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Rhinacanthus nasutus – Its protective role in oxidative stress and antioxidant status in streptozotocin induced diabetic rats

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ABSTRACT

Objective: To find out the protective action of methanolic extract of *Rhinacanthus nasutus* (*R. nasutus*) on lipid peroxidation (LPO) activities and enzymatic antioxidants of liver in streptozotocin (STZ) induced diabetic rats. **Methods:** Experimental diabetes was induced by a single dose of STZ (50 mg/kg) through intraperitoneal injection. The oxidative stress was measured by tissue LPO level and by enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in liver. **Results:** The raise in LPO levels with reduction in enzymatic activities was the outstanding features observed in diabetic control rats. Administration of *R. nasutus* (200 mg/kg body weight per day) for 30 days caused a significant reduction in LPO level in STZ induced diabetic rats (group IV) when compared with diabetic control rats (group III). Moreover, *R. nasutus* treated diabetic rats (group IV) showed significant increase in the activities of enzymatic antioxidants when compared to diabetic control rats (group III). **Conclusions:** The results obtained specify the ameliorating effects of *R. nasutus* in the role of oxidative stress created in the experimental diabetic rats.

1. Introduction

Diabetes mellitus is a heterogeneous metabolic disorder with different etiologies. It is characterized by disarrangements in carbohydrates, proteins and fat metabolism caused by the complete or relative deficit of insulin secretion and /or insulin action. During diabetes mellitus stable hyperglycemia causes an increased production of free radicals via autoxidation of glucose and non enzymatic protein glycation, which leads to the disruption of cellular functions and oxidative damage to the membranes. Oxidative stress is defined as the disturbance in the balance of homeostatic rate between the production of reactive oxygen species (free radicals) and antioxidant defense mechanism, which may lead to tissue injury[1]. Free radicals play an important role in causation of diabetes. Antioxidants are the molecules wrestle against free radicals such as superoxide, hydroxyl radicals, peroxyl radicals, singlet oxygen and peroxynitrite, which leads to oxidative stress and thereby to cell damage. If antioxidant defense system is not sufficient against the formed free radicals then damage may occur in a variety of tissues. Hence, these antioxidants can scavenge most of the free radicals have a vast importance in the enhancement of the recovery from the diseased condition.

Free radicals are naturally unstable molecules because of the presence of unpaired electrons. Consequently, they can be highly reactive, while the radicals vary from each other and they react with the neighbor molecules to accept or donate electrons to achieve the more stable state. Lipid peroxidation is perchance the most widely studied concept of free radical attack and is of great significance in diabetic vascular damage. Methane Dicarboxylic Aldehyde (MDA) is end product of lipid peroxidation and the increased MDA production plays a vital role in the progression of diabetes[2] by altering the transbilayer fluidity gradient, which could impede the activities of membrane-bound receptors and enzymes. Moreover, in diabetes, there is a chance to promote inflammatory changes in the vessel wall, increase oxidative stress and create a state of widespread inflammation, thereby incline to atherosclerosis and cardiovascular disease[3]. The plants contain different types

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of free radical scavenging compounds such as flavonoids, polyphenols, triterpenes, vitamins which are well-off in antioxidant activity[4]. The preliminary studies on *in vitro* antioxidant studies of *Rhinacanthus nasutus* (Linn) (*R. nasutus*) have been studied and showed significant activity[5]. There are mp systemic studies of *R. nasutus* on antioxidant studies invitro and invivo. Keeping in view of the antioxidant studies *in vitro*, this study aims to counteract the lipid peroxidation through antioxidant defense system in diabetic rats by using a rare medicinal plant *R. nasutus*.

R. nasutus belongs to Acanthaceae family. It is a flowering plant and is well recognized for its remedial uses commonly called as Nagamalli in Telugu, Jupani in Hindi, Doddapatika in Kannada, Kaligai, Anichi in Tamil, Yuthikaparni in Sanskrit and Gajakarni in Marathi[6]. It has been used as Thai traditional medicine for the treatment of various cancers, *e.g.* cervical and liver cancers. Different parts of this plant have been also used for the treatment in a range of other diseases such as diabetes, hypertension, eczema, pulmonary tuberculosis, herpes, hepatitis, and various skin diseases, and the active components of this plant have been widely investigated[7]. *R. nasutus* has been found to possess antimicrobial properties that can control a variety of infecting organisms, anti diabetic and hypolipidemic activities[8-10].

