. An enzyme unit was defined as the amount of enzyme that catalyzes the release of one µmol of \( \text{H}_2\text{O}_2 \) per min at 20 °C.

**Estimation of Total Antioxidant capacity by phosphomolybdenum method:** Based on the reduction of molybdate to molybdenum & the subsequent estimation of a green phosphomolybdenum complex at acidic pH. Briefly an aliquot of 0.1ml of sample solution containing a reducing species i.e., ethanol combined into an eppendorf tube with 1 ml reagent solution. The tubes were capped and incubated in a thermal block at 95°C for 90 mins. After the samples had cooled to room temperature, the aqueous solution was read at 695nm against a blank. A typical blank solution contained 1 ml of reagent solution (0.6m \( \text{H}_2\text{S}_0\text{4} \), 28mm sodium phosphate and 4mm ammonium molybdate) and appropriate volume of the same reducing species used for the sample and it was also incubated under the same condition as that of the test sample. The antioxidant capacities were expressed as equivalent of α–tocopherol.

**Statistical analysis:** The quantitative measurements were made on six animals in each group and the values were expressed as mean ± SE. Statistical analysis was performed using One-way analysis of variance (ANOVA) followed by Scheffe’s post hoc test. \( P <0.05 \) was considered statistically significant. All the statistical methods were carried out through the SPSS for Windows (version 16).

**Results:**

The mean blood glucose levels in diabetic control group (group II) and MK group (group III) on 8th day were 272±19 and 247±17 mg/dL. The difference is not statistically significant (\( P>0.5 \)). The mean blood glucose levels in diabetic rats were elevated 2-folds as compared to the control group (group I). Figure 1 shows the mean blood glucose levels in the groups at the end of 30 days. The difference between the glucose levels between diabetic control group and MK group is very highly significant (\( P<0.001 \)).

Malondialdehyde content in erythrocytes of diabetic control group was significantly (\( P<0.001 \)) elevated compared to that of the control group and MK group. Catalase activity in the diabetic group was increased compared to
control group and MK group (P<0.001). The total antioxidant activity in the diabetic control group was significantly lower than the control group and MK group (p<0.01). Data tabulated in table 1.

Table-1: Effect of MK leaves on blood glucose levels and antioxidant parameters on control and experimental rats during the study. * p<0.001

<table>
<thead>
<tr>
<th></th>
<th>Non diabetic Control</th>
<th>Diabetic Control</th>
<th>MK group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Glucose level (in mg/dl)</td>
<td>83±10</td>
<td>275±13*</td>
<td>99±10*</td>
</tr>
<tr>
<td>Malondialdehyde (mmol thiobarbituric acid reactive substances/mg of protein)</td>
<td>1.17±0.21</td>
<td>2.11±0.14*</td>
<td>1.45±0.23*</td>
</tr>
<tr>
<td>Catalase activity (mmol H₂O₂/ min/ mg protein)</td>
<td>20.5±2</td>
<td>35.3±1.8*</td>
<td>21.9±2.3*</td>
</tr>
<tr>
<td>Total antioxidant capacity (α-tocopherol)</td>
<td>9.5±2.1</td>
<td>5.33±1*</td>
<td>8.81±1.2*</td>
</tr>
</tbody>
</table>

Figure 1: Blood glucose levels in the groups studied at the end of 30 days.

Figure 2: Tabulation of total antioxidant capacity of the blood of the rats studied. (mmol thiobarbituric acid reactive substances/mg of protein).
Figure 3: Tabulation of catalase activity in blood of rats studied. (mmol H2O2/min/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non diabetic Control</td>
<td>20.5</td>
<td>2</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>35.3</td>
<td>1.8</td>
</tr>
<tr>
<td>MK group</td>
<td>21.9</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Figure 4: Tabulation of total antioxidant capacity of the blood of the rats studied. (α – tocopherol)

<table>
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<tr>
<th></th>
<th>Mean</th>
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<tr>
<td>Non diabetic Control</td>
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<td>MK group</td>
<td>8.81</td>
<td>8.81</td>
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Discussion:

