Original Article

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Olive and ginkgo extracts as potential cataract therapy with differential inhibitory activity on aldose reductase

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Summary **Aldose reductase (AR) has been the leading target in the treatment of diabetic cataract. Although numerous synthetic AR inhibitors (ARI) have been identified, their adverse side effects currently preclude their use. Olive leaf extract (OLE) as well as ginkgo leaf extract (GLE) are natural supplements that have wide therapeutic indices and a plethora of salutary effects during diabetes that so far untested on sugar cataract progression. As such, the present study sought to evaluate the AR-inhibiting properties of OLE and GLE using the isolated enzyme from rabbit lens. Biochemical analyses revealed that both OLE and GLE inhibited rabbit lens AR activity in a concentration-dependent manner with half maximal inhibitory concentration (IC_{50}) 65 µg/mL and 72.5 µg/mL, respectively. Interestingly, the results of kinetic studies exhibited a differential pattern of inhibition by these two extracts. While an non-competitive inhibition of AR was promoted by OLE recognized by significant decrease in the apparent maximum velocity (V_{max}) (0.12 ± 0.009677 µM/min *versus* 0.278 ± 0.0013677 µM/min) without significant change in Michaelis constant (Km), the GLE showed a competitive pattern of inhibition characterized by significant increase in apparent Km (4.4 ± 0.0068 µM), without change in V_{max} value. It would appear that these classes of natural extracts represent effective and safe therapeutic options that hold the great promise for treatment not only diabetic cataract, but also other ocular diseases characterized by uncontrolled AR activity.**

Keywords: Rabbit lenses, polyol pathway, aldose reductase, NADPH, enzyme inhibiting, cataract, olive extract, ginkgo extract

1. Introduction

Diabetes, the silent killer, is the biggest national health threat due to its deadly and costly complications despite appropriate therapeutic measures. Among the most common secondary diabetic complications is cataract, the opacity of the lens that produces painless gradual loss of vision (*1*). Diabetic cataract is usually acquired during persistence hyperglycemia through increasing the glucose level greatly in eye lens where glucose entry is independent of insulin. This excess glucose is metabolized *via* an accessory pathway known as the polyol pathway. Activation of this pathway leads to the accumulation of the osmolyte sorbitol in eye lens resulting in osmotic swelling and subsequent hydropic lens fibers that degenerate to form sugar cataracts (*2*). Aldose reductase (AR) is the rate-limiting enzyme of the polyol pathway that catalyzes a NADPH-dependent reduction of a glucose to sorbitol and is found abundantly in eye lens (*3*). Nevertheless, this enzyme has a low affinity for glucose, and little substrate is processed under physiological conditions but its activity is more pronounced with chronic hyperglycemia (*4*). As such, pharmacologic regulation of AR activity is a rational approach to modulate early pathological pathways associated with cataract genesis long before the occurrence of vision loss among diabetics. This view has been strengthened recently by the finding

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that the administration of AR inhibitors at the onset of diabetes prevents diabetic cataract formation in experimental animals (*5*).

While a number of structurally diverse compounds inhibit AR significantly, many of these compounds possess poor pharmacokinetic properties or side-effects not associated with the specific inhibition of AR (*6*). These side effects range from severe allergic reactions with sorbinil (*7*), impaired kidney function with zenarestat (*8*), to alteration of liver function with tolrestat (*9*). These problems reflect a need for the development of new, more effective and safe drug which may rise the therapeutic benefits for diabetic patients. As there is still no safe AR inhibitor (ARI), there is a renewed interest in recent times to use plant-based medicine in the area of health care owing to its efficient, safe, and economic features.

Several plant species have been used for prevention or managing diabetes by the native Americans, Chinese, Africans, South Americans, and Asian Indians. However, a limited number of medicinal plant species have been studied and validated for their hypoglycemic properties using laboratory diabetic animal models and in clinical studies using human subjects. Among the antidiabetic plants recommended by traditional practitioners are olive and ginkgo on which the retrieved research has focused to utilizing them as alternatives to current antidiabetic therapies. However, they have not been investigated for their beneficial effects on diabetes cataract.

There are several studies have been conducted on the efficacy of the olive leaf extract (OLE). These studies proved that OLE has a powerful effect on lowering blood glucose level (*10*). Similarly, ginkgo (*Ginkgo biloba*) is one of the oldest living tree species and its leaves are among the most extensively studied herbs in use today. In Europe and the United States, ginkgo supplements are among the best-selling herbal medications. Interestingly, Kudolo and his colleagues provided evidence for lowering hyperglycemia among diabetic patients treated by *Ginkgo biloba* (*11-12*). However, no thoroughly study to date has investigated the independent and combined effects of these extract on polyol pathway and its subsequent sugar cataract progression. Therefore, this study sought to pursue the protective effect of both OLE and GLE on AR activity and to gain insight into the inhibition mechanism involved therein.

