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Evaluation of *in vitro* aldose reductase inhibitory potential of different fraction of *Hybanthus enneaspermus* Linn F. Muell

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ABSTRACT

Objective: To evaluate the aldose reductase inhibitory (ARI) activity of different fractions of *Hybanthus enneaspermus* for potential use in diabetic cataract. **Methods:** Total phenol and flavonoid content of different fractions was determined. ARI activity of different fractions in rat lens was investigated *in vitro*. **Results:** The results showed significant level of phenolic and flavonoid content in ethyl acetate fraction [total phenol (212.15±0.79 mg/g), total flavonoid (39.11±2.27 mg/g)] and aqueous fraction [total phenol (140.62±0.57 mg/g), total flavonoid (26.07±1.49 mg/g)] as compared with the chloroform fraction [total phenol (68.56±0.51 mg/g), total flavonoid (13.41±0.82 mg/g)] and petrolium ether fraction [total phenol (36.68±0.43 mg/g), total flavonoid (11.55±1.06 mg/g)]. There was a significant difference in the ARI activity of each fraction, and it was found to be the highest in ethyl acetate fraction [IC_{50} (49.26±1.76 μg/mL)] followed by aqueous extract [IC_{50} (70.83±2.82 μg/mL)] and it was least in the petroleum ether fraction [IC_{50} (118.89±0.71 μg/mL)]. Chloroform fraction showed moderate activity [IC_{50} (98.52±1.80 μg/mL)]. **Conclusions:** Different fractions showed significanct amount of ARI activity, where in ethyl acetate fraction it was found to be maximum which may be due to its high phenolic and flavonoid content. The extract after further evaluation may be used in the treatment of diabetic cataract.

1. Introduction

Diabetes mellitus is a chronic disorder of carbohydrate, lipid and protein metabolism characterized by persistent elevation of blood glucose level in the body. It is a major risk factor of cataract which is the leading cause of blindness over the world. Various pharmacological strategies are used to prevent the cataract formation, among them aldose reductase inhibitors have received much attention because of its involvement in the pathophysiology of diabetic complications including cataract[1,2]. The clinical consequences of these complications include lower limb amputation, end-stage renal failure, and loss of vision[3]. Prolonged exposure to chronic hyperglycemia in diabetes can lead to various complications, affecting the cardiovascular, renal, neurological and visual systems, such as lens, retina, nerves and kidney, which are insulin-insensitive, and are the target organs for complications such as cataracts, retinopathy, neuropathy and nephropathy[4]. Several mechanisms such as increased aldose reductase-related polyol pathway, increased advanced glycation end product formation, and excessive oxidative stress are involved in this process[5,6]. Aldose reductase is found in almost all mammalian cells, but at high levels in organs such as the cornea, lens, retina, kidney, myelin sheath and sciatic nerves, which are affected by diabetic complications[7]. When polyol pathway activity is increased, it causes accumulation of polyol in lens fibers, influx of water and generation of osmotic stress, osmotic swelling, changes in membrane permeability, and oxidative stress culminating in tissue injury, that's why it has got special attention in the clinical treatment of secondary complications of diabetes[2]. Formation of sorbitol is a result of enzymatic degradation of glucose through polyol pathway which is a tissue poison whose accumulation increases osmotic pressure and may damage the tissues by causing them to swell. Since aldose reductase is localized primarily in lens epithelial cells, it is possible to prevent cataract *via*

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inhibition of the activity of aldose reductase[8].

According to World Health Organization (WHO) reports that about 80% of the world's population in 2001 used herbal medicine for health[7]. A number of studies have been undertaken to identify natural and synthetic compounds that inhibit aldose reductase and reduce oxidative stress, and the flavonoids are among the most potent aldose reductase inhibitors known. Moreover, of these quercetrin (a glycoside of quercetin), quercetin is believed to be among the strongest of the aldose reductase inhibitors, though their anticataractous activities in animal studies remain controversial. Due to the undesirable side effects of available drugs, therapeutic drugs against diabetic cataract from natural source are still required. Recently, some indigenous plants were reported to have potent aldose reductase inhibitory activities and their anticataract potentials were evaluated against galactose-induced biochemical changes in rat lens organ culture. *Ocimum sanctum* was the most effective aldose reductase inhibitor *in vitro* with an IC_{50} value of 20 μg/mL, which is comparable to that of *Aralia* extract[9].

