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Inhibition of aldose reductase by tannoid principles of *Emblica officinalis*: Implications for the prevention of sugar cataract

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Purpose: Aldose reductase (AR) has been a drug target because of its involvement in the development of secondary complications of diabetes including cataract. Although numerous synthetic AR inhibitors (ARI) have been tested and shown to inhibit the enzyme, clinically synthetic ARIs have not been very successful. Therefore, evaluating natural sources for ARI potential may lead to the development of safer and more effective agents against diabetic complications. In the present study we have assessed the inhibition of AR by constituents of *Emblica officinalis* both in vitro and in lens organ culture.

Methods: *E. officinalis* is widely used against many chronic ailments including diabetes. Aqueous extract of *E. officinalis* and its major constituent tannoids were tested for inhibition against both rat lens and purified recombinant human AR. ARI potential of isolated tannoids of *E. officinalis* were also investigated against osmotic stress in rat lens organ culture.

Results: *E. officinalis* extract inhibited rat lens and recombinant human AR with IC₅₀ values 0.72 and 0.88 mg/ml respectively. Since *E. officinalis* is a rich source of ascorbic acid, we investigated whether ascorbic acid was responsible for AR inhibition by *E. officinalis* extract. However, ascorbic acid did not inhibit AR even at 5 mM concentration. Further, we demonstrate that the hydrolysable tannoids of *E. officinalis* were responsible for AR inhibition, as enriched tannoids of *E. officinalis* exhibited remarkable inhibition against both rat lens and human AR with IC₅₀ of 6 and 10 µg/ml respectively. The inhibition of AR by *E. officinalis* tannoids is 100 times higher than its aqueous extract and comparable to or better than quercetin. Furthermore, the isolated tannoids not only prevented the AR activation in rat lens organ culture but also sugar-induced osmotic changes.

Conclusions: These results indicate that tannoids of *E. officinalis* are potent inhibitors of AR and suggest that exploring the therapeutic value of natural ingredients that people can incorporate into everyday life may be an effective approach in the management of diabetic complications.

Cataract, the leading cause of blindness world wide, is associated with many risk factors. Diabetes has been considered to be one of the major risk factors of cataract [1,2]. Many experimental studies in vitro and in vivo support the view that diabetes is a cause of cataract. Under normal conditions cellular glucose is phosphorylated by hexokinase for further utilization through glycolysis or the HMPS pathway. During hyperglycemia cellular levels of glucose greatly increase in tissues where glucose entry is independent of insulin. In these tissues, which include lens, retina, kidney, and peripheral nerves, this excess glucose is metabolized via an accessory pathway known as the polyol pathway [3]. Aldose reductase (AR: EC 1.1.1.21) is the rate-limiting enzyme of the polyol pathway. AR catalyzes glucose to sorbitol and sorbitol dehydrogenase, the second enzyme of the pathway, further converts sorbitol to fructose [3,4]. An AR-catalyzed formation of sorbitol was also observed in a number of tissues and the polyol pathway was recognized as an essential component of intermediary metabolism [4-6]. Although some recent studies implicate a role for AR in the detoxification of aldehydes, the major bioactive products of lipid peroxidation [7,8], the physi-

ological relevance of this pathway and its role in overall carbohydrate metabolism remains unclear. Under normoglycemic conditions, approximately 3% of the glucose metabolized is routed via the polyol pathway [9]. However, under hyperglycemic conditions, this pathway accounts for more than 30% of the glucose utilized [10]. The observations of van Heyningen [11] that production of galactitol and sorbitol (polyols of galactose and glucose) in large quantities in rat lens during sugar-induced cataractogenesis stimulated intense interest in the pathological role of polyol pathway in the development of cataract. Reduction of excess glucose to the osmolyte sorbitol leads to osmotic swelling, changes in membrane permeability, and subsequent cataract formation. Therefore, prevention of polyol accumulation by inhibiting AR to prevent cataract and other diabetes-associated ocular pathology has received considerable attention.

