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Research Article

Inhibitory Effects of Ethanolic Extract of *Raphanus sativus* on Aldose Reductase Enzyme

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Abstract: Aldose reductase enzyme plays an important role in diabetic complications such as cataracts. The purpose of this study was to look into plant extract used for the treatment of diabetes, and their principal fraction for Aldose reductase (AR) inhibitory activity and to find out their influence in diabetic complications. Thus, ethanolic extracts of *Raphanus sativus* L. and their respective its organic solvent soluble fractions, including the ethyl acetate (EtOAc), *n*-butanol (BuOH) and water layers, using DL-glyceraldehyde as a substrate, were studied for their inhibitory activity against rat lens AR, rat kidney AR. In addition, *in vivo* inhibition of lens galactitol accumulation by the major soluble fraction of the plant extract in galactose-fed rat model has been studied. The results shows that all the tested soluble fraction of extracts possess significant AR inhibitory actions in both *in vitro* and *in vivo* assays with n-butanol showing the most the most potent effect. The results obtained in this study give a new dimension to the hitherto unknown activity of the plants as possible protective agents against long-term diabetic complications.
Keywords: aldose reductase, antidiabetic, galctose rat model and extras.

INTRODUCTION

Hyperglycemia plays an important role in the pathogenesis of long term complications, and diabetic patients with poor blood-glucose control are particularly at risk. Persistent hyperglycemia induces abnormal changes such as the formation of advanced glycation endproducts (AGEs) and activation of aldose reductase (AR) which is implicating the diabetic or other pathogenic complications. AR is a cytosolic enzyme and is small monomeric protein composed of 315 aminoacid residues. It is the key enzyme of the polyol pathway which catalyzes the conversion of glucose and galactose to the sugar alcohols sorbitol and galactitol respectively [1].

Under hyperglycemia, because of the saturation of hexokinase with ambient glucose, the increased flux of glucose through the POP accounts for as much as one-third of the total glucose turnover [2]. This leads to overflow of the products of the POP along with depletion in reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the oxidized form of nicotinamide adenine dinucleotide (NAD), the cofactors used in the pathway. The acceleration of the POP thus elicits various metabolic imbalances in those tissues that undergo insulin independent uptake of glucose. Such metabolic perturbation provokes the early tissue damage in the "target" organs of diabetic complications, such as ocular lens, retina, peripheral nerve, and renal glomerulus [3, 4].

Increased extracellular glucose levels as well as fructose from the POP leads to the formation of AGEs, due to non-enzymatic glycation reactions such as Maillard's reaction. The Maillard's reaction takes place between the sugars and the tissue proteins, aminoacids. This leads to the formation of reactive compounds through Schiff's base formation and amodari rearrangement. The compounds resulted are finally converted to fluorescent compounds that are further more reactive to the tissue proteins and aminoacids in the body. These reactive, fluorescent substances are known as AGEs. These are responsible for subsequent damage of the tissues and severe complications of DM [5]. Therefore, there is growing interest in drugs that inhibit AR.

The present project directed towards discovery of preventive agents for the diabetic complications from herbal medicine as they are in most cases lack toxic and side effects. *Raphanus sativus* (Radish) is an annual or biennial bristly herb with a white or brightly colored tuberous tap root and coarsely toothed leaves. It belongs to the family *Brassicaceae*. It showed cardio-protective activity in the rabbits. Gastrointestinal and uterine tone modulatory activities of *R. sativus* are reported. Its antioxidant effects are reported in alimentary

hyperlipidemic rats. Study of extract from radish sprouts in rats showed antioxidant properties and significantly induced bile flow. Its root extract peroxidation *in vivo* showed protective effect on paracetamol- induced hepatotoxicity in albino rats. Its ethanolic extract showed inhibition of lipid and *in vitro* in albino rats. Hexane extract of *Raphanus sativus* L. roots inhibits cell proliferation and induces apoptosis in human cancer cells by modulating genes related to apoptotic pathway. Its leaves protect experimentally induced gastric ulcers in rats. Its root juice showed an antidiabetic effect. In the present study, an effort has been made to evaluate the effect of *Raphanus sativus* L. against AR [6-11].

