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Inhibitory Effects of *Zingiber officinale* Roscoe Derived Components on Aldose Reductase Activity in Vitro and in Vivo

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Ginger (*Zingiber officinale* Roscoe) continues to be used as an important cooking spice and herbal medicine around the world. Scientific research has gradually verified the antidiabetic effects of ginger. Especially gingerols, which are the major components of ginger, are known to improve diabetes including the effect of enhancement against insulin-sensitivity. Aldose reductase inhibitors have considerable potential for the treatment of diabetes, without increased risk of hypoglycemia. The assay for aldose reductase inhibitors in ginger led to the isolation of five active compounds including 2-(4-hydroxy-3-methoxyphenyl)ethanol (**2**) and 2-(4-hydroxy-3-methoxyphenyl)ethanoic acid (**3**). Compounds **2** and **3** were good inhibitors of recombinant human aldose reductase, with IC_{50} values of 19.2 ± 1.9 and 18.5 ± 1.1 μM, respectively. Furthermore, these compounds significantly suppressed not only sorbitol accumulation in human erythrocytes but also lens galactitol accumulation in 30% of galactose-fed cataract rat model. A structure−activity relationship study revealed that the applicable side alkyl chain length and the presence of a C3 OCH_3 group in the aromatic ring are essential features for enzyme recognition and binding. These results suggested that it would contribute to the protection against or improvement of diabetic complications for a dietary supplement of ginger or its extract containing aldose reductase inhibitors.

KEYWORDS: ***Zingiber officinale*; ginger; aldose reductase inhibitor; erythrocyte; sorbitol; galactosemia; diabetes**

INTRODUCTION

Ginger, the underground stem or rhizome of the plant *Zingiber officinale* Roscoe (Zingiberaceae), continues to be used as an important cooking spice around the world. Furthermore, ginger has been used as a medicine in Asia, Indian, and Arabic herbal traditions since ancient times. In China, ginger has been used to aid digestion and treat stomach upset, diarrhea, and nausea for more than 2000 years. In recent times there has been scientific research undertaken to test the validity of the medicinal claims made about ginger, and some exciting results with respect to the medicinal properties of ginger have been obtained. One of these properties is the effect of the juice of *Z. officinale* on streptozotocin-induced type 1 diabetic rats (*1*). Treatment with *Z. officinale* produces a significant increase in insulin levels and a decrease in fasting glucose levels in diabetic rats. Furthermore, treatment with ginger affected a significant reduction in fructose-induced elevation in lipid levels, body weight, hyperglycemia, and hyperinsulinemia and also caused a decrease in serum cholesterol, serum triglyceride, and blood pressure in diabetic rats (*2*). Ginger rhizome yields two primary extracts: oleoresin and essential oil. The oleoresin is a solvent extract containing both the essential oil and the phenolic compounds responsible for the pungency of ginger. The main pungent compounds in fresh ginger are a series of homologous phenolic ketones known as gingerols, such as [6]-, [8]-, and [10]-gingerols.

Current scientific evidence demonstrates that morbidity and mortality of diabetes can be eliminated by aggressive treatment with diet, exercise, and new pharmacological approaches to achieve better control of blood glucose levels. In recent years, the possibility of preventing the onset of diabetes using dietary