2. Materials and methods

2.1. Materials

2.1.1.Plant materials

The fresh leaves of *R. nasutus* were collected from Tirumala Hills, Tirupati, Chittoor district of Andhra Pradesh in the month of July – October and identified by a Botanist, Department of Botany, S.V.University, Tirupati. The leaves were shade dried and ground into fine powder and used for preparation of the extracts.

2.1.2. Preparation of extract

Fresh leaves of *R. nasutus* (L) were shade dried and milled to fine powder using a mechanical grinder. The powdered plant material was macerated and shaken in methanol for 48 h using a bath shaker. The extract was then filtered with filter paper (Whatman No.1) and concentrated to dryness under vacuum and reduced pressure using Rota evaporator at 40 ℃. The concentrate was then layered on aluminum foil and freeze dried for further use.

2.1.3. Chemicals

Streptozotocin (STZ) was purchased from Sigma (Germany) and all other chemicals and reagents used in this study were of analytical grade. Glibenclamide was purchased from a local drug store.

2.2. Methods

2.2.1. Experimental design

Adult, male rats of Wistar strain weighing 150-180g obtained from Sri Venkateswara Enterprises, Bangalore-21, were chosen as animal model for this study. They were housed individually in clean, sterile, polypropylene cages under standard conditions (12 h light/dark cycles) with free access to standard chow (Hindustan Lever Ltd., Bangalore, India) and water ad libitum. The animals were acclimatized to the laboratory for one week prior to the start of experiments. The animal experiments were designed and performed in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines. The rats were divided into 5 groups comprising of 6 animals in each group as follows:

Group I: Normal rats (Controls).

Group II: Normal + *R. nasutus* treated rats (200 mg/kg body weight per day).

Group III: Diabetic untreated rats.

Group IV: Diabetic + *R. nasutus* treated rats (200 mg/kg body weight per day).

Group V: Diabetic + Glibenclamide treated rats.

2.2.2. Induction of experimental diabetes

Diabetes was induced by a single intraperitoneal injection of a freshly prepared STZ solution (Sigma, no. 242-646-8) (50 mg/kg in citrate buffer 0.01 M, pH 4.5) to overnight-fasted rats. Control rats received only the buffer. Diabetes was identified by polydipsia, polyurea and by measuring non-fasting plasma glucose levels 48 h after injection of STZ. Animals which show blood glucose levels more than 250 mg/dL were considered as diabetic rats and used as the experimental animals[11].

2.2.3. MDA content (lipid peroxidation LPO)

This assay is used to determine MDA levels in liver tissue as described by Okhawa *et al*[12]. The tissue was homogenized (5%W/V) in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA). The homogenates were centrifuged at 10 000 rpm for 10 min at 0 oC in cold centrifuge. The separated supernatant part was used the estimation 200 μL of the tissue extract was added to 50 μL of 8.1% sodium dodecyl sulphate (SDS), vortexed and incubated for 10 min at room temperature. 375 μL of 20% acetic acid (pH 3.5) and 375 μL of thiobarbituric acid (0.6%) were added and placed in boiling water bath for 60 min. the sample were allowed cool at room temperature. A mixture of 1.25 ml of butanol: phyridine (15: 1) was vortexed and centrifuged at 1000 rpm for 5 min. the colored layer (500 μL) was measured at 532 nm using 1,1,3,3,- tetraethoxypropane as a standard. The values were expressed in μ moles of malondialdehyde formed/ gram wet weight of the tissue.

2.2.4. Superoxide dismutase (SOD – EC: 1.15.1.6)

Superoxide dismutase activity was determined in the liver tissue according to the method of Misra and Fidrovich[13] at room temperature. The liver tissue was homogenized in ice cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% homogenate (w/v). The homogenates were centrifuged at 10 000 rpm for 10 min at 0 ℃ in cold centrifuge. The supernatant was separated and used for enzyme assay. 100 μL of tissue extract was added to 880 μL (0.05 M, pH 10.2, containing 0.1 mM EDTA) carbonate buffer and 20 μL of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture. The optical density was measured at 480 nm for 4 min by using 01 Hitachi U-2000 Spectrophotometer. Activity expressed as the amount μmoles of enzyme that inhibits the

oxidation of epinephrine by 50%, which is equal to 1 unit.