MATERIALS AND METHODS

Chemicals

DL-glyceraldehyde, NADPH, quercetin, phenylisocynate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Extract action

Fresh *Raphanus sativus* radish (5 kg) was purchased from Warangal local market and was extracted with 95% ethanol for 2 h at 60 °C. The combined filtrates were concentrated to dry. The extract was suspended in distilled water and partitioned sequentially with methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc) and n-butanol (BuOH), respectively.

Experimental animals

Wister albino rats purchased from Mahaveer Enterprises, Hyderabad, India, were housed under standard laboratory conditions and fed commercial rat feed and tap water *ad libitum*. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996). All experimental study protocols were approved by the Institutional Animal Ethical Committee (IAEC) of S.R.R. College of Pharmaceutical Sciences, Valbhapur (V), Elkathurthy (M), Karimnagar- 505476, Telangana, INDIA.

In vitro AR inhibitory activity using rat lens homogenate

Preparation of lens homogenate

Male Wistar albino rats weighing 150 g were used in the study. The rats were sacrificed by spinal nerve dislocation. After collection of the eye balls, the lences were enucleated through posterior approach and homogenized with 3 volumes of 0.1 M sodium phosphate buffer, pH 6.2, using tissue homogenizer (Zentrifugen, Micro 220R, Hettich, Germany). The homogenate was centrifuged for 30 min at 16,000 rpm at 4 °C, the supernatant collected was used as crude AR preparation [12-13].

The protein content, enzyme activity and specific activity of the enzyme preparation were determined using reported methods [14-15].

The AR activity was calculated as:

$$\text{Activity (U/ml)} = \frac{\left[\Delta A\frac{\text{Enzyme}}{\text{min}} - \Delta A\frac{\text{Control}}{\text{min}}\right]}{(6.2\ \text{X Volume of enzyme for analysis})\ \text{X Total volume}}$$

Where, 6.2 is the micro molar extinction coefficient of NADPH at 340 nm.

$$\text{Specific activity (U/mg protein)} = \frac{\text{Activity (U/ml)}}{\text{Protein conc. (mg/ml)}}$$

Enzyme inhibitory assay

AR inhibitory activity was assayed spectrophotometrically. Quercetine each at concentrations of 1.0, 5.0, and 10.0 µg/mL and extract at concentrations of 10, 50, and 100 µg/mL were prepared in 10% dimethyl sulfoxide (DMSO). The blank reaction mixture consisted of 200 µL of 0.15 mM NADPH, rat lens homogenate (enzyme preparation), double-distilled water instead of substrate (10 mM DL-glyceraldehyde), and DMSO instead of test sample, and the final volume was made up to 2 mL with sodium phosphate buffer (pH 6.2). Absorbance was read at 340 nm, and the reading was recorded as a correction factor. The control consisted of 200 µL of 0.15 mM NADPH, 10 mM DLglyceraldehyde (substrate), enzyme preparation, and DMSO (instead of test sample). The test samples consisted of all the components of the reaction mixture of control along with 200µL of different concentrations of the test sample solutions, individually. These samples were read against the control. The reaction was initiated by the addition of 200 µL of DL glyceraldehyde, and absorbance was measured at 340 nm using double-beam UV–visible spectrophotometer (SL210, Elico, India), for 1 min at 5-sec interval. Absorbance was recorded for all concentrations, which were employed in triplicate. The ARI activity of each inhibitor sample was calculated using the formula:

$$\%\ \text{Inhibition} = 1 - \left[\frac{\Delta\ \text{A Sample/min} - \Delta\ \text{A Blank/min}}{\Delta\ \text{AControl/min} - \Delta\ \text{A Blank/min}}\right]\ \text{X}\ 100$$

Where, ΔA sample/min is decrease of absorbance for a minute with a test sample, while ΔA blank/ min is with DMSO and double-distilled water instead of a sample and a substrate, respectively. ΔA control/min is with 10% DMSO in place of the sample [13].

In vivo method

In vivo experiments were conducted on 6-week-old male Wistar albino rats weighing 180-200 g. The animals were divided into 4 groups, each containing six animals. All the sample solutions for

dosing were prepared in normal saline and dosing was done for 14 days.

