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In vitro bioefficacy of Hydro-ethanolic extracts of *Ricinus communis* bark and root for Antioxidative and Antihyperglycemic potential.

Veena Garg[1],Nitika Verma[2], Swaati Sharma[3]
[1]Department of Bioscience and Biotechnology, Banasthali University, Banasthali, Rajasthan-304022,INDIA

ABSTRACT

Hyperglycemia due to absolute or relative insulin deficiency, along with a condition of oxidative stress is a typical characteristic of diabetes. Antioxidants play a vital role in the alleviation of these. The present study was designed to study the in vitro antioxidant and antidiabetic potentials of the hydro-ethanolic extracts of Ricinus communis (RC) bark and root. The models used for the antioxidant ability were non enzymatic (DPPH radical scavenging assay, Hydroxyl Radical Scavenging assay, Reducing power assay) and enzymatic antioxidants (Catalase(CAT), Superoxide Dismutase(SOD) and Peroxidase(Px)) whereas, antidiabetic potential was estimated by measuring the α-amylase and α-glucosidase activities. Both the extracts exhibited a dose dependent increase in the percentage inhibition and enzyme activities thus, showing an increment in the presence of antioxidants and antidiabetic activity with rising concentration. RC root exhibited IC_{50} values of 401 µg/ml for α-amylase and 598 µg/ml for α-glucosidase which were lower as compared to RC bark. However, the amount of antioxidants enzymatic as well as non-enzymatic were found to be higher in RC bark; the IC_{50} values being lesser; 204.79 µg/ml for DPPH, 192.67 µg/ml for hydroxyl radical and 188.34 µg/ml for metal chelating activity. These results indicate R. communis root as a good anti-diabetic agent whereas its bark is having more potent antioxidant system.

Keywords: Diabetes Mellitus, Oxidative stress, Antioxidant, *in vitro*, *Ricinus communis*

INTRODUCTION

Impaired insulin secretion affects carbohydrate, fat and protein metabolism in diabetics (1). Diabetes is thus described as a state of disordered metabolism causing hyperglycemia. Enzymes α-amylase and α-glucosidase function in metabolism of carbohydrates. It is proposed that inhibition of their activity would postpone the breakdown of carbohydrates, which would thereafter decrease the glucose absorption, and will thus lower down the postprandial blood glucose level elevation (2).

Oxygen is the basic requirement for the continuity of all the life forms. Under normal physiological conditions univalent oxygen is reduced to oxygen involved free radicals called the Reactive Oxygen Species (ROS), like superoxide (O2•-), hydroxyl (OH•-), and nitric oxide radicals (NO•). These free radicals, when accumulate into abundance, lead to a state of imbalance called as Oxidative Stress

(OS). OS is directly associated to the pathogenesis of different diseases like Diabetes Mellitus (DM) (3).

In diabetes during glucose oxidation, electrons initially make a molecule unstable which initiate a chain reaction resulting in over production of free radicals (4), leading to OS, which is implicated for the development and progression of many complications.

Antioxidants are agents that prevent or defer the oxidation of other molecules by scavenging the free radicals and thus restraining the oxidizing chain reactions. They may also be referred to as oxidation inhibitors (5). A molecule of antioxidant, is capable of reacting with a single free radical, neutralizing it by donating electrons and ending the carbon-stealing reaction. It thus acts as a scavenger and prevents cell and tissue damage. It has been demonstrated that antioxidants play major part in management of OS during DM (4) and therefore, have gained utmost therapeutic importance (6).

Rich sources of antioxidants, with good effectiveness, fewer side effects in clinical experience and relatively low costs are therefore sought after for diseases involving OS. Attributing to the presence of antioxidants such as flavonoids, phenolic compounds, coumarins, terpenoids etc, in herbal products, these are used in many countries from a long time as treatments of DM (7).