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supplements and/or herbal medicines has attracted increasing attention. The most serious problem in diabetes is that complications develop slowly and cause significant tissue damage before clinical signs appear. It is well-known that prolonged hyperglycemia is a primary factor of several diabetic complications and careful control of the blood glucose levels delays or protects against development of severe complications. However, some patients develop several complications at an early stage in spite of careful control of their glucose levels (*3*), because development of complications is influenced by various other factors, for example, autoimmunity and hereditary factors. It also affects many promoters such as activation of polyol metabolism, glycation, protein kinase C, or oxidative stress. The polyol pathway plays an important role in these. The enzymes aldose reductase [alditol:$NADP^+$ oxidoreductase (EC 1.1.1.21)] and sorbitol dehydrogenase [L-iditol:NAD^+ 2-oxidoreductase (EC 1.1.1.14)] play key roles in the polyol pathway (*4*). Aldose reductase is a superfamily of NADPH-dependent oxidoreductases (*5*−*7*) and catalyzes the reduction of various sugars to sugar alcohols, including the reduction of glucose to sorbitol. Aldose reductase is cell-based and has a low affinity for glucose and, thus, this pathway plays a minor role in glucose metabolism in blood. In a diabetic condition, however, sufficient glucose can enter the tissues, and the pathway operates to produce both sorbitol and fructose. These cells accumulate them because of their poor penetration across the membranes. These abnormal metabolic results have been reported to be factors responsible for diabetic complications such as cataracts (*8*, *9*), retinopathy (*10*, *11*), neuropathy (*12*), and nephropathy (*13*). Therefore, aldose reductase inhibitors have considerable potential for the treatment of these diseases, without increasing the risk of hypoglycemia. At present, the inhibitors belong to two different chemical classes. One is the hydantoin derivatives, such as sorbinil, Dilantin, and Minalrestat, and the other is the carboxylic acid derivatives, such as Epalrestat, Alrestatin, Tolrestat, and Zopolvestat (*14*−*19*). However, it is known that most of the compounds have unacceptable side effects. Hydantoin-type inhibitors have a risk of Steven−Johnson syndrome and hypersensitivity reaction (*20*, *21*). On the other hand, many carboxylic acid derivatives have also been withdrawn from clinical trials due to lack of efficacy or toxicity (*22*, *23*). Many laboratories have synthesized various types of aldose reductase inhibitors, but only a few compounds can affect them without serious side effects. The carboxylic acid derivatives Tolrestat and Epalrestat are two of the most effective aldose reductase inhibitors, with no serious side effects, and they are approved for marketing in several countries.

Many kinds of aldose reductase inhibitors have been found from natural sources (*24*). Medicinal herbal and edible plants might be expected to yield less toxic inhibitors of diabetic complications. In our search for aldose reductase inhibitors, we found that a hot water extract of *Z. officinale* exhibited significant inhibitory activity. Ginger has been commonly used not only as a food spice but also as an important component in Japanese herbal medicine (Kampo). Investigations of the constituents of *Z. officinale* have shown a large number of terpenoid and pungent principles (*25*−*28*). In this paper, we report the inhibition of aldose reductase by water extracts of gingers for the first time and the isolation of the active compounds and their structure−activity relationships. In addition, we investigated the effects of these compounds on the accumulation of sorbitol in human erythrocyte and accumulation of galactitol in the lenses of galactose-fed rat model for diabetes.

MATERIALS AND METHODS

General Experimental Procedures. The purity of samples was checked by HPTLC on silica gel $60F_{254}$ (E. Merck) using the solvent system PrOH/AcOH/H_2O (4:1:1), and was detected by iodine vapor. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker DRX500. Chemical shifts were expressed in parts per million downfield from tetramethylsilane in CD_3OD as an internal standard. The standard samples **1** and **7** were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Compounds **5**, **14**, and **15** and quercetin were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other standard samples **2**−**4**, **6**, and **8**−**13** were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The purity of the compounds isolated was checked by HPLC analysis.

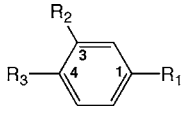
Plant Material. Rhizomes of *Z. officinale* Roscoe were purchased at a crude drug shop in Japan (Tochimoto Tenkaido Co., Osaka, Japan). A voucher specimen (TMPW No. 730) of the *Z. officinale* was deposited at the Herbarium of the Institute of Natural Medicine, University of Toyama.

Extraction and Isolation. The rhizomes of *Z. officinale* (3 kg) were extracted with hot water for 40 min. The hot water extracts were filtered through Celite and applied to a Diaion HP-20 (75−150 μm, Mitsubishi Chemical Industries, Ltd., Tokyo, Japan) column (500 mL) and eluted with 0−100% MeOH. Active fractions were detected by enzyme assays. The 100% MeOH and H_2O fractions were inactive. The aqueous MeOH eluate was divided into two active pools, A (50% aqueous MeOH eluate, 588.4 mg) and B (75% MeOH eluate, 347.1 mg). Pool A was chromatographed on a Sephadex LH-20 (25−100 μm, Amersham Biosciences Corp, Piscataway, NJ) column (1.9 × 92 cm) with 50% aqueous MeOH as eluant and gave an active fraction (fractions 37−68, 204.5 mg). The fraction size was 8 mL. This fraction was further chromatographed with a Toyopearl HW-40S (20−40 μm, Tosho Corp., Tokyo, Japan) column (3.0 × 60 cm) with 50% aqueous MeOH as eluant to give (4-hydroxy-3-methoxyphenyl)methanol (43.8 mg, **1**), 2-(4-hydroxy-3-methoxyphenyl)ethanol (35.5 mg, **2**), and 2-(4-hydroxy-3-methoxyphenyl)ethanoic acid (30.8 mg, **3**). Pool B was purified by a Sephadex LH-20 (1.9 × 92 cm) with 50% aqueous MeOH as eluant to give 2-(3,4-dimethoxyphenyl)ethanoic acid (6.1 mg, **4**) and 4-(4-hydroxy-3-methoxyphenyl)-2-butanone (80.5 mg, **5**).

