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Effects of Furanocoumarins from *Angelica Dahurica* on Aldose Reductase and Galactosemic Cataract Formation in Rats*

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The ether extract from the roots of *Angelica dahurica* was found to inhibit bovine lens aldose reductase (BLAR) activity *in vitro* by 100% at 100 μg/ml. Systematic fractionation of the ether soluble fraction and subsequent active fractions monitored by bioassay led to isolation of four furanocoumarins, isoimperatorin (I), imperatorin (II), ter-O-methyl byakangelicin (III) and byakangelicin (IV), among which compound III and IV were identified as potential AR inhibitors, their IC_{50} values being 2.8 μM and 6.2 μM, respectively. Galactosemic cataract formation in rats treated with 40 g/kg/day of galactose was blocked almost completely throughout the experimental periods up to 44 days by i.p. administrations of byakangelicin (IV) at 50 mg/kg/day. In coincidence with the inhibitory action on cataract formation, the galactitol accumulation in rats treated with byakangelicin (IV) was found to be markedly prevented by approximately 80.5% compared to those of the control. These results indicate that byakangelicin (IV), as a main principle of this plant, possesses high potential for a clinically useful drug of the future which prevents and/or improves sugar cataract as well as diabetic complications.

Key words : *Angelica dahurica*, Umbelliferae, furanocoumarins, byakangelicin, aldose reductase, galactosemic cataract, galactitol

INTRODUCTION

The enzyme aldose reductase (AR) which brings about intracellular accumulation of sorbitol or galactitol in the polyol pathway of aldose metabolism has been demonstrated to play important roles not only in the cataract formation in the lens (Van Heyningen, 1959; Pirie and Van Heyningen, 1964) but also in the pathogenesis of diabetic complications such as neuropathy (Ward, 1973), retinopathy (Engerman and Kern, 1984) and nephropathy (Beyer-Mears *et al.*, 1984), *etc.*.

Evidence, therefore, suggests that compounds which inhibit AR are expected to be effective in preventing sugar cataract formation as well as in diabetic complications.

Recently, a number of structurally diverse AR inhibitors (ARI) not only of synthetic but also of natural origin, thus have been extensively studied to clarify their *in vivo* efficacies for prevention of cataract formation as well as diabetic complications in experimental animals (Dvornic *et al.*, 1973) and even in clinical trials (Handelsman and Turtle, 1981).

In a previous communication (Shin *et al.*, 1993), on a survey for potential ARI's from medicinal plants, we have shown that some hot water extracts from herbal medicines exhibited a significant inhibition of bovine lens AR (BLAR) *in vitro*, among which the roots of *Angelica dahurica* (Umbelliferae) was evaluated to have relatively potent AR inhibitory activity (>90% at 100 μg/ml).

This report deals with identification and characterization of potential ARI's from this plant and their effects on galactose-induced cataract formation in rats.

MATERIALS AND METHODS

Plant Material

The dried roots of *Angelica dahurica* Benth, et. Hook (Umbelliferae) were purchased from a local market (Kyong Dong, Seoul) and botanically identified by Dr. H. J. Chi of this Institute. A voucher specimen was

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*Part 3 of Studies on the Inhibitory Effects of Medicinal Plant Constituents on Cataract Formation.

Table I. ^{1}H-NMR data of coumarins (Comp. I-IV)

Compound	H-3	H-4	H-5	H-8(6)	H-2′	H-3′	H-a	H-b	H-c	H-d	H-e
I	6.26	8.15	–	7.10	6.86	7.60	4.90	5.50	1.70(s)	–	–
	d(9.8)	d(9.8)		s	d(2.0)	d(2.0)	d(7.0)	tq(7.1)	1.82(s)		
II	6.35	7.74	7.34	–	6.79	7.67	4.99	5.60	1.72	–	–
	d(9.6)	d(9.6)	s		d(2.0)	d(2.0)	d(7.0)	tq(7.1)	d(1)		
III	6.25	8.10	–	–	7.00	7.60	3.85-4.30	4.60	1.23	3.23	4.15
	d(9.8)	d(9.8)			d(2.4)	d(2.4)	m	dd	s	s	s
IV	6.27	8.11	–	–	7.00	7.63	4.14-4.36	4.60	1.31(s)	–	4.17
	d(9.8)	d(9.8)			d(2.4)	d(2.4)	m	dd	1.27(s)		s

Spectra were measured at 80 MHz in $CDCl_3$ and chemical shifts were expressed in δ scale.
Signals are designated as follows: s, singlet; t, triplet; q, quartet.
Figures in parentheses are coupling constants in Hz.

deposited in the Herbarium of this Institute.

