

Original Article

Dietary sources of aldose reductase inhibitors: prospects for alleviating diabetic complications

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Activation of polyol pathway due to increased aldose reductase activity is one of the several mechanisms that have been implicated in the development of various secondary complications of diabetes. Though numerous synthetic aldose reductase inhibitors have been tested, these have not been very successful clinically. Therefore, a number of common plant/ natural products used in Indian culinary have been evaluated for their aldose reductase inhibitory potential in the present study. The aqueous extracts of 22 plant-derived materials were prepared and evaluated for the inhibitory property against rat lens and human recombinant aldose reductase. Specificity of these extracts towards aldose reductase was established by testing their ability to inhibit a closely related enzyme *viz.*, aldehyde reductase. The *ex vivo* incubation of erythrocytes in high glucose containing medium was used to underscore the significance in terms of prevention of intracellular sorbitol accumulation. Among the 22 dietary sources tested, 10 showed considerable inhibitory potential against both rat lens and human recombinant aldose reductase. Prominent inhibitory property was found in spinach, cumin, fennel, lemon, basil and black pepper with an approximate IC₅₀ of 0.2 mg/mL with an excellent selectivity towards aldose reductase. As against this, 10 to 20 times higher concentrations were required for 50% inhibition of aldehyde reductase. Reduction in the accumulation of intracellular sorbitol by the dietary extracts further substantiated their *in vivo* efficacy. The findings reported here indicate the scope of adapting life-style modifications in the form of inclusion of certain common sources in the diet for the management of diabetic complications.

Key Words: aldose reductase, aldehyde reductase, sorbitol, dietary inhibitors, red blood cells

INTRODUCTION

Long-term secondary complications are main cause of morbidity and mortality in diabetic patients.¹ The major microvascular complications of diabetes include nephropathy, neuropathy, retinopathy while cataract is, however, an avascular complication.² Several metabolic factors contribute to the dysfunction observed in diabetic vasculopathy,³ which include increased glucose flux through the polyol pathway, increased production of reactive oxygen species by the mitochondrial respiratory chain, nonenzymatic glycations, protein kinase-C activation and increased flux through the hexosamine pathway.¹ The polyol pathway has received considerable attention.

Aldose reductase (ALR2; EC: 1.1.1.21) belongs to aldo-keto reductases (AKR) super family. It is the first and rate-limiting enzyme in the polyol pathway and reduces glucose to sorbitol utilizing NADPH as a cofactor. Sorbitol is then metabolized to fructose by sorbitol dehydrogenase.⁴ Normally, the polyol pathway represents a minor route of glucose utilization, accounting for <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.⁵ Abnormal activation of the polyol pathway during diabetes leads to accumulation of osmotically active sorbitol leading to osmotic as well as oxidative stress,

culminating in tissue injury.⁵ Evidence for the involvement of ALR2 in diabetic neuropathy, retinopathy, nephropathy and cataract emerged from several independent studies.⁴⁻⁸ Mice, which are devoid of lens ALR2, do not develop sugar cataract in hyperglycemic conditions.⁸ In addition, transgenic mice, which over express ALR2 develop cataract in hyperglycemic conditions.⁹ Genetic deletion of ALR2 in mice, prevents all early effects of diabetes on neural, glial and vascular cells of retina¹⁰ reinforced the credentials of the polyol pathway as a target for devising the strategies to prevent diabetic complications. Thus inhibiting ALR2 activity appears to be an effective means to prevent the diabetic complications.

Experimental animal models suggest that the compounds that inhibit ALR2 could be effective in the prevention of certain complications.¹¹ Although, a wide variety of compounds have been synthesized to inhibit ALR2

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and studied in experimental models, only limited number of drugs have reached clinical trial.¹² Largely two chemical classes of ALR2 inhibitors (ARI) have been tested in phase III trials. While carboxylic acid inhibitors (zopolrestat, ponalrestat, tolerestat) have shown poor tissue penetration and are not very potent in vivo, spiroimide (spirohydantoin) inhibitors penetrate tissues more efficiently, but many have caused skin reactions and liver toxicity. This restricted the doses that could be used in humans to levels that were sub therapeutic in animal models.^{13,14,15} Relatively poor selectivity for ALR2 versus aldehyde reductase (ALR1; EC: 1.1.1.2),^{16,17} a closely related enzyme of AKR family that plays a role in the detoxification of reactive aldehyde, might be one of the reasons for not being successful in clinical trials.¹⁸