Assay of Enzyme Activity. Recombinant aldose reductase, which retains the same properties exhibited by human muscle and retina, was purchased from Wako Pure Chemical Industries (Osaka, Japan). Aldose reductase activity was spectrophotometrically measured at 37 °C by using 100 mM D,L-glyceraldehyde as the substrate (*29*).

Determination of Sorbitol in Human Erythrocytes. Human blood was obtained from a healthy female volunteer, who was fully informed of this study and gave written consent. Erythrocytes from heparinized blood were separated from the plasma and buffy coat by centrifuging at 3000*g* for 30 min. The cells were routinely washed three times with isotonic saline at 4 °C. In the final washing, the cells were centrifuged at 2000*g* for 5 min to obtain a consistently packed cell preparation. The packed cells (1 mL) were then incubated in a Krebs−Ringer bicarbonate buffer (pH 7.4) (4 mL) containing 28 mM glucose in the presence or absence of 40 μM samples at 37 °C in 5% CO_2 for 60 min. The erythrocytes were washed with cold saline by centrifuging at 2000*g* for 5 min, precipitated by adding 6% of cold perchloric acid (3 mL), and centrifuged again at 2000*g* for 10 min. The supernatant was neutralized with 2.5 M K_2CO_3 at 4 °C and used for sorbitol determination (*30*, *31*). The reaction mixture contained the appropriate protein-free supernatant, 50 mM glycine buffer (pH 9.4), 0.2 mM NAD^+, and 1.28 units of sorbitol dehydrogenase. The incubations were performed at 37 °C for 30 min, and the relative fluorescence due to NADH was measured by a fluorescence spectrometer at an excitation wavelength of 366 nm and an emission wavelength of 452 nm (*32*).

Animal Experiments. The protocol was approved by the Animal Experiments Committee of the University of Toyama. Male Wistar rats with a body weight of 130 g were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The rats were kept in wire-bottomed cages under a conditional lighting regimen with dark light. The room temperature (≈23 °C) and humidity (≈60%) were controlled automatically. The rats were divided into four groups (group A−D), and the blood glucose



1: $R_1 = CH_2OH$, $R_2 = OCH_3$, $R_3 = OH$
2: $R_1 = (CH_2)_2OH$, $R_2 = OCH_3$, $R_3 = OH$
3: $R_1 = CH_2COOH$, $R_2 = OCH_3$, $R_3 = OH$
4: $R_1 = CH_2COOH$, $R_2 = OCH_3$, $R_3 = OCH_3$
5: $R_1 = (CH_2)_2COCH_3$, $R_2 = OCH_3$, $R_3 = OH$
6: $R_1 = OH$, $R_2 = OCH_3$, $R_3 = OH$
7: $R_1 = (CH_2)_3OH$, $R_2 = OCH_3$, R_3= OH
8: $R_1 = (CH_2)_4OH$, $R_2 = OCH_3$, $R_3 = OH$
9: $R_1 = CH_2NH_2$, $R_2 = OCH_3$, $R_3 = OH$
10: $R_1 = CH_2CN$, $R_2 = OCH_3$, $R_3 = OH$
11: $R_1 = CH_2COOH$, $R_2 = NO_2$, $R_3 = OH$
12: $R_1 = CH_2COOH$, $R_2 = OH$, $R_3 = OH$
13: $R_1 = CH_2COOH$, $R_2 = H$, $R_3 = OH$
14: $R_1 = (CH_2)_2COCH_2CH(OH)(CH_2)_4CH_3$, $R_2 = OCH_3$, $R_3 = OH$
15: $R_1 = (CH_2)_2COCH{=}CH(CH_2)_4CH_3$, $R_2 = OCH_3$, $R_3 = OH$

Figure 1. Structure of active compounds from *Z. officinale* Roscoe.

levels were measured by portable kit, Antsence II (Sankyo Co. Ltd., Tokyo, Japan) every 7 days. Normal rats (group A) were fed on a basal 18% casein diet. The other three groups were fed on 18% casein diet containing 50% galactose and were given 5% gum arabic (group B) or 2-(4-hydroxy-3-methoxyphenyl)ethanol (**2**) (400 mg/kg of body weight/day) or 2-(4-hydroxy-3-methoxyphenyl)ethanoic acid (**3**) (400 mg/kg of body weight/day) orally via a stomach tube.