Instruments and Reagents

The melting points were taken on a Mitamura Riken (Model MRK No. 4204) melting point determining apparatus and uncorrected. ^{1}H-NMR spectra were taken with a Varian FT-80A NMR spectrometer. UV spectra were obtained by a Gilford system (No.2600) UV spectrophotometer. Sorvall Ultracentrifuge, OTD65 B (Dupont); Gas chromatograph, Hewlett Packard 5840 and Lyophilizer, FTS system were also used. Column chromatography was performed using 70-230 mesh silica gel 60 (E.Merck 7734) and vacuum liquid chromatography using silica gel G (E.Merck 7731 or 7729). DL-glyceraldehyde, NADPH, galactose, and galactitol were purchased from Sigma Chem. Co. (ST Louis, M. O., USA). All other chemicals and reagents used were of the first grade commercially available. Bovine eyes for preparation of enzyme source were obtained from a local abattior soon after slaughtering. The intact lenses removed were frozen at −60℃ until used.

Isolation of Compounds

The dried and coarsely powdered roots (1 kg) were extracted 3 times with hot methanol refluxing for 5 hrs on a water bath. The methanol extract was concentrated under reduced pressure and fractionated into hexane, ether and ethylacetate fractions. Column chromatography of the hexane fraction over silica gel using hexane-ether as an eluent (gradient) gave 13 subfractions. Preparative VLC of subfr. 9 and 10 with (hexane →ether) gave compound I as colorless needle crystals (mp. 108℃, 0.5 g).

Preparative VLC of subfr. 12 gave compound II as colorless needle crystal (mp. 106℃, 0.2 g). The ether fraction was concentrated to a dark brown residue which was subjected to SiO2 column chromatography and VLC using benzene-ether-ethylacetate (gradient) gave subsequently compound III as pale yellow powders (mp. 88℃, 0.15 g) and compound IV as pale yellow powders (mp. 118℃, 0.8 g). The compounds isolated were identified by NMR data (Table I).

Preparation of Crude Aldose Reductase

Aldose reductase from bovine lens was partially purified according to the method described by Hayman and Kinoshita (1965) with slight modification. Lenses were homogenized in 5 volumes of ice cold 5 mM phosphate buffer (pH 7.4) in a Waring blender (Omni mixer 17105, Dupont instrument) and centrifuged at 18,000 g for 15 min to remove insoluble materials. The supernatant fluid was added with saturated ammonium sulfate until 40% saturation.

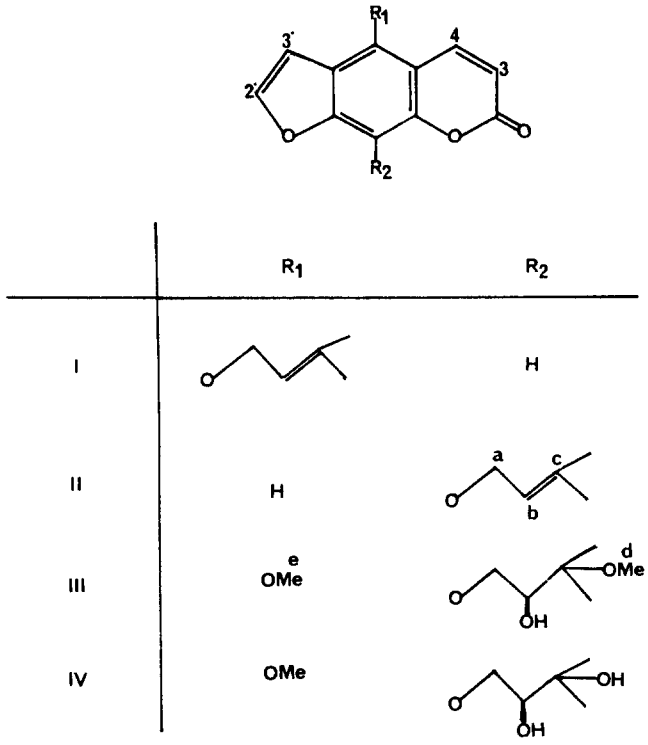
The thick suspension was allowed to stand for 15 min with occasional stirring to ensure complete precipitation and centrifuged.

The supernatant fraction thus obtained was used for AR enzyme sources without further purification.