2.2.5. Catalase (CAT – EC: 1.11.1.6)

Catalase activity was assayed by a slightly modified version of Aebi[14] at room temperature. The liver tissue was homogenized in ice cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% homogenate (w/v). The homogenates were centrifuged at 10 000 rpm for 10 min at 0 ℃ in cold centrifuge. The resulting supernatant was used as enzyme source. 10 μL of 100% EtOH was added to 100 μL of tissue extract and then placed in an ice bath for 30 min. After 30 min the tubes were kept at room temperature followed by the addition of 10 μL of Triton X-100 RS. In a cuvette containing 200 μL of phosphate buffer and 50 μL of tissue extract was added 250 μL of 0.066 M H2O2 (in phosphate buffer) and decreases in optical density was measured at 240 nm for 60 sec in a UV Spectrophotometer. The molar extinction coefficient of 43.6 M cm-1 was used to determine CAT activity. One unit of activity is equal to the moles of H_2O_2 degraded / mg protein / min.

2.2.6. Se-dependant glutathione peroxidase (Se-GSH-Px – EC: 1.11.1.9)

Se-dependant glutathione peroxidase (Se-GSH-Px) was determined by a modified version of Flohe L and Gunzler[15]. 5% (w/v) of liver tissue homogenate prepared in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenates were centrifuged at 10 000 rpm for 10 min at 0 ℃ in cold centrifuge. The resulting supernatant was used as enzyme source. The reaction mixture consist 500 μL of phosphate buffer, 100 μL of 0.01 M GSH (reduced form), 100 μL of 1.5 mM NADPH and 100 μL of GR (0.24 units). The 100 μL of tissue extract was added to the reaction mixture and incubated at 37 ℃ for 10 min. Then 50 μL of 12 mM t-butyl hydroperoxide was added to 450 μL of tissue reaction mixture and measured at 340 nm for 1 min. The molar extinction coefficient of 6.22 X 103 M cm^{-1} was used to determine the activity. One unit of activity is equal to the mM of NADPH oxidized / mg protein / min. The enzyme activity was expressed in moles of NADPH oxidized / mg protein / min.

2.2.7. Statistical analysis

The results were expressed as mean ± SD (n=6). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered to be statistically significant.

3. Results

Table 1 illustrates the variations in lipid peroxidation, SOD, CAT, GPx in normal control, normal controls treated with *R. nasutus*, diabetic control, diabetic controls treated with *R. nasutus* extract, diabetic controls treated with an oral hypoglycemic drug Glibenclamide for 30 days. The values of lipid peroxidation were expressed in μ moles of malondialdehyde formed/gram wet weight of the tissue. The liver MDA contents were significantly increased in diabetic control rats (Group III). The treatment with leaf extract of *R. nasutus* at a dose of 200 mg/kg body weight showed significant reduction in the lipid peroxidation (MDA content) in diabetic control (Group IV). The significant fall to the normal level was observed in the lipid peroxidation level in diabetic control when treated with glibenclamide.

In addition, there were marked decrease in liver SOD, CAT and GPx levels in diabetic rats (Group III). The significant elevation in the antioxidant levels of SOD, CAT and GPx was observed in the diabetic rats treated with *R. nasutus* (Group IV). The significant increase in the above antioxidant enzymes was seen in the diabetic rats treated with glibenclamide also (Group V).

Table 1
Changes in SOD, CAT, GPx and lipid peroxidation in the liver tissue.

Groups	SOD	CAT	GPx	LPO
Normal control	1.17±0.30	0.48±0.38	1.23±0.07	48.04±3.46
Normal + PE (200mg/kg body weight)	1.17±0.51	0.53±0.23	1.26±0.10	47.31±2.93
Diabetic control (STZ – 50 mg/kg body weight)	0.42±0.34[b]	0.23±0.19[b]	0.83±0.12[b]	69.707±2.91[b]
Di + PE	1.37±0.42[a]	0.44±0.30[a]	1.09±0.06[a]	52.942±1.75[a]
Di + Gli	1.02±0.48[a]	0.44±0.44[a]	1.15±0.10[a]	55.962±2.06[a]

The values are expressed as units of superoxide anion reduced / mg protein / minute, μ moles of H_2O_2 degraded / mg protein / minute, μ moles of NADPH oxidized / mg protein / minute, μ moles of malondialdehyde formed / gram wet weight of the tissue respectively.
All the values are mean±SD of six individual observations.
a: $P<0.05$ significance when compare to normal control rats.
b: $P<0.05$ significance when compare to diabetic rats.

4. Discussion

The present study demonstrates that STZ induced hyperglycemia accompanied by the presence of oxidative damage in the liver of diabetic rats. The use of medicinal plants and phytomedicine in most of the developing countries is well recognized. Traditional medicines like ayurveda, unani, Sidha have been used for centuries in the treatment of diabetes. But a few medicinal plants have been scientifically proved to be anti diabetic potential. Therefore, we have investigated the effect of *R. nasutus* on oxidative stress through antioxidant defense mechanism. The beneficial effects of *R. nasutus* provide an additional support to finding from previous studies that *R. nasutus* is free radical scavenger with powerful antioxidant capacity *in vitro*[5]. In diabetic condition, there is a excessive oxidative stress is created and it is likely involved in progression of pancreatic β-cell dysfunction[15]. There is widespread

support that lipid peroxidation and protein oxidation lead to loss of membrane integrity, an important factor in acceleration of diabetes mellitus[17]. Lipid peroxidation is commonly used as an indicator of tissue oxidative stress, in which oxygen intermingles with polyunsaturated fatty acids and leads to the formation of lipid products such as MDA and 4-HNE, and boost up the generation of free radicals, which leads to the damage of cell membrane, cell necrosis and inflammation. A recent study by Natheer H Al-Rawi showed the elevated levels of MDA in the saliva of diabetic patients[18]. SOD and CAT are the two major scavenging antioxidants which remove free radicals *in vivo*. A decrease in these antioxidant activity can lead to a surplus availability of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which sequentially generate hydroxyl radicals (·OH), resulting in initiation and propagation of lipid peroxidation. SOD dismutases the $O_2^{\cdot-}$ free radical into H_2O_2, which is then converts into H_2O by CAT or glutathione peroxidase[19]. GPx catalyzes the reaction of hydroperoxides with GSH to form glutathione disulphide. A significant decrease in GPx activity in diabetic rats suggests that inactivation by reactive oxygen species. The decrease may also be due to the decreased availability of its substrate, GSH, which has been depleted during diabetes condition[20]. It has been confirmed that the activity of these antioxidant enzymes (SOD, CAT, GPx) decrease in diabetic rats[21].

Furthermore, *R. nasutus* had a potent increasing effect on liver SOD, CAT and GPx activities when compared to diabetic control group. Oxidative stress due to free radical mediated lipid peroxidation has been implicated in the pathogenesis of several diseases including diabetes mellitus[22]. Flavonoids, phenols and lignin precursors are regarded to be beneficial antioxidants as they exhibit scavenging activity of harmful active oxygen species.

The antioxidant property of *R. nasutus* extract certainly is due to the presence of flavonoids and polyphenolic components as main active ingredients having potent antioxidant activities. In conclusion, *R. nasutus* offers a promising therapeutic value in prevention of diabetes[9]. These effects could be mainly endorsed to its antioxidant properties as shown by significant quenching impact on the amount of lipid peroxidation along with, enhancement of antioxidant defense systems in liver tissue.

The present study reveals that the plant *R. nasutus* exhibits significant scavenging effect in all the radical scavenging assays. This is the first report on the antioxidant activity of this plant *in vivo* in STZ induced diabetic rats. The methanolic extract of this plant possesses good antioxidant activity. Further studies on isolation and characterization the compounds which are responsible for their potential antioxidant activity are in progress.

Conflict of interest statement

We declare that we have no conflict of interest.

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