Thus, there is a need for developing and evaluating new ARI considering efficacy, selectivity and safety issues. In the present study we have evaluated the ARI potential of the aqueous extract of some commonly consumed traditional plant/diet sources using rat lens and recombinant human ALR2. The specificity of these sources towards ALR2 was evaluated using ALR1. Further, *ex vivo* incubation of RBC under high glucose conditions was utilized to investigate the effect on intracellular sorbitol accumulation. Based on these results we have identified some dietary agents with definite ARI potential that may allow a diet-based approach to combat diabetic complications.

MATERIALS AND METHODS

Materials

DL-glyceraldehyde, glucose, lithium sulfate, 2-mercaptoethanol, NADPH, quercetin, rutin, dimethylsulfoxide, sorbitol, sorbitol dehydrogenase, and NAD were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of analytical grade and were obtained from local companies.

Rat lens ALR2: Crude ALR2 was prepared from rat lens as described previously.¹⁹ Lenses were homogenized in 10 volumes of 100 mM potassium phosphate buffer pH 6.2. The homogenate was centrifuged at 15,000 g for 30 min at 4°C and the resulting supernatant was used as the source of ALR2.

Purification of recombinant human ALR2

Recombinant human ALR2 was expressed and purified from bacterial cultures essentially as described previously²⁰ with the exception that affinity chromatography over AffiGel Blue (Bio-Rad) was used in final purification step.

Purification of ALR1

Aldehyde reductase was partially purified from goat liver as described previously.²¹ Goat liver was obtained from local slaughterhouse within 30 min of killing. Liver was homogenized in 100 mM phosphate buffer pH 7.2. The homogenate was centrifuged at 15,000xg for 30 min and the supernatant was subjected to ammonium sulfate precipitation. Precipitate obtained between 35-65% saturation was dissolved in 10 mM Tris-Cl buffer pH 8.0, dialyzed for two days against the same buffer and the desalted solution was applied on to a DEAE-cellulose col-

umn equilibrated with 10 mM Tris-Cl buffer pH 8.0. Two distinct peaks were resolved between 0-500 mM NaCl. The major activity was eluted at 30 mM NaCl and fractions corresponding to this peak were pooled. The pooled fractions were dialyzed and used as ALR1 for inhibition studies.

Preparation of aqueous extract from dietary agents

Dietary material was obtained afresh from the local market. The eatable parts of the materials were freeze-dried and powdered. Aqueous extract (5%) was prepared from these materials stirring at room temperature for 3 h. Insoluble material was removed by centrifugation followed by filtration. The water extract was freeze-dried and stored under desiccation at 4°C. The selection criteria of plants relied on their hypoglycemic activity²²⁻²⁵ and also the fact that they are commonly consumed in the diet or used as herbal remedies in various pathological conditions including diabetes. Only those parts of the plants, which are commonly consumed, were used in the study (Table 1).

Aldose reductase assay

ALR2 activity was assayed as described previously.¹⁹ The assay mixture in 1ml contained 50 mM potassium phosphate buffer pH 6.2, 0.4 M lithium sulfate, 5 mM 2-mercaptoethanol, 10 mM DL-glyceraldehyde, 0.1 mM NADPH and enzyme preparation (rat lens or recombinant enzyme). Appropriate blanks were employed for corrections. The assay mixture was incubated at 37°C and the reaction was initiated by the addition of NADPH at 37°C. The change in the absorbance at 340 nm due to NADPH oxidation was followed in a Lambda35 spectrophotometer (Perkin-Elmer, Shelton, U.S.A.).

Aldehyde reductase assay

The activity of ALR1 was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm as a function of time at 37°C using glyceraldehyde as substrate. The assay mixture in 1ml contained 50 mM sodium phosphate buffer of pH 7.2, 0.2 M lithium sulfate, 10 mM DL-glyceraldehyde, 5mM β -mercaptoethanol and 0.1 mM NADPH.

