Original Research Article

Vernonia anthelmintica (L.) Willd. Prevents Sorbitol Accumulation through Aldose Reductase Inhibition

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Abstract: Inhibition of Aldose reductase (AR) of polyol pathway delays the development of secondary diabetic complications in diabetes patients. This study analyses the potential of *Vernonia anthelmintica* (L.) Willd., an anti-diabetic plant used in traditional medicine in inhibiting Aldose reductase. Aldose reductase inhibition(ARI) assay, IC_{50}, kinetic analysis, specificity and cytotoxicity studies were performed with the methanolic extract of *V. anthelmintica* seeds. The sub-fractions obtained on column chromatography and HPTLC were studied for their ARI potential. The ethyl acetate fraction of *V. anthelmintica* exhibited promising AR inhibition against both goat lens AR and recombinant human AR. The inhibition was of uncompetitive type implying its advantage in hyperglucose conditions. The extract did not considerably influence goat liver aldehyde reductase and showed no toxicity to normal cells at minimum inhibitory doses. The results project the possibility of developing new lead ARI molecules from *V. anthelmintica*.

Keywords: Aldose reductase inhibitor(ARI), *Vernonia anthelmintica*, Enzyme kinetics, Secondary diabetic complications, Sorbitol

INTRODUCTION

Enzyme inhibitors are inevitable in the treatment regimen of the metabolic imbalances resulting from unregulated enzyme action. Upregulation of Aldose reductase (AR) enzyme is correlated with several pathological processes such as cardiac disorders, inflammation, mood disorders, renal insufficiency and ovarian abnormalities, but prime attention was paid to its role in the development of secondary diabetic complications [1]. Eventhough diabetic complications are proposed to be caused by multiple aetiologies like hexosamine pathway activation, nonenzymatic glycation, mitochondrial respiratory chain disruption and protein kinase C activation, hyperglycemia induced AR activation is held as the foremost cause for secondary diabetic complications [2]. AR catalyzes NADPH–dependent reduction of glucose to sorbitol, the first step of the polyol pathway; which is subsequently metabolized into fructose by sorbitol dehydrogenase (SDH). The inability of sorbitol to cross cell membrane and its slow degradation by SDH leads to sorbitol accumulation in tissues like retina, renal glomeruli and neurons resulting in hyperosmotic stress. This leads to the development of diabetic complications such as

cataract, retinopathy, nephropathy and neuropathy [3]. The overexpression of aldose reductase gene in diseased conditions and its role in generating oxidative stress and inflammatory responses points to the need for curbing this enzyme under such conditions.

Several synthetic ARIs and compounds with ARI potential from natural sources has been studied, but very few has been able to successfully make it through the clinical trials. Of the two major classes of ARI molecules, carboxylic acids possessed lower activity *in vivo* and lacked the ability to penetrate physiological membranes [4]. Spirohydantoins, on the other hand, showed higher activity *in vivo*, but evoked hypersensitivity reactions [5, 6]. Considering these facts, there is an urgent need to discover and develop new aldose reductase inhibitors (ARIs) which show good inhibitory potential with lesser side-effects. Ayurveda and traditional medicines presents a large repertoire of medicinal plants used for the effective control and treatment of diabetes and these plants can be explored for unidentified ARIs with more efficacies and less side-effects. AR inhibitory action showed by the tannoid principles of *Emblica officinalis* and

Lithospermic acid B from *Origanum vulgare L. ssp hirtum* are some promising examples [7, 8].

*Vernonia anthelmintica (L.) Willd (*bitter cumin), a shrub belonging to the family Asteraceae has been reported to be effective in management of type 2 diabetes. The plant inhibits α-glucosidase more specifically than α amylase and exerts its anti-hyperglycemic effect by suppressing maltose digestion and absorption. Compared to the antidiabetic drug glibenclamide, it was reported to be more beneficial with fewer side effects in effective treatment of diabetes [9, 10]. Methanolic extract of the plant also possessed insulinogenic effect in streptozotocin induced diabetic rat models making it more suitable for managing diabetes [11]. Screening of thirteen plants with reported antihyperglycemic activity in our laboratory projected ARI potential of *Vernonia anthelmintica* (L.) Willd. Therefore, the present study was conducted to analyse the feasibility of the plant as a source of lead ARIs. Other reported bioactivities of *V. anthelmintica* include antihelminthic [12], larvicidal, antihyperglycemic [13], antipyretic, antifilarial, anti-inflammatory [14], antimicrobial and anticancer [15] activities.

