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Oriental Pharmacy and Experimental Medicine 2009 **9(3)**, 245-251
DOI 10.3742/OPEM.2009.9.3.245

Aldose reductase inhibitory activity and anti catraract potential of some traditionally acclaimed antidiabetic medicinal plants

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Received for publication August 11, 2008; accepted March 13, 2009

SUMMARY

Aldose reductase (AR) has been reported to play an important role in sugar-induced cataract. In the present study, the AR inhibitory activity of *Enicostemma hyssopifolium* (EH), *Gymnema sylvestre*, *Eclipta alba*, and *Tinospora cordifolia* (TC) were studied along with their effect on sugar-induced cataractogenic changes in sheep lenses *in vitro*. AR inhibitory activity of the aqueous extracts of plants and their anticataract potentials were evaluated *in vitro* in sheep lenses, considering the activity of normal sheep lenses as 100%. The concentration of the plant extract that showed maximum activity was selected to further study its effect on galactose-induced polyol accumulation *in vitro*. The IC_{50} values of EH and TC were calculated to be 102 and 85 μg/ml, respectively. EH showed a significant inhibition (61.3%) in polyol accumulation followed by TC (53.1%). EH and TC possesses a significant anticataract activity *in vitro* and its anticataract potential could be related with its AR inhibitory effect.

Key words: Aldose reductase; Cataract; *Eclipta alba*; *Enicostemma hyssopifolium; Gymnema sylvestre;* Polyol; *Tinospora cordifolia*

INTRODUCTION

Increased blood sugar levels influence the refractive power of the lens in diabetics, and can lead to diabetic cataract. Surplus glucose induces accumulation of the sugar alcohol within the cells, thus generating disturbances of the osmotic balance and finally causing cataract. The enzyme aldose reductase is the first enzyme of the polyol pathway (Fig. 1) that reduces excess D-glucose into D-sorbitol with concomitant conversion of NADPH into $NADP^+$ (Kador, 1988; Carper *et al*., 1989; Tomlinson *et al*., 1994; Crabbe and Goode, 1998; Yabe-Nishimura, 1998).

Experiments with animals have shown that aldose reductase inhibitors can prevent the formation of such opacities in streptozotocin-induced diabetic rats (Kim *et al*., 2008). Aldose reductase is the key enzyme of polyol pathway and has been a drug target in the clinical treatment of secondary complications of diabetes including cataract (Tomlinson *et al*., 1994). Literature review showed that the cataract progression can be slowed or prevented by the use of natural therapies, particularly with those plants having high flavonoids content and have shown considerable *in vivo* AR inhibiting effect (Lim *et al*., 2001) and hypoglycemic

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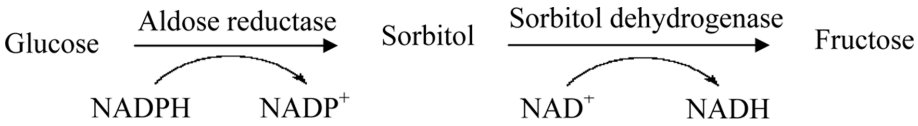

Fig. 1. Polyol pathway.

activity. Some aldose reductase inhibitors from natural sources like vitamin C, ellagic acid, dehydrocorydaline, maesanin, flaviolin, salvianolic acid, perillosides A-D etc. are known and studied well (Fuente and Manzanaro, 2003). In the search of newer aldose reductase inhibitors from natural source, the present study was undertaken to explore the aldose reductase inhibitory activity of 4 well reported hypoglycemic plants. In the present study, aqueous extracts of four reputed antidiabetic plants viz. *Enicostemma hyssopifolium* (Vijayvargia *et al*., 2000; Vasu *et al*., 2002; Srinivasan *et al*., 2005), *Gymnema sylvestre* (Baskaran *et al*., 1990; Shanmugasundaram *et al*., 1990), *Eclipta alba* (Ananthi *et al*., 2003), and *Tinospora cordifolia* (Wadood *et al*., 1992; Stanely *et al*., 2000) were selected to evaluate their AR inhibiting potential and anticataract activity *in vitro*. The selection criteria of aqueous extracts of plants relied on their reported hypoglycemic activity and their usage as common ingredient of many antidiabetic polyherbal formulations.

MATERIALS AND METHODS

Plant extracts

The fresh plants were collected from the village nearby Junagadh district, Gujarat, India, and were authenticated as *Enicostemma hyssopifolium* Verdoon (Gentianaceae), *Tinospora cordifolia* (Willd.) Miers ex Hook .F. and Thoms (Menispermaceae), *Gymnema sylvestre* (Retz.) R.Br. ex Schult (Asclepediaceae), and *Eclipta alba* (L.) Hassk. (Compositae) at Botanical Survey of India, Pune. Voucher specimen has been deposited in herbarium of our laboratory for future reference and numbers are given as EH1/Samp1, TC1/samp3, GS1/Samp4, and EA1/Samp2 respectively. Aerial parts of all the plants were dried under shade and powdered. Aqueous extracts were prepared by cold maceration for 24 h. They were filtered and filtrates were dried under vacuum at 50°C using rota-evaporator. Dried extracts were kept in dessicator overnight and then were stored at low temperature (2 - 8°C) in refrigerator. Extracts were reconstituted in phosphate buffer saline (PBS) for subsequent experimental studies.

Chemicals

D-xylose, sodium carbonate, galactose, galactitol, quercetin, Dulbecco's modified eagle's medium (DMEM), fetal calf serum, adenine dinucleotide phosphate reduced form (NADPH) were purchase from Himedia, India. All other chemicals like, ammonium sulfate, dimethyl sulfoxide, lithium sulfate, perchloric acid, potassium hydroxide, periodic acid, stannous chloride and chromotropic acid were of analytical grade and procured from Qualigens, India.

Enzyme preparation from lens homogenate

Sheep eyes were obtained from a local abattoir soon after slaughtering, and the lenses were enucleated by making lateral incision and frozen until needed. The supernatant fraction of the sheep lens homogenate was prepared according to the procedures of Hayman and Kinoshita (Hayman and Kinoshita, 1965). Briefly Lenses (10 - 12 g) were homogenized in 3 volumes of cold distilled water in a teflon paddled homogenizer and centrifuged at 10,000 × *g* for 15 min to remove insoluble material. Saturated ammonium sulfate was added to the supernatant fluid to 40% saturation. The thick suspension had been allowed to stand with occasional stirring for 15 min to ensure complete precipitation. It was centrifuged and the precipitate was discarded. Additional inert protein was removed by

increasing the ammonium sulfate concentration to 50% saturation and centrifuging the mixture. Aldose reductase was then precipitated from the 50% supernatant solution by the addition of saturated ammonium sulfate solution up to 75% saturation and was recovered by centrifugation. The pellet obtained by centrifugation was dissolved in the phosphate buffer (0.067 M). Further purification of the fraction was done with the use of Sephadex LH 20 (Sigma-Aldrich, USA). Enzyme preparation was analyzed for its protein content using Lowry's method (Lowry *et al*., 1951).

Sample preparation

Dried extracts were reconstituted in 5% DMSO in phosphate buffer to prepare stock solutions. Various dilutions of final concentration 25, 50, 75, 100, 200, 300 and 400 μg/ml were prepared.

Determination of AR activity

Lens AR activity was measured according to the method of Hayman and Kinoshita with slight modification. In short, the reaction mixture was prepared at 25°C, with a total volume of 3.0 ml, containing 50 mM Na-phosphate buffer (pH 6.2), 0.125 mM NADPH, 400 mM LiSO4, 0.3 ml of enzyme preparation and 10 mM D-xylose as a substrate with or without plant extract. The reaction was initiated by addition of NADPH and continued for 5 min. Absorbance was recorded in a double beam spectrophotometer at 340 nm, at every 30 s intervals for 5 min. A negative control was prepared using 5% DMSO in phosphate buffer (pH 6.2). The bioassays were run in triplicate and the average inhibitory activities of the extracts were calculated using the following formula.

$$\% \text{ AR Inhibition} = \frac{\Delta\text{Abs. (Negative Control)} - \Delta\text{Abs. (Extract)}}{\Delta\text{Abs. (Negative Control)}}$$

To determine the AR inhibiting activity, 0.3 ml of plant extract from various stock solutions was added to both the reference and sample cuvettes. The reaction was initiated by the addition of 0.3 ml of 0.125 mM NADPH in sample cuvette and the rate of reaction was measured as described above. ΔOD/min/mg protein was calculated for each sample. Percent inhibition of AR activity was calculated and IC_{50} value for each extract was obtained from a dose–response curve.

Effect on polyol level under osmotic stress

Eyes were enucleated and lenses were dissected from the sheep eyes. Each isolated lens was incubated in 5 ml of DMEM at 37°C in an incubator at 95% air and 5% CO_2 (Halder *et al*., 2003). The medium was supplemented with 10% fetal calf serum and 0.9 g/l sodium bicarbonate. Streptomycin, 100 μg/ml and penicillin, 100 IU/ml were also added to prevent bacterial contamination. After 2 h of incubation, opaque lenses which were damaged during dissection were discarded and transparent lenses were taken for the subsequent experimental studies. Osmotic stress was generated by supplementing galactose (GAL; 30 mM) in the DMEM culture medium. To standardize the incubation period in presence of GAL, enucleated clear lenses were incubated for different time periods such as 1, 3, and 5 h at 37°C with 5% CO_2 and 95% air. The time period of maximum polyol accumulation was calculated to be 3 h, which was considered for the subsequent experiments. The transparent sheep lenses were divided into negative control, positive control and treated groups. Negative control, positive control and treated group's lenses were incubated for 3 h in DMEM, DMEM supplemented with 30 mM of GAL and quercetin (33.3 mM), and DMEM supplemented with 30 mM of GAL as well as the concentration of the extract with maximum AR inhibitory activity shown in the previous experiment, respectively. Post-incubation, lenses from different groups were washed, fresh weight recorded and processed for polyol estimation by the reported method (West and Rapoport, 1949). Briefly, the lenses were homogenized in 9 volumes

of 0.6 N perchloric acid and centrifuged at 5,000 × *g* for 20 min. The supernatant so obtained was neutralized with 2 N KOH and again centrifuged. The supernatant was reacted with 0.25 ml of periodic acid (0.03 M) for 10 min followed by the addition of freshly prepared 0.25 ml of stannous chloride (0.125 M) and 2.5 ml of chromotropic acid (0.2%). The reaction mixture was heated on a boiling water bath for 30 min. The absorbance of the purple-colored complex was measured at 570 nm using spectrophotometer. A parallel standard was also prepared using galactitol.

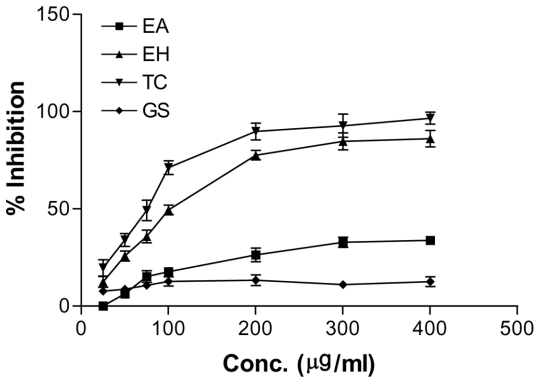

Fig. 2. The effect of TC, EH, EA and GS on AR activity in sheep lenses. Each value is mean ± S.E.M. of three observations.

RESULTS

The phytochemical screening of aqueous extracts revealed that EH contains significant amount of flavonoids, coumarins, bitter principles and carbohydrates, EA found to have phenolics, mainly coumestans as well as carbohydrates and proteins, TC contains terpene glycosides, alkaloids, flavonoids and carbohydrates, GS found to be rich in saponin content and also shows presence of carbohydrates and proteins.

Inhibition of lens AR

The effects of the aqueous extract were estimated with the aldose reductase enzyme, using D-xylose as a substrate. Their inhibitory potencies and IC_{50} values on the AR enzyme were estimated. The average inhibitory activities of the extracts were calculated and shown in Table 1. Aqueous extracts of TC and EH were found to inhibit lens AR. It is evident from the dose response curve that TC offered maximum AR inhibiting activity followed by EH. Inhibitory activity of EA and GS was not significant when compared with negative control. At the concentration of 200 µg/ml, TC showed AR inhibition by 89.8%. The AR inhibitory activity of TC, EH, EA, and GS at different concentrations is presented in Fig. 2. It is evident from the DRC that the maximum inhibitory effect of TC and EH was produced at the concentration of 200 µg/ml for both, and IC_{50} value was calculated to be 85 and 102 µg/ml respectively.

Effects on lens polyol

Polyol content in sheep lenses maintained in organ

Table 1. AR Inhibitory activity of extracts

Concentration (µg/ml)	% Inhibition			
	EA	EH	TC	GS
25	0.00 ± 0.00	12.45 ± 1.57	19.66 ± 2.42	7.70 ± 0.32
50	6.29 ± 0.73	25.75 ± 1.47	34.00 ± 1.91	8.70 ± 0.71
75	15.16 ± 1.74	35.82 ± 1.90	49.15 ± 3.02	10.70 ± 0.83
100	17.75 ± 0.72	49.43 ± 1.43	71.20 ± 2.04	12.70 ± 1.37
200	26.31 ± 2.03	77.62 ± 1.42	89.80 ± 2.49	13.27 ± 1.59
300	32.82 ± 1.48	84.67 ± 2.55	92.72 ± 3.44	11.05 ± 0.94
400	33.82 ± 1.18	86.03 ± 2.45	96.58 ± 1.77	12.60 ± 1.44

Each value is mean ± S.E.M. of three observations.

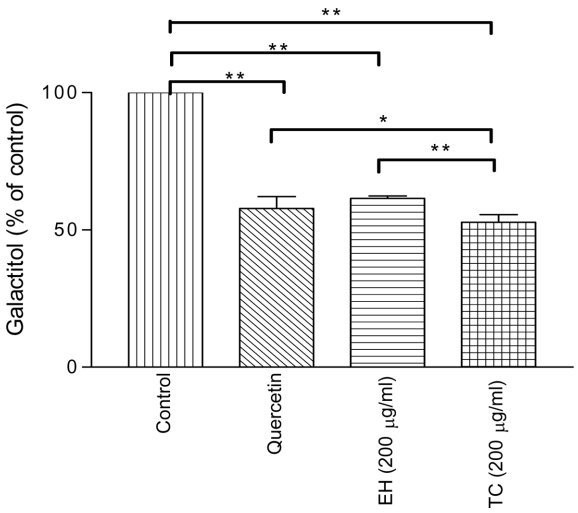

Fig. 3. The effect of EH and TC on polyol accumulation in lenses cultured in a GAL- medium. $^{*}P < 0.05$, $^{**}P < 0.001$. (n = 5, data analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparision Test).

culture under high galactosemic condition is presented in Fig. 3. The effect of herbal extracts on polyol levels was studied at 3 h, the time of maximum polyol accumulation. The most effective AR inhibitory concentration of TC and EH i.e. 200 μg/ml was used to see their effect on polyol accumulation. EH present in a GAL medium significantly decreased the lens polyol to 61.3% in comparison to control group whereas the polyol level in TC treated lenses was found to be 53.1 in comparison to the control lenses. EA and GS were excluded from this experiment.