Hybanthus enneaspermus (*H. enneaspermus*) (L) F. Muell (Violaceae), is a herb distributed in different parts of India such as in Bundelkhand, Agra, Bengal, Tamilnadu, Gujarat, Karnataka, and it is also found in the other parts of the world such as Sri Lanka, tropical Asia, Africa, and Australia. This plant has been reported to contain phytoconstituents *viz.* dipeptide alkaloids, aurantiamide acetate, isoarborinol and β-sitosterol[10]. Traditionally *H. enneaspermus* is used as aphrodisiac, demulcent, tonic, and used to treat various diseases such as urinary infections, diarrhea, cholera, leucorrhoea, gonorrhea, dysuria, inflammation and sterility[11,12]. Pharmacologically this plant has been screened for anti-inflammatory, antitussive, antiplasmodial, antimicrobial, and anticonvulsant activities[13].

Based upon the ethnopharmacological reports we have earlier reported that this plant showed significanct activity in the treatment of diabetes[14]. So the present study was aimed to evaluate the protective effects of different fractions of *H. enneaspermus* on diabetic complications such as aldose reductase inhibitory activity using rat lens[15]. Moreover, relationship between total phenol, flavonoid and its aldose reductase inhibitory potential was also investigated. To the best of our knowledge, this is the first report on the aldose reductase inhibitory effect of different fractions of *H. enneaspermus*.

2. Materials and methods

2.1. Chemicals and instrument

DL-glyceraldehyde, NADPH, quercetin, rutin were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents used were of analytical grade. The absorbance measurements were recorded using the ultraviolet-visible spectrophotometer (Shimadzu, Pharmaspec-1700).

2.2. Collection of plant material

H. enneaspermus plant material was purchased from herbal vendors in Chennai. The plant was identified and authenticated by the chief botanist Tampcol Anna, Hospital Chennai. A voucher specimen (Cog/HE/01/08) was deposited in Department of Pharmaceutics, Institute of Technology Banaras Hindu University, Vanarasi, India for further reference.

2.3. Preparation of extract, and its fractions

The ethanolic extract was prepared by Soxhlet extraction method by taking 1 kg of the powdered plant material extracting with ethanol. The extract was filtered, concentrated and finally dried in vacuo. The ethanol extract was then fractionated by the use of different solvents of varying polarity as shown in the flowchart (Figure 1).

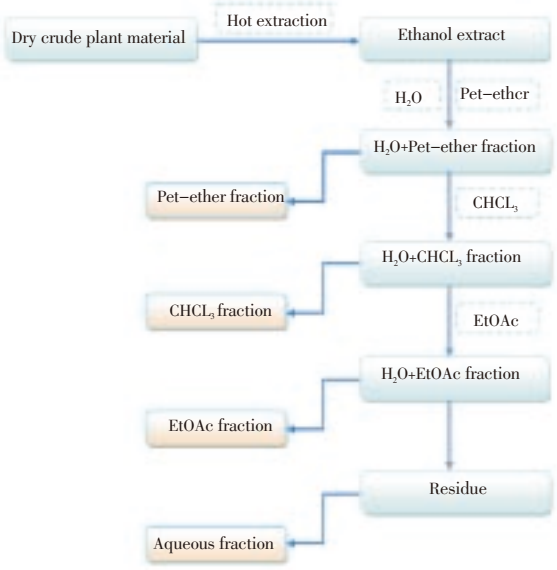

Figure 1. Schematic diagram of fractionation of *H. enneaspermus* extract.