V. anthelmintica is commonly known as wild cumin/purple fleebane and as kalijeeri in Hindi. Scientific synonyms of the plants are *Centratherum anthelminticum* (L.) Kuntze, *Baccharoides anthelmintica* (L.) Moench. and *Conyza anthelminticum*. Bioactivities of the plant are mainly reported in seeds that are known for its astringent properties. Identification of more than 120 bioactive compounds from *V. anthelmintica* suggests scientific evidence for its traditional uses [16]. In addition to ARI potential of the seed extract of *V. anthelmintica*, this paper reports its kinetic parameters, IC$_{50}$, cytotoxic analysis and specificity for AR.

MATERIALS AND METHODS
Materials
DL-glyceraldehyde, NADPH and quercetin were purchased from Himedia (India), MTT was purchased from Sigma(USA). Human recombinant Aldose reductase enzyme was purchased from BioVision (BioVision, USA). All other chemicals and solvents were of analytical grade and were obtained from Himedia (India) and Merck (India).

Selection of plant
For this study, the plant *Vernonia anthelmintica* was selected based on primary screening for aldose reductase inhibition. The plant was authentified as *Vernonia anthelmintica (L.) Willd*-RHK 6351 of family *Asteraceae* and voucher specimens were deposited at the Regional Herbarium of Kerala, St. Berchmans College, Chaganasserry, Kerala, India. This plant was also listed in www.plantlist.org (accessed on 3 November 2015).

Extraction and phytochemical analysis
The methanolic extract was prepared by Soxhlet extraction from 100 g of the cleaned and powdered seeds of *V. anthelmintica*. The extract was filtered, concentrated, dried and one g/ml extract in DMSO was prepared for the inhibition assay. The concentration of DMSO in assay mixture was not more than 1 %. The extract was analysed for the presence of alkaloids, flavonoids, tannins, phenols, saponins, steroids and cardiac glycosides.

Aldose reductase assay
Fresh goat eyeballs were obtained from the slaughter house immediately after slaughtering and stored at 0-4 °C until use. Aldose reductase enzyme was prepared from goat eye lenses and assayed according to the method of Hayman and Kinoshita with slight modifications [17]. Activity was assayed spectrophotometrically by measuring the decrease in the absorption of NADPH at 340 nm over a 4-minute period using DL-glyceraldehyde as the substrate. One ml of assay mixture contained 0.1 M phosphate buffer solution (pH 6.2), 10 mM DL-glyceraldehyde, 0.1 mM NADPH and the enzyme for aldose reductase activity assay. 10 µl of the extract at two different concentrations was also added to the assay mixture for aldose reductase inhibition assay. Quercetin, a known aldose reductase inhibitor was used as the positive control. 1% DMSO in phosphate buffer (pH 6.2) was taken as the negative control to rule out the influence of DMSO on AR inhibition [18, 19]. The protein estimation in the enzyme preparation was performed by Bradford method. Similarly, the inhibitory capacity of the extract was also tested using the pure enzyme AKR1B1 (Aldose reductase, human recombinant). One unit of enzyme activity corresponded to ΔA /min/ml, where ΔA represents the change in absorbance per minute due to utilization of NADPH.

IC$_{50}$ and Kinetic analysis
Half maximal inhibitory concentration (IC$_{50}$) of the extracts was determined by checking the activity of enzyme in the presence of different concentrations of the extracts with a fixed concentration of substrate (10 mM).

For studying the effect of fractions on the enzyme kinetics of aldose reductase, the values of K_m and V_{max} were determined using varied concentration of DL- Glyceraldehyde in the absence and presence of different concentrations of the extracts by Lineweaver-Burk double reciprocal plots.

Aldehyde reductase assay
Aldehyde reductase was isolated from goat liver [20]. Briefly, liver was homogenized in 100 mM phosphate buffer (pH 7.2) and centrifuged at 12,000 rpm for 30 min. The precipitate obtained between 55-65% saturation of ammonium sulphate was dissolved in 10 mM Tris-HCl buffer (pH 8.0) and dialyzed for two

days against the same buffer. The desalted solution was then run on a DEAE cellulose column equilibrated with 10 mM Tris HCl buffer and eluted out with 0-50 mM NaCl. Based on activity, fraction eluted at 30mM NaCl was used for enzyme assay and the protein concentration was estimated by Bradford method.