The use of traditional medicines, mainly derived from plant sources, has been a major part in the management of many chronic ailments including diabetes, particularly in countries like India [12-14]. Moreover, there is a renewed interest in recent times to identify as many plant sources as possible for their therapeutic value [14,15]. A large number of plants/spices are now well recognized to possess hypoglycemic potential [12-14,16]. Most of the studies have focused only at containing blood glucose levels, mainly as an alternative to

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exogenous insulin. However, many of these hypoglycemic agents have not been investigated for their beneficial effects on secondary complications of diabetes like cataract, retinopathy, nephropathy, and neuropathy. As AR inhibitors (ARIs) have considerable clinical value in ameliorating the secondary complications of diabetes, it would be of great importance to investigate ARI potential and to test the anticataractogenic effect of these natural sources. Since most of the plant products including natural spices are largely free from adverse effects and are being used as a source of diet and traditional medicine [15], testing the ARI potential of these sources may lead to a better management of secondary complications of diabetes.

Emblica officinalis Gaertn., commonly known as amla, is extensively used in many preparations of Ayurveda and also against many chronic ailments including diabetes [17-19]. In the present study we have assessed the ARI potential of amla and report that tannoid principles of amla are potent inhibitors of rat lens AR and also human recombinant AR. We also show that tannoid principles of amla prevent the sugar-induced

polyol stress in cultured rat lenses and together these results imply that amla ingredients may be explored as an anticataractogenic agent for diabetic cataract.

METHODS

Materials: DL-glyceraldehyde, glucose, lithium sulfate, 2-mercaptoethanol, NADPH, quercetin, dimethylsulfoxide, TC-199 medium (M-3769), sorbitol, sorbitol dehydrogenase, NAD, and glutathione reductase were purchased from Sigma Chemical Company (St. Louis, MO). Tannoid principles from amla were isolated according to Ghosal et al. [20] and obtained in the form of a standardized mixture of emblicanin A, emblicanin B, punigluconin, and pedunculagin from Indian Herbs Research & Supply Company (Saharanpur, India). The U. S. patent number for the product is 6,124,268 and further details can be obtained from the U. S. Patent Office.

Rat lens aldose reductase: Crude aldose reductase was prepared from rat lens. Eyeballs were removed from 9 week old WNIN male rats obtained from National Center for Laboratory Animal Services, National Institute of Nutrition, Hyderabad. Animal care and protocols were in accordance with and approved by Institutional Animal Ethics Committee. Lenses were dissected by posterior approach and homogenized in 10 volumes of 100 mM potassium phosphate buffer pH 6.2. The homogenate was centrifuged at 15,000x g for 30 min at 4 °C and the resulting supernatant was used as the source of AR.

Purification of recombinant human aldose reductase: Recombinant human aldose reductase was purified from bacterial cultures. Enzyme from expression cultures was extracted and purified essentially as described previously [21] with the exception that affinity chromatography over AffiGel Blue (Bio-Rad) was used as a final purification step.

Extraction of *E. officinalis* fruit: Fresh fruits of *E. officinalis* were obtained from the local market. The pericarp was collected and freeze-dried. Dried material was powdered and a water extract was prepared by 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.

Aldose reductase assay: AR activity was assayed according to the method described by Hayman and Kinoshita [22]. The assay mixture in 1 ml contained 50 µM potassium phos-

