Isolation of Aldose Reductase Inhibitors from the Flowers of *Chrysanthemum boreale*

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The methanol extract from the whole parts of the flowers of *Chrysanthemum boreale* was found to exhibit a significant inhibition of rat lens aldose reductase (RLAR) activity *in vitro*. Bioassay guided systematic fractionation of the methanol extract led to the isolation of four flavonoids which were identified as acacetin (I), apigenin (II), luteolin (III) and linarin (IV). Compounds I-III were demonstrated to exhibit a significant inhibition of RLAR. Luteolin (III) was found to be the most potent AR inhibitor with IC_{50} value of 5×10^{-7}M.

Key works : *Chrysanthemum boreale*, Aldose reductase inhibition, Flavonoids, Luteolin, Acacetin, Apigenin

INTRODUCTION

The enzyme, aldose reductase (AR), together with sorbitol dehydrogenase, which catalyzes the reduction of aldose to polyol, have been demonstrated to play a central role in the cataract formation in galactosemia and diabetes (Kinoshita and Nishimura, 1988). AR inhibitors, therefore, are considered to be effective in preventing cataract onset and various diabetic complications. In the course of a series of studies for the purpose of evaluating naturally occurring AR inhibitors, we found that the methanol extract from the flowers of *Chrysanthemum boreale* to be used in the treatment of headache, eye diseases, etc. in Chinese medicines exhibited a significant inhibitory activity against rat lens AR *in vitro*. Based on this result, bioassay directed fractionation of the methanol extract led to isolation of four flavonoid compounds, among which luteolin (III) was found to be the most potent AR inhibitor with an IC_{50} value of 5×10^{-7}M.

MATERIALS AND METHODS

Plant Materials

The whole parts of the flowers of *Chrysanthemum boreale* (Compositae) were collected in late Sept. 1992 in the Jin-Bu area, Kangwon province. The bo-

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tanical identity was confirmed by Dr. Cho, S.H. and the voucher specimens are deposited in the herbarium of this Institute.

Instruments and Reagents

Melting points were determined on a Mitamura Riken (No.4204) apparatus and are uncorrected. ^{1}H-and ^{13}C-NMR spectra were measured on a Varian FT-80A (80 MHz) or a Bruker AM-300 (300 MHz) spectrometer. MS were measured on a Hewlett Packard Model 5985B GC/MS system. UV absorption spectra were determined by Gilford 2600 UV-Vis spectrophotometer. NADPH, phenylmethylsulfonylfluoride (PMSF), 2-mercaptoethanol, DL-glyceraldehyde and tetramethyleneglutaric acid (TMG) were purchased from Sigma Chem. Co. (St. Louis). Other reagents were the first grade commercially available.

Fractionation and Isolation of Compounds

The dried flowers (265 g) were extracted with hot methanol three times and concentrated under reduced pressure to dryness, which was again separated into chloroform (25.7 g), ethylacetate (2.6 g) and n-butanol (7.9 g) soluble fractions. The ethylacetate soluble fraction which had the most potent AR inhibitory activity was subjected to silca gel column chromatography by eluting with ethylacetate, methanol-ethylacetate (gradient, 1-20%) to obtain 7 subfractions. Further fractionation of subfr. No. 2 on a silica gel column by eluting with

methanol-chloroform (gradient, 5-9%) and subsequently with Sephadex LH-20, led to isolation of compounds I-IV. These four compounds were identified as acacetin (I), apigenin (II), luteolin (III) and linarin (IV) by spectral data and comparing with those in the literature (Mabry *et al.*,1970; Harborne *et al.*, 1975; Harborne and Mabry, 1982; Do *et al.*, 1991).

Compound I (acacetin): Yellow powder (recrystallized from methanol); m.p. 266-268°C; EIMS (70 eV) m/z (rel. int., %): 284 (M^+, 90), 269 (4.9), 256 (13.6), 241 (63.8), 213 (19.1), 153 (5.8), 152 (38.2), 132 (100.0), 124 (14.9), 117 (38.0); ^{1}H-NMR (80 MHz, DMSO-d_6)δ: 3.85 (3H, s, OMe), 6.19 (1H, br.s, H-6), 6.49 (1H, br.s, H-8), 6.84 (1H, s, H-3), 7.10 (2H, d, J =8.0Hz, H-3', H-5'), 8.02 (2H, d, J =8.0Hz, H-2', H-6'), 12.89 (1H, br.s, 5-OH).

Compound II (apigenin): Yellow powder (recrystallized from methanol); m.p. 345-350°C; EIMS (70 eV), m/z (rel. int., %): 270 (M^+, 100.0), 269 (16.8), 242 (39.1), 241 (13.6), 153 (60.4), 152 (37.4), 124 (44.0), 121 (40.1), 118 (32.1).; ^{1}H-NMR (300 MHz, DMSO-d_6)δ:6. 19(1H, d, J=1.9Hz, H-6), 6.48 (1H, d, J=1.9Hz, H-8), 6.76 (1H, s, H-3), 6.93 (2H, d, J=8.8Hz, H'-3, H-5'), 7.92 (2H, d, J=8.7Hz, H-2', H-6'), 10.77 (1H, br.s, OH), 10.30 (1H, br.s, OH), 12.94 (1H, s, 5-OH).