The activity of ALR1 was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm, using DL-glyceraldehyde as substrate. The assay mixture in 1ml contained 50 mM sodium phosphate buffer of (pH 7.2), 10 mM DL-glyceraldehyde and 0.1 mM NADPH along with the enzyme preparation. For inhibition studies, the extracts in DMSO were added to aldehyde reductase assay mixture and incubated for 5 min before the initiation of reaction by NADPH. A negative control was prepared using DMSO in phosphate buffer. The percentage of inhibition was calculated using the formula.

% Inhibition= {[Abs. (negative control) - Abs. (extract)]/ Abs. (negative control)} ×100

Cytotoxicity

Cytotoxicity of the extract was assessed in Chang liver cells by analyzing morphological changes and MTT assay. For studying morphological changes, 100μl cell suspension ($5x10^4$ cells/ well) was seeded in 96 well tissue culture plates and incubated at 37ºC in a humidified 5% CO_2 incubator. After 24 hours, the growth medium was removed and 100μl of different concentrations of extract in 5% DMEM were added and incubated at 37ºC with 5% CO_2. Cells were observed every 24 hours up to 72 hours for morphological changes.

For MTT assay, after 24 hours of incubation, $5x10^4$ cells/well were added with 0.15 mg MTT after removing the medium and incubated at 37ºC with 5% CO_2 for 4 hours. Formazan formed was then solubilized in DMSO and was measured at a wavelength of 570 nm [21].

Sorbitol estimation

RBC was separated by centrifuging ACD treated blood collected from healthy volunteers after an overnight fast. The cells were washed thrice using isotonic saline, suspended in Kreb's Ringer bicarbonate buffer (pH 7.4, pre-equilibrated with 5% CO_2) and incubated under either normal (5.5 mM) or high glucose (55 mM) condition in the presence or absence of the extract at 37°C in 5% CO_2. After 3 hrs, the cells were homogenized in nine volumes of 0.8 M perchloric acid, centrifuged at 5000 *g* at 4°C for 10 min and pH of the protein filtrate was adjusted to 3.5 with 0.5 M potassium carbonate. The sorbitol content of the protein filtrate was measured by SDH enzyme assay. The reaction mixture consisted of 1.0 ml of 0.05 M glycine buffer (pH 9.4) containing 0.2 mM nicotinamide adenine dinucleotide (NAD) and 0.64 U of sorbitol

dehydrogenase (Sigma Chemical Co., lot 94C-0237, from sheep liver) and 0.5 ml protein-free filtrate. Assay mixture without NAD, sorbitol dehydrogenase or filtrate was run simultaneously as blanks. The relative fluorescence due to NADH production was measured with an excitation wavelength of 366 nm and an emission wavelength of 452 nm and concentration was calculated from sorbitol standards (0.06 to 50.0 nmol/ml) [22].

Chromatography

The methanol extract (1 gm/ml) of the dry seeds of *V. anthelmintica* was fractionated by silica gel column chromatography using different solvents of varying polarity viz., hexane, chloroform, ethyl acetate, butanol and acetonitrile. In between the use of each solvent, 1:1 mixture of the corresponding solvents were also used for fractionation. The fractions obtained were analysed for their aldose reductase inhibitory potential and the potent fraction was analysed by HPTLC, using the solvent system chloroform: methanol (8:2).

Statistical Analysis

All experiments were done at least in triplicates and the data were expressed as mean ± standard deviation. Graphs were plotted using Graphpad Prism version 6.

RESULTS AND DISCUSSION

Information suggesting association between ARI inhibition and delayed development of secondary diabetic complications as well as the failure of synthetic/natural compounds in view of toxicity and hypersensitivity lead to more researches on the identification and validation of natural compounds for lead ARI molecules. Considering the immense potential of plant kingdom in providing lead drug molecules, independent groups studied several plants for the presence of ARI molecules.