2. Materials and Methods

2.1. *Materials*

DL-glyceraldehyde (GA), lithium sulfate, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and Dimethylsulfoxide (DMSO), were obtained from Sigma Chemical Company (Sigma-Aldrich, St Louis, MO, USA). All other chemicals and solvents were of analytical grade and were obtained from local company (Al-Gomheria Co., Mansoura, Egypt).

2.2. *Extracts preparation*

Total extract of olive leaf was obtained by macerating dried leaves with 70% methanol at 25°C for 48 hours, the extract was evaporated under vacuum at 45°C; then the dried powder was dissolved in DMSO to prepare the stock solution of 1 mg/mL. Extract of Ginkgo leaves was ready obtained as dried powder from (Pharco-Pharm Company, Cairo, Egypt) and then dissolved in DMSO to prepare the stock solution of 1 mg/mL.

2.3. *Preparation of crude AR from rabbit lenses*

Following the method of Pottinger, 1967, with some modifications (*13*), twenty rabbits of 2 months old and average weight of 1.5 kg were purchased from local Cuniculture (Mansoura, Egypt). Animal care and protocols were in accordance with and approved by Institutional Animal Ethics Committee. Immediately after rabbits were killed by decapitation, the eyes were removed and placed in a dish of isotonic saline. 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 15,000× g for 30 min at 4°C and the resulting supernatant was used as the source of AR and stored in -20°C until used.

2.4. *Determination of anti-AR activity in vitro*

The reaction mixture was prepared at (25 ± 1°C), with a total volume of 2.3 mL cuvette, containing Na-K phosphate buffer (pH 6.8), 0.05 mM NADPH, 0.02 M LiSO4, enzyme preparation equivalent to 0.8 g protein, and 0.01 M GA as a substrate with or without plant extracts. The reaction was initiated by addition of NADPH and continued by 10 min. The change in the absorbance (Abs) at λ_{max}, 340 nm due to NADPH oxidation was followed in a Bio-lab spectrophotometer (Biolab Scientific Ltd, Ontario, Canada). A negative control (Neg. Ctrl) was prepared using DMSO (the solvent of extracts) in phosphate buffer (pH 6.8). 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. At the end, the inhibitory activity of the extracts was calculated using the following formula:

$$\% \text{ ARI} = \frac{\Delta\text{Abs. (Neg. Ctrl.)} - \Delta\text{Abs. (Extract)}}{\Delta\text{Abs. (Neg. Ctrl.)}}$$

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.

2.5. *Kinetic parameters*

K_m and V_{max} of rabbit lens AR were determined with varying concentrations of glyceraldehyde in the absence and presence of OLE or GLE. K_m and V_{max} were estimated by Lineweaver-Burk double reciprocal plots. Inhibition constant (Ki) for each extract was determined using the following formulas:

$$\text{Ki for competitive inhibitor} = \frac{\text{Km [I]}}{(\text{Km}_{app}\text{-Km})}$$

$$\text{Ki for non-competitive inhibitor} = \frac{\text{Vmax}_{app}\text{ [I]}}{\text{Vmax- Vmax}_{app}}$$

2.6. *Data analysis*

The results were expressed as mean ± SD. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by the post hoc test (Fisher's PLSD) when indicated. Significance was defined as $p < 0.05$.

3. Results

3.1. *Validation of the analytical method used*

AR which is found primarily in the eye lens is the key relay in the polyol pathway that is initiating sugar cataract formation. Rodent lens is known to have the highest AR activity compared to other species (*14*). Therefore, we have assessed the inhibitory potential of OLE and GLE against AR isolated from the eye lens of rabbits. In order to evaluate the activity of the isolated enzyme, we have successfully validated an analytical method by the adaptation of the procedure given by Hayman and Kinoshita with some modifications (*15*). Firstly, the validation of this method was based on the consumption of the co-factor, reduced NADPH, in the AR catalyzed-NADPH-dependent conversion of glucose to sorbitol. Under the used analytical conditions, enzymatic curves were done by monitoring the absorbance change of NADPH to NADP+ using absorbance spectroscopy. The starting spectrum was characteristic of NADPH, which absorbs light at both λ_{max}, 260 and 340 nm, whereas NADP+ only absorbs light at λ_{max}, 260 not 340 nm (Figure 1A). Moreover, linear and concentration-dependent dose-response trends of the absorbance were seen at 340 using the values obtained from the standards NADPH (Figure 1B). This dose-response curve was then used to calculate the concentration of the consumed NADPH during AR activity and hence the velocity of the enzymatic reaction which was monitored by measuring the λ_{max} 340 nm absorbance decrease as a function of time (Figure 1C).