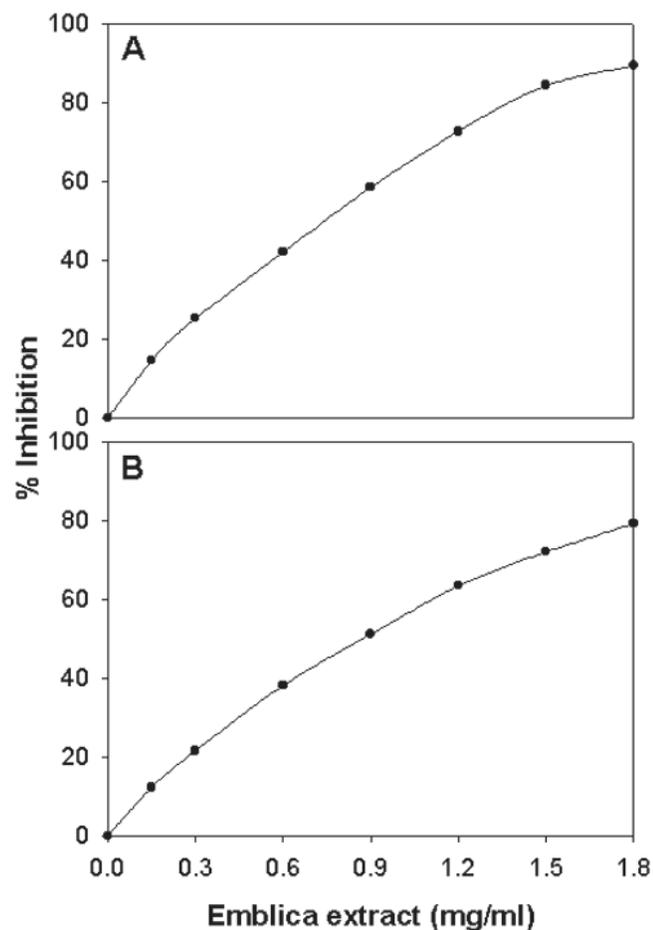


Figure 1. Inhibition of aldose reductase by aqueous extract of *E. officinalis*. Representative plot of inhibition of rat lens AR (A) and human recombinant AR (B) by aqueous extracts of *E. officinalis*. AR activity in the absence of *E. officinalis* extract was considered as 100%.

TABLE 1. INHIBITION OF ALDOSE REDUCTASE (AR) BY ASCORBIC ACID

| Ascorbic acid (mM) | Rat lens AR | Human recombinant AR |
|--------------------|-------------|----------------------|
| 0 | 100 | 100 |
| 0.01 | 98.6 | 98.3 |
| 0.1 | 97.8 | 98.8 |
| 1.0 | 95.2 | 96.6 |
| 5.0 | 96.2 | 95.8 |

Values are expressed as percent residual activity and are averages of three independent experiments.

phate buffer pH 6.2, 0.4 mM lithium sulfate, 5 μ M 2-mercapto ethanol, 10 μ M DL-glyceraldehyde, 0.1 μ M NADPH, and enzyme preparation (rat lens or recombinant enzyme). Appropriate blanks were employed for corrections. The assay mixture was incubated at 37 °C and 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 Cary Bio 100 spectrophotometer.

Inhibition studies: For inhibition studies concentrated stocks of aqueous extract of amla or mixture of isolated tannoid principles were prepared in water and quercetin was prepared in DMSO. Various concentrations of inhibitors were added to the assay mixture and incubated for 5-10 min before initiating the reaction by NADPH as described above. The percent of inhibition with test compounds was calculated considering the AR activity in the absence of inhibitor was 100%. The concentration of each test sample giving 50% inhibition (IC_{50}) was then estimated.

Kinetic parameters: K_m and V_{max} of rat lens AR and recombinant AR were determined with varying concentrations of glyceraldehydes in the absence and presence of tannoid principles of amla. K_m and V_{max} were estimated by Lineweaver-Burk double reciprocal plots.

Lens organ culture studies: Eyes were enucleated from 2 month old WNIN rats immediately after sacrifice by cervical dislocation. Lenses were dissected from the eyes by posterior approach. Each isolated lens was incubated in 2 ml modified TC-199 medium with antibiotics (filtered through 0.2 μ m Millipore disc filters) according to the method of Zigler and

Hess [23]. They were incubated at 37 °C under 95% air and 5% CO₂ with and without 55 mM glucose for a period of 18 h (for sorbitol estimation) or for 4 days (for lens morphology). Medium was changed for every 48 h. Lenses incubated in the medium containing a normal glucose concentration (5.5 mM) and 30 mM fructose (for maintaining similar osmolarity) were considered as control lenses. When added to medium, a stock mixture of tannoids of amla was prepared in water and filtered through 0.2 μ m filters (Millipore).