V. anthelmintica had been studied for its anthelminthic [12], anticancer [15], anti-inflammatory [14] activity and many more. Report on the antihyperglycemic activity of the plant [13] prompted us to study its ARI activity, as it would be an added advantage to the diabetes patients. Use of *V. anthelmintica* seed extract in treating diabetes by increasing serum insulin, C protein, total protein and albumin levels as well as normalizing the elevated blood sugar, glycated hemoglobin, lipids and key enzymes in diabetes has been validated. The methanolic extract of *V. anthelmintica* inhibited the goat lens AR with a percentage inhibition of 69.2 ± 2.90 %. The extracted goat lens aldose reductase showed a specific activity of 0.056 ± 0.003 U/min/mg and normal activity of 0.019 ± 0.001 U/min/ml which was taken as 100 % activity. The IC_{50} value of methanolic extract against goat lens AR was 9.3 ± 1.042 µg/ml and that of the known AR inhibitor Quercetin was 3.9 ± 0.085 µg/ml (figure 1).
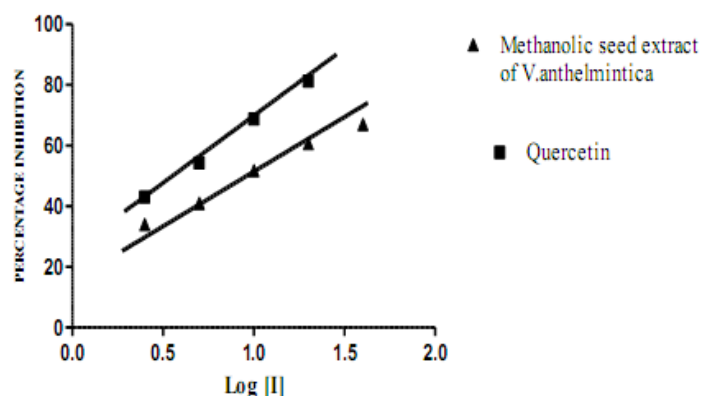
Fig-1: Dose dependant effect of methanolic seed extract of *V.anthelmintica* and Quercetin on goat eye lens aldose reductase activity. The experiment was done in triplicate and values expressed as mean ± SD

Several medicinal plants have been investigated for their ARI potential. Kumar *et al*. [23] reported ARI potential of *Houttuynia cordata* Thunb with IC$_{50}$ values of 64.62 ± 3.90, 90.69 ± 7.50, 134.59 ± 4.90 and 151.58 ± 3.30 µg/ml for its aqueous, ethyl acetate, chloroform and hexane fractions respectively. ARI potential with an IC$_{50}$ of 49.46± 2.26 µg/ml was reported for *Hybanthus enneaspermus* (Linn) F. Muell. [24]. Ethanolic extract of *Pedalium murex* fruit gave ARI potential with an IC$_{50}$ of 57.20 ± 3.68 µg/ml [19]. Another potential plant - derived ARI candidate reported is the tannoid principles of *Emblica officinalis* with an IC$_{50}$ of 6 µg/ml against rat lens AR (7). The IC$_{50}$ value (9.3 ± 1.042 µg /ml) of the methonolic *V. antihelmintica* extract was comparatively less than most of the already reported plant extracts and can be considered as a promising candidate for ARI molecule development. For the best of our knowledge, this is the first report on the ability of *V. anthelmintica* in inhibiting aldose reductase.

Methanolic *V. anthelmintica* extract caused a decrease in V$_{max}$ and K$_m$ (0.0081 ± 0.0001 mM/min and 0.0528 ± 0.0057 mM respectively) of goat aldose reductase when compared with that in the absence of the extract (0.0184 ± 0.0002 mM/min and 0.0953 ± 0.0100 mM). Thus, there was a 56 % decrease in V$_{max}$

and 45 % decrease in K$_m$. When the extract concentration was doubled, there was 72.82 % decrease in V$_{max}$ (0.0050 ± 0.0001 mM/min) and 80 % decrease in Km (0.0194 ± 0.0093 mM). The decrease in V$_{max}$ and K$_m$ was also evident in the Lineweaver-Burk plot suggesting an uncompetitive type (Figure 2) of inhibition by the extract. Thus, the inhibitor present in the extract is binding neither to the enzyme nor to the substrate DL-glyceraldehyde; rather it is binding to the enzyme - substrate complex thereby rendering it inactive. Though rare, generally uncompetitive inhibition was shown by several inhibitors of aldose reductase. Among reports on plant-derived aldose reductase inhibitors, isoquercitrin present in English tea exhibited a mix of uncompetitive and noncompetitive inhibitions [25]. Different fractions of *Houttuynia cordata viz*. aqueous and ethyl acetate fractions yielded non-competitive and uncompetitive type of enzyme inhibition mechanisms whereas its chloroform and hexane fractions exhibited a competitive type of inhibition [23]. Uncompetitive inhibition shown by *V. anthelmintica* extract is advantageous as the inhibition is not reversed or diluted at high glucose concentration. Furthermore, Aldose reductase inhibition with uncompetitive inhibitors like zopolrestat prevented glucose accumulation and corrected the abnormal glycolytic pathway in diabetes individuals [26].
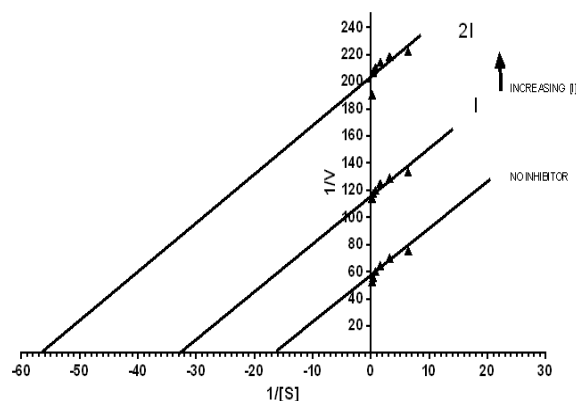
Fig-2: Line-weaver Burk plot of methanolic extract of *V. anthelmintica* extract. The parallel slopes indicate an uncompetitive type of inhibition for partially purified goat lens aldose reductase enzyme. Each value represents mean ± standard deviation of three independent measurements

