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Aldose reductase inhibitory principles from the whole plant of *Hybanthus enneaspermus* (Linn) F. Muell

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ABSTRACT

Objective: To evaluate active aldose reductase (AR) inhibitory principles from ethanolic extract and one isolated compound beta sitostirol from *Hybanthus enneaspermus* (*H. enneaspermus*) Linn F. Muell. (Violaceae), used in the treatment of various complications including diabetes. **Methods:** AR inhibitory activities of *H. enneaspermus* and isolated compound beta sitosterol against rat lens AR enzyme were investigated *in vitro* using quercetin as a standard drug. **Results:** There was a significant difference in the AR inhibitory activity, and it was found to be more in *H. enneaspermus* [IC_{50} (49.46±2.26 μ g/mL)] followed by beta sitosterol [IC_{50} (82.71±0.42 μ g/mL)] but was less compared to standard quercetin [IC_{50} (3.326±0.11 μ g/mL)]. From the value of V_{max}, K_m and K_i it was found that *H. enneaspermus* inhibited enzyme in a non competitive manner, whereas beta sitosterol inhibited enzyme in a competitive manner. **Conclusions:** *H. enneaspermus* showed significant AR inhibitory activity compared to the isolated compound beta sitosterol, which may be due to its high phenolic and flavonoid content. So this plant can be used to treat diabetic complications in the future, but further evaluation is necessary to know the exact mode of action involved in this process.

1. Introduction

Chronic hyperglycemia can cause various complications including blindness, renal failure, neuropathy, limb amputation, myocardial infarction and stroke, which are mainly due to elevated levels of glucose in tissue such as the nerve, kidney, retina and lens. Many biochemical pathways are involved in the process associated with this complication such as polyol pathway[1,2]. Aldose reductase (AR) which belongs to aldo–keto reductase is the first and rate–limiting enzyme in the polyol pathway which converts glucose to sorbitol utilizing Nicotinamide Adenine Dinucleotide Phosphate (NADPH) as a cofactor. Accumulation of sorbitol leads to osmotic swelling, changes in membrane permeability, and also oxidative stress culminating in tissue injury. It also changes the reduction potential of the cell and induces oxidative stress. Oxidative stress is mainly due to depleted intracellular levels of reduced glutathione, increased non–enzymatic glycation

and activation of protein kinase C[2]. From earlier study it was proven that inhibition of AR enzyme could be effective in the treatment of cataract, retinopathy, nephropathy and neuropathy. Eventhough both synthetic and natural AR inhibitors have been reported to prevent some diabetic complications in animal models and clinical trials, still very limited number of AR inhibitor is there due to deleterious side effects and poor penetration of target tissues such as nerve and retina[3].

Many AR inhibitors obtained from natural sources such as quercitrin, guaijaverin, and desmanthin–1 showed significant inhibitory activity against AR enzyme as mentioned in some other study[4]. In the earlier studies it has been shown that cataract progression can be slowed down or prevented by the use of natural therapies. Plants which have high flavonoid content were found to have significant inhibitory potential against AR enzyme[5]. Quercitrin–3–rhamnosid a type of flavonoid was the first synthetic AR inhibitor tested *in vivo* against AR enzyme. In another study it was found that oral consumption of quercitin and naringin decreases sorbitol accumulation in lenses of diabetic rats[6]. From the data obtained in earlier study it was found that, 4–oxo–4h–chromen ring in flavonoid structure is necessary to inhibit the AR enzyme. High ascorbic acid concentration

can also inhibit erythrocyte AR where aminoguanidine prevented the retinopathy in diabetic rats[7]. Two other isoflavone compounds, tectorigenin and irigenin, were also shown strong inhibitiory activity against AR enzyme[8].

Hybanthus enneaspermus (*H. enneaspermus*) Linn F. Muell. (Violaceae) is an herb distributed in the tropical and subtropical regions of world. The plant is used to treat urinary infections, diarrhea, cholera, leucorrhoea, gonorrhea, dysuria, inflammation, sterility and diabetes in the different systems of medicine[9,10]. Pharmacologically it has been tested for anti−inflammatory, antitussive, antiplasmodial, antimicrobial, anticonvulsant and antidiabetic activities[11,12]. Due to presence of significant amount of phenol and flavonoid contents with relevent antioxidant and antidiabetic potential, the aim of the present study was to evaluate a potential new source of AR inhibitors from the ethanolic extract of *H. enneaspermus* wich will prove beneficial for treatment of diabetes related secondary complication such as cataract.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade and procured from Sigma Chemical Co., New Delhi and Merck India Ltd. For absorbance measurements ultraviolet−visible spectrophotometer (Shimadzu, Pharmaspec−1700) was used.