Estimation of sorbitol in the lens: For extraction of sorbitol the lens 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 an enzymatic method as described previously [24].

Estimation of vitamin C in amla extract: The amount of vitamin C present in aqueous extracts of amla used for AR inhibition was estimated by HPLC according to a previously described method [25]. One ml of 1% aqueous extract of amla was precipitated with 0.35 M perchloric acid and centrifuged at 10,000x g for 15 min. The supernatant was filtered through

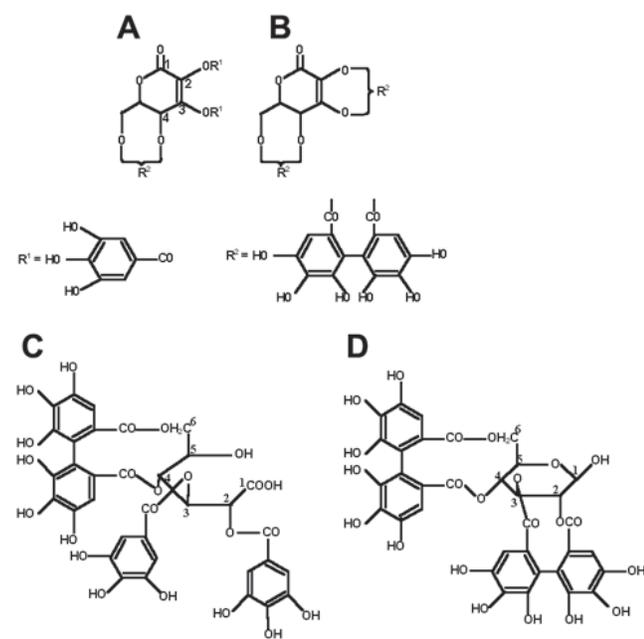


Figure 2. Major hydrolysable tannoids of *E. officinalis*. Structures of the major tannoids of *E. officinalis*, emblicanin A (A), emblicanin B (B), punigluconin (C), and pedunculagin (D). While emblicanin A has one R1 and one R2 side groups, emblicanin B has two R2 side groups.