2.4. Phytochemical analysis

Total phenolic content of different fractions of *H. enneaspermus* was determined using Folin-Ciocalteu method. Absorbance of the final solution mixture was measured at 765 nm, gallic acid was used as a standard and results were expressed as mg of gallic acid equivalent per gram (mg GAE/g) of dried extract[16]. For the determination of the total flavonoid content the aluminium chloride method was incorporated using rutin as the standard. The absorption at 415 nm was read for determination of total flavonoid content. The amount of flavonoid in plant extracts was calculated using rutin as a standard[17].

2.5. Lens aldose reductase activity

2.5.1. Animals

Healthy adult Wistar albino rats (150-200 g) aged between 2 and 3 months were taken for the study. They were housed under standard environmental conditions [12 h light and 12 h dark cycle, (25±30) ℃, (35-60)% relative humidity] in polypropylene cages with free access to pelleted food (Mona laboratoty animal feed) and water *ad libitum* throughout the experimental period. The experimental protocol has been approved by the Institutional Animal Ethics Committee of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

2.5.2. Preparation of lens homogenate

Eyes of normal Wistar albino rats were removed immediately after sacrifice. The lenses were removed from the eye, washed with saline and fresh weights of lens were measured. Transparent lenses free from any disease were pooled and a 10% homogenate was prepared in 0.1 M phosphate buffer saline (pH 7.4). The homogenate was then centrifuged in a refrigerated centrifuge at 5 000 × g for 10 min, and then supernatant was collected and kept in ice. Protein content of the lens homogenate was determined[2].

2.5.3. Determination of aldose reductase activity

For the determination of the aldose reductase inhibitory activity of the different fractions, a sample cuvette was taken 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 (substrate) (5×10^{-4} M) and final volume was made to 1 mL. Absorbance was taken against a reference cuvette containing all components but not DL-glyceraldehyde. The final pH of the reaction mixture was adjusted to the pH 6.2. When substrate was added to the solution mixture, enzymatic reaction started, and absorbance (OD) was recorded at 340 nm for 3 min at 30 sec interval. Aldose reductase activity was calculated and expressed as ΔOD/min/mg protein[2].

2.5.4. Lens aldose reductase activity and plant extract

A stock solution of the different fractions was prepared by dissolving the extract into phosphate buffer saline (PBS). For determination of the aldose reductase inhibiting activity, 0.1 mL of each fraction from various stock solutions (final concentrations: 25, 50, 75, 100, 200 and 300 μg/mL) 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 mentioned above.

For each sample, ΔOD/min/mg protein was calculated. Percentage inhibitions of aldose reductase activity of the extract were calculated assuming normal rat lens having 100% activity. IC_{50} values were calculated for each sample by ploting a graph between log dose concentrations versus percent inhibition[2].

2.5.5. Kinetic studies of inhibitory activity against aldose reductase

The kinetic studies of inhibitory activity against aldose reductase of different fractions were analyzed using the Lineweaver-Burk plot.

2.6. Statistical analysis

Results were expressed as mean value ± standard error mean (SEM) of triplicate. Linear regression analysis was performed, quoting the correlation coefficient r^2. Two-way 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. Extraction yields

The yield in the ethanolic extract and its sub-fractions was given in Table 1. The yield percentages of different fractions in decreasing order were as follows: aquous fraction (5.1%) > chloroform (4.5%) > petrolium ether (3.6%) > ethyl acetate (2.8%).

3.2. Phytochemical analysis

The total amount of phenolic and flavonoid was determined in different fraction equivalent to gallic acid and rutin, respectively used as a standard (Table 1). Ethyl acetate fraction contained the maximum amount of phenol and flavonoid [total phenol (212.15±0.79 mg/kg) and total flavonoid (39.11±2.27 mg/kg)], whereas petrolium ether fraction contained the least amount [total phenol (36.68±0.43 mg/kg) and total flavonoid (11.55±1.06 mg/kg)].