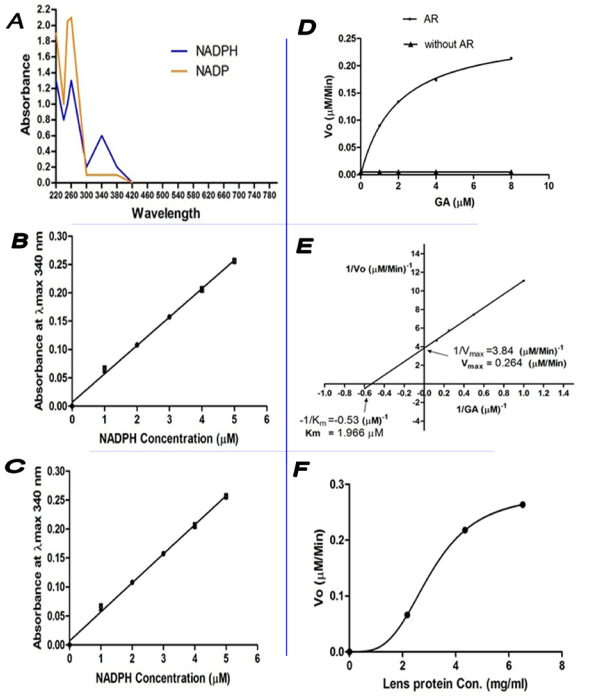

Figure 1. Validation of the analytical method used. (A) Absorbance spectra of NADPH and NADP. **(B)** Dose response at λ_{max}, 340 nm, for different NADPH concentrations. **(C)** Time resolved absorbance response of NADPH at λ_{max}, 340 nm obtained for 1, 2, 4, 8 mM of DL-glyceraldehyde (GA) as a substrate in aldose reductase (AR) catalyzed reactions. **(D)** Hyperbolic curve of AR-catalyzed NADPH-dependent reactions in which the reaction velocity (Vo) is dependent on GA concentration. **(E)** Lineweaver-Burk plot of the AR catalyzed reaction with variable substrate concentrations of GA (0.05-8 µM) for determination of Vmax, the reaction velocity at saturated substrate concentration, and the Michaelis constant, K_m. **(F)** AR activity at different concentrations of lens protein measured by the rate of NADPH oxidation at λ_{max} 340 nm.

Secondly, the initial velocity (Vo) of AR-catalyzed reaction was dependent on GA concentration; this relationship is typically hyperbolic, with a linear increase at lower concentrations until the reaction approaches saturation, at which point further increases in substrate will not increase reaction velocity (Figure 1D). Two important parameters are typically calculated using kinetic assay data: V_{max} (0.264 ± 0.0137 µM/min), the reaction velocity at saturated substrate concentration, and the Michaelis constant, K_m (1.996 µM), which is a measure of the affinity of the enzyme for the substrate. These parameters are obtained through a double-reciprocal plot of velocity against GA concentration for the linear portion of the original curve (Figure 1E).

To further validate the activity of the isolated AR, we have measured Vo at various enzyme concentrations, using a substrate concentration that is well above the K_m (Figure 1F). As shown in Figure 1F, increasing the added enzyme concentration to 6 mg/ml lens protein caused a progressive increase in the rate of NADPH consumption and thereby the velocity of the reaction.

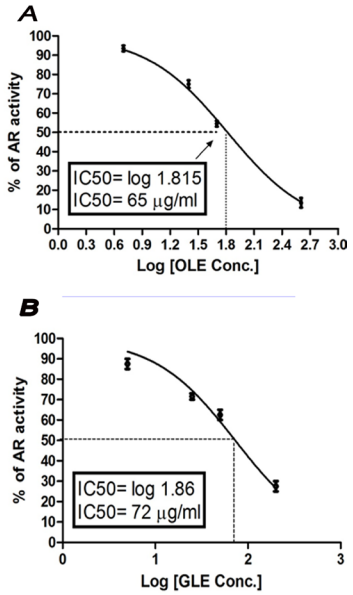

Figure 2. Representative graph for inhibiting rabbit lens aldose reductase activity by (A) olive leaf extract (OLE) and (B) ginkgo leaf extract (GLE) and the IC_{50} of each extract.