Inhibition studies

For inhibition studies concentrated stocks of aqueous extract of dietary agents were prepared in water. Various concentrations of these extracts were added to assay mixture (of ALR2 or ALR1) and incubated for 5 min before initiating the reaction by NADPH as described above. The percent of inhibition with test compounds was calculated considering the ALR1 or ALR2 activity in the absence of inhibitor was 100%. The concentration of each test sample giving 50% inhibition (IC_{50}) was determined by plotting log concentration of aqueous extract versus percentage inhibition.

In vitro incubation of RBC

Five mL blood was collected from healthy male volunteers on overnight fasting in heparinized tubes. Red blood cells were separated by centrifugation and washed three times with isotonic saline at 4°C. Washed RBC were sus-

Table 1. Inhibition of rat lens and human recombinant ALR2 (hrALR2) by aqueous extracts of dietary agents common names, scientific names and the part of the plants evaluated for the inhibition along with IC₅₀ values are given. Data for IC₅₀ values are the average of three independent experiments.

S. No.	Common name	Scientific name	Parts Tested	IC ₅₀ Value (mg/mL)	
				Rat ALR2	hrALR2
1	Spinach	<i>Spinaceae oleracea</i>	Leaves	0.10 ± 0.01	0.11 ± 0.005
2	Cumin	<i>Cuminum cyminum</i>	Seeds	0.17 ± 0.02	0.15 ± 0.01
3	Fennel	<i>Foeniculum vulgare</i>	Seeds	0.18 ± 0.02	0.19 ± 0.02
4	Basil	<i>Ocimum sanctum</i>	Leaves	0.20 ± 0.01	0.12 ± 0.01
5	Black pepper	<i>Piper nigrum</i>	Seeds	0.22 ± 0.02	0.22 ± 0.03
6	Fenugreek	<i>Trigonella foenumgraceum</i>	Seeds	0.24 ± 0.03	0.30 ± 0.03
7	Lemon	<i>Citrus lemon</i>	Fruit	0.25 ± 0.01	0.18 ± 0.01
8	Bitter gourd	<i>Momordica charantia</i>	Fruit	0.28 ± 0.02	0.25 ± 0.01
9	Orange	<i>Citrus sinensis aurantium</i>	Fruit	0.28 ± 0.04	0.25 ± 0.02
10	Curry leaves	<i>Murraya koenigii</i>	Leaves	0.31 ± 0.01	0.28 ± 0.03
11	Cinnamon	<i>Cinamomum zeylencium</i>	Bark	0.4 ± 0.02	0.19 ± 0.02
12	Ajwain	<i>Trachyspermum ammi</i>	Seeds	0.65 ± 0.03	N.T.
13	Gauva	<i>Psidium guajava</i>	Fruit	0.70 ± 0.03	N.T.
14	Aegle	<i>Marmelos bael</i>	Leaves	>1	N.T.
15	Mustard	<i>Brassica nigra</i>	Seeds	>1	N.T.
16	Apple	<i>Malus pumila</i>	Fruit	>1	N.T.
17	Wet ginger	<i>Zingiber officinalis</i>	Root	>1	N.T.
18	Dry ginger	<i>Zingiber officinalis</i>	Root	>1	N.T.
19	Onion	<i>Allium sepa</i>	Bulb	>1	N.T.
20	Garlic	<i>Allium sativum</i>	Bulb	>1	N.T.
21	Coriander	<i>Coriander sativum</i>	Leaves	>1	N.T.
22	Grapes	<i>Vitis vinifera</i>	Fruit	>1	N.T.

pended in Krebs's-ringer bicarbonate buffer, pH 7.4 (pre-equilibrated with 5% CO₂) and incubated at 37°C in presence of 5% CO₂ for 3 h under normal (5.5 mM) and high glucose (55 mM) conditions. The effect of aqueous dietary extracts on sorbitol accumulation was evaluated by incubating the RBC with different concentrations of extracts.