The pharmacological use of any extract can be made permissible only after assessing its side effects and toxicity. Aldose reductase inhibitor molecules have been shown to inhibit aldehyde reductase, another member of the aldo– keto reductase family that is involved in the detoxification of reactive aldehydes. Here, same concentration (10 µg/ml) of extract caused 39.6 ± 0.85 % inhibition of aldehyde reductase, compared to 69.2 ± 2.90 % of inhibition against aldose reductase, indicating its higher affinity for AR enzyme. The extract did not show considerable cytotoxicity against Chang liver cells at a concentration of 6.25 µg/ml which is near to IC_{50} of extract against goat lens aldose reductase. Also even at doses of about fifteen folds of IC_{50}, there was more than 50 % normal cell viability, ruling out chances of cell toxicity at possible drug doses. *In vitro* sorbitol accumulation study in erythrocytes also suggested reduced sorbitol accumulation in the presence of the extract, which was particularly evident in hyperglucose treated cells. At the same time, normal glucose treated erythrocytes maintained the normal sorbitol level when treated with extract implying safety of the extract (Table 1).

Table 1: Red blood cell sorbitol expressed as µg/ml RBCs were measured after incubating RBCs under normal (5 mM) and hyperglucose (50mM) condition in the presence and absence of extract (5 µg/ml) and quercetin (5µg/m)l. The experiments were done in triplicate and values expressed as mean± SD

| Sample	Red blood cell sorbitol (µg/ml RBC)
Glucose (5 mM)	1.1 ± 0.09
Glucose (50 mM)	7.1 ± 0.12
Glucose (50 mM) + extract (5 µg/ml)	1.3 ± 0.09
Glucose (50 mM) + Quercetin (5 µg/ml)	1.4 ± 0.08