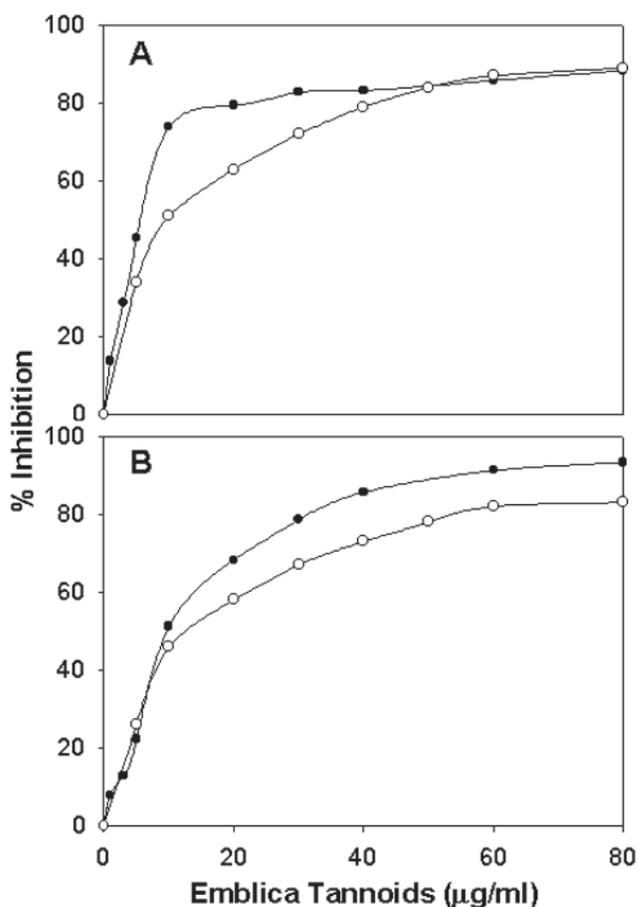


Figure 3. Inhibition of aldose reductase by tannoids of *E. officinalis*. Representative graph of inhibition of rat lens AR (A) and human recombinant AR (B) by isolated tannoids of *E. officinalis* (solid circles) and quercetin (open circles).

0.45 μm filters (Millipore) and loaded onto a C_{18} reversed phase column as described in [25].

RESULTS & DISCUSSION

E. officinalis is extensively found throughout India and some other Asian countries. The fruits are widely consumed raw, cooked, or pickled. The fruits of the plant form a major constituent of many potent Ayurveda preparations [17]. These preparations are widely used for their preventive, curative, and health restorative properties. It possesses very good hypocholesterolemic [18], anticarcinogenic [26], antidiabetic [19], and antioxidant properties [19,27-29]. Therefore, we have investigated amla for its inhibitory potential against rat lens and human recombinant AR.

As shown in Figure 1A, aqueous extracts of *E. officinalis* inhibited rat lens AR, with an IC_{50} value of 0.72 mg/ml. However, rat lens is known to have the highest AR activity compared to other species [30]. Moreover, the other constituents in the crude preparation of rat lens AR could complicate the results of AR inhibition by amla. Thus the relevance of inhibition of rat lens AR by *E. officinalis* may have limited application to human diabetic cataract. Therefore, we have also assessed the inhibitory potential of *E. officinalis* against purified human recombinant AR expressed in *E. coli*. Interestingly aqueous extracts of *E. officinalis* inhibited human recombinant AR to the same extent as that of rat lens AR with an IC_{50} value of 0.88 mg/ml (Figure 1B). The results using a pure preparation of human AR not only substantiate the inhibition of rat lens AR by amla, but also indicate that any artifacts that could have possibly occurred with crude preparations of rat lens AR are not significant. Since amla is a rich source of vitamin C (ascorbic acid), it is believed that the major constituent responsible for most of the biological actions of amla is vitamin C. Further, it was reported that vitamin C can inhibit human erythrocyte AR [31]. Nevertheless, the reported vitamin C content in amla varies from negligible to 0.7% [20]. Hence, we have estimated the amount of vitamin C present in the water extract of amla that was used for AR inhibition studies by HPLC. One gram of amla extract was found to have 1.2 mg of vitamin C (0.12%). We then assessed the inhibitory effect of ascorbic acid against both rat lens and human recombinant AR. As shown in Table 1, vitamin C did not inhibit AR at concentrations as high as 5 mM, thus suggesting that AR inhi-

bitation by *E. officinalis* is due to some other constituents other than vitamin C.

It is interesting to note other studies indicating that the biological actions, particularly antioxidant activities, of amla can not be attributed to ascorbic acid alone [27-29]. The potent vitamin C-like activity, antioxidative effect of amla fruit against reactive oxygen species, was observed with low molecular weight hydrolysable tannins [20]. Four such compounds, emblicanin A, emblicanin B, punigluconin, and pedunculagin have been isolated from amla pericarp and their structures have been established [20]. The first two compounds are naturally occurring galloellagi-tannins, which were neither identified earlier in nature nor were prepared synthetically. The other two compounds (punigluconin and pedunculagin) were identified by correspondence of their properties to those of previously reported from other plant species [20]. The chemical nature of the major tannoids of amla, emblicanin A, emblicanin B, punigluconin, and pedunculagin, are 2,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-2-ketoglucono- δ -lactone, 2,3,4,6-bis-(*S*)-hexahydroxydiphenoyl-2-keto- glucono- δ -lactone, 2,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl gluconic acid, and 2,3,4,6-bis(*S*)-hexahydroxydiphenoyl-D-glucose, respectively [20] (Figure 2). Recently, Bhattacharya et al. [28,29] demonstrated the antioxidant property for the tannoid rich fraction of *E. officinalis*