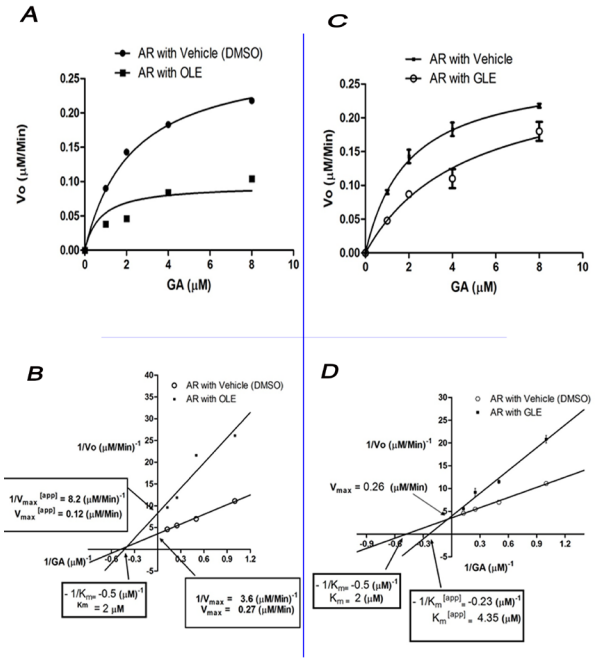

Figure 3. Kinetic data for determination the type of inhibiting aldose reductase by either OLE or GLE. (A) and **(B)** Representative hyperbolic and double-reciprocal plots, respectively, for the inhibitory effect of OLE on rabbit lens AR in the presence or absence of OLE (65 µg/mL) in three independent experiments (uncompetitive type of inhibition). **(C)** and **(D)** Representative hyperbolic and double-reciprocal plots, respectively, for the inhibitory effect of GLE on rabbit lens AR in the presence or absence of GLE (72.5 µg/mL) in three independent experiments (competitive type of inhibition).

3.2. *Olive leaf and ginkgo leaf extracts mitigated lens AR activity in a dose dependent and differential manner*

Given the primary role played by AR in the initiation of the entire sequence of cataractous change during diabetes and the fact that GLE and OLE exhibited a plethora of benefits in experimental diabetes (*16*), we aimed to investigate whether GLE and OLE, may also be effective in attenuation of AR activity. To address this point, the ability of both extracts to reduce AR activity was determined using the enzyme pre-incubated with indicated concentrations of OLE or GLE for half an hour before the addition of the substrate (GA). As shown in Figure 2A and B, extracts of both olive leaf and ginkgo inhibited rabbit lens AR activity in a concentration-dependent manner with IC_{50} values 65 µg/mL and 72.5 µg/mL, respectively.

In light of olive leaf and ginkgo extracts' anti-AR effect, interest in their mechanisms of inhibition has been expanded to explore the type of inhibition. Consequently, kinetic studies were performed and Lineweaver-Burk plots were constructed. Interestingly, the results obtained from enzyme kinetic studies exhibited a differential pattern of inhibition by these two extracts. As shown in Figures 3A and 3B, an non-competitive inhibition of AR was promoted by OLE which was recognized by its characteristic effect on the V_{max} (0.278 ± 0.0013677 µM/min) that was decreased significantly in the apparent maximum velocity (56%; 0.12 ± 0.009677 µM/min) while K_m (2 ± 0.0017 µM) did not differ as compared with the substrate GA (1.996 ± 0.0012 × 10^{-3} µM). On the other hand, GLE showed a competitive inhibition with an increased (220%) apparent K_m for GA (4.4 ± 0.0068 µM), while V_{max} remained unchanged (Figures 3C and 3D).

Next, from the aforementioned differential AR inhibitory properties of both OLE and GLE, a renewed interest in the combination anti-AR therapies has been stimulated. To underscore this point, the capacity of both extract to mitigate AR activity was evaluated individually as well as in combination therapy. As shown in Figure 4A, the combination ratio of 3:1, GLE:OLE was much more effective than either GLE or OLE alone in inhibiting AR. This finding was supported by the kinetic data in that showed a mixed type of inhibition at the ratio used because it influences both K_m and V_{max} (Figures 4B and 4C).