Determination of Aldose Reductase Activity *in vitro*

The AR activity was estimated at room temperature *in vitro* according to the method of Brubaker *et al.* (1986) with slight modification with a reaction mixture of 1 ml containing 0.4 M ammonium sulfate, 0.16 mM NADPH, 10 mM DL-glyceraldehyde in 0.1 M phosphate buffer (pH 6.2). The enzymatic reaction was initiated by the addition of substrate and the activity was measured by recording the decrease in absorbance at 340 nm with a Gilford 2600 UV spectrophotometer. Nonspecific reduction of NADPH in the absence of substrate was subtracted from the total activity. Enzyme activity was adjusted by dilution with water so that 0.2 ml of enzyme solution gave an average reaction rate for the control reactions of 0.055± 0.005 absorbance unit/5 min.

The effect of an inhibitor on the enzyme activity was determined by the addition of the inhibitor solu-



tion in dimethylsulfoxide (DMSO) at the desired concentration (0.1%). IC_{50} values, the concentration of inhibitors required to produce 50% inhibition, were calculated from regression equations obtained by plotting% inhibitory activity against at least three graded log concentrations of an inhibitor.

Induction of Galactosemic Cataracts and Treatment of Drugs

The galactose-induced cataracts in rats were triggered by the method employed by Okuda (1985). Male rats weighing 80-100 g were administered orally with galactose solution suspended in 0.9% NaCl at doses of 20 or 40 g/Kg/day twice a day (10 : 00 AM and 7 : 00 PM) for upto 44 days. The galactose-treated rats were administered i.p. once a day with byakangelicin (IV) or imperatorin (II) suspended in 0.5% CMC from day 1 throughtout the experimental period. The control rats received vehicle only. The development of cataract was examined by observing the lens periphery every two days using a pen light. The appearance of apparant opalcification in the peripheral region of a lens was regarded as the onset of cataract formation.

Measurement of Galactitol Accumulation in Lens

Galactitol accumulated in freshly excised lenses were analyzed by gas-liquid chromatography. At the end of the experimental period or at definite intervals, rats were killed by ether anesthesia and their lenses were removed and weighed. Excised lenses were prepared by homogenization in a solution of 0.3 N $ZnSO_4$, deproteinized with 0.3 N $Ba(OH)_2$ and rehomogenized. The resulting precipitate was centrifuged and the supernatant containing lens sugars was lyophilized for 18 hr. The lyophilized samples were then derivatized with acetic anhydride in pyridine. Standards of galactitol were prepared either individually or in a mixture, lyophilized and derivatized as the lens samples.

Table II. Effects of various fractions from A.dahuricae Radix on lens AR

Treatment	AR inhibition (%)		
	1.0	0.5	0.1 (mg/ml)[a]
n-Hexane fr.	100	100	25.5
Ether fr.		100	100
n-Butanol fr.	100	100	76.3
H_2O fr.		58.2	34.2

[a] Final concentration in the reaction mixture.

Table III. IC_{50} values of furanocoumarins from A. dahuricae Radix against BLAR

Compound No.	IC_{50} (μM)[a]
I	22
II	10
III	2.8
IV	6.2
TMG	~10[b]

[a] IC_{50} values were calculated by regression analysis of data ploted on logit-log paper.
[b] IC_{50} value of tetramethyleneglutaric acid (TMG), a reference compound.

RESULTS

Evaluation of Active Principles for Inhibition of AR *In vitro*

The effect of various fractions obtained by partitioning the methanol extracts successively with n-hexane, ether, n-butanol on BLAR were evaluated *in vitro* and the results were shown in Table II.

A complete inhibition of AR activity was shown at 500 μg/ml of all fractions except H_2O fraction, among which the ether soluble fraction exhibited the most potent inhibitory activity, i.e., 100% inhibition even at a 100 μg/ml concentration. Further fractionation and chromatographic separations of ether soluble fraction resulted in the isolation of four pure compounds (Comp. I-IV) and their structure elucidation demonstrated that those are known furanocoumarins already separated from this plant (Hata *et al.*, 1963). The four furanocoumarins isolated were subjected to test their inhibitory activities of BLAR *in vitro* and the results