Estimation of sorbitol in RBC

At the end of incubation period, RBC was homogenized in 9 volumes of 0.8 M perchloric acid. The homogenate was centrifuged at 5,000x g at 4°C for 10 min and the pH of the supernatant was adjusted to 3.5 with 0.5 M potassium carbonate. The sorbitol content of the supernatant was measured by fluorometric method as described previously using a spectrofluorometer (Jasco-FP-6500).²⁶

RESULTS

We have evaluated 22 plant/spice materials that are commonly consumed in Indian culinary preparations for their potential to inhibit ALR2 (Table 1). In addition to the selection criteria described above, most of these traditional medicinal plant extracts are known to have chemotherapeutic, antioxidant and other beneficial effects, and it was postulated that they might inhibit the polyol pathway. Compared to other tissues, ALR2 is higher in the eye lens, particularly rat lens.²⁷ Hence WNIN rat lens was used as ALR2 source for initial screening of ARI activity of aqueous extracts of dietary agents. Aqueous extracts were found to inhibit rat lens ALR2 to various extents with IC₅₀ values ranging from 0.1 mg/ml to >1 mg/mL (Table 1 and Figure 1). Aqueous extracts of bitter gourd, black pepper, cinnamon, curry leaves, cumin, fennel, fenugreek, lemon, orange, spinach and basil revealed considerable

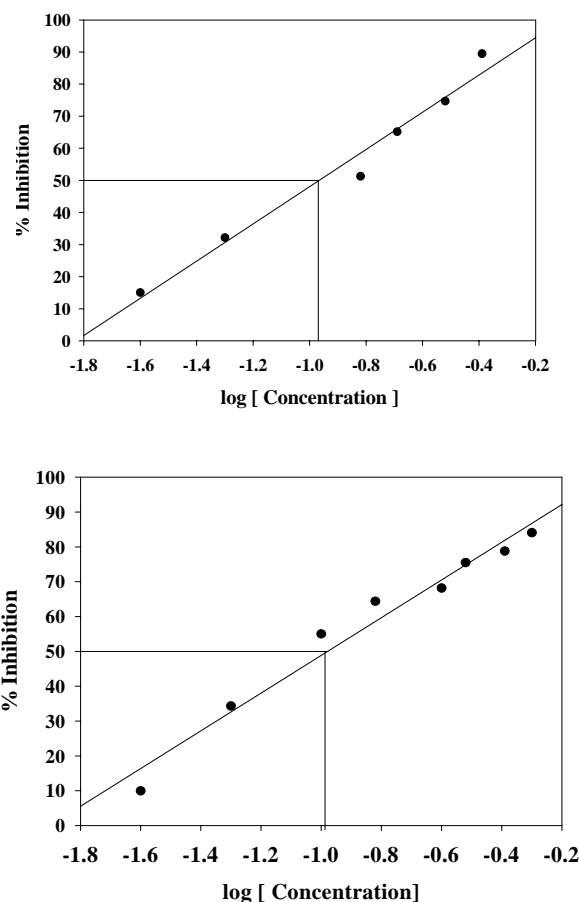


Figure 1. Representative inhibition curve of rat lens ALR2 (top panel) and human recombinant ALR2 (bottom panel) by the aqueous extract of cumin. IC₅₀ value was calculated by linear regression analysis of log inhibitor (extract) concentration versus percentage of inhibition. ALR2 activity in the absence of extract was considered as 100%.

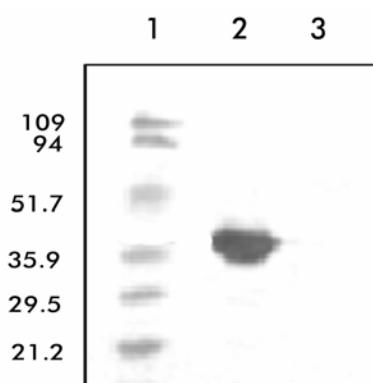


Figure 2: Immunodetection with anti-human recombinant ALR2 antibody. To rule out possible contamination of partially purified aldehyde reductase (ALR1) from goat liver with ALR2, immunodetection of goat liver ALR1 preparation was carried out using anti-recombinant ALR2 antibody. Lane 1: molecular weight markers, Lane 2: recombinant human ALR2 (positive control) and Lane 3: partially purified ALR1 from goat liver.

inhibitory activities with IC_{50} values <0.5 mg/mL (Table 1). Moderate (IC_{50} : ~ 0.7 mg/mL) or no significant inhibition (IC_{50} : >1.0 mg/mL) was found with aqueous extracts of rest of the materials (Table 1).