3.3. Aldose reductase inhibitory activity

Different fractions were found to inhibit rat lens aldose reductase activity to various extents with IC_{50} values ranging from 2 μg/mL to >100 μg/mL (Table 1 and Figure 2). The aldose reductase activity in normal rat lens was found to be (0.014 4±0.000 7 μg/mL). Among the fractions, ethyl acetate fraction showed higher percentage of inhibition [IC_{50} (49.26 ±1.76 μg/mL)] followed by aqueous fraction [IC_{50} (70.83± 2.82 μg/mL)]. Chloroform fraction [IC_{50} (98.52±1.80 μg/mL)] also showed significant inhibition but was less as compared with the ethyl acetate fraction. Petrolium ether fraction [IC_{50} (118.89±0.71 μg/mL)] was found to have the least inhibition potential against aldose reductase enzyme. The aldose reductase inhibitory activities of different fractions were presented in Table 1 and Figure 2. Quercetin was used as a

Table 1
Phytochemical analysis and aldose reductase inhibitory activity of different fractions of *H. enneaspermus* (mean±SEM).

Extract / fractions	Yield (%)	Total phenol (mg/g)	Total flavonoid (mg/g)	Aldose reductase inhibitory activity IC_{50} (μ g/mL)
Petrolium ether	3.6	36.68±0.43	11.55±1.06	118.89±0.71
Chloroform	4.5	68.56±0.51	13.41±0.82	98.52±1.80
Ethyl acetate	2.8	212.15±0.79	39.11±2.27	49.26±1.76
Aqueous	5.1	140.62±0.57	26.07±1.49	70.83±2.82
Quercetin	NA	NA	NA	2.74±0.04

NA: not analyzed.

Table 2
Kinetic parameters of rat lens aldose reductase (mean±SEM).

Group	Aldose reductase activity	V_{max}	$K_m \times 10^{-3}$ mM	K_i
1	DL-glyceraldehyde	0.1746±0.0017	0.6089±0.0012	0.0000±0.0000
2	DL-glyceraldehyde + petrolium ether	0.3840 ± 0.0004^{a}	4.5916 ± 0.0035^{a}	1.8670 ± 0.0060^{a}
3	DL-glyceraldehyde + chloroform	0.3315 ± 0.0004^{ab}	3.9620 ± 0.0210^{b}	1.3349 ± 0.0043^{ab}
4	DL-glyceraldehyde + ethyl acetate	0.1544 ± 0.00029^{abc}	0.5849 ± 0.0017^{abc}	0.0985 ± 0.0003^{abc}
5	DL-glyceraldehyde + aqueous	0.1978 ± 0.0004^{abcd}	1.2600 ± 0.0021^{abcd}	0.3052 ± 0.0010^{abcd}

a: comparing with the DL-glyceraldehyde; b: comparing with the DL-glyceraldehyde + petrolium ether; c: comparing with DL-glyceraldehyde + chloroform; d: comparing with the DL-glyceraldehyde + ethyl acetate fraction.

standard in the experiments.

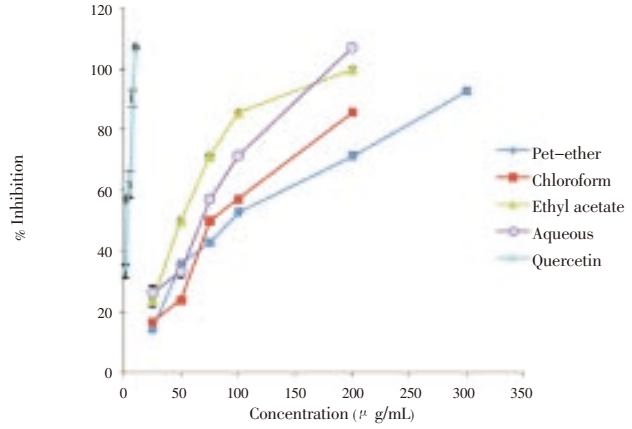

Figure 2. Effect of different fractions of *H. enneaspermus* and quercetin on aldose reductase activity.