Ricinus communis is a renowned plant from the family Euphorbiaceae, commonly called as the 'Castor oil plant' in English and 'arand', 'erand', 'andi' or 'rend' in Hindi. It is distributed widely across the world and is a tropical plant. It is a 4-5 meters tall, woody tree which grows where there is access to water. It is a monoecious species, which has separate male and female flowers on the same plant (8).
Medicinally *R. communis* is known for maintaining a disease free healthy life since ages. The plant is reported to possess anti-oxidant, antihistaminic, antinociceptive, antiasthmatic, antiulcer, immunomodulatory, antidiabetic, hepatoprotective, antifertility, anti-inflammatory, anti-microbial, neuro stimulator, lipolytic, wound healing, insecticidal activity. The pharmacological activities are due to the presence of variety of phytoconstituents in the plant. (9)

MATERIALS AND METHOD

Chemicals

All the chemicals were bought from HIMEDIA (India), SRL (India), CDH (India), SD Fine (Mumbai, India) and Qualigens (India/ Germany) and only analytical grade chemicals were used for this study.

Collection of Plant material and Preparation of Extract

Ricinus communis (RC) bark and roots were procured from Agricultural Research Institute, Mandor (Jodhpur, Rajasthan, India). The bark and roots of the plants were taxonomically identified and shade dried. These were then reduced in size using a grinder, into a coarse powder of uniform particle size by sieving it through sieve no. 80. The powder so obtained was packed into a thimble for its extraction in 50% ethanol using soxhlet apparatus, and was extracted till solution became clear. The extract was then concentrated to dryness on a waterbath. The solid to semisolid mass obtained thereafter was then preserved in refrigerator below 10°C, in an air-tight container (10). The suspension of the hydroethanolic extract was used for further biochemical studies.

***In vitro* antioxidant activity**

Assay of Non enzymatic antioxidants

DPPH Radical scavenging assay

3 ml of the plant extract was added to 1ml of 0.1 mM solution of DPPH made in methanol. This was incubated at 37°C. After 30 min, its absorbance was measured against control (Ascorbic acid and BHT) using a spectrophotometer (Hitachi) at 517 nm. The absorbance values of the test samples were compared with those of the controls (without extract) to calculate the percentage inhibition(11) The radical scavenging activity was then estimated by calculating the inhibition percentage (I) as follows

I= (Abs control – Abs sample) / Abs control X 100

Hydroxyl Radical Scavenging Assay

The competition between hydroxyl radicals generated from deoxyribose and the extract was measured from Fe3+/ascorbate/EDTA/H_2O_2 system(12). A reaction mixture was prepared with 3.0 mM deoxyribose, 0.1 mM $FeCI_3$, 0.1 mM EDTA, 0.1 mM ascorbic acid, 1 mM H_2O_2 and 20 mM phosphate buffer (pH 7.4) and 0.1ml of extract was added to it making a final volume of 3.0 ml. . Reactions were carried out in triplicate. These were incubated for 1 hr at 37ºC after which 1 ml of thiobarbituric acid (1%) and 1.0 ml trichloroacetic acid (2.8%) were added and then incubated for another 20 min at 100ºC. The test tubes were cooled and the absorbance of the reaction mixture was measured at532 nm. Deoxyribose and buffer were taken as blank. Inhibition percent (I) of deoxyribose degradation was calculated using the formula

I= (Abs control – Abs sample) / Abs control.

Ascorbic acid was used as a positive control

Metal Chelating Activity

To a solution of 1 ml of ferrous sulphate (0.125 mM), 1 ml of the extract was added. 1 ml of ferrozine (0.3125 mM) was then added to initiate the reaction. After this the reaction mix was incubated at room temperature for 10 min. Thereafter, absorbance was measured at 562 nm, while EDTA or Citric acid was used as positive control. The ability of sample to chelate ferrous was calculated relative to the control using formula

Chelating effect = (Abs control – Abs sample) / Abs control (13)

Assay for Enzymatic antioxidants

Superoxide dismutase

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 to 0.5 ml of plant extract. 0.4 ml of 1 mM Hydroxylamine hydrochloride was added to initiate the reaction after which absorbance was taken at 5 min intervals at 560 nm. SOD activity was expressed as the amount of enzyme required for inhibiting the reduction of NBT by 50% (14). The specific activity was expressed in terms of units per mg of protein.