2.2. Procurement of plant material

Plant material (*H. enneaspermus*) was procured from herbal vendors in Chennai, and authenticated by the chief botanist TAMPCOL Anna Hospital Chennai, voucher specimen (Cog/HE/01/08) was kept for further reference at Laboratory herbarium, Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Vanarasi, India.

2.3. Preparation of extract

The air−dried whole plant parts of *H. enneaspermus* (1 kg) were extracted with ethanol in a Soxhlet apparatus for 12 h. After filtration of the solvent, the organic phases were concentrated under a vacuum to get dry extract.

2.4. Phytochemical evaluation

2.4.1. Fingerprint analysis
HPTLC fingerprint analysis of *H. enneaspermus* was performed in chloroform : methanol (8:2) solvent system, mode of application was automatic, volume of sample was 5 μL, development mode was ascending and scanning was done at 254 nm using win cats software.

2.4.2. Isolation process
Powdered material of *H. enneaspermus* was refluxed with n−Hexane for 2 h, after filtration organic solvent was concentrated under vacuum to get dry extract. n−Hexane soluble fraction (15 g) was packed on column with silica gel used as a stationary phase and successively eluted with a stepwise gradient of n−Hexane−ethyl acetate (0, 1, 2, 5, 10, 20 and so on up to 100%) and then were washed with methanol. The active guided fractions (6:4) were colleced, mix all the same fractions which was checked by TLC, concentrated and finally kept for 5 day. Solid mass was dissolved in the methonal and were separated. This process was then repeated until the colour of the solution became colourless. Finally the colourless solid mass was dissolved in the chloroform and 2 mL of methanol was further added to it and was kept at room temerature for 5 days from which white soft crystal were obtained. For the determination of the structure and nature of compound, various type of data analysis such as melting point, infrared spectrum, NMR spectral analysis have been done. Finally the isolated compound was confirmed by TLC fingerprint analysis using standard beta sitosterol as a reference compound.

2.5. Measurement of AR inhibitory activity in vitro

2.5.1. Experimental animals
Healthy albino rats of Charles foster strain (140−180 g) were used in this study, they were housed under standard laboratory conditions [12 h light/12 h darkness, (21 ± 2) ℃]. Animals were given standard pellets diet (Mona laboratoty animal feed) and water *ad libitum* throughout the experimental period. Experimental study was approved by the Institutional Animal Ethical Committee of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

2.5.2. Purification of lens AR
Eyes of normal Wistar rats were removed immediately after sacrificing them, washed with saline and their fresh weights were measured. 10% homogenate was prepared from cleared lenses in 0.1 M phosphate buffer saline (PBS, pH 7.4). After centrifugation at $5\,000 \times$ g for 10 min in a refrigerated centrifuge, the supernatant was collected and kept in ice[5].

2.5.3. Determination of protein content
Protein content of supernatant was determined by the method of Lowry *et al*[5].

2.5.4. Determination of AR activity
A sample cuvette containing 0.7 mL of phosphate buffer (0.067 M), 0.1 mL of NADPH (25×10^{-5} M), 0.1 mL of lens supernatant, 0.1 mL of DL−glyceraldehyde (5×10^{-4} M) as a substrate to a final volume of 1 mL was read against a reference cuvette containing all components but the substrate, DL−glyceraldehyde. The final pH of the reaction mixture was 6.2. The enzymatic reaction was started by

the addition of the substrate and the absorbance (OD) was recorded at 340 nm for 3 min at 30 second interval. AR activity was expressed as OD/min/mg protein[5].

2.5.5. Determination of AR activity and compound

Dried extracts and beta sitosterol were reconstituted in PBS to prepare stock solutions. To determine their AR inhibiting activity, 0.1 mL of each concentration (25, 50, 75, 100, 200 and 300 μg/mL) from various stock solutions prepared in the PBS was added to both the reference and standard cuvettes. The reaction was initiated by the addition of 0.1 mL DL–glyceraldehyde and the rate of reaction was measured as described above[5].