TABLE 2. IC_{50} VALUES OF *E. OFFICINALIS* TANNOIDS AND QUERCETIN FOR INHIBITION OF AR

| Inhibitor | Rat lens AR | Human recombinant AR |
|------------------|-------------|----------------------|
| Emblica Tannoids | 6.1 | 9.8 |
| Quercetin | 9.2 | 13.5 |

Values are in $\mu\text{g}/\text{ml}$ and are an average of three independent experiments.

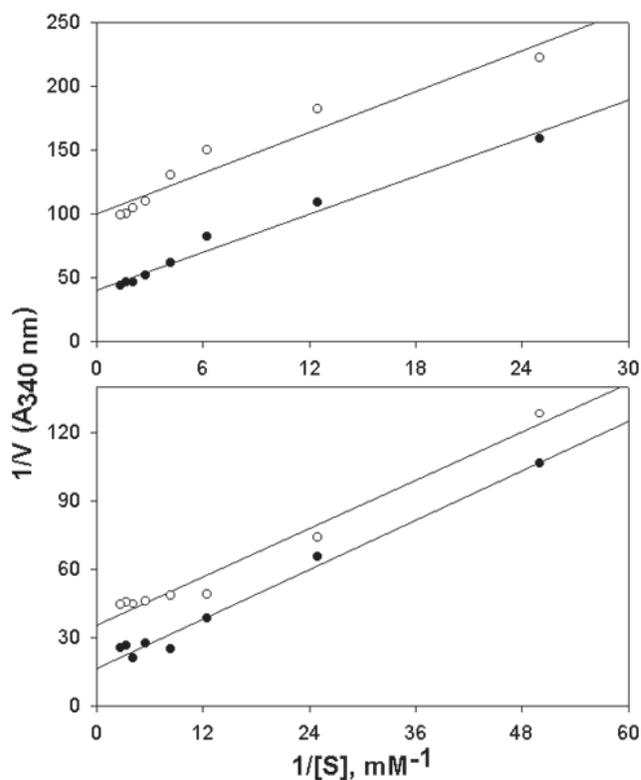


Figure 4. Nature of inhibition of aldose reductase by *E. officinalis* tannoids. Representative double-reciprocal plots of rat lens AR (upper graph) and human recombinant AR (lower graph) in the presence (open circles) and absence (closed circles) of tannoids of *E. officinalis*.

in a stress induced oxidative damage model in rat brain. Therefore, we have obtained the enriched fraction of *E. officinalis* juice with the above tannoids as a standardized extract and investigated the inhibitory effects against AR in vitro and in lens organ culture. The relative proportions of different tannoids in the standardized extract are, emblicanin A and B-35-55%, punigluconin-4-15%, pedunculagin-10-20%, rutin-3%, and gallic acid-1%.

As shown in Figure 3, a mixture of tannoid principles isolated from amla exhibited remarkable inhibition against both rat lens AR and human recombinant AR. The inhibitory potential of isolated tannoids of amla was about 100 times greater compared to the aqueous extract of amla and suggests their potential application in the prevention of sugar cataract. The IC₅₀ values obtained with rat lens and recombinant AR for amla tannoids were 6 and 10 µg/ml respectively (Table 2). On comparison with IC₅₀ values of quercetin, a well known natural flavonoid with aldose reductase inhibitory potential [32],

TABLE 3. KINETIC PARAMETERS OF RAT LENS AND HUMAN RECOMBINANT AR IN THE ABSENCE AND PRESENCE OF TANNOIDS OF *E. OFFICINALIS*

| Kinetic parameter | Rat lens AR | | Human recombinant AR | |
|-------------------|-----------------|------------------|----------------------|------------------|
| | (-) | (+) | (-) | (+) |
| | Tannoids | Tannoids | Tannoids | Tannoids |
| Km (mM) | 0.279 ±0.032 | 0.161* ±0.022 | 0.085 ±0.017 | 0.037* ±0.013 |
| Vmax | 24.2 ±1.57 | 10.6* ±1.42 | 3.88 ±0.219 | 1.72* ±0.231 |