Determination of Lens Galactitol Levels. After 21 days, rats were sacrificed by CO_2 asphyxiation, and their eyeballs were surgically excised. The lenses were carefully dissected under sterile conditions (*33*). Lens material was weighed and homogenized in 20% ice-cold acetonitrile (1 mL). The sample and methyl α-D-mannopyranoside (0.1 μM) as internal standard were mixed and centrifuged to eliminate proteins. The resulting supernatant was lyophilized. Sugar alcohols were trimethylsilylated using Tri-Sil reagent (GL Sciences, Inc., Tokyo, Japan). After the addition of 1 mL of the silylating reagent, the tube was placed in an incubating oven at 60 °C for 30 min. Analyses were performed on a GL Science GC353 and Supelco SPB-1 capillary column (30 mm × 0.32 mm × 0.25 μm) coated with cross-linked methyl silicone. Nitrogen was used as the carrier gas, and column temperature was raised from 120 to 265 °C at 5 °C/min and then to 295 °C at 10 min (*34*, *35*).

RESULTS AND DISCUSSION

Isolation and Characterization of Active Compounds. Bioassay-guided fractionation of the extract was performed. Hot water extracts of *Z. officinale* were filtered through Celite, applied to a Diaion HP-20 column, and eluted with 0−100% MeOH. Among them, 50 and 75% aqueous MeOH extracts showed significant inhibitory activity against aldose reductase (88.1 and 88.0% at 100 μg/mL, respectively), whereas H_2O and 100% MeOH extracts were ineffective. Further chromatography of these active fractions using Sephadex LH-20 and Toyopeal HW-40S gave five compounds active against aldose reductase. By direct comparison with authentic samples, their structures were identified as (4-hydroxy-3-methoxyphenyl)methanol (**1**), 2-(4-hydroxy-3-methoxyphenyl)ethanol (**2**), and 2-(4-hydroxy-3-methoxyphenyl)ethanoic acid (**3**), 2-(3,4-dimethoxyphenyl)-ethanoic acid (**4**), and 4-(4-hydroxy-3-methoxyphenyl)-2-butanone (**5**) (**Figure 1**).

Inhibition of Aldose Reductase. The inhibitory activities of compounds **1**−**5** against aldose reductase were compared with that of quercetin, a natural aldose reductase inhibitor (*36*). As shown in **Table 1**, compounds **1**, **2**, and **3** exhibited slightly better inhibition than quercetin ($IC_{50} = 27.0 \pm 3.8$ μM), with IC_{50} values of 24.4 ± 4.6, 19.2 ± 1.9, and 18.5 ± 1.1 μM, respectively, and the inhibitory potentials of compound **4** and quercetin were almost the same. On the other hand, compound **5** ($IC_{50} = 197 \pm 12$ μM) was a much weaker inhibitor than quercetin.

Table 1. Concentration of the Phenolic Compounds Giving 50% Inhibition of Aldose Reductase Activities

compound	IC_{50}[a] (μM)	compound	IC_{50}[a] (μM)
1	24.4 ± 4.6	**9**	76.9 ± 6.3
2	19.2 ± 1.9	**10**	63.4 ± 7.4
3	18.5 ± 1.1	**11**	NI[b]
4	30.3 ± 2.5	**12**	NI
5	197 ± 12	**13**	NI
6	273 ± 16	**14**	NI
7	283 ± 37	**15**	NI
8	642 ± 26	quercetin	27.0 ± 3.8

[a] Inhibitory activity is expressed as the mean of 50% inhibitory concentration (IC_{50}). Expressed as mean ± SD ($n = 3$). [b] NI, <50% inhibition at 1000 μM.