Catalase

Titrimetric method was used to determine the Catalase activity (15). 5 ml of 300 µM phosphate buffer (pH 6.8) containing 100 µM hydrogen peroxide (H_2O_2) was added to 1ml plant extract and the reaction mixture was left at 25°C for 1 min. 10 ml of 2% sulphuric acid was then added to arrest the reaction. The residual H_2O_2 was titrated against 0.01N potassium permanganate till pink colour was obtained. Enzyme activity was estimated by calculating the decomposition of µM H_2O_2 per min per mg protein.

Peroxidase

Peroxidase activity was estimated by adding3.5 ml of phosphate buffer (pH 6.5) and 0.1 ml of O-dianisidine solution to 0.2 ml of plant extract. 0.2 ml of 0.2 mM H_2O_2 was added to initiate the reaction and absorbance was then measured every 30sec intervals upto 3 min. The extinction

coefficient of oxidized O-dianisidine was calculated for estimating peroxidase activity. The enzyme activity was expressed as units per mg of protein (16).

***In vitro* antidiabetic activity**

α- amylase assay

α- amylase activity was measured by using the DNS method. To 500 µL of plant extract500 µL of 0.02 M sodium phosphate buffer with 6 mM sodium chloride and 0.04 units of α-amylase solution were added. The reaction mixture was then incubated for 10 min at 37°C. After this, 500 µL of 1% starch solution dissolved in 0.02 M sodium phosphate buffer was added. 1.0 ml of 3, 5 dNSA reagent then stopped the reaction. The test tubes were then incubated in a boiling bath water for 5 min and cooled to room temperature. The absorbance at 540 nm was measured after diluting the reaction mixture by adding 10 ml distilled water (17). Similarly, control samples were prepared, but without plant extracts and were compared with test samples containing. The results were expressed as calculated using the formula:

I= (Abs control – Abs sample) / Abs control

α- glucosidase assay

1 ml solution of starch substrate (2 % w/v maltose or sucrose) was incubated with 0.2 M Tris buffer pH 8.0 and the plant extract at 37°C for 5 min. 1 ml of alpha-glucosidase enzyme (1U/ml) was added to this to start the reaction, the test tubes were incubated for 40 min at 35°C thereafter.

2 ml of 6N HCl was then added to stop the reaction. The absorbance was then measured at 540nm to estimate the intensity of the colour (18). The activity was estimated by calculating % inhibition using the same formula as above.

Calculation of IC_{50}:

The antiradical and antidiabetic activity of tested compounds is worked out by calculating IC50 (concentration of a compound inhibiting the activity of a test solution by 50%) using MS Excel.

Statistical Analysis

Results are expressed as mean ± 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 students' 't'-test using Sigma Plot (version 8.0). The values of $P<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Enzymatic antioxidant activity

The hydro-ethanolic extracts of root and bark both showed presence of potent enzymatic antioxidants. Table 1 displays that RC root exhibited specific enzyme activities of 11.82 U/mg (SOD), 14.27 U/mg (CAT) and 11.6 U/mg (Peroxidase) at a concentration of 1mg/ml; whereas in RC bark these were observed as 17.20 U/mg (SOD), 15.98 U/mg (CAT) and 16.2 U/mg (Peroxidase),which were higher as compared to RC root.

TABLES:

Table1: Enzyme activity of antioxidants in hydro-ethnolic extracts of *R. communis* bark and root.

