

TITLE: *IN VITRO* AND *IN VIVO* ANALYSIS OF ENZYMATIC ANTIOXIDANT POTENTIAL OF *RICINUS COMMUNIS* LEAVES IN ALLOXAN INDUCED DIABETIC SWISS ALBINO MICE.

Nitika Verma*, Swaati Sharma, Veena Garg

Department of Bioscience and Biotechnology, Banasthali University, Rajasthan-304022.

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***Correspondence for
Author**

Dr. Nitika Verma

Department of
Bioscience and
Biotechnology,
Banasthali University,
Rajasthan-304022.

ABSTRACT

The antioxidative bioefficacy of hydro-ethanolic extract of *Ricinus communis* (RC) leaves was tested by evaluating its enzymatic antioxidant potential both *in vitro* and *in vivo*. The *in vitro* tests included assay for Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxide (GPx) activity. The *in vivo* experiments were performed on male Swiss albino mice. The experimental animals were divided in to 4 groups of 7 mice each viz.: Normal Control (NC), Diabetic Control (DC), Leaf extract Treated (LT) and Glibenclamide Treated (GT). The animals of groups DC, LT and GT were made diabetic by a single intra-peritoneal injection of Alloxan monohydrate (150mg/kg body weight) and thus were under diabetes caused

oxidative stress. In a 45 day long experiment, the group NC and DC were kept untreated and were given only normal saline. Whereas, group LT was treated with *Ricinus communis* Leaf Extract (RCLE) (300mg/kg body weight) and group GT with a standard drug Glibenclamide for 45 days. The antioxidant assays for CAT, SOD and GPx were carried out on Liver, Kidney and Pancreas homogenates of experimental animals after the 45 day treatment. The *in vitro* assays demonstrated that the antioxidant ability of the extract increased with its rising concentration. *In vivo* assays showed significantly reduced enzymatic antioxidants in mice of diabetic group, as compared to the normal group. The treatment with hydro-ethanolic leaf extract helped in significantly normalizing the disturbed enzymatic antioxidants in diabetic mice which was not possible by Glibenclamide treatment.

INTRODUCTION

Oxido-reductive reactions occur normally inside cells of the body resulting into production of free radicals. Free radicals are particles or sub-atomic sections that contain single electrons in

their furthest nuclear or sub-atomic orbital and are fit for autonomous presence. The reactivity of radicals is for the most part stronger than non-radical species.^[1] Reactive oxygen species (ROS) refers to oxygen determined species like oxygen radicals, responsive nitrogen species (RNS) and some non-radical receptive subsidiaries that are oxidizing executors and/or are effectively changed over to radicals. In a state of imbalance, which occurs due to exposure to unfavourable conditions, disordered metabolism and various diseases, there is over production of ROS, RNS and free radicals, which are accountable for peroxidation of lipids and for reduction in the the activity of antioxidant defense enzymes, and may be described as Oxidative stress. Thus, there becomes a need for supplementing the diet with foodstuffs rich in antioxidants to combat the distress.^[1, 2]

Antioxidants can be described as oxidation inhibitors as they prevent the propagation of chain reactions involving oxidation of other molecules and thus help in scavenging the reactive molecules.^[3] These may be enzymatic or non-enzymatic based on their nature. Enzymatic antioxidants are proteinaceous molecules capable of catalyzing reactions involving break down of highly toxic free radicals into less reactive species by reducing them. Major antioxidant enzymes known are Catalase (CAT), Superoxide Dismutase (SOD), Peroxidase, Oxidoreductase etc.