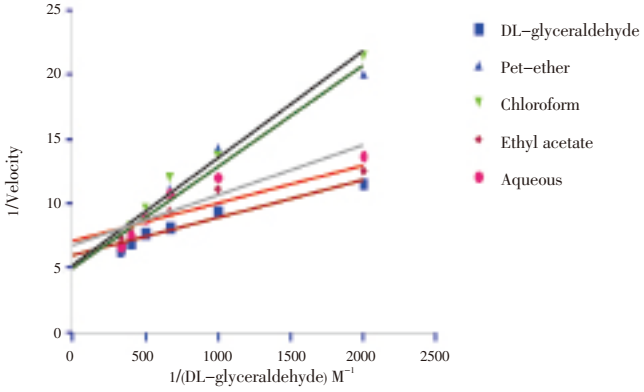

Figure 3. Effect of different fractions of *H. enneaspermus* on the Lineweaver-Burk plot of aldose reductase activity with DL-glyceraldehyde as substrate.

Kinetic study was performed for the entire fraction to understand exactly the type of inhibition, and based upon the data it is clear that ethyl acetate fraction showed non compititive inhibition, because the value of V_{max} (0.1544± 0.00029] and k_m (0.5849±0.0017×10^{-3} mM) did not differ as compared with the substrate DL-glyceraldehyde (0.1746± 0.0017, 0.6089±0.0012×10^{-3} mM). In all other fractions *viz.* petrolium ether, aqueous and chloroform fraction the value of the V_{max} and K_m differs significantly as compared with DL-glyceraldehyde which reveled that these fraction showed competitive inhibition. The values of V_{max} and K_m for all fractions were given in Table 2 and Figure 3.

4. Discussion

H. enneaspermus is used to treat various types of ailments in the traditional system of medicine including the diabetes. It contains good amount of phenol and flavonoid contents. It is used in the treatment of diabetes as well as an antioxidant, so on the basis of this data, the present study was carried out to determine the protective effects of different fractions of *H. enneaspermus* on aldose reductase enzyme system of rat lens to determine the potential anticataract effect of this plant[14,15].

The phytochemical value reported in this paper might be used as an analytical tool for the standardization of *H. enneaspermus*, if any adulterants or contaminants are there in the material it can be easily identified by the use of these parameters. This data can also be used for the comparative analysis of the sample which is collected in different place as well as time. The result reveals that ethyl acetate was the most effective solvent for extracting phenolics from *H. enneaspermus*. The total phenolic and flavonoid content of the ethyl acetate fraction was higher than those of the petroleum ether, chloroform and aqueous fraction. This could be explained by the possible formation of complexes by certain part of the phenolic compounds with other components, which are more extractable in ethyl acetate

than those of other fractions[18,19]. Different fractions showed significanct aldose reductase inhibitory activity however, it was maximum in the case of ethyl acetate fraction followed by aqueous fraction (quercetin used as standard). The aqueous fraction also showed comparatively significant inhibitory activity. In case of petroleum ether and chloroform fractions the activity was found to be less.

Aldose reductase reduces glucose to sorbitol and then metabolized to fructose by sorbitol dehydrogenase. Normally this accounts for less than 3% of glucose consumption. However, in the presence of high glucose, the activity of this pathway is substantially increased and could represent up to 30% of total glucose consumption. So aldose reductase inhibition in the early onset of the secondary complication in the diabetic mellitus will be beneficial, as ethyl acetate fraction showed good inhibitory activity against aldose reductase so it can be potentially used to treat diabetic complication in the early stage. The inhibitory effects of plant phytochemicals, including polyphenols which are currently regarded as natural antioxidants, and their antioxidant activities are important for human health, against carbohydrate hydrolyzing enzymes which contribute to the lowering of blood glucose in diabetics and are capable of reducing oxidative stress by scavenging reactive oxygen species and preventing cell damage[20,21]. In addition, some flavonoids and polyphenols as well as sugar derivatives are found to be effective in inhibiting α-glucosidase and aldose reductase enzyme[22]. Previous study shows this plant is safe and has no toxic effect in animal at a dose level of 5 g/kg b.w., and has good amount of phenol content as well[14].