Sample	Conc (µg/ml)	Specific activity of enzymatic antioxidants (Units/mg)		
		SOD	Catalase	Peroxidase
RC Bark	200	3.94±4.105	5.97 ± 2.2	4.6±6.4

	400	8.0±14.062	8.96 ± 2.5	7.9±6
	600	11.6±6.196	11.66 ± 5.7	11.0±1.4
	800	13.73±3.402	13.48±5.3	13.5±3.3
	1000	17.20±8.994	15.98± 7.3	16.2±3. 9
RC Root	200	2.79±11.654	4.86±8.87	3.4±5.4
	400	5.33±10.495	7.66 ± 7.9	5.4±3.5
	600	7.19±41.788	9.66 ± 8.87	6.9±2.02
	800	9.76±7.440	12.6 ± 0.7	9.4±1.10
	1000	11.82±92.656	14.27± 3.23	11.6±2.1

The enzyme activity values are Mean ± SD (n=3)

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 (19). The major defense antioxidant enzyme Superoxide dismutase (SOD), scavenges the superoxide anions by catalyzing their conversion into H_2O_2 which is less toxic (20). 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 (21). 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 (22), without the production of more free radicals (23). The CAT activity also saw proportional increment with the rising concentration of both the extracts.

The third category of enzymatic antioxidants studied is Peroxidase. Peroxidases are oxidoreductases which use H O as electron acceptor for their mode of action in catalyzing different oxidative reactions and reducing H O to water while oxidizing a variety of substrates (24). Our study demonstrated that the Peroxidase activity was also concomitant to higher extract concentrations.

Non-enzymatic antioxidant activity

All concentrations of both the extracts showed significantly ($P < 0.05$) higher activities than their controls. Like enzymatic antioxidants, the potential of non-enzymatic antioxidants was also seen to be higher in RC bark than in RC root. According to Table 2, the IC_{50} values calculated were found to be 204.7 µg/ml (DPPH), 192.677 µg/ml (Hydroxyl radical) and 188.34 µg/ml (Metal chelation) for RC bark, which were lower than those of RC root: 297.34 µg/ml (DPPH), 298.3 µg/ml (Hydroxyl radical), and 179.45 µg/ml (Metal chelation). The IC_{50} values for controls were calculated as 625 µg/ml (DPPH), 168.3 µg/ml (Hydroxyl radical) and 162.82 µg/ml (Metal chelation).

Table 2: Non-enzymatic antioxidants in hydro-ethnolic extracts of *R. communis* bark and root.

Sample	Conc (µg/ml)	% Inhibition		
		DPPH	Hydroxyl Radical	Metal Chelation
RC Bark	200	48.7 ± 7.3	53.76±15.678	71.82±9.58
	400	68.58± 5.1	88.64±32.612	86.21±8.463
	600	82.116 ± 7.2	113.67±32.580	112.25±11.299
	800	98.93 ± 8.7	142.02±36.747	136.66±1.052
	1000	134.39 ± 1.25	215.02±18.118	179.13±8.11

	IC_{50} (µg/ml)	**204.79**	**192.67**	**188.34**
	200	43.2 ± 2.2	21.10±0.837	40.32±1.30
	400	59.09 ± 6.1	87.43±1.674	98.38±0.312
	600	76.98 ± 8.6	109.02±19.675	124.62±1.398
	800	91.28 ± 1.7	145.85±9.641	147.63±88.199
	1000	133.8 ± 5	183.64±38.074	162.68±2.2
RC Root	IC_{50} (µg/ml)	**297.34**	**298.3**	**179.45**
Control*	200	6.87 ± 3.88	64.2 ± 4.73	60.68 ± 0.02
	400	21.33 ± 2.34	70.4 ± 2.34	69.9 ± 0.08
	600	46.78 ± 7.8	76.01 ± 1.59	83.78 ± 0.07
	800	87.3 ± 8.78	82.63 ± 2.78	95.9 ± 0.07
	1000	130.4 ± 10.18	88.87 ± 3.07	104.87 ± 0.02
	IC_{50} (µg/ml)	**625**	**168.3**	**162.82**

The % inhibition values are Mean ± SD (n=3)
*The Control used for DPPH scavenging and hydroxyl radical scavenging assays was Ascorbic acid and EDTA for Metal chelating activity.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay is most commonly estimated for the study of natural antioxidants. DPPH is a free radical which is quite stable. It loses its characteristic deep purple (λmax 515–517 nm) colour when it accepts hydrogen from a corresponding donor. (25). The increased inhibition of DPPH with higher concentration of plant extracts displayed their increasing antioxidant ability.