Oxidative stress has been implicated in the development of many pathophysiological conditions including hypertension, diabetes, atherosclerosis and cancer, as well as the process of aging. From the current study, it is evident diabetic rats had much higher glucose levels (Figure 1) than of control rats (275 vs 83 mg/dL). Oral administration of aqueous MK leaves extract statistically significantly (p<0.001) decreased the blood glucose levels in diabetic rats (275 vs 99 mg/dL). This effect is usually dependent on the number of β cells left in the islets of Langerhans of pancreas. MK leaves may increase the functionality of β cells. It has been suggested that MK leaves acts by decreasing glycogenesis and gluconeogenesis. Many other plant extracts are also reported to possess such
hypo-glycemic properties.\textsuperscript{13} It shows that \textit{Murrayakoenigii} has hypoglycemic activity \& also has shown significantly better glycemic control than the standard drug Glibenclamide (p < 0.05). From the current study it can be stated that MK leaves can be used as substitute for conventionally used antidiabetic drugs.

In the present study we examined the antioxidant status in streptozotocin induced diabetic rats. The reactive oxygen species have been shown to play a prime role in cellular damage in diabetic microvascular complications. Intracellular antioxidant mechanism will scavenge the reactive oxygen radicals. The imbalance in handling the oxygen free radicals will result in many clinically detectable changes in the organ system concerned.\textsuperscript{15,16,17} Oxidative stress induced by excessive production of superoxide and an imbalance in antioxidant enzymes has been linked to the development of diabetic complications. Our results confirm previous data of enhanced reactive oxygen species levels in diabetes mellitus.\textsuperscript{18,19,20} Determination of malondialdehyde by thiobarbituric acid is used as an index of the extent of lipid peroxidation.\textsuperscript{18} In the present study, the level of malondialdehyde is increased in the diabetic rats (2.11mmol thiobarbituric acid reactive substances/mg of protein). In rats receiving MK leave extract the levels are significantly lower (1.45 mmol thiobarbituric acid reactive substances/mg of protein) indicating that the MK leaves extract is protective (figure 2). Increased levels of malondialdehyde may be due to free oxygen radical induced membrane lipid damage. Similar pro-antioxidant results were reported by others using garlic oil\textsuperscript{21} in the diabetic and hypertensive rats. MK leaves were also shown to be protective in diabetic rats with possible antioxidant mechanism by others\textsuperscript{22} using thiobarbituric acid reactive substance estimation. These studies hypothylates MK leaves extract prevents the formation of glycosylated haemoglobin. Therefore MK leaves eventually protect from the formation of reactive oxygen species and induction of lipid peroxidation by STZ in diabetic rats. Presence of antioxidant carbazole alkaloids of MK leaves might be involved in stabilization glycemic level and free radical scavenging mechanisms.\textsuperscript{8, 22, 23}

In the present study the erythrocyte catalase activity was increased in diabetic group (35.3 mmol H$_2$O$_2$ /min/ mg protein) (figure 3). The catalase activity was significantly lower in control as well as rats fed with MK leaves (20.5 \& 21.9 mmol H$_2$O$_2$ /min/ mg protein). The increase in the erythrocyte antioxidant enzymes such as catalase is related to the oxidative damage of membrane protein and lipid by increased oxygen free radicals in the body. Similar results were recorded in literature.\textsuperscript{24}

The total antioxidant capacity is estimated using phosphomolybdenum method (figure 4), which is based on reduction of molybdate to molybdenum. The measure of antioxidant capacity considers the cumulative action of all
the antioxidants present in the plasma. In the present study the antioxidant capacity of the rat plasma were significantly lower in diabetic group (5.33 $\alpha$ – tocopherol) in comparison to the control group and MK group (9.5 and 8.81 $\alpha$ – tocopherol). Beneficial effects of garlic oil in terms of increase in total antioxidant capacity have been reported in the similar lines of the present study using aqueous extract of MK leaves.

Figure 5: Blood glucose levels in the groups studied at the end of 30 days.

Conclusion:

Diabetes induced in rats by streptozotocin is indeed a high oxidative stress state. Aqueous extract of MK leaves has resulted in lower blood glucose levels. Also MK leaves are beneficial in diabetes as it has antioxidant effects as shown by the parameters in the present study.

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