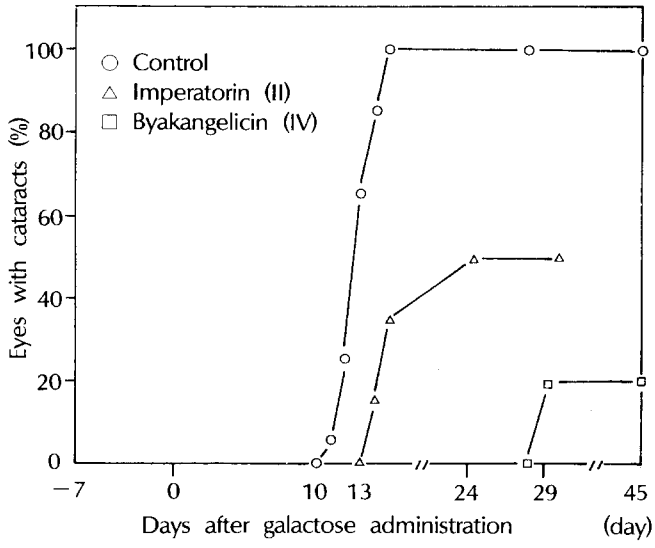


Fig. 1. Effect of pretreatments on cataract formation induced by galactose in rats.
Rats were administered with furanocoumarins (50 mg/kg/day, i.p., suspended in 0.5% CMC. ○, Control; □, byakangelicin (IV); △, imperatorin (II)) from 7 days prior to the onset of galactose administrations (40 g/kg/day, p.o.) throughtout the experimental periods upto 44 days. Each group consisted of 6 rats. The appearance of vacuoles at the lens periphery was judged with a slit lamp.

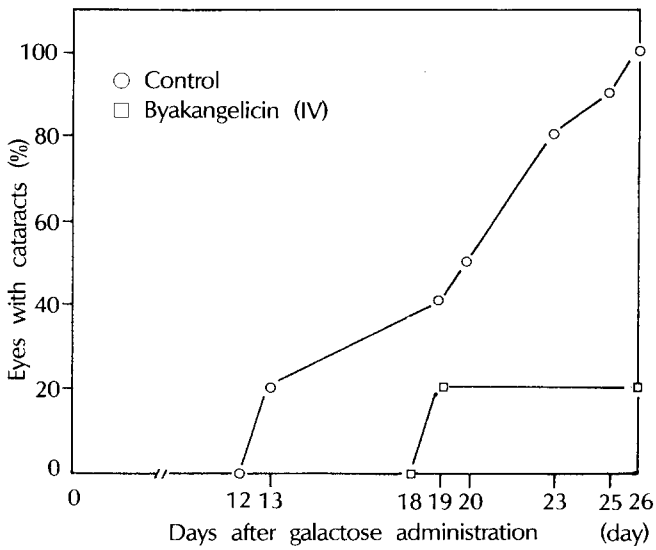


Fig. 2. Effect of simultaneous treatments with galactose on cataract formation in rats. Rats were administered with byakangelicin (IV) (50 mg/kg/day, i.p.) and galactose (20 g/kg/day, p.o.) concommitantly throughtout the experimental periods upto 25 days.

were shown in Table III. It was demonstrated that all of the compounds exhibited inhibition of BLAR in a concentration-dependent manner, and comparison of their inhibitory potencies by IC_{50} values indicated that ter-O-methyl byakangelicin (III) was the most potent ($IC_{50}=2.8$ μM) and the potencies were decreased in the order of comp. IV, II, I. Byakangelicin (IV), although a little less potent ($IC_{50}=6.2$ μM) than ter-O-methyl byakangelicin (III), was almost equipotent to TMG, known as one of typical AR inhibitors *in vitro*.

Effects of Furanocoumarins on Galactosemic Cataract Formation *In vivo*

The effects of pretreatment of byakangelicin (IV) and imperatorin (II) on cataract formation in galactose-treated rats were tested and the results were illustrated in Fig. 1. By day 15, on galactose treatments (40 g/kg/day), all of the eyes of the control animals developed an early-stage cataract which was judged by the appearance of vacuoles at the lens periphery. Only two of twelve eyes of rats dosed with byakangelicin (IV) at 50 mg/kg/day, i.p., from 7 days prior to galactose administration showed a cataract formation on day 29, but no further vacuole formation in other animals was observed during the period of experiment upto 45 days. Imperatorin (II) was far less effective and with its treatment at the same dosage, 50% of the animals showed the development of cataract on day 24.

In another series of experiment, rats were administered with the test compound concommitantly with the onset of galactose administrations and were observed their effects on cataract formation. As indicated in Fig. 2, even though all of the eyes of the control rats received galactose (20 g/kg/day) have developed a cataract by day 26, only two of ten eyes of rats given byakangelicin (IV) at 50 mg/kg/day, i.p., showed lenticular vacuole formation on day 19, but no further development of the cataract could be observed throughout the experimental periods.