The phytochemical analysis has revealed the presence of alkaloids, flavonoids, tannins, phenols, saponins, steroids and cardiac glycosides. The methanolic extract of *V. anthelmintica* was fractionated in an attempt to purify and concentrate the active components and reducing the effective concentration needed for its action. Fractionation of methanolic extract was by column chromatography using different eluting solvents and the yields of each fraction were as follows: hexane (0.6 %), hexane: chloroform (2 %), chloroform (2.6 %), chloroform: ethyl acetate (10 %), ethyl acetate (37.3 %), ethyl acetate: butanol (12.6 %), butanol (11.3 %), butanol: acetonitrile (6 %) and acetonitrile (10.6%). Hexane, hexane: chloroform, chloroform and chloroform: ethyl acetate fractions exhibited only very less AR inhibitory potential compared to ethyl acetate, ethyl acetate: butanol, butanol, butanol: acetonitrile and acetonitrile fractions. Ethyl acetate fraction showed the highest activity with an IC_{50} of 6.5 ± 1.09 µg/ml.
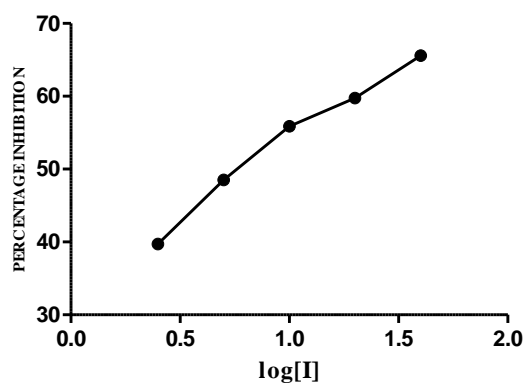
Fig-3: Dose dependant effect of ethyl acetate fraction of *V.anthelmintica* on goat eye lens aldose reductase activity. The log values of varied concentrations of the inhibitor were plotted against percentage enzyme inhibition. Each value represents mean ± standard deviation of three independent measurements
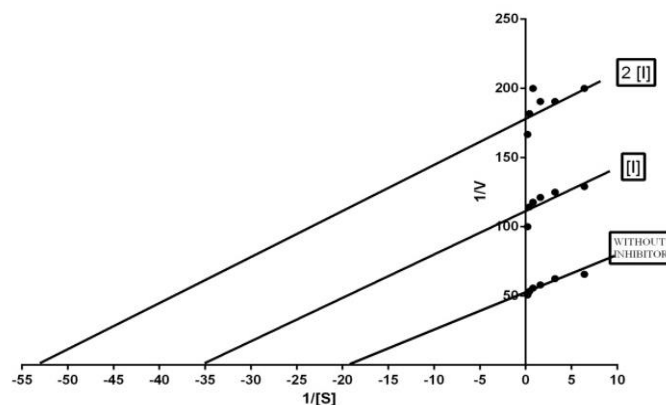
Fig-4: Line-weaver Burk plot ethyl acetate fraction of *V. anthelmintica* extract. The parallel slopes indicate an uncompetitive type of inhibition for partially purified goat lens aldose reductase enzyme. Each value represents mean ± standard deviation of three independent measurements

Kinetic study showed a 52 % decrease in V_{max} (0.0091 ± 0.0004 mM/min) and 25.5 % decrease in K_m (0.035 ± 0.0017 mM) in the presence of the ethyl acetate fraction of *V.anthelmintica*; compared to the V_{max} and K_m in the absence of the extract (0.0191 ± 0.0004 mM/min and 0.047 ± 0.0010 mM respectively) (Figure 3). When the inhibitor concentration was doubled, there was 71 % decrease in V_{max} (0.0055 ± 0.0002 mM/min) and 58.72 % decrease in K_m (0.0194 ± 0.0014 mM) (Table 2). Ethyl acetate fraction also

showed an uncompetitive type of inhibition (Figure 4). The ARI potential of the ethyl acetate fraction was also substantiated with recombinant human aldose reductase enzyme. Assay using AKR1B1 (Aldose reductase, human recombinant) with methanolic seed extract of *V.anthelmintica* gave an ARI inhibition of 71.43 % and the ethyl acetate fraction exhibited an ARI inhibition of 85.72 %. The ethyl acetate fraction gave an IC 50 value of 4.5 ± 0.02 µg/ml with the recombinant enzyme.