V_{max} is reported as µmoles NADPH oxidized/h/100 mg for rat lens AR or µmoles NADPH oxidized/min/mg for human recombinant AR. Asterisks (*) indicate a statistically significant difference from the values in the absence of tannoids (Student's t-test, p<0.001). Values are mean±standard deviation (n=5).

tannoids of amla appear to be more potent (Table 2). We have estimated the kinetic parameters, K_m and V_{max}, to understand the nature of inhibition of AR by tannoids. Decreased V_{max} and K_m with glyceraldehyde as substrate indicate that tannoids inhibit AR in an uncompetitive manner (Figure 4 and Table 3). To rule out the possibility that tannoids may also inhibit other enzymes in a non-specific manner, we have tested whether tannoids inhibit glutathione reductase and rat lens glucose-6-phosphate dehydrogenase. Neither aqueous extract nor the tannoid mixture of amla inhibited these enzymes (data not shown).

TABLE 4. EFFECT OF *E. OFFICINALIS* TANNOIDS ON ALDOSE REDUCTASE ACTIVITY AND SORBITOL LEVELS IN LENS CULTURE

| Group | AR activity | Sorbitol |
|--|----------------|------------------|
| Control | 26.4 ±2.45 | 19.4 ±1.74 |
| Glucose 55 mM | 36.2* ±3.51 | 83.1* ±9.13 |
| Glucose 55 mM + 50 µg/ml Tannoid mixture | 29.8# ±2.82 | 67.2*# ±10.27 |

AR activity and sorbitol levels were measured in rat lens incubated in the presence of normal (5.5 mM) and high (55 mM) glucose for 18 h. AR activity is expressed as µmoles NADPH oxidized/h/100 mg protein and sorbitol is expressed as nmoles/100 mg lens. The asterisks (*) indicate a statistically significant difference from the control group and the sharps (#) indicate a statistically significant difference from the glucose 55 mM group (ANOVA, p<0.05). Values are mean±standard deviation of three independent experiments.