Most plants have defensive biochemical capacities of owing to the presence of naturally occurring antioxidants in them. Numerous auxiliary mixes and proteins of higher plants have been shown with *in vitro* examinations to secure against oxidative harm by hindering or extinguishing free radicals and ROS.^[4]

Oxidative anxiety is expanded in diabetic patients since prolonged hyperglycemic status results into an expanded creation of oxygen free radicals due to glucose autoxidation and protein glycation non-enzymatically. There is extensive proof proposing that oxidative anxiety assumes a part in tissue damage connected with diabetes.^[5] Expanded levels of the results of oxidative damage caused to lipids and proteins can be distinguished in the serum of diabetics.^[6]

Popularly known as the ‘Castor oil plant’, *Ricinus communis* is a soft wooded tree belonging to the family Euphorbiaceae. It is a widespread tropical plant found in the warm temperature regions of the world. Medicinally, its various parts have been reported for being capable of treating inflammation,^[7] liver disorders,^[8, 9] diabetes^[10] and as a laxative,^[11] and antifertility

agent^[12] (antinociceptive RC leaves.pdf). The *R. communis* leaves have been reported to possess phyto-constituents like flavonoids, tannins, steroids, saponins, alkaloids, terpenoids and glycosides^[13]

MATERIAL AND METHODS

Chemicals: All the chemicals accumulated for utilization in this study were of analytical value and were acquired from HIMEDIA (India), SRL (India), CDH (India), SD Fine (Mumbai, India), Qualigens (India/ Germany) and Lobachemie Pvt. Ltd.

Preparation of extract: Leaves of *R. communis* were gathered from the plants accessible at Agricultural Research Institute, Mandor (Jodhpur, Rajasthan, India). They were distinguished taxonomically and shade dried. They were thereafter diminished into a coarse powder utilizing an electric grinder. The powder was then subjected to soxhlet extraction utilizing 50% ethanol as solvent. The concentrate so acquired was then dried in a vacuum rotatory evaporator under diminished weight at $60 \pm 1^\circ\text{C}$. The concentrated extract was kept in a hot air broiler at $40-45^\circ\text{C}$ till it dried to a semisolid mass. As contrasted with powder the yield of concentrate was 6.3% w/w. Dilutions from 5-25 $\mu\text{g/ml}$ were readied for in vitro measures. While, the doze for treating the experimental animals, was made by dissolving the extract in 20% Tween 20 (in 0.9% saline)

In vitro estimation of enzymatic antioxidant potential

Superoxide dismutase: Superoxide dismutase activity was determined according to the method of Beauchamp and Fedovich; 1976. 0.5 ml of plant extract was taken and 1 ml of 125 mM sodium carbonate, 0.4 ml of 25 μM NBT and 0.2 ml of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1 mM Hydroxylamine hydrochloride and the absorbance was read at 560 nm using spectrophotometer at 5 min intervals. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg of protein.^[15]

Catalase: Catalase activity was determined by the titrimetric method (Chance & Maehly; 1995) To 1ml plant extract, 5 ml of 300 μM phosphate buffer (pH 6.8) containing 100 μM hydrogen peroxide (H_2O_2) was added and left at 25°C for 1 min. The reaction was arrested by adding 10 ml of 2% sulphuric acid, and residual H_2O_2 was titrated with potassium permanganate (0.01N) till pink colour was obtained. Enzyme activity was estimated by calculating the decomposition of μM H_2O_2 per min per mg protein.^[16]

Glutathione Peroxidase: Method of Rotruck et al. was referred to estimate the activity of Glutathione peroxidase (GPx). 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H₂O₂, 0.2 ml of water and 0.5 ml of plant extract were taken in a reaction mix. This was then incubated at 0, 30, 60, 90 seconds successively. 0.5 ml of 10% TCA was added to stop the reaction and the mixture was centrifuged. 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate) were added to 2ml of the supernatant so obtained such that a color was developed, which was read at 412 nm. The enzyme activity was expressed in terms of μg of glutathione utilized/min/mg protein. ^[17]