Interestingly, [6]-gingerol (**14**) and [6]-shogaol (**15**), well-known as major products of *Z. officinale*, exhibited <50% inhibition, even at concentrations as high as 1 mM. These results mean that the presence of the side alkyl chains is essential for recognition of aldose reductase. As shown in **Table 1**, 4-hydroxy-3-methoxyphenol (**6**) ($IC_{50} = 273 \pm 16$ μM), which does not have the side alkyl chain, was a much weaker inhibitor than compounds **1** and **2**. On the other hand, the extension of the alkyl chain length, such as in 3-(4-hydroxy-3-methoxyphenyl)-propanol (**7**) ($IC_{50} = 283 \pm 37$ μM) and 4-(4-hydroxy-3-methoxyphenyl)butanol (**8**) ($IC_{50} = 642 \pm 26$ μM), apparently depressed the inhibitory potential. From a comparison of the potency of these compounds, it appears that an increasing length of the side alkyl chain is associated with a depressed inhibitory potential, and the C_1-C_2 alkyl length is suitable for potent inhibition.

The replacement of the substitution at side alkyl chain terminal of **1** by the NH_2 and CN groups to give (4-hydroxy-3-methoxyphenyl)methamine (**9**) and (4-hydroxy-3-methoxyphenyl)acetonitrile (**10**) interfered with their binding to the enzyme and showed about a 4-fold lower potency (IC_{50} values of 76.9 ± 6.3 μM for **9** and 63.4 ± 7.4 μM for **10**). In contrast, the replacement of the terminal OH group of **1** by the COOH group to give **3** did not affect the inhibitory potential. The replacement of the C3 OCH_3 group in **3** by the NO_2 and OH groups to give 2-(4-hydroxy-3-nitrophenyl)ethanoic acid (**11**) and 2-(3,4-dihydroxyphenyl)ethanoic acid (**12**) or the removal of the C3 OCH_3 group to give 2-(4-hydroxyphenyl)ethanoic acid (**13**) abolished their inhibition toward the enzyme. In contrast, the replacement of the C4 OH group in **3** by the OCH_3 group to give **4** did not affect the inhibitory potential ($IC_{50} = 30.3 \pm 2.5$ μM). These results suggest that the presence of the C3 OCH_3 group in the aromatic ring is an essential feature for recognition and strong binding.

Inhibition of Sorbitol Accumulation on Erythrocytes. Orally active aldose reductase inhibitors belong to two different chemical classes including hydantoin derivatives and carboxylic acid derivatives. Even though both types show almost the same significant effect on enzyme prperties, their cell and in vivo results are often quite different (*22*, *23*). Therefore, we investigated the effect of active compounds isolated from *Z. officinale* on the accumulation of sorbitol in human erythrocytes. It has already been reported that the activity of erythrocyte aldose reductase increases in diabetic patients (*37*) and that erythrocyte sorbitol levels in rats are positively correlated with the levels in the lens, sciatic nerve, and retina (*38*, *39*). The

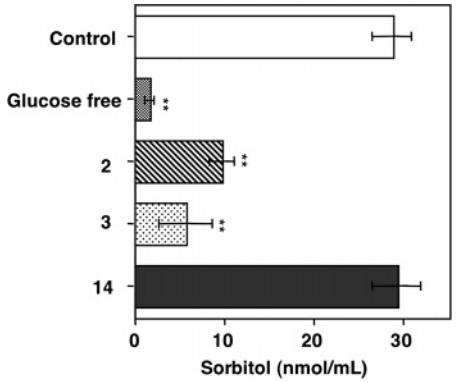


Figure 2. Effects of 2-(4-hydroxy-3-methoxyphenyl)ethanol (**2**) and 2-(4-hydroxy-3-methoxyphenyl)ethanoic acid (**3**) on sorbitol accumulation in human erythrocyte. Erythrocyte was incubated for 60 min in a Krebs–Ringer bicarbonate buffer containing 28 mM glucose and in the presence or absence of 40 μM compounds. Each value represents the mean ± SEM ($n = 5$). **, significant difference ($p < 0.01$) compared with the control.