4. Discussion

Biochemical studies have shown that activation of AR in the eye lens is an early event that occurs in response to diabetes prior to mature cataract formation resulting in the accumulation of high concentrations of polyols. These polyols lead to excessive hydration, loss of membrane permeability, and leakage of free amino acids, glutathione, myoinositol, and others. The sequelae is a hyper-osmotic associated oxidative insult that is postulated to be the primary cause for the development of diabetic cataract (*17*). Thus, by targeting AR, the onset and progression of sugar cataract can be delayed or even

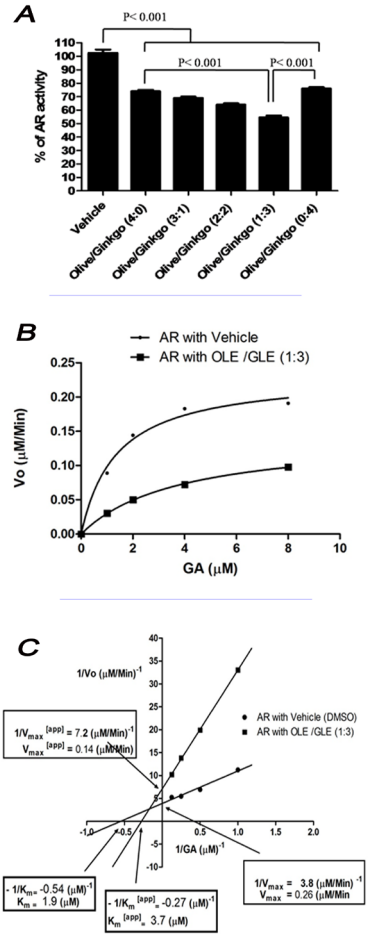

Figure 4. Representative graph for inhibiting rabbit lens aldose reductase activity by (A) different combination ratios between OLE and GLE. (B) and (C) Representative hyperbolic and double-reciprocal plots, respectively, for the inhibitory effect of the combination between OLE and GLE in 1:3 ratio on rabbit lens AR in the presence or absence of this combination in three independent experiments (mixed type of inhibition).

prevented. Unfortunately, AR inhibitors have fallen short. The problem is that AR inhibitors are very effective at preventing cataracts but they have been eliminated from any eye disease-related clinical trials because of adverse side effects (*6*). When diabetic patients were treated with the AR inhibitor sorbinil, enhancement of the nerve conduction velocity was observed (*18*), but generally, the effect had shown to be modest with major adverse reaction of hypersensitivity similar to that seen with other hydantoins. Even though no clinically important adverse reaction was observed with ponalrestat, the subsequent AR inhibitor of a different structure, it failed to provide beneficial effects in randomized controlled study (*19*). Tolrestat, another class of inhibitors, showed increased serum levels of alanine aminotransferase or aspartate aminotransferase as side effect (*20*). Because of the inability to demonstrate efficacy in the multicenter double blind studies, the clinical development of tolrestat was eventually withdrawn.

In the present study, we put forward a new evidence for introducing Olive and Ginkgo leaf extracts as natural AR inhibitors. These natural extracts have wide therapeutic index with very low toxicity (*21-23*) and their use have shown a plethora of salutary effects in different animal models of experimental diabetes.

Chronic treatment with OLE inhibited the high glucose-induced neural damage and suppressed diabetes-induced thermal hyperalgesia observed in diabetic rat (*24*). Additionally, the chronic treatment with GLE improved the vascular function in diabetic nephrotic patients (*25*). Recently, OLE has been reported for its potential hypoglycemic activity following chronic systemic administration in overweight middle-aged men who are at risk of developing the metabolic syndrome as well as in experimental animals (*10,26*). Likewise, GLE has been shown to possess anti-hyperglycemic activity in streptozotocin (STZ)-induced diabetic rats (*27*). These anti-diabetic effects are mediated *via* preservation of insulin positive β-cells and restoration of the glucose metabolic enzyme activities (*28, 29*).

In our *in vitro* studies, GLE showed a remarkable inhibition of AR suggesting the presence of potential enzyme inhibiting compound(s) in the extract. To find the mechanism of inhibition, we have formulated double reciprocal plot from the kinetics data and the results indicate the competitive mode of inhibition by GLE with a Ki value of 62.5 μM. On the other hand, OLE inhibited the AR activity non-competitively with a Ki value of 114.36 μM. In other words, although AR inhibition by GLE can be overcome by adding higher concentrations of substrate, no amount of substrate can overcome AR inhibition by OLE. Moreover, their combination was much more effective than either GLE or OLE alone, a finding that was supported by the kinetic data.

Collectively, the experiments in this study provide new insights into the mechanisms of anti-cataraceous effects of both GLE and OLE by attenuating AR activity in a differential manner. Consequently, it would appear that these classes of natural extract represent effective, safe and well tolerated therapeutic options that hold the best hope for treatment lens diseases characterized by uncontrolled NADPH-dependent AR activity.

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