***In vivo* estimation of enzymatic antioxidant activity**

Animal care and monitoring: Healthy 6-8 months old, 25-35 gm by weight, male, Swiss albino mice (*Mus musculus*) were procured from C.C.S. Haryana Agricultural University (Hissar, India). The experimental animals were kept under standard laboratory conditions: Light (12:12 h L: D cycle), Temperature ($23 \pm 2^\circ\text{C}$) and Relative Humidity ($55 \pm 5\%$). They were fed standard rat pellet feed and tap water *ad libitum*. The treatment and maintenance of all the animals was as per the directions of Institutional Animal Ethics Committee of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Introduction of diabetes and treatment

For this study, experimental animals were segregated into following groups of seven mice each:

NC	DC	GT	LT
Normal Control	Diabetic Control	Glibenclamide Treated	Diabetic + RCLE Treated

Alloxan monohydrate, freshly dissolved in normal saline was injected (150 mg/kg body weight) intra-peritonally to induce diabetes in mice of groups DC, GT and LT after overnight fasting. ^[7] Subsequently, free access to food and 10% glucose solution was provided to the mice to counter the hypoglycemic shock after Alloxan injection. After one week of Alloxan injection, the Fasting Blood Glucose (FBG) concentration was observed by using Dr. Morepen's One Touch Ultra glucometer and compatible blood glucose strips by Johnson & Johnson Company, USA. ^[18] Mice with FBG level greater than 140 mg/dl were considered to be diabetic 14 and were therefore selected for treatment. The drug (10 mg/kg body wt) and the leaf extract (300 mg/kg body wt) were administered orally, once in a day, for 45 days.

Antioxidant assays: After the 45 day experiment, the animals were sacrificed by cervical dislocation. The organs: Liver, pancreas and kidney were collected, washed off the adhering tissues and blood with ice-cold normal saline solution (0.9%). Dry weight of all the organs was noted. Each tissue was then homogenized in 0.2 M tris-HCl (10 ml of tris-HCl for every 1 g of tissue). The homogenates were subjected to centrifugation at 10,000 rpm for 20 minutes at 4°C. The supernatants collected were used for estimation Superoxide Dismutase (SOD), ^[19] Catalase (CAT), ^[20] Glutathione Peroxidase (GPx). ^[21]

Statistical Analysis: Results are expressed as mean $\pm$ Standard Error of Mean (SEM). Statistical analysis was performed using one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc multiple comparison test using SPSS (version 16.0) and student's 't'-test using SigmaPlot (version 8.0). The values of $P < 0.05$ were considered as statistically significant.'

RESULTS

Table 1: *In vitro* enzymatic antioxidant potential of enzymatic antioxidants in RCLE

Conc. of RCLE ($\mu\text{g/ml}$)	Specific activity of enzymatic antioxidants (Units/mg of protein)		
	SOD	CAT	GPx
200	46.30 $\pm$ 8.61	68.7 $\pm$ 6.30	73 $\pm$ 5.9
400	73.87 $\pm$ 2.784	97.45 $\pm$ 2.5	105 $\pm$ 3.0
600	102.26 $\pm$ 5.75	125 $\pm$ 5.0	123 $\pm$ 2.2
800	146.86 $\pm$ 2.53	146 $\pm$ 2.3	158 $\pm$ 7.5
1000	182.60 $\pm$ 8.17	168.7 $\pm$ 3.5	180 $\pm$ 5.3

Values are mean $\pm$ SEM; n= 3.

The activities of enzymatic antioxidants were tested at various extract concentrations ranging from 200 $\mu\text{g/ml}$ to 1000 $\mu\text{g/ml}$. The activity of SOD rose to 182.60 units/mg at 1000 $\mu\text{g/ml}$, which was higher than 46.30units/mg at 200 $\mu\text{g/ml}$. The CAT activity was recorded as 68.7units/mg at 200 $\mu\text{g/ml}$ and increased to 168.7units/mg at 1000 $\mu\text{g/ml}$. Similarly GPx also saw an increment in its activity proportional to increasing concentration of the extract, being 73units/mg at 200 $\mu\text{g/ml}$ and 180units/mg at 1000 $\mu\text{g/ml}$. (Table 1)

Thus it was observed that the enzyme activities increased with increasing concentrations of the extract indicating it has a considerable enzymatic potential.