In the second stage, we have assessed the inhibitory potential of those extracts with IC_{50} values <0.5 mg/mL against purified recombinant human ALR2 (Figure 1). Inhibitory potential of these extracts was either similar for human recombinant ALR2 or even better than for rat lens ALR2 (Table 1). The findings with a pure preparation of human recombinant ALR2 confirmed the inhibition of rat lens ALR2 and indicated their potential to inhibit human ALR2.

The specificity of those aqueous extracts with IC_{50} values <0.5 mg/ml in ALR2 assay was assessed by using partially purified ALR1 from goat liver. It has been reported that mammalian liver is rich in ALR1, but low in ALR2.²⁸ Nonetheless, to rule out the contamination of partially purified goat ALR1 preparation with ALR2, western blotting was done using antibody against human recombinant ALR2.²⁹ No signal was observed with goat liver ALR1 preparation when probed with anti-ALR2 antibody (Figure 2) suggesting the absence of ALR2 in ALR1 preparation. Since ALR1 does not appear to utilize glucose to any detectable extent, we also assayed goat liver ALR1 with glucose as substrate. No significant activity was observed with glucose (data not shown). Most of the dietary extracts exhibited selectivity with ALR2 in that they did not inhibit of ALR1 with similar IC_{50} values. About 10 to 20 times higher concentrations required for 50% inhibition of ALR1 as compared to ALR2 (Table 2). On comparing the IC_{50} value of extracts against rat lens or human recombinant ALR2 with goat liver ALR1, selectivity ratio was calculated for these extracts (Table 2). This selectivity ratio indicates relative specificity of dietary extracts towards ALR2. Based on this spinach was found to have the highest specificity towards ALR2 with a ratio of 32, followed by basil, cinnamon, lemon, cumin, and fennel. In fact most of the extracts with IC_{50} value <0.5 mg/ml have a selectivity ratio >10 , as an indication of exceptionally higher specificity towards ALR2.

Table 2. Inhibition of goat liver ALR1 by aqueous extracts of dietary agents. Data for IC_{50} values are the average of three independent experiments. Specificity of the extracts towards rat lens and human recombinant ALR2 was determined based on selectivity ratio of IC_{50} values with ALR1 and ALR2.

Extracts	IC_{50} Value for ALR1 (mg/mL)	Selectivity ratio, ALR1/ratALR2	Selectivity ratio, ALR1/hrALR2
Spinach	3.2	32	32
Basil	3.2	16	26
Lemon	3.2	13	18
Cinnamon	3.2	8	17
Cumin	2.4	14	16
Bitter gourd	3.2	11	13
Fennel	3.2	18	12
Curry leaves	3.2	10	11
Black pepper	2.0	9.1	9.0
Orange	2.0	7.1	8.0
Fenugreek	2.0	8.3	6.6

The above in vitro studies might not be able to predict specificity of dietary extracts with regard to other AKR or the significance of their ARI potential. Under hyperglycemic conditions, accumulation of intracellular sorbitol is the triggering factor responsible for the diabetic complications like cataract, neuropathy and some vascular complications.^{4,30} Among human AKR, ALR2 is unique in its ability to catalyze the NADPH-dependent conversion of glucose to sorbitol.³¹ In addition to lens, retina, nerve and kidney, activation of ALR2 in RBC leads to the accumulation of sorbitol,³⁰ and significant positive correlation has been established between erythrocyte ALR2 level and human diabetic cataract,³² retinopathy,^{33,34} and neuropathy.³⁵ We have also found a direct correlation between erythrocyte ALR2 and sorbitol levels.³⁴ Therefore, we assessed accumulation of sorbitol in RBC under high glucose conditions (ex vivo) to understand the significance of dietary agents with ARI potential. In vitro incubation of RBC with 55 mM glucose resulted in the accumulation of sorbitol three to four fold higher than the control (Figure 3). Incubation of RBC in the presence of dietary extracts under high glucose conditions lead to reduction in the accumulation of intracellular sorbitol (Figure 3). Though, degree of inhibition varied with different extracts, on average there was 40-50% reduction with the concentrations equal to their IC_{50} value (Figure 3). These results indicate the significance of these dietary agents in terms of preventing the accumulation of intracellular sorbitol.