All groups were labeled according to the samples given. The control and all the test groups were orally fed with galactose at a dose of 10 mg/kg body weight. The quercetin was given at a dose of 10mg/kg body weight. Extracts were given at a dose of 200mg/kg body weight.

The animals were kept in standard laboratory conditions, with standard rat pellet diet and water *ad libitum*, in a 12 h light and 12 h dark cycle at a constant temperature of 25±5 °C. Relative humidity was maintained at 35-60%. All the animals were sacrificed on the 15th day by spinal nerve dislocation, both eye balls were collected from each rat, and lens was enucleated through posterior approach. After washing with saline, the pair of lenses of each rat homogenized with 1ml of ice cold water, individually. The proteins were precipitated with ethanol (70% of the final volume) and removed by centrifugation (30 min at 16,000 rpm). Centrifugation was done at 4 °C, and the supernatant was collected as described by Kato *et al*., (2006) [16] and lyophilized at -40 °C using Lyodel freeze-drier (Delvac Pumps Pvt. Ltd., Chennai, Tamil Nadu, India).

Estimation of lens galactitol levels by RP-HPLC

Lyophilized samples of all groups of rats were derivatized by adding 250µl of pyridine and 500µl of phenylisocynate and then incubated for 1h at 55 °C in water bath with mechanical shaking. After incubation period, reactor flasks were cooled and 250µl of methanol was added to remove excess of phenylisocynate, which otherwise could react with water of the eluent. The clear solutions obtained were then diluted twice with pyridine to decrease the interferences due to absorption of the reagents [17].

Derivatized samples of each group were analyzed by HPLC with Photo Diode Array Detector (Schimadzu LC-10 AT, Japan), Hamilton Rheodine injector syringe (Sigma Aldrich), Chemsil ODS-2 C18 HPLC- column, 250 mm-4.6 mm (Chem India Pvt. Ltd., Hyderabad). A mixture of 60% acetonitrile and 40% of double distilled water was used as mobile phase. Flow rate was adjusted to 1ml/min, and the injection volume was 20µl. detector wavelength was adjusted to 240 nm. A standard graph was plotted by analyzing solutions of different concentrations of galactitol using glucose as internal standard.

Statistical analysis

The data was presented as a mean ± SD. The significance of the difference was analysed by one-way analysis of varience.

RESULTS

***In vivo* inhibition of AR**

RLAR inhibitory activities of the plant extracts and the fractions were compared with quercetin. RLAR enzyme protein concentration, enzyme activity and specific activity of the lens homogenate were found to be 1.24 mg/mL, 3.58 U/mL and 2.91 U/mg respectively. AR inhibitory values (ARI %) and IC_{50} values (mg/mL) of the test compounds against lens homogenate were given in Table 1.

***In vivo* inhibition of AR**

The derivatized lens homogenates of each test as well as control group rats were analyzed by HPLC. The retention time of galactitol was found to be 7.1 min and that of glucose 4.5 min. Galactitol concentration was calculated using a standard graph, and values were shown in Figure-1.

Table 1: *In vitro* AR inhibitory activity of the plant extracts and fraction on RLAR

Extract and Fraction	Concentration (µg/mL)	Aldose Reductase Inhibition (%)	IC_{50} (µg/mL)
95% EtOH extract	10	43.66 ± 0.41	-
CH_2Cl_2 fr.	10	13.05 ± 0.12	-
EtOAc fr.	5	74.89 ± 1.17	3.07 ± 0.02
	2.5	54.83 ± 1.27	
	1	30.78 ± 0.64	
n-BuOH fr.	10	84.02 ± 0.97	1.05 ± 0.55
	5	51.57 ± 0.53	
	1	20.33 ± 1.15	
Water fr.	10	24.3 ± 0.64	-
Quercetin	2.5	69.64 ± 0.72	1.09 ± 0.01
	1	48.9 ± 0.51	
	0.5	31.62 ± 0.09	