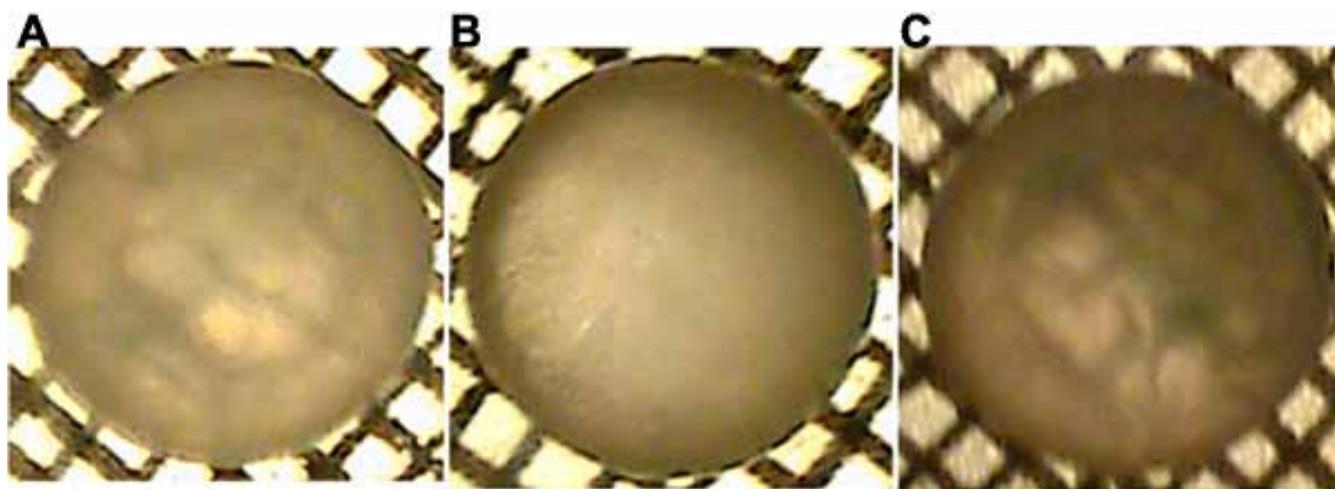


Figure 5. Effect of *E. officinalis* tannoids on lens transparency under osmotic conditions. Morphology of lens in organ culture: Rat lenses were cultured in modified TC-199 as described in Methods in the presence of 5.5 mM glucose and 30 mM fructose, control lens (A), 55 mM glucose (B), and 55 mM glucose along with 50 µg/ml tannoid mixture of amla (C).

Accumulation of high concentrations of polyols in the lens leads to excessive hydration, gain of sodium, and loss of potassium ions due to an increase in intracellular ionic strength [3]. Also there is a loss of membrane permeability and leakage of free amino acids, glutathione, myoinositol, and other small molecular weight substances. The resulting hyper osmotic stress associated oxidative insult is postulated to be the primary cause for the development of diabetic complications such as cataract, retinopathy, neuropathy, and nephropathy [33]. To understand the significance of in vitro inhibition of AR by amla, the effect of isolated tannoids against osmotic stress were investigated in lens organ culture. Rat lenses incubated with 55 mM glucose for 18 h developed vacuoles and AR activity significantly increased compared to lenses incubated with a normal glucose (5.5 mM) concentration along with fructose (30 mM, Table 4). Incubation of lenses with 55 mM glucose also resulted in increased production of sorbitol compared to control lenses (Table 4). While the lens incubated with 55 mM glucose for 4 days became cloudy and lost transparency, the morphology of the lens incubated with 55 mM glucose along with 50 µg/ml of tannoid mixture of amla appears similar to control lenses even after 4 days (Figure 5). More importantly, activation of AR and increased sorbitol levels due to hyperglycemic stress was prevented when lenses were incubated with 55 mM glucose in the presence of 50 µg/ml amla tannoids in the medium (Table 4). While the mixture of *E. officinalis* tannoids was not effective at concentrations <10 µg/ml, a concentration >100 µg/ml in the medium imparts some color to lenses (data not shown). The results indicate that the in vitro inhibition of AR by *E. officinalis* could be correlated with in vivo conditions as it prevented changes associated with AR activation in lens organ culture.

Due to its proposed involvement in the development of diabetic complications, AR has been a drug target in the clinical management of secondary complications of diabetes including cataract [34]. Structurally distinct compounds such as flavonoids, benzopyrans, spirohydantoin, alkaloids, nonsteroidal anti-inflammatory agents, and quinones have all been shown to inhibit the enzyme with various degrees of efficacy and specificity [6,35]. Sorbinil, statil, tolrestat, alrestatin, epalrestat, and ALO1576 are some of the well-studied inhibitors that have also been clinically tested. However, to date, none of the currently available synthetic ARIs have proved clinically effective and some have in fact had deleterious side effects. Moreover there is an increased interest in recent times to identify many natural (plant/spice) sources for their therapeutic properties, mainly because most of the plant and plant products (including natural spices) are largely free from adverse effects and are being used as a source of diet and traditional medicine. Recently a few other plant products have been reported to inhibit crude rat lens AR [36-38]. Therefore, we are interested in screening plant/spice sources for their anticataractogenic potential and in this context the results of inhibition of AR by *E. officinalis* suggest that screening of dietary components for AR inhibition could lead to the development of possibly safer and more effective agents than other known ARIs against diabetic cataract. Studies are underway

to investigate the anticataractogenic effect of *E. officinalis* and its constituent tannoids against diabetic cataract in animal models.

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