Compound III (luteolin): Yellow powder (recrystallized from methanol); m.p. 328°C; EIMS (70 eV), m/z (rel. int., %): 286 (M^+, 63.8), 258 (37.4), 229 (19.3), 153 (100.0), 152 (21.9), 137 (9.7), 134 (41.5), 124 (31.9), 123 (16.8); ^{1}H-NMR (300MHz, DMSO-d_6)δ:6.18 (1H, d, J=2.0Hz, H-6), 6.43 (1H, d, J=2.0Hz, H-8), 6.63 (1H, s, H-3), 6.89 (1H, d, J=9.0Hz, H-5'), 7.38 (1H, s, H-2'), 7. 45 (1H, dd, J=2.2, 9.0Hz, H-6'), 9.31, 9.81, 10.74 (br.s, 3xOH), 12.96 (1H, s, 5-OH).

Compound IV (linarin): Yellow powder (recrystallized from methanol); m.p.269-271°C; ^{1}H-NMR (300 MHz, DMSO-d_6)δ: 1.09 (3H, d, J=6.2Hz, Me of rhamnose), 3.87 (3H, s, OMe), 4.57 (1H, s, anomeric H of rhamnose), 5.06 (1H, d, J=7.2Hz, anomeric H of glucose), 6.46 (1H, d, J=1.9Hz, H-6), 6.79 (1H, d, J=1.9Hz, H-8), 6.91 (1H, s, H-3), 7.15 (2H, d, J=8.9Hz, H-3', H-5'), 8.04 (2H, d, J=8.9Hz, H-2', H-6'), 12.88 (1H, s, 5-OH);^{13}C-NMR (75.5MHz, DMSO-d_6) : 17.65 (C-6'''), 55.45 (OCH$_3$), 66.04 (C-6''), 68.21 (C-5'''), 69.57 (C-4'''), 70.26 (C-2'''), 70.71 (C-3'''), 72.01 (C-4'''), 73.02 (C-2''), 75.63 (C-5''), 76.22 (C-3''), 94.73 (C-8), 99.61 (C-1''), 100.00 (C-6), 100.43 (C-1'''), 103.74 (C-3), 105.40 (C-10), 114.61 (C-3', C-5'), 122.62 (C-1'), 128. 32 (C-2', C-6'), 156.88 (C-9), 161.06 (C-5), 162.34 (C-7), 162.88 (C-4'), 163.87 (C-2), 181.88 (C-4).

Measurement of Aldose Reductase Activities

The effects of the test materials on rat lens AR were estimated as described previously (Brubaker *et al.*, 1986). In brief, rat lenses were homogenized and centrifuged at 100,000g and the supernatant was used as an enzyme source. AR activities were measured using 10 mM DL-glyceraldehyde as substrate, by determining the decrease in absorbance (340 nm) of NADPH (16 mM) for 5 min in the presence or absence of the test materials. Appropriate blanks contained all reagents except the substrate. The percent inhibition of each sample was calculated by comparing the reaction rate of the solution containing both substrate and inhibitor with that of the control solutions containing only the substrate. IC$_{50}$ values, the concentration of the inhibitor which caused 50% inhibition was calculated from regression equations.

RESULTS AND DISCUSSION

Various fractions obtained by partitioning the methanol extract of the flower successively with chloroform, ethylacetate and n-butanol were subjected to an assay for AR inhibitory activity *in vitro*. As shown in Table I, the ethylacetate soluble fraction was found to exhibit the most potent inhibitory activity, its IC$_{50}$ values being 8×10^{-7} g/ml. Further fractionation of the ethylacetate fraction by SiO$_2$ column chromatography gave 7 subfractions (Fig. 1) and among which subfr. 2 was shown to exhibit the most potent inhibitory activity (IC$_{50}$=5×10^{-8} g/ml) and subfr. 6, being 10 times less potent. Gradient elution of subfr.2 and 6 by silica gel and Sephadex LH-20 gave four crystalline compounds identified as acacetin (I), apigenin (II), luteolin (III) and linarin (IV). These are the same flavonoids first isolated from this plant. These four compounds were subjected to tests for RLAR inhibitory activity at three graded concentrations and the results are shown in Table II. Luteolin (III) was found to exhibit the most potent AR inhibitory activity, its IC$_{50}$ being 5×10^{-7} M, which is approximately 100 times more potent than TMG, a typical prototype AR inhibitor *in vitro*. Acacetin (I) and apigenin (II) were approximately 10 times and 60 times less potent, respectively, than luteolin (III). Linarin (IV), the diglycoside of acacetin