2.5.6. Determination of enzyme kinetic

Enzyme kinetics K_m and V_{max} of AR were determined with varying concentrations of DL–glyceraldehyde as substrate in the absence and presence of different concentrations of *H. enneaspermus* and beta sitosterol by Lineweaver– Burk double reciprocal plots. Inhibitory constant (K_i) was derived by plotting slopes obtained from Lineweaver–Burk plots versus *H. enneaspermus* and beta sitosterol concentration[5].

2.6. Statistical analysis

Results are expressed as Mean $\pm$ SEM of three independent determinations. Linear regression analysis was performed, quoting the correlation coefficient r^2. Two–ways ANOVA followed by Bonferroni post test was performed for evaluation of all data. GraphPad Prism (version 5) software was used for all statistical analysis, and $P < 0.05$ was considered as significance.

3. Results

3.1. Phytochemical evaluation

From the column chromatography study it was found that *H. enneaspermus* contain significant amount of beta sitosterol, % yield and structure of the isolated compound

were presented in Table 1 and Figure 1. HPTLC fingerprint analysis showed that *H. enneaspermus* contain nine spots, where two spots were found to be prominent, which was presented in the Figure 2.
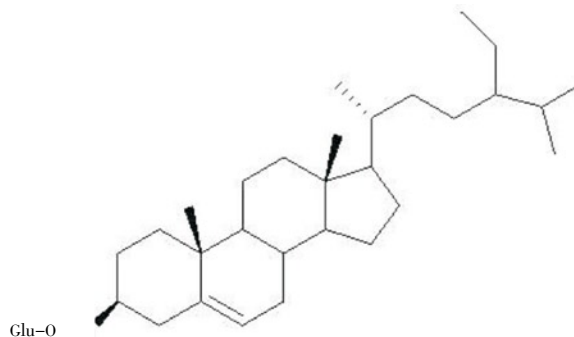
Figure 1. Structure of the isolated compound (Beta sitosterol).

3.2. AR inhibitory activity

The present study deals with AR inhibitory potential of *H. enneaspermus* and isolated compound beta sitosterol on rat lens AR enzyme. *H. enneaspermus* have been chosen in the prsent study based on the ethnopharmacological uses. From the data obtained in the present study it was found that the inhibitory potential of *H. enneaspermus* and beta sitosterol in the form of IC_{50} value was found to be significant against AR enzyme. The AR concentration in normal rat lens was found to be 0.0148 $\pm$ 0.0006 which was considered 100%. At the concentration of 100 μg/mL *H. enneaspermus* extract showed more percentage of inhibition (86.61%) compare to the isolated beta sitosterol (78.78%). The IC_{50} value of the *H. enneaspermus* and beta sitosterol were presented in Table 1 and Figure 3.

3.3. Study of enzyme kinetic

Study of the enzyme kinetic was performed in Lineweaver–Burk plot using Dl–glyceraldehyde as a substrate. The Lineweaver–Burk plot was made in between 1/velocity Vs

Table 1
AR inhibitory potential of *H. enneaspermus* and beta sitosterol.

S. No	Extract/compounds	% yield	AR inhibitory activity* [IC_{50} (μg/mL)]
1	*H. enneaspermus*	13.45	49.460 $\pm$ 2.260
2	Beta sitosterol	5.98[#]	82.710 $\pm$ 0.420
3	Quercetin	NA	3.326 $\pm$ 0.110

*– Values represent Mean $\pm$ SEM of three independent measurements; NA– Not analysed; #– compared to the n–hexane extract .

Table 2
Kinetic parameter of rat lens AR.

S. No	AR activity	V_{max}	K_m	K_i
1	DL–glyceraldehyde	0.1686 $\pm$ 0.0006	0.6562 $\pm$ 0.0045	0.0000 $\pm$ 0.0000
2	DL–glyceraldehyde + Beta sitosterol	0.1393 $\pm$ 0.0003[a]	0.6903 $\pm$ 0.0035[a]	0.1945 $\pm$ 0.0004
3	DL–glyceraldehyde + *H. enneaspermus*	0.1429 $\pm$ 0.0030[a]	0.9627 $\pm$ 0.0004[ab]	0.1622 $\pm$ 0.0003[b]

Values are Mean $\pm$ SEM of three independent data in each group; a: compare to the DL–glyceraldehyde, b: compare to the DL–glyceraldehyde + Beta sitosterol (Two–way ANOVA followed by Bonferroni post test, $P < 0.05$ was considered as significance).