All values were expressed as mean ± S.D, n = 3

IC_{50}, 50% Inhibitory concentration; SD, Standard deviation

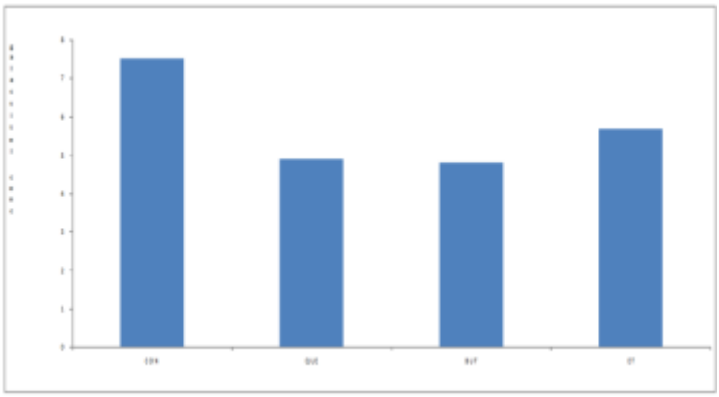

Fig-1: *In vivo* rat lens galactitol levels of plant extract fractions determined by RP-HPLC. Column: ODS-2 C18, 250 mm-4.6 mm; Detector: Photo Diode Array; Mobile phase: 60% acetonitrile and 40% double distilled water; Flow rate: 1 ml/min; Injection volume: 20µl; CON: control group; QUE: quercetine; BUT: n-butanol fraction; ET: ethyl acetate fraction

DISCUSSION

The AR activity of the plant extracts and fractions were compared with quercetin, a potent natural AR inhibitor. Specific activity was calculated from the protein concentration and enzyme activity, this concentration influences the optimization condition for AR by taking saturation concentration of NADPH and glyceraldehydes, i.e. 0.15mM and 10 mM, respectively.

For years, many medicinal plants and their extracts have been demonstrated to effectively treat diabetes. The purpose of this study was to identify new AR inhibitors (ARI) from *R. sativus* for the treatment of diabetic complications. A 95% ethanol extract of *R. sativus* was found to exhibited inhibitory activity against crude rat lense aldose reductase (rAR). Consequently, the 95% ethanol extract of *R. sativus* was further partitioned by systematic fractionation. Among the resulting fractions, the ethyl acetate (EtOAc) and n-butanol (BuOH) soluble fractions exhibited potent inhibitory activity against rAR with IC_{50} values of 2.07 and 1.05 µg/mL, respectively, compared with the positive control quercetin IC_{50} = 1.09 µg/mL.

In view of the powerful *in vitro* AR inhibitory effects of the tested extract, their *in vivo* inhibitory activity was studied by estimation of rat lens galactitol levels in galactose fed rat model using RP-HPLC. The accumulation of polyols such as galactitol was considered to be responsible for the development of cataracts [18]. Lens changes occur more quickly under galactosemic conditions because glucose is converted to sorbitol by AR and then to fructose by sorbitol dehydrogenase in polyol pathway. However, since galactitol is not further metabolized, accumulation of galactitol will take place in a relatively short period of time. Genesis of such galactosemic conditions was reported to result in more speedy onset and progression of retinal change than other diabetic models [19]. As shown in Figure-1, quantification of galactitol by RP-HPLC indicated that all the studied extracts significantly suppressed galactitol accumulation.

Literature reveals that, Poly phenol could elicit AR inhibition by preventing the enzymatic conversion of glyeraldehyde to glycerol and also glucose to sorbitol thereby increasing levels NADPH, a coenzyme highly involved in protecting against the toxicity of reactive oxygen species [20]. This is in agreement with the present findings, *Raphanus sativus* n-butanol and EtOAc fraction may be possing AR inhibitory activity is significantly high, which may be due to the presence of poly phenolic compounds in the *Raphanus sativus* L., which is supported from Beevi et al. 2010, 2011[6, 7].

CONCLUSION

It is believed that the data described in this study provide new evidence and highlight the significance of the studied medicinal plant extracts and their pure compounds in preventing diabetic complications. However, further evaluation is warranted to determine the beneficial effects on whole range of chronic diabetic complications.