Generation of hydroxyl radicals is crucial for the irreversible damage inflicted by oxidative stress (26). The hydroxyl radical is an extremely reactive species and reacts at a high rate with all surrounding molecules — proteins, lipids, nucleic acids and sugars. Scavenging of hydroxyl radicals is therefore important to suppress the condition of oxidative stress. The dose dependent increase in the % inhibition of hydroxyl radicals thus elucidates the antioxidant potential of RC.

Oxyradical generation can be prevented by metal ion chelation. (27) On chelating ferrous ions Fe++ Ferrozine is capable of forming a red color complex which is quantified. In the presence of other chelating agents, this reaction is limited such that there is a decrease in the red color intensity. The color reduction gives an estimate of the chelating activity.(28) The metal ion chelating activity of the extracts increased with increasing amount of sample.

Anti-diabetic activity

All concentrations of both the extracts showed significantly ($P < 0.05$) higher activities than control. The estimated IC_{50} values (Table 3) of RC bark and RC root for α-amylase inhibition were 668 µg/ml and 401 µg/ml respectively; and those for α-glucosidase inhibition were found to be 624 µg/ml and 598 µg/ml respectively. Lower IC_{50} values of RC root reveal it as an antidiabetic with greater potential than RC bark. However the IC_{50} values for control, Acarbose were 325.50 µg/ml for α-amylase and 230.71 µg/ml for α-glucosidase.

Table 3: Anti-diabetic potential of hydro-ethanolic extracts of *R. communis* bark and root

Sample	Conc. (µg/ml)	% Inhibition	
		α-amylase	**α-glucosidase**
RC Bark	200	23.02 ± 5.722	18.02 ± 5.22
	400	34.05 ± 16.125	29.45 ± 16.15
	600	45.75 ± 7.308	46.55 ± 7.38
	800	68.20 ± 9.07	62.28 ± 9.07
	1000	80.90 ± 8.105	83.08 ± 10.5
	IC_{50} (µg/ml)	**668**	**624**
RC Root	200	39.77 ± 4.973	23.77 ± 4.973
	400	49.45 ± 15.910	39.45 ± 15.910
	600	62.116 ± 5.722	52.116 ± 5.722
	800	83.93 ± 18.227	78.93 ± 18.227
	1000	114.23 ± 31.522	94.23 ± 31.522
	IC_{50} (µg/ml)	**401**	**598**
Control*	200	40.3±6.929	49.34 ± 1.04
	400	52.0±3.323	63.48 ± 0.91
	600	64.38±1.202	47.9±0.73
	800	76.95±0.565	72.56 ± 1.22
	1000	89.265±1.279	91.58 ± 1.3
	IC_{50} (µg/ml)	**325.50**	**230.71**

The % inhibition values are Mean ± SD (n=3)
*The control used for both the assays was Acarbose

The main physiological role of enzymes α- glucosidase and α- amylase is the breakdown of dietary starch into maltose which is further broken down into glucose for absorption and assimilation (28, 29). These enzymes help by catalyzing the hydrolysis of the internal α-1, 4 glucosidic linkages in polysaccharides taken in diet. Therefore, their activity is enhanced in diabetics. Thus, food stuffs which inhibit such enzymes may prove to of value as novel therapeutic anti-diebetic agents (30). The hydro-ethanolic extracts of *R. communis* root and bark have shown a considerable inhibition of these enzymes, which is comparable to that of the standard Acarbose.

CONCLUSION

In conclusion, data presented here rationalizes the bioefficacy of hydro-ethanolic extracts of *R. communis* root and bark as potential remedy for treatment of hyperglycemia and accompanying oxidative stress during diabetes mellitus. Drawing a comparision, it can be deduced that the roots of *R. communis* exhibit a higher hypoglycemic nature. While, the *R. communis* bark proves to be of greater antioxidative value. The different capabilities of both the extracts can be attributed to the presence of various phyto-constituents in them. Further studies on their *in vivo* behavior and their mechanism of action can be worked out.

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