1/ [Dl–glyceraldehyde]. From the value of V_{max}, K_m and K_i it was found that *H. enneaspermus* inhibited enzyme in non competitive manner, whereas beta sitosterol inhibited enzyme in competitive manner. Values of V_{max}, K_m and K_i were presented in the Table 2, where the Lineweaver–Burk plot was presented in Figure 3.
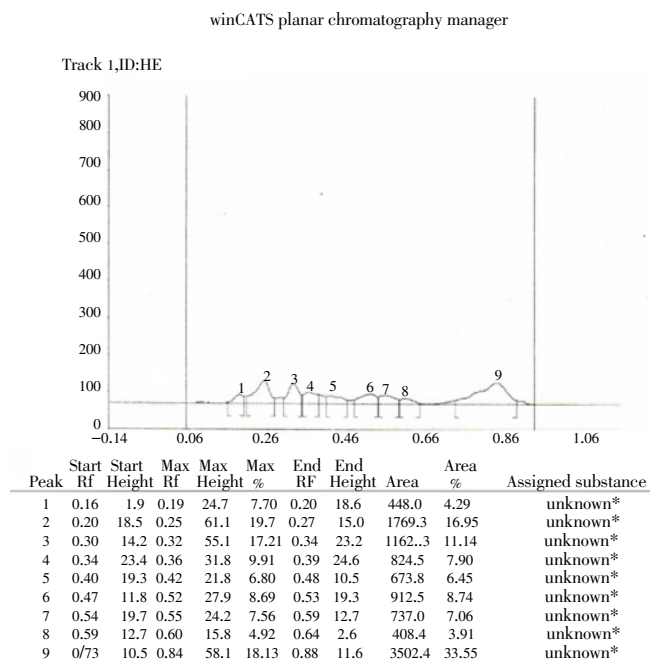

winCATS planar chromatography manager

Track 1,ID:HE

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End RF	End Height	Area	Area %	Assigned substance
1	0.16	1.9	0.19	24.7	7.70	0.20	18.6	448.0	4.29	unknown*
2	0.20	18.5	0.25	61.1	19.7	0.27	15.0	1769.3	16.95	unknown*
3	0.30	14.2	0.32	55.1	17.21	0.34	23.2	1162..3	11.14	unknown*
4	0.34	23.4	0.36	31.8	9.91	0.39	24.6	824.5	7.90	unknown*
5	0.40	19.3	0.42	21.8	6.80	0.48	10.5	673.8	6.45	unknown*
6	0.47	11.8	0.52	27.9	8.69	0.53	19.3	912.5	8.74	unknown*
7	0.54	19.7	0.55	24.2	7.56	0.59	12.7	737.0	7.06	unknown*
8	0.59	12.7	0.60	15.8	4.92	0.64	2.6	408.4	3.91	unknown*
9	0/73	10.5	0.84	58.1	18.13	0.88	11.6	3502.4	33.55	unknown*

Figure 2. HPTLC fingerprint analysis of *H. enneaspermus*.
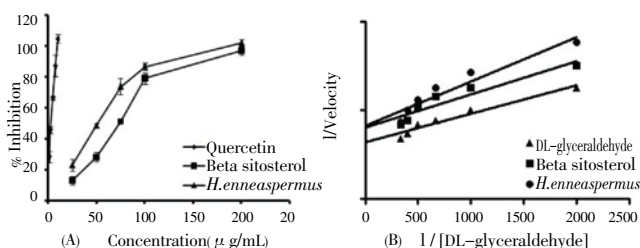

Figure 3. (A) Effect of *H. enneaspermus*, Beta sitosterol and quercetin on AR inhibitory activity. (B) Effects of *H. enneaspermus* and Beta sitosterol on the Lineweaver–Burk plot of AR activity with DL–glyceraldehyde as substrate.