REFERENCES

1. Sun G, Ma Y, Gao X, Ko¨nig S, Fales HM, Kador PF; Method for isolating tight-binding inhibitors of rat lens aldose reductase. Experimental Eye Research, 2004; 79: 919–926.
2. Gonza´ lez RG, Barnett P, Aguayo J, Cheng HM, Chylack LTJ; Direct measurement of polyol pathway activity in the ocular lens. Diabetes, 1984; 33:196–199.
3. Kinoshita JH, Nishimura C; The involvement of aldose reductase in diabetic complications. Diabetes Metab Rev, 1988; 4:323–337.
4. Pugliese G, Tilton GT, Williamson JR; Glucose-induced metabolic imbalances in the pathogenesis of diabetic vascular disease. Diabetes Metab Rev, 1991; 7:35-59.
5. Jung HA, Jung YJ, Yoon NY, Jeong DM, Bae HJ, Kim DW, Na DH, Choi JS; Inhibitory effects of Nelumbo nucifera leaves on rat lens aldose reductase, advanced glycation end products

formation and oxidative stress. Food and Chemical Toxicology, 2008; 46: 3818-3826.
6. Beevi SS, Mangamoori LN and Gowda BB; Polyphenolics profile and antioxidant properties of Raphanus sativus L. Nat Prod Res, 2012; 26(6): 557-563.
7. Beevi SS, Narasu ML and Gowda BB; Polyphenolics profile, antioxidant and radical scavenging activity of leaves and stem of Raphanus sativus L. Plant Foods Hum Nutr, 2010; 65(1): 8-17.
8. Zaman RU; Study of Cardioprotective Activity of Raphanus sativus L. in the Rabbits. Pakistan J Biological Sci,2004; 7(5): 843-847.
9. Lugasi A, Blazovics A, Hagymasik K, Kocsis I and Kery A; Antioxidant effect of squeezed juice from black radish (R. sativus L var niger) in alimentary hyperlipidaemia in rats. Phytother Res, 2005; 19: 587-591.
10. Chaturvedi P, George S and Machacha CN; Protective role of Raphanus sativus root extract on paracetamol-induced hepatotoxicity in albino rats. Int J Vitam Nutr Res, 2007; 77(1): 41-45.
11. Devaraj VC, Gopala Krishna B, Viswanatha GL, Satya Prasad V and Vinay Babu SN; Protective effect of leaves of Raphanus sativus Linn on experimentally induced gastric ulcers in rats. Saudi Pharmaceutical Journal, 2011; 19(3): 171-176.
12. Hayman S, Kinoshita H; Isolation and properties of lens aldose reductase. J Biol Chem, 1965; 240: 877-882.
13. Jung HA, Yoon NY, Kang SS, Kim YS, Choi JS; Inhibitory activities of prenylated flavanoids from Sophora flavescens against aldose reductase and generation of advanced glycation endproducts. J Pharm Pharmacol, 2008; 60: 1227-1236.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ; Estimation of Total Protein Content. Journal of Biological Chemistry, 1951; 193: 265.
15. Chetan S, Shylaja MD, Nagappa GM; Inhibition of aldose reductase from cataracted eye lenses by finger millet (Eleusine coracana) polyphenols. Bioorg Med Chem, 2008; 16: 10085-10090.
16. Kato A, Higuchi Y, Goto H, Kizu H, Okamoto T, Asano N, Hollinshead J, Nash RJ, Adachi I; Inhibitory effects of Zingiber officinale Roscoe derived components on aldose reductase activity in vitro and in vivo. J Agric Food Chem, 2006; 54: 6640-6644.
17. Dethy JM, Deveen BC, Janssens M, Lenaers A; Determination of Sorbitol and Galactitol at the Nanogram Level in Biological Samples by High-Performance Liquid Chromatography. Analytical Biochemistry, 1984; 143: 119-124.
18. Kinoshita JH; Mechanism initiating cataract formation. Invest Opthalmol, 1974; 28: 713-724.
19. Kador PF, Inoue J, Secchi EF, Lizak MJ, Rodriguez L, Mori K, Greentree W, Blessing K, Lackner PA, Sato S; Effect of sorbitol dehydergenase inhibitors on sugar cataract formation in galactose fed and diabetic rats. Exp Eye Res, 1998; 67: 203 -208.
20. Rao AR, Veeresham C, Asres K; In vitro and in vivo inhibitory activities of four Indian medicinal plant extracts and their major components on rat aldose reductase and generation of advanced glycation endproducts. Phytotherapy Research, 2013; 27(5):753-60.