Kinetic studies suggest that these compounds can interact and inhibit enzyme in both compititive and uncompititve manner, appearing to interact with the enzyme at a site independent of either substrate or enzyme. Based upon the data obtained, it was found that ethyl acetate fraction had significant activity against aldose reductase enzyme, this may be due to the presence of the phenol and flavonoid contents. Moreover, from the pioneering studies on sorbinil and alrestatin to recent investigation on zopolrestat and zenarestat, several compounds in clinical trials or in the market for the treatment of the diabetic complications have been developed but were subsequently withdrawn, suggesting that no universally potent inhibitor currently exists[3].

Kinetic study was performed for all the fraction to understand the type of inhibition. The ethyl acetate fraction shows non comptitive inhibition whereas petrolium ether and chloroform and aqueous fraction shows competitive inhibition. Effect of different fractions on rat lens aldose reductase activity in Lineweaver-Burk plot using DL-glyceraldehyde as a substrate was established. The Lineweaver-Burk plot was made in between 1/velocity *vs* 1/DL-glyceraldehyde. From the value of V_{max} and K_i it was concluded that ethyl acetate fraction shows the maximum inhibitory potential whereas petrolium ether fraction shows the least inhibition in the entire tested fraction. Moreover, aqueous and chloroform fraction also shows significant inhibition but less as compared with the other fractions. From k_m value it was concluded that affinity towards substrate for enzyme in case of ethyl acetate fraction was maximum and for petrolium ether fraction it was found to be less.

There are reports of aldose reductase inhibiting activity of few natural products such as root of *Salacia oblonga* and *Salviae multiorrhizae*, *Glycerrhiza uralensis*, *Radix astragali* and puerarin. These plants are rich in bioflavonoids, which are reported to reduce the aldose reductase activity. It further strengthens our study because *H. enneaspermus* also contains flavonoid and other phytochemicals[2]. Aldose reductase inhibitors including quercetin are currently the most commonly used oral agents for their good penetrations through cellular membranes and fast metabolism of sorbitol by sorbitol dehydrogenase considered for treatment of diabetic complications. Further it was concluded that flavonol and flavanone having the 7-hydroxy and/or catechol moiety at the B ring exhibit the strong activity in inhibition of aldose reductase. *In vitro* inhibitory activity of quercitirin (a glycoside of quercetin) was reported and also compared with cinnamaldehyde from *Cinnamomum* that showed higher activity[8]. Similarly, flavanone and flavonol glucosides isolated from a plant popularly known as 'plant insulin'(*Myrcia multiflora*) have been reported to possess aldose reductase inhibitory activity[23].

Recent studies show that majority of the plasma antioxidants are depleted in type 2 diabetes patients[24]. It has been suggested from the ESR studies that patients with diabetes mellitus are susceptible to higher levels of oxidative stress[25]. This oxidative stress causes imbalance between the oxidant and antioxidant defense mechanisms resulting in lipid peroxidation, DNA damage, and enzyme inactivation, including free radical scavenger enzymes contributing to tissue damage[26-28]. Because *H. enneaspermus* extract had significant antioxidant activity so it may be used to treat the imbalance of antioxidant and cure different diabetic complications[29].

In conclusion, the present study was carried out to determine the inhibitory potential of different fractions of *H. enneaspermus* on aldose reductase enzyme. The relationship between phenolic, flavonoid and aldose reductase inhibition was also investigated. The ethanolic extract has been chosen for fractionation due to its expected flavonoid contents that were reported to have antidiabetic activity. To evaluate the aldose reductase inhibitory activity, activity guided fractionation of *H. enneaspermus* was carried out on rat lens aldose reductase enzyme. The ethyl acetate fraction of the ethanol extract was found to exihibit maximum rat lens aldose reductase inhibitory activity as compared to the other fractions.

Conflict of interest statement

We declare that we have no conflict of interest.

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