Table: 2 Percentage AR inhibition of goat lens aldose reductase by ARI assay by different fractions of methanolic seed extract of *V. anthelmintica* and the IC_{50} values based on dose dependent assay, kinetic parameters such as V_{max} and K_m of Aldose reductase enzyme at different concentrations of ARI potent fractions of *V.anthelmintica* under varied substrate concentrations and their mechanism of enzyme inhibition from LB plot. The experiments were done in triplicate and values expressed as mean± SD
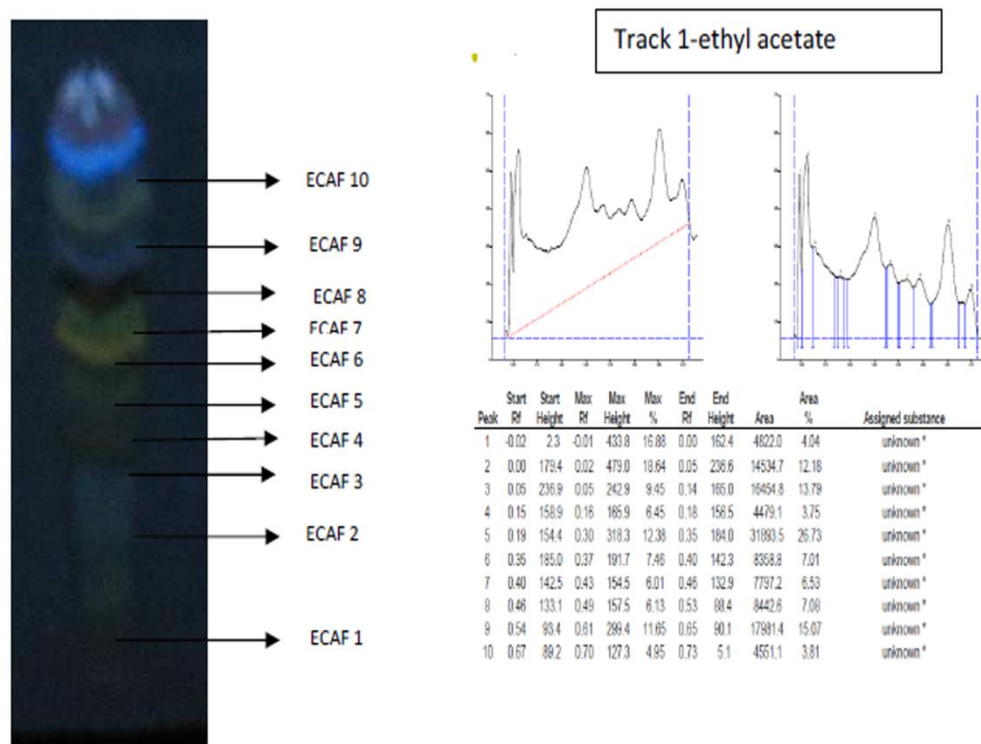
Fraction	Percentage of enzyme inhibition	IC 50	Kinetic parameters						
			Vmax (mM/min) (without inhibitor)	Km (mM) (without inhibitor)	Vmax (mM/min) (at [I])	Km (mM) (at [I])	Vmax (mM/min) (at [2I])	Km (mM) (at [2I])	Type of inhibition
Hexane	14.83 ± 0.87	–	–	–	–	–	–	–	–
Hexane chloroform (1:1)	18.03 ± 0.72	–	–	–	–	–	–	–	–
Chloroform	24.47 ± 0.85	–	–	–	–	–	–	–	–
Chloroform –ethyl acetate (1:1)	29.60 ± 1.05	–	–	–	–	–	–	–	–
Ethyl acetate	65.60 ± 1.25	6.5 ± 1.09	0.019 ± 0.0004	0.047 ± 0.001	0.009 ± 0.0004	0.035 ± 0.002	0.006 ± 0.0002	0.019 ± 0.001	Uncompetitive
Ethyl acetate – butanol	59.03 ± 1.10	11.3 ± 0.02	0.019 ± 0.0004	0.072 ± 0.009	0.009 ± 0.0002	0.067 ± 0.013	0.005 ± 0.0001	0.075 ± 0.015	Non-competitive
Butanol	55.30 ± 1.22	13.1± 0.02	0.019 ± 0.0004	0.071 ± 0.009	0.007 ± 0.0002	0.058 ± 0.013	0.005 ± 0.0001	0.057 ± 0.011	Non-competitive
Butanol-acetonitrile	52.73 ± 0.78	15.7± 0.02	0.019 ± 0.0004	0.081 ± 0.012	0.008 ± 0.0002	0.066 ± 0.010	0.007 ± 0.0001	0.081 ± 0.010	Non-competitive
Acetonitrile	53.03 ± 0.70	15.4± 0.03	0.019 ± 0.0004	0.081 ± 0.012	0.008 ± 0.0002	0.089 ± 0.014	0.006 ± 0.0001	0.094 ± 0.010	Non-competitive

HPTLC analysis of the ethyl acetate fraction yielded ten bands, of which, two bands showed

considerable aldose reductase inhibition (Figure 5). Among these two bands, ARI activity of one band (EAF

10; Rf 0.67; 55.82 ± 0.50% inhibition) decreased with time, which may be due to the volatile nature of the compound present and is under analysis in the laboratory. The second band EAF 8 with an Rf of 0.46 exhibited an ARI potential of 56.18 ± 0.78 %. The

compounds present in these bands can be regarded primarily responsible for aldose reductase inhibition of the plant. Further purification and identification of the respective compounds may reveal new compounds with potential aldose reductase inhibitory activity.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.02	2.3	-0.01	433.8	16.88	0.00	162.4	4822.0	4.04	unknown *
2	0.00	179.4	0.02	479.0	18.64	0.05	236.6	14534.7	12.18	unknown *
3	0.05	236.9	0.05	242.9	9.45	0.14	165.0	16454.8	13.79	unknown *
4	0.15	158.9	0.16	165.9	6.45	0.18	158.5	4479.1	3.75	unknown *
5	0.19	154.4	0.30	318.3	12.38	0.35	184.0	31893.5	26.73	unknown *
6	0.35	185.0	0.37	191.7	7.46	0.40	142.3	8358.8	7.01	unknown *
7	0.40	142.5	0.43	154.5	6.01	0.46	132.9	7797.2	6.53	unknown *
8	0.46	133.1	0.49	157.5	6.13	0.53	88.4	8442.6	7.08	unknown *
9	0.54	93.4	0.61	299.4	11.65	0.65	90.1	17981.4	15.07	unknown *
10	0.67	89.2	0.70	127.3	4.95	0.73	5.1	4551.1	3.81	unknown *