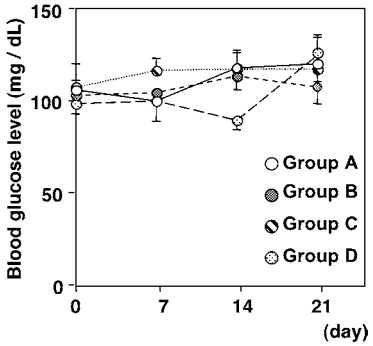


Figure 3. Effects of 2-(4-hydroxy-3-methoxyphenyl)ethanol (**2**) and 2-(4-hydroxy-3-methoxyphenyl)ethanoic acid (**3**) on blood glucose level. Blood glucose levels were monitored by portable kit, Sankyo Antsence II, every 7 days. Group A, normal diet; group B, 30% galactose-fed; group C, 30% galactose-fed and compound **2** (400 mg/kg of body weight/day); group D, 30% galactose-fed and compound **3** (400 mg/kg of body weight/day). Each value represents the mean ± SEM ($n = 3$).

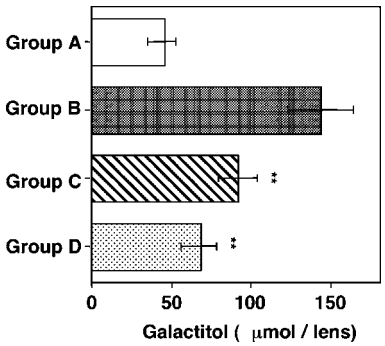


Figure 4. Effects of 2-(4-hydroxy-3-methoxyphenyl)ethanol (**2**) and 2-(4-hydroxy-3-methoxyphenyl)ethanoic acid (**3**) on lens galactitol accumulation in 30% galactose-fed rats. Galactitol was measured as described under Materials and Methods. Group A, normal diet; group B, 30% galactose-fed; group C, 30% galactose-fed and compound **2** (400 mg/kg of body weight/day); group D, 30% galactose-fed and compound **3** (400 mg/kg of body weight/day). Each value represents the mean ± SEM ($n = 3$). **, significant difference ($p < 0.01$) compared with the control.

effects of **2**, **3**, and **14** on sorbitol accumulation in human erythrocytes are shown in **Figure 2**. Sorbitol accumulation was 18-fold greater when the cells were incubated in high-glucose medium, compared to that in glucose-free incubation. Compounds **2** and **3** inhibited sorbitol accumulation by almost 66.2 ± 1.4 and 80.1 ± 2.9% at 40 μM, respectively. On the other hand, compound **14**, with no inhibitory activity against aldose reductase, gave no effect on sorbitol accumulation in the cells. Sorbitol dehydrogenase catalyzes the conversion of sorbitol to fructose in the presence of NADH. Although we furthermore studied their effect on sorbitol dehydrogenase activity, they gave no effect on the activity. These results suggest that these compounds have good absorption and tissue penetration, because their inhibitory potencies are the same as those in the enzyme assays.

Inhibition of Lens Galactitol Accumulation in Galactose-Fed Rat. The accumulation of polyols such as sorbitol or galactitol are thought to be responsible for the development of cataracts (*40*). Lens changes occur more rapidly under galactosemic conditions because glucose is converted to fructose by aldose reductase and sorbitol dehydrogenase in the sorbitol pathway, but galactitol is not further metabolized by sorbitol dehydrogenase. Moreover, the onset and progression of retinal changes are more rapid in galactosemia than other diabetes models (*41*). There was no significant effect of feeding galactose or test samples on body weight of the animals during this experiment. Although blood glucose level was monitored at 7 day intervals for 21 days, compounds **2** and **3** did not affect this parameter, as shown in **Figure 3**. Lens galactitol levels were significantly higher in group B (30% galactose-feeding group), compared to group A (normal diet-feeding group). Compounds **2** (group C) and **3** (group D) significantly inhibited galactitol accumulation by 38.7 ± 5.4 and 58.7 ± 5.2% at 400 mg/kg of body weight/day, respectively (**Figure 4**). Moreover, groups C and D did not get opacification of the lens, but group B developed cataract after two weeks. These results clearly indicate that 30% galactose feeding promotes the accumulation of lens galactitol, as seen in group B, and compounds **2** and **3** can suppress this formation, as seen in groups C and D.

In summary, we discovered a new biological activity, an aldose reductase inhibitory activity, of five components isolated from ginger. Some of them significantly suppressed not only sorbitol accumulation in human erythrocytes but also galactitol accumulation in the lens in animal models. The clinical studies have shown that the degree of aldose reductase inhibition required for long-term protection is much greater than that required for a short-term effect (*42*). As a dietary supplement, ginger or its extract containing aldose reductase inhibitors would be potentially useful in the prevention and self-medication of diabetic complications.

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