DISCUSSION

Results of the present study showing the inhibition of ALR2 by dietary agents gain attention in many respects. Most of these plant/spice sources are expected to be largely free from adverse effects as they are being used in a variety of dietary preparations and as traditional medicines as well. For example, spinach, bitter gourd and curry leaves are very commonly used as vegetables. While lemon and orange are commonly available citrus

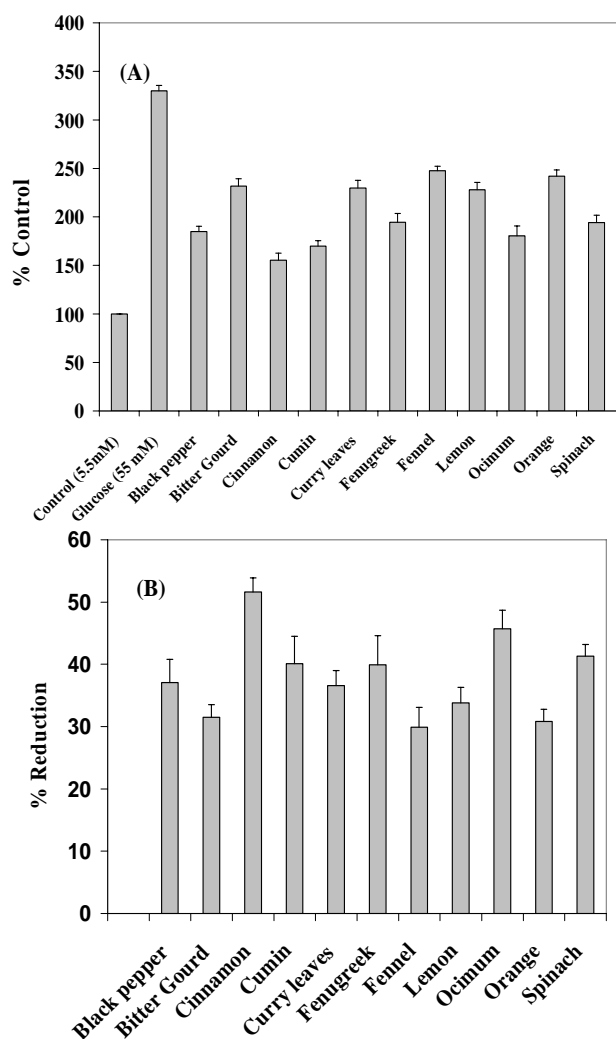


Figure 3: Panel A: Accumulation of sorbitol in the presence of 55mM glucose in the absence and in the presence of extracts. Sorbitol level in RBC under normal glucose concentration (5.5 mM) was considered as 100%. Panel B: Percentage reduction in the accumulation of sorbitol in RBC in the presence of extracts. Sorbitol level in RBC under high (55 mM) glucose concentration in the absence of extracts was considered as 100%. Data are mean \pm SE of three independent experiments. Sorbitol levels were significantly higher under high glucose (55 mM) conditions compared to normal (5.5 mM) conditions (ANOVA $p < 0.05$). Sorbitol levels were significantly lower in the presence of extracts as compared to in the absence of extracts under high glucose (55 mM) conditions (ANOVA $p < 0.05$).

fruits, cumin, cinnamon, fennel, black pepper, amla (Indian gooseberry) are used both as culinary spices and as traditional medicine, whereas others are used as traditional medicines (e.g., basil and ajwain, also known as *Ajowan caraway*).

Additional to the safety issue, specificity of inhibition towards the target enzyme is another concern with most of the available inhibitors. In fact, some ARI are known to equally interact with ALR1. For example, while a widely used inhibitor like sorbinil inhibits ALR2 and ALR1 with comparable IC_{50} values, zopolrestat, tolrestat and ponalrestat are marginally better in having higher specificity towards ALR2.^{36,37} Nonspecific behavior towards ALR1 is due to the high homology it shares with ALR2 in structure and function.³⁸ Inhibition of closely

related enzymes not involved in the polyol pathway might be one of the possible reasons for the multiple side effects of ARI therapy. Only a few studies focused on specificity aspects of ARI. Hence, the high specificity shown by dietary extracts in the present study may have a role to play in the development of ARI. Further, inhibition of accumulation of sorbitol in RBC under hyperglycemic conditions by dietary agents substantiate their potential to reduce diabetic complications.