4. Discussion

Through the literature review it was found that *H. enneaspermus* has impressive ethnopharmacological profile which is used in the different systems of the medicine for the treatment of the various type of complication including diabetic[10]. On the basis of these ethnopharmacological data present work dealt with the evaluation of AR inhibitory potential of the *H. enneaspermus* and isolated compound beta sitosterol on rat lens AR enzyme. For this purpose extraction was performed, further isolation was done through column chromatography. Further HPTLC fingerprint analysis of ethanolic extract of *H. enneaspermus* was carried out followed by performing the AR inhibitory activity of *H.*

enneaspermus and beta sitosterol on rat lens AR enzyme.

The phytoconstituents reported in the plant revealed the presence of majorly flavonoids, terpenes, phenols, anthraquinones, glycosides, polyoses, alkaloids, and saponins, tannins. Among these compond phenolic compounds (71.95 mg/g) and flavonoids (20 mg/g) were found to be in abundant quantity[12]. The above phytoconstituents could be used as an analytical tool for the standardization of *H. enneaspermus*, if any adulterants or contaminants were present in the plant material it can be identified by the use of this parameter. HPTLC fingerprint analysis data can also be used for the comparative analysis of the sample, collected in different places and time interval. Ethanol was used for the extraction process due to its high extractability and less toxicity. For the isolation purpose n–Hexane was used as a solvent due the non polar nature of the isolated compound beta sitosterol. In the ethanol extract total phenol and flavonoid was found to be at significant level which could be explained by the possible formation of complexes between phenolic compounds with other components, which are more extractable in ethanol[13,14].

Since mice are devoid of lens AR enzyme, which do not develop sugar cataract in hyperglycemic conditions therefore, Wister rat lens were used as AR enzyme source for initial screening of *H. enneaspermus* and beta sitosterol[15]. Further compared to other tissues, AR enzyme was found to be in higher percentage in the eye lens, particularly rat lens[16]. The inhibitory effects of plant phytochemicals including polyphenols, known as natural antioxidants, and their antioxidant activities are important for human health, which can lower the blood glucose level in diabetes and is capable of reducing oxidative stress by scavenging reactive oxygen species, and prevent cell damage[17–19]. Some flavonoids and polyphenols were found to be effective against α –glucosidase and AR. For the development of effective, safe and potentially useful products such as commercial α –glucosidase and AR inhibitors from natural sources, various researches have been done[20]. From our earlier study it was found that *H. enneaspermus* up to 5 g/kg bw has no toxic effect in animal experimental study suggesting that this plant is safe for human being.

From earlier work it was found that root of *Salacia oblonga*, *Salviae multiorrhizae*, *Glycerrhiza uralensis*, *Radix astragali*, puerarin and curcumin have significant inhibitory potential against AR enzyme. Further, theses plants have also been reported to contain sigificant amount of bioflavonoids, which have also been reported to have AR inhibitory potential against AR enzyme. So in the present study AR inhibitiry activity of the *H. enneaspermus* might be due to the presence of these compounds[3,5]. In another study it was found that flavonoids are among the most potent AR inhibitors known, whereas *Ocimum sanctum* was found to be most effective against AR enzyme compared to other extract such as *Aralia* extract. Other compound such as quercetin, flavanone and flavonol glucosides has also been reported to have sigificant AR inhibitory activity[21,22]. Some of the

AR inhibitors (zopolrestat, ponalrestat, tolerestat, sorbinil, eparetat, and ranireatat) have been developed in the past years but have some side effects and limited efficacy[23]. The depletion of plasma antioxidants is a major cause of diabetes related complications such as atherosclerosis and coronary heart disease. From our earlier study it was found that *H. enneaspermus* has significant antioxidant activity, so it can be used to treat such type of complication[12].

In conclusion, the present study reveals that *H. enneaspermus* has significant AR inhibitory potential compared to the isolated compound beta sitosterol. Further, the relationship between phenolic, flavonoid and AR inhibitory activity was also investigated. The ethanolic extracts have been chosen due to its expected flavonoid contents that were reported to have AR inhibitory activity. The drug has shown significant AR inhibitory potential but, further *in vivo* study is necessary to know the exact mechanism of action and safety profile of the *H. enneaspermus* and beta sitosterol.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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