Statistical analysis

Data of polyol content in lenses were analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparision Test. *P* values less than 0.05 were considered as significant.

DISCUSSION

Significant production of polyol in the lens during sugar-induced cataractogenesis stimulated intense interest in the pathological role of polyol pathway in the development of cataract. Polyol accumulation in the lens leads to an increase in the intracellular ionic strength resulting in excessive hydration, eventually loss of membrane integrity and leakage of free amino acids, glutathione and myo-inositol (Heyningen,1959) etc. Aldose reductase is found to be the primary factor responsible for this pathological condition (Heyningen, 1959). Human genetic and biochemical data also suggested a strong link between raised AR activity and strongly altered risk of diabetic complications such as cataract, nephropathy, retinopathy and neuropathy (Collier and Small, 1991). These results together with recent clinical, experimental and pharmacological data provide powerful support for research and development of AR Inhibitors. There are many reports of AR inhibiting activity of natural products such as *Eugenia borinquensis, Mangifera indica, Eucalyptus deglupta,* and *Syzygium malaccense* (Guzman and Guerrero, 2005). Ellagic acid and two of its derivatives were found as a potent aldose reductase inhibitors in the leaves of *Myrciaria dubia* (Ueda *et al*., 2004). Three flavonoids isolated from *Brickellia arguta* showed anticataract activity in rats (Rosler *et al*., 1984). Acteoside, an active aldose reductase inhibitor phenolic glycoside was discovered from a 70% acetone extract of *Monochasma savatierii* (Kohda *et al*., 1989). Monoterpene glycosides, perillosides A and C isolated from the leaves of *Perilla frutescens* were found to be excellent aldose reductase inhibitor (Kohda *et al*., 1995). Some sulfated flavonoids in *Polygonum hydropiper* were discovered to show potent inhibition against bovine lens aldose reductase (Haraguchi *et al*., 1996). Other studies showed that flavonoid glycosides (Haraguchi *et al*., 1998), isoflavonoids (Jung *et al*., 2002), flavanone glucosides (myrciacitrins III) (Suryanarayana *et al*., 2004), protocatechualdehyde (Suryanarayana *et al*., 2002) and tannoid principles (Lee *et al*., 2005) had strong inhibitory activity. Plants which are rich in polyphenols and bioflavonoids are reported to reduce the AR activity. Diabetes being the major risk factor for cataract, the plants for the present study has been

selected on the basis of their reported hypoglycemic property. Selected plant's aqueous extracts were studied for their AR inhibiting activity and, subsequently, their anticataract potential was evaluated against GAL-induced biochemical changes in sheep lenses maintained in organ culture in the presence and absence of the extracts. The results of the present study suggest that TC and EH possess AR inhibiting activity of different magnitude. Most of the reported natural AR inhibitors are phenolic compounds and in addition to inhibition of the polyol pathway, they are also involved in anti oxidant mechanisms, like the flavonoids and vitamin C, in the inhibition of the overproduction of superoxide (Brownlee, 2001) or in metal ion chelation processes (Crabbe and Goode, 1998). The aqueous extract of *T. cordifolia* has shown the anti-oxidant action in diabetic rats (Prince and Menon, 1999; Stanely *et al*., 1999). High flavonoids content of *E. hyssopifolium* (Ghosal and Jaiswal, 1980) impart a good anti oxidant property which might be in turn responsible for its AR inhibiting potential. These plants could be further evaluated for bio guided fractionation to emerge out with a new AR inhibitor molecule.

ACKNOWLEDGEMENTS

One of the authors, Mr. Mayurkumar Patel, would like to thank All India Council for Technical Education (AICTE), New Delhi, for providing the financial assistance, in the form of National Doctoral Fellowship (NDF) to carry out this work.

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