Table 2: FBG levels during the 45 day treatment.

Day	NC	DC	LT	GT
Before induction	106.14 ± 13.18	102 ± 3.36	105.29 ± 5.71	99 ± 17.54
After induction	106.14 ± 13.18 b	185.57 ± 13.91 a	186.0 ± 3.43 a	206 ± 35.37 a
45	103.71 ± 9.8 b	163.71 ± 16.96 a	89.2 ± 8.73 b c	95.7 ± 18.1 b c

Values are mean ± SEM; n= 7.

*Before induction (Basal values); Student's 't'-test is significant at $P < 0.05$. a: significant ($P < 0.05$) difference, b: insignificant difference ($P > 0.05$) compared to basal values; c: significant ($P < 0.05$) difference compared to values obtained after alloxan injection

Alloxan injection to DC, LT and GT groups resulted in significant ($P < 0.05$) aggravation of blood glucose levels or hyperglycemia unlike the normal control group, NC. Hyperglycemia is the major symptom which leads to diabetic complications¹⁵. Treatment with RCLE helped in significantly lowering the glucose level by 52.15% (i.e. from 186.0 ± 3.43 mg/dl to 89.2 ± 8.73 mg/dl) to normalization. The reduction in the FBG levels by RCLE can be compared to the hypoglycemic effect of glibenclamide, which produced 53.54% reduction (from 206 ± 35.37 mg/dl to 95.7 ± 18.1 mg/dl) in blood glucose level of group GT after 45 days of dosage (Table2)

Table 3: *In vivo* enzymatic antioxidant potential of RCLE in Liver, Pancreas and Kidney of Alloxan induced diabetic mice after the 45 day treatment.

Parameters		NC	DC	LT	GT
Liver	CAT	208.2 ± 9.43	136.3 ± 6.6 a	372.1 ± 2.7 *bc	150.5 ± 7.8a b*
	GPx	217.5 ± 12.7	122.7 ± 10.3 a	229.73 ± 7.5 a*bc	148.4 ± 6.5a b*
	SOD	190.2 ± 15.4	140.7 ± 5.14 a	189.5 ± 45.07 a*bc	132.6 ± 2.51a b*
Pancreas	CAT	214.3 ± 8.9	153.3 ± 2.85 a	315.2 ± 8.5 a*bc	194.2 ± 9.5a* b
	GPx	172.1 ± 22.3	89.01 ± 9.75 a	181.22 ± 11.27abc*	105.16 ± 20.2ab*
	SOD	220.3 ± 14.5	197.4 ± 27.9 a	216.9 ± 9.72 ab*c	208.4 ± 15.9a b*
Kidney	CAT	268 ± 2.64	146.4 ± 5.7 a	421.6 ± 16.6 a*bc	196.5 ± 3.9a b
	GPx	167.8 ± 8.6	84.23 ± 14.6 a	184.46 ± 15.5 a*bc	96.2 ± 5.7 a b*
	SOD	198.5 ± 26.07	124.2 ± 12.17 a	182.3 ± 6.02a b*c*	113.8 ± 9.13a b*

Values are mean ± SEM; n= 7.

GSH: mg/gm tissue, TBARS: nM TBARS/mg protein, CAT: μ moles H₂O₂ decomposed/min/mg protein, GPx: μ g GSH consumed/min/mg protein, SOD: Units/min/mg protein; Student's 't'-test is significant at $P < 0.05$. a: significant ($P < 0.05$) difference, a*: insignificant ($P > 0.05$) difference compared to NC; b: significant ($P < 0.05$) difference, b*: insignificant ($P > 0.05$) difference compared to DC; c: significant ($P < 0.05$) difference, c*: insignificant ($P > 0.05$) difference compared to GT.