Fig-5: HPTLC chromatogram and spectral data of the ethyl acetate extract of *Vernonia anthelmintica* using the solvent system (Chloroform: methanol 8:2)

Plant extracts contain several bioactive compounds and various flavonoids, polyphenols and sugar derivatives that are reported to have aldose reductase inhibitory activity [24]. Aqueous extracts of *Emblica officinalis* and its major constituent tannoids exhibited inhibition against both rat lens and human AR with IC_{50} of 6µg/ml and 10µg/ml respectively. The ether and methanolic extracts of *Gentiana lutea* roots dose dependently inhibited sorbitol accumulation in human erythrocytes under high glucose conditions [27]. Curcumin analogs with dihydroxyl groups also showed considerable ARI activity [28].

V. anthelmintica seeds are rich in secondary metabolites and mainly contain glycosides, phenolic compounds, tannins, flavonoids, saponins and sterols apart from primary metabolites. The higher inhibitory potential of ethyl acetate fraction can be attributed to the presence of these phytochemicals. Flavonoids such as 2,3,4,4-tetrahydroxychalcone (Butein); 5,6,7,4-tetrahydroxy flavone and 7,3',4'-trihydroxydihydroflavone; sterols such as sterol -4-alpha-methylvernosterol, vernosterol, avernosterol and steroids such as (24a/R)-stigmasta- 7-en-3-one, 24(a/R)-stigmasta-7, 9(11)-dien-3-one, 24(a/S)-stigmasta-5 and 22-dien-3ß-ol, stigmasta-7 and 22-dien-3ß-ol are some of the secondary metabolites reported in *V. anthelmintica* seeds [15].

The deranged metabolism of the body affects the normal physiology in more than one way as seen in diabetes. Secondary metabolites are shown to possess many significant bioactivities with pharmacological effects. The result from this study showing ARI potential of *V. anthelmintica*, along with its already reported antidiabetic properties can envisage it as evolving treatment strategy in managing diabetic complications. In addition, antioxidative and anti inflammatory properties of *V. anthelmintica* can be an added advantage in combating the oxidative stress and accelerated inflammation arising due to diabetes thus making it a powerful drug aspirant. Based on the results of this study, the ethyl acetate fraction of *V. anthelmintica* becomes a potent candidate for further analyses and isolation of active molecule with less side effects and more potency. Analyses of the structure and docking potential of the metabolites are underway in the lab and this will throw more light into the mechanism and efficacy of its action.

CONCLUSION

This study reports a very promising potential of *V. anthelmintica* to inhibit aldose reductase enzyme involved in the development of secondary diabetic complications. The investigation has unveiled the partially purified ethyl acetate fraction exhibiting a promising IC_{50} value of 4.5 ± 0.02 µg/ml with the recombinant AR enzyme. Kinetic studies showed that these compounds can interact with and inhibit enzyme in an uncompetitive manner. Also the fact that it has proved to have negligible side effects and cytotoxicity also makes it a potent candidate for drug development. Isolation and characterisation of the active component of this extract may be helpful in developing a lead ARI molecule which will be beneficial in alleviating the secondary diabetic complications.

STATEMENT OF AUTHORSHIP

This work is conceptualized and designed by Hazeena VN and Anie Y. The experimental procedures were performed by Hazeena VN, Sruthi CR and Soumya CK. Interpretation and analysis of data, drafting of the article and proof reading were done by Hazeena V N, Haritha V H, Jayachandran K and Anie Y. All authors have read and approved the final manuscript.

ABBREVIATIONS

AR – Aldose Reductase, ARI- Aldose Reductase Inhibitor, *V. anthelmintica – Vernonia anthelmintica (L.) willd.,* IC_{50} – Half maximal inhibitory concentration, NADPH- Nicotinamide adenine dinucleotide phosphate (reduced).

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