Table I. Effects of various fractions on RLAR activity *in vitro*

Fractions	IC$_{50}$(ug/ml)[a]
MeOH fr.	4.7
CHCl$_3$ fr.	15.0
EtOAc fr.	0.8
BuOH fr.	5.0
H$_2$O fr.	60.0

[a]Final concentrations in the reacton mixtures. IC$_{50}$ values were calculated from the least-squares regression equations in the plot of the logarithm of at three graded concentrations vs % inhibition.
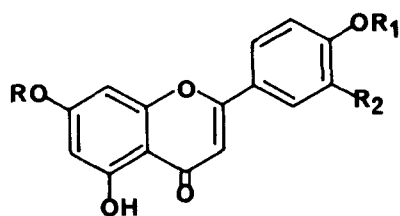
I $R = R_2 = H$, $R_1 = CH_3$

II $R = R_1 = R_2 = H$

III $R = R_1 = H$, $R_2 = OH$

IV $R = Glc\underline{^6\quad^1}Rha$, $R_1 = CH_3$, $R_2 = H$

Fig. 1. Structures of flavonoids

Table II. Effects of various column chromatographic subfractions on RLAR activity *in vitro*

Fractions	$IC_{50}(ug/ml)^a$
Subfr. No. 1	15.0
2	0.05
3	4.0
4	40.0
5	5.0
6	0.5
7	4.0

[a]Final concentrations in the reaction mixtures. IC_{50} values were calculated from the least-square regression equations in the plot of the logarithm of at three graded concentrations vs. % inhibition.

Table III. Effects of flavonoids isolated from the flowers of *Chrysanthemum boreale* on RLAR activity *in vitro*

Compounds	$IC_{50}(uM)^a$
Acacetin(I)	6.0
Apigenin(II)	30.0
Luteolin(III)	0.5
Linarin(IV)	1300.0
TMG[b]	10.0

[a]IC_{50} values representing the concentration which caused 50% inhibition of enzyme activity were calculated from the least-square regression equations in the plot of the logarithm of at three graded concentrations vs. % inhibition.
[b]Tetramethyleneglutaric acid, a reference compound as one of typical AR inhibitors.

(I), exhibited extremely far weaker activity, its IC_{50} being 1.3 mM. There have been many studies for evaluation of naturally occurring compounds which possess inhibitory activity of AR as well as cataract formation and structurally diverse flavonoid compounds have been evaluated as potent AR inhibitors *in vitro* (Okuda *et al.*, 1984; Shimizu *et al.*, 1987; Shimizu *et al.*, 1984; Hirono, 1987; Pathak *et al.*, 1991; Mingzhi and Zhufang, 1986). In the studies on the structure-activity relationships of flavonoids to the AR inhibitory activities, it has been shown that the inhibitory potencies are different from one structural moiety to another (Shimizu *et al.*, 1984); flavones are more active than those with other flavonoid skeletons, glycosides are more active than the corresponding aglycones, the number of sugars present affects activity and further, inhibition is greater in trihydroxy than dihydroxy flavones and the hydroxylation in the 4'-position has beneficial effects. Among four flavones with the 5,7-dihydroxy group in ring A tested in the present study, luteolin (III) possessing the 3',4'-dihydroxy group was far more active than the those with

a 4'-hydroxyl or methoxyl group. This result clearly indicated that the dihydroxy moiety at 3',4'-position gave a beneficial effect in exerting activity. Unexpectedly, linarin (IV), an oligosaccharide showed far weaker activity than its corresponding aglycone, a-cacetin (I), and furthermore, acacetin (I), with a 4'-methoxyl group instead of a hydroxyl group was far more potent than apigenin (II) possessing a hydroxyl group at the 4'-position. The reason for such discrepancies are unknown so far as the present results are concerned. In a previous communication (Shin *et al.*, 1994), we demonstrated that lueolin (III) was the main active principle for inhibition of AR activity of the fruits of *Vitex rotundifolia*. The present result also demonstrates that luteolin (III) is one of the main active materings of the flowers of *Chrysanthemum boreale*. It, therefore, might play a key role in the manifestation of activity when *Chrysanthemii flos* was used as a crude drug. Varma *et al.* (1977) reported that the cataract formation in the diabetic *degus* was retarded by treatment with quercitrin, a potent naturally occurring flavonoidal AR inhibitor, and the delay was accompanied by a decrease in the level of lens sorbitol. Luteolin (III) was demonstrated to be almost equipotent with quercitrin in the inhibition of AR *in vitro*. From these results, it is postulated that luteolin (III) can also be effective in preventing and/or retarding cataractogenic or diabetic complications. To confirm this, the effects of luteolin (III) on cataract development and accompanied polyol accumulation in the lens of both galactosemic and diabetic rats are currently under study the results of which will be reported elsewhere.

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