In coincidence with the inhibitory action on the cataract formation, galactitol accumulation in rats treated with byakangelicin (IV) was demonstrated to be prevented significantly as illustrated in Table IV.

The galactitol contents measured in lens obtained from rats after consecutive treatments with byakangelicin (IV) from 7 days prior to the start of galactose administrations upto 44 days, was found to be markedly decreased by approximately 80.5% compared to those of the control.

DISCUSSION

It has been reported that lens AR plays a central role in the reduction of aldose to polyol and cataract formation in diabetes and galactosemia is triggered by the accumulation in the lens of excessive sorbitol or galactitol synthesized by the action of AR (Kinoshita, 1974). ARIs thus have been shown to prevent or delay significantly sugar-induced cataractogenesis in rats and

Table IV. Effect of byakangelicin (IV) on galactitol accumulation in rat lens with cataract induced by galactose

Treatment	Dose (mg/kg/day)	Galactitol (μmoles/ g lens)[a]	% of control
Control		17.4± 2.7	100
Byakangelicin (IV)	50	3.4± 0.6*	19.5

Rats were injected i.p. with byakangelicin (IV) from 7 days prior to onset of galactose administrations (40 g/kg/day) throughout the experimental periods upto 44 days, and galactitol contents were assayed on the 45th day.
[a] Data are means± S.E. of 4 rats. Significantly different from the control: *$p<0.01$.

several synthetic ARIs are currently available and many have been tested for their clinical use, albeit with limited success (Raskin and Rosenstock, 1987), i.e.; Synthetic compounds with diverse structures such as sorbinil (Beyer-Mears and Cruz, 1985), epalrestat (Terashima *et al.*, 1982) other hydantoin derivatives (Inagaki *et al.*, 1982) etc., and flavonoids (Shimizu, 1984) and isoliquiritigenin (Aida *et al.*, 1990) from natural origin have been extensively studied and reported to inhibit AR. The present study was carried out in a search for a new potential AR inhibitors useful for the treatment of galactosemic as well as for diabetic cataract from *Angelica dahurica* roots and we found that byakangelicin (IV) the most promising active principles utilizable as a lead compound, because this compound not only inhibited AR *in vitro* but also prevented from the formation of galactosemic cataract *in vivo*, and furthermore, it was a main component peculiar to this plant part.

Recently, although ONO-2235, a synthetic compound being utilized for treatment of diabetic peripheral neuropathy, has been reported to possess a strong AR inhibitory activity *in vitro*, it shows a very weak suppressive effect on galactose-induced cataract formation in rats *in vivo* (Kato, 1991).

Byakangelicin (IV), on the contrary, which was approximately 100 times less potent in AR inhibition than that of ONO-2235 ($IC_{50}=7\times10^{-8}$ M level) *in vitro*, was found to exhibit a highly potent inhibitory action of galactosemic cataract formation in rats *in vivo*. These results strongly suggested that AR inhibitory activity *in vitro* does not necessarily directly related to the *in vivo* efficacy for prevention of cataract formation.

The reason for such differences in direct enzyme inhibitory potencies and *in vivo* efficacies between byakangelicin (IV) and ONO-2235 may be explained by the facts that AR enzymes are only the initiating factor in sugar cataract formation and possess rather broad substrate specificity (Hayman *et al.*, 1966) and thus their susceptibilities are different from one substrate to others (Poulsom, 1987). It can also be postulated that byakangelicin (IV) might have effects on other metabolic pathways and, thus, reach the target tissue *in vivo* more easily than ONO-2235.

Protective effects against lipid peroxidation (Jacques *et al.*, 1988) or protein denaturation caused by quinoid derivatives produced by abnormal metabolism of amino acids in the lens (Nakagaki *et al.*, 1965) might be considered as other possible factors pertaining to *in vivo* efficacy of byakangelicin (IV), although its true mechanism of actions are remained to be elucidated.

We already demonstrated, however, that byakangelicin (IV) exhibited a significant inhibition of rat hepatic microsomal lipid peroxidation *in vitro* (Shin *et al.*, 1993).

It is concluded, therefore, that byakangelicin (IV), as a main principle of this plant, could be offered as a leading compound for further study as a new drug for sugar cataract and possibly for diabetic complications.

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