It was observed that Alloxan administration to mice lead to a significant ($P < 0.05$) decrement in the antioxidant enzymes: CAT, GPx and SOD in all the three studied tissues- liver, kidney and pancreas (Table 3). 45 days of Glibenclamide treatment could not restore the enzymatic antioxidant level, as significantly ($P < 0.05$) lower values of enzyme activities can be seen under GT. However, the 45 days treatment with RCLE showed an overwhelming significant ($P < 0.05$) increment in most of the cases in LT, nearly normalizing or increasing the amount of antioxidant enzymes in some cases.

DISCUSSION

The *in vitro* studies reveal that the hydroethanolic extract of *R. communis* leaves (RCLE) has a considerable enzymatic antioxidant potential. This was also proved *in vivo*, by treating experimental mice under oxidative stress with RCLE for a period of 45 days and estimating the antioxidant enzymes in their liver, pancreas and kidneys. The oxidative stress was induced by administration of Alloxan rendering the experimental mice diabetic.

Alloxan intoxicates the pancreatic β -cells. Inside the body, it affinitively accumulates in the β -cells and consecutively causes their destruction by over-production of Reactive Oxygen Species (ROS), superoxide radicals and peroxides. ^[22, 23] β -cell cytotoxicity results into deficiency of insulin which plays a major role in causing hyperglycemia. ^[24] Persistent hyperglycemia leads to diabetic complications. ^[22] It is reported that enhanced glucose metabolism via the Polyol (sorbitol) pathway is accompanied with hyperglycemia, which also leads to further over-production of Oxygen free radicals. ^[24] An aggravated glucose in the mitochondria causes rapid transfer of electrons through the Electron Transport Chain (ETC) and finally to oxygen and thus leading to over production of oxygen free radicals.

Superoxide anions are a species produced by various enzyme systems by auto-oxidation reactions or by non-enzymatic electron transfers reducing molecular oxygen univalently. These are basically oxygen centered radicals with selective reactivity, their reductive capability can also reduce certain iron complexes. ^[25] The major defense antioxidant enzyme Superoxide dismutase (SOD), scavenges the superoxide anions by catalyzing their conversion into H_2O_2 which is less toxic. ^[26] The SOD activity observed a rising trend with respect to concentration of the extracts, which suggests an increase in their antioxidant nature with increasing concentration. However, hydrogen peroxide is also toxic. ^[27] Catalase is another antioxidant enzyme which is important for the defense mechanism against the H_2O_2 radicals. CAT catalyzes the dismutation of H_2O_2 into water and oxygen ^[28], without the production of

more free radicals. ^[29] The CAT activity also saw proportional increment with the rising concentration of both the extracts. The third category of enzymatic antioxidants studied is Peroxidase. GPx is an example of selenium-containing peroxidases, which are known to catalyze the reduction of a variety of hydroperoxides (ROOH and H₂O₂) using GSH, thereby protecting mammalian cells against oxidative damage. ^[30]

Patients suffering from diabetes are reported to experience more oxidative stress than normal individuals, as the formation of ROS is uncontrollable in case of diabetics. ^[31] The damage gets more severe, as the key antioxidant enzymes are lost consecutively. Glycation renders SOD and CAT inactive, ^[32, 33] leading to an imbalanced oxido-reductive environment in the cell. ^[32]

The *in vivo* study revealed that treatment with standard hypoglycemic drug Glibenclamide in group GT failed to restore the antioxidative status of tissues. Whereas, RCLE treatment not only lowered but has helped in normalizing the blood glucose levels, portraying it as a potent antihyperglycemic, but also normalized the enzymatic antioxidant activity in group LT. In some cases the enzymatic antioxidant activities found in animals of group LT were more as compared to the Normal control group NC.

CONCLUSION

It can be concluded from the findings of the study that the leaves of *R. communis* are identified to possess antioxidant potential which was found absent in standard drug Glibenclamide. Hence, may be prescribed as adjunct to dietary therapy to ameliorate oxidative stress caused in diabetic patients.

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