There have been reports indicating the hypoglycemic effect of some of these culinary items such as fenugreek, cinnamon, basil and bitter melon, but their ARI potential has not been investigated previously, and particularly their specificity and significance.

Accumulation of advanced glycation end products (AGEs) due to non-enzymatic glycation of proteins per se and/ or activation of ALR2 has also been implicated in the development of diabetic complications.³⁹ Inhibition of AGE formation is believed to play a role in prevention of diabetic complications. In addition to the ARI property described in the present study, we have demonstrated previously that some of these agents including cinnamon, cumin, green tea, basil and black pepper inhibit AGE formation.⁴⁰ Multiple properties of these agents might make them more effective to reduce diabetic complications. We have also demonstrated that curcumin from turmeric^{41,42} and tannoids of *Emblca officinalis*^{19,43} delays diabetic cataract formation in rats, mainly through inhibition of eye lens ALR2. Attempts are underway to isolate the active principles responsible for ALR2 inhibition by the dietary agents used in the study and to investigate their ability to delay or prevent diabetic complications. Other studies^{44,45} have also examined plant/ natural sources for their ARI activity to identify treatment strategies that pose less of a risk for diabetes. However, these studies have not evaluated the specificity, selectivity and significance of the natural sources.

Hyperglycemia is the major determinant of microvascular complications in diabetes. Although, there have been major advances in the control of hyperglycemia (diabetes) through dietary changes, hypoglycemic agents, insulin and islet transplantation, the long term complications of diabetes remain serious problems to be addressed. Dietary intervention with traditional foods and medicines derived from natural sources could be a mainstay in the management of diabetes. Therefore, the ARI potential of commonly used foods or traditional medicinal plants is of value. Subject to clinical studies and epidemiological evidence, these plants could be promoted as preferred food adjuvants for control of the diabetic complications and given preference in the design of diabetic diets. The findings encourage a diet-based approach to combat diabetic complications. They also point to selective ALR2 inhibitors for pharmacological intervention. The dietary ARI sources may allow a more diet-based approach or novel pharmacotherapy to preventive diabetes management.

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AUTHOR DISCLOSURES

The authors declare no conflict of interest whatsoever.

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Original Article

Dietary sources of aldose reductase inhibitors: prospects for alleviating diabetic complications

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來自飲食的醛糖還原酶抑制劑：減緩糖尿病併發症的展望

當醛糖還原酶活性上升會活化多元醇代謝路徑，這可能是糖尿病多種併發症發展相關的機制之一。多種人工醛糖還原酶抑制劑已經被測試過，但是並沒有很成功的通過臨床測試。因此本研究評估在印度料理普遍使用的幾種植物/天然產物的醛糖還原酶抑制劑之潛在性。準備 22 種植物性材料的水溶性萃取物，並評估它們對抗老鼠水晶體及人類重組醛糖還原酶之抑制效能。專一性評估是藉由測試這些萃取物對很近似的的醛類還原酶的抑制能力來比較。在活體外試驗中培養紅血球於高葡萄糖含量基質中被用來顯示防止細胞內山梨醇堆積的效能。在 22 種被測試的食物材料中，有 10 種對於老鼠的水晶體及人體重組醛糖還原酶具有潛在的抑制作用。在波菜、小茴香、茴香、檸檬、羅勒及黑胡椒，發現顯著的抑制能力，IC₅₀ 約為 0.2 mg/mL，且對於醛糖還原酶有良好的選擇性。亦即抑制 50% 的醛類還原酶需要高出 10 到 20 倍的濃度。透過膳食萃取物減少細胞內山梨醇的累積，進一步證實它們在有機體的效用。本篇報告指出納入一些普遍食物的生活型態修飾可能可以管控糖尿病之併發症。

關鍵字：醛糖還原酶、醛類還原酶、山梨醇、膳食抑制劑、紅血球