Comparative Study of Seventeen *Salvia* Plants: Aldose Reductase Inhibitory Activity of Water and MeOH Extracts and Liquid Chromatography–Mass Spectrometry (LC-MS) Analysis of Water Extracts

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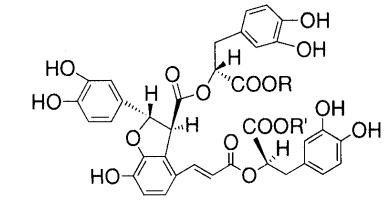
The dry root and rhizome of *Salvia miltiorhiza* (Lamiaceae) are used as a crude drug Danshen, while those of *S. deserta* (Xinjiang-Danshen) are mixed in Danshen at Xinjiang province when the former is in short supply. The water and MeOH extracts of *S. deserta* showed strong aldose reductase (AR) inhibitory activity, and their active constituents were determined to be polar compounds different from "tanshinones" of *S. miltiorhiza*, *i.e.*, lithospermic acid B (1), salvianolic acid K (2), salviaflaside (3), and rosmarinic acid (4) (IC_{50}, 2.63—3.91 μM).

We also examined the AR inhibitory activity of water and MeOH extracts of seventeen *Salvia* plants, including ten species of Danshen resources (*S. bowleyana*, *S. deserta*, *S. miltiorhiza*, *S. miltiorhiza* var. *miltiorhiza* f. *alba*, *S. paramiltiorhiza*, *S. paramiltiorhiza* f. *purpureo-rubra*, *S. przewalskii*, *S. przewalskii* var. *mandarinorum*, *S. sinica* f. *purpurea*, *S. trijuga*), and their water extracts were also analyzed by liquid chromatography–mass spectrometry (LC-MS). The results indicated that there were four types with regard to the AR inhibitory activity and three types with regard to the amount of 1. Ten species used as Danshen resources showed good correlation between the AR inhibitory activity and the morphological classification. However, the intensities of their AR inhibitory activity varied, and they contained 1 in varying amounts. These facts suggested that the ten species were not the same, and thus their use as a Danshen resource should be based on their activity and/or active constituents.

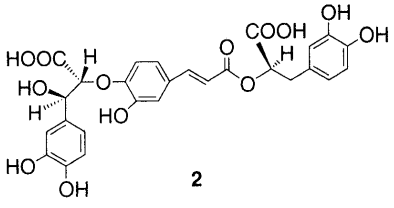
Key words *Salvia*; aldose reductase inhibitory activity; LC-MS; *Salvia deserta*; lithospermic acid B; tanshinone

Danshen (丹参, Radix Salviae miltiorhizae) is the dry root and rhizome of *Salvia miltiorhiza* BUNGE (Lamiaceae). It is officially listed in the Chinese Pharmacopoeia[1] and used for treatment of menstrual disorder, menostasis, menorrhalgia, insomnia, blood circulation diseases, and angina pectoris as well as against inflammation.[2] Moreover, Danshen was reported to strongly inhibit aldose reductase (AR)[3] and salvianolic acid A, one of the Danshen constituents, was reported to have AR inhibitory activity.[4] In the course of our study on *Salvia* plants, we examined the components of roots of *S. miltiorhiza* (Danshen)[5,6] and *S. deserta* (Xinjiang–Danshen).[7] *Salvia miltiorhiza* contained "tanshinones"[8] [tanshinone I, tanshinone IIA, dihydrotanshinone I, and cryptotanshinone] as abietane-type diterpenoids and a tetramer [magnesium lithospermate B (**1a**)] as the main caffeic acid derivative, while *S. deserta* contained "royleanones"[8] [6,7-dehydroroyleanone, royleanone, 7-*O*-methylhorminone, 7-*O*-acetylhorminone, horminone] as abietane-type diterpenoids and a trimer [salvianolic acid K (**2**)] as the main caffeic acid derivative. The "tanshinones" in *S. miltiorhiza* showed inhibitory activity against AR from rat eye lens, and their activity was considered to be due to the *o*- or *p*-naphthoquinone group. We thus examined the activity of the extracts of *S. deserta* containing very different constituents from *S. miltiorhiza*.

In China one hundred and ten *Salvia* species are grown, and twelve of them (*S. bowleyana*, *S. deserta*, *S. miltiorhiza*, *S. miltiorhiza* var. *miltiorhiza* f. *alba*, *S. paramiltiorhiza*, *S. paramiltiorhiza* f. *purpureo-rubra*, *S. przewalskii*, *S. przewalskii* var. *mandarinorum*, *S. sinica*, *S. sinica* f. *purpurea*, *S. trijuga*, *S. yunnanensis*) are used as resources of Danshen.[9] Few comparative studies have been made of their composition and activities, however.[9] Thus, we



1 R = R' = H
1a R, R' = Mg^{2+} salt



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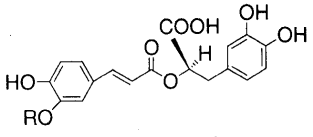

3: R = Glc.
4: R = H

Chart 1

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Table 1. List of Plant Name, Locality, and Voucher Sample Number Used in This Study

Sample No.	Plant name	Locality	Voucher sample No.
1	*S. bowleyana* DUNN	Gaoan, Jiangxi province	CPU 646
2	*S. bowleyana* DUNN	Kaihua, Zejiang province	CPU 647
3	*S. bulleyana* DIELS	Dali, Yunnan province	CPU 692
4	*S. deserta* SCHANG.	Urumuqi, Xinjiang province	TMPW 15403
5	*S. flava* FORREST *et* DIELS	Lijiang, Yunnan province	CPU 690
6	*S. meiliensis* S. W. SU	Huoshan, Anhui province	CPU 701
7	*S. miltiorhiza* BUNGE	Chuxian, Anhui province	CPU 698
8	*S. miltiorhiza* BUNGE (cultivated)	Zhongjiang, Sichuan province	CPU 653
9	*S. miltiorhiza* BUNGE	Heze, Shandong province	TMPW 15481
10	*S. miltiorhiza* BUNGE var. *miltiorhiza* f. *alba* C. Y. WU *et* H. W. LI (cultivated)	Zhangqiu, Shandong province	CPU 648
11	*S. paramiltiorhiza* H. W. LI *et* X. L. HUANG	Shucheng, Anhui province	CPU 649
12	*S. paramiltiorhiza* f. *purpureo-rubra* H. W. LI	Tongling, Anhui province	CPU 686
13	*S. przewalskii* MAXIM.	Lijiang, Yunnan province	CPU 654
14	*S. przewalskii* MAXIM. var. *mandarinorum* STIB.	Saotong, Yunnan province	CPU 651
15	*S. przewalskii* MAXIM. var. *mandarinorum* STIB.	Dali, Yunnan province	CPU 694
16	*S. sinica* MIGO f. *purpurea* H. W. LI	Chongyang, Anhui province	CPU 695
17	*S. trijuga* DIELS	Lijiang, Yunnan province	CPU 700

Table 2. Yields (%, against Roots) and Inhibitory Activities (IC_{50}, μg/ml) of Extracts and Fractions

Sample No.	Plant name	Water extract		MeOH extract		AcOEt-soluble fraction		AcOEt-insoluble fraction	
		Yield	IC_{50}	Yield	IC_{50}	Yield	IC_{50}	Yield	IC_{50}
1	*S. bowleyana*	23.7	364.7	2.02	99.3	1.37	70.2	0.59	90.0
2	*S. bowleyana*	27.6	365.1	3.02	98.0	1.99	60.9	0.72	86.1
3	*S. bulleyana*	11.7	96.9	2.91	99.8	2.18	93.3	0.65	87.1
4	*S. deserta*	23.4	84.3	3.34	78.5	2.02	76.8	1.16	7.2
5	*S. flava*	21.4	228.2	2.14	98.6	1.18	86.3	0.86	70.0
6	*S. meiliensis*	14.6	298.0	3.34	97.2	2.39	61.0	0.87	92.6
7	*S. miltiorhiza*	25.2	223.6	3.38	93.1	2.37	10.8	0.87	89.0
8	*S. miltiorhiza* (cultivated)	24.6	199.8	3.89	93.8	2.53	11.2	1.10	91.3
9	*S. miltiorhiza*	21.6	211.5	3.56	93.0	2.34	9.9	1.04	91.9
10	*S. miltiorhiza* var. *miltiorhiza* f. *alba* (cultivated)	40.4	197.3	5.17	95.2	3.53	12.5	1.48	88.6
11	*S. paramiltiorhiza*	40.5	348.7	3.35	96.6	2.22	40.1	0.92	84.0
12	*S. paramiltiorhiza* f. *purpureo-rubra*	19.7	346.5	2.91	99.1	1.78	41.2	0.99	87.8
13	*S. przewalskii*	23.4	83.5	3.34	33.1	2.20	8.6	1.07	8.0
14	*S. przewalskii* var. *mandarinorum*	16.4	84.2	2.55	27.9	1.75	9.3	0.77	8.3
15	*S. przewalskii* var. *mandarinorum*	14.5	86.2	3.06	29.8	2.05	7.9	0.87	7.2
16	*S. sinica* f. *purpurea*	28.8	213.1	2.37	97.5	1.30	86.7	0.98	90.9
17	*S. trijuga*	23.3	79.9	5.88	98.8	4.37	19.5	1.26	70.1

also examined AR inhibitory activity of seventeen *Salvia* plants (Table 1), including *S. miltiorhiza* and *S. deserta*, among which ten species are used as Danshen resources. In addition, their water extracts were examined by liquid chromatography–mass spectrometry (LC-MS).[10] This paper deals with the AR inhibitory activity of the water and MeOH extracts and LC-MS analysis of the water extracts.[10]

Results and Discussion

AR Inhibitory Activity of *Salvia deserta* The MeOH and water extracts of *S. deserta* (No. 4) inhibited AR from rat eye lens concentration-dependently, and their activities (IC_{50}, 78.5 and 84.3 μg/ml, respectively) were almost the same as the MeOH extract of *S. miltiorhiza* (No. 9; IC_{50}, 93.0 μg/ml). Then, the activity of the MeOH extract was transferred into the AcOEt-insoluble fraction (IC_{50}, 7.2 μg/ml), and that of *S. miltiorhiza* (No. 9) into the AcOEt-soluble fraction (IC_{50}, 9.9 μg/ml). This suggested that the active constituent of *S. deserta* would be a polar compound(s), different from that of *S. miltiorhiza* of which the active constituents were less-polar "tanshinones".[5]

We measured the AR inhibitory activity of the eleven compounds isolated from *S. deserta*. The less-polar diterpenoids (mainly "royleanones"), having no *o*- or *p*-naphthoquinone group, showed only weak activity ($IC_{50} > 10$ μM; inhibition rate at 10 μM was 8.6—36.1%), while the polar constituents [lithospermic acid B (**1**), salvianolic acid K (**2**), salviaflaside (**3**), and rosmarinic acid (**4**)] showed inhibitory activity (IC_{50}, 2.63, 2.81, 3.15, and 3.91 μM, respectively), weaker than epalrestat (IC_{50}, 0.04 μM), a strong AR inhibitor in clinical use,[11] but stronger than quercetin (IC_{50}, 5.20 μM), a natural AR inhibitor.[12] This result would support the previous consideration that the activity of "tanshinones" was due to the *o*- or *p*-naphthoquinone group. In addition, the results also explained the difference in the activities of

the water extracts, because the constituents of the water extract were almost the same as those of the AcOEt-insoluble fraction by TLC and LC-MS analyses.

AR Inhibitory Activity of Seventeen *Salvia* Plants The results mentioned above suggested that there are at least two types in the genus *Salvia*. We then measured the AR inhibitory activity of seventeen *Salvia* plants, including *S. miltiorhiza* (No. 9) and *S. deserta* (No. 4) (Table 2). The MeOH extracts inhibited AR more strongly (IC_{50}, 27.9—99.8 μg/ml) than the water extracts, and the MeOH extracts of *S. przewalskii* (No. 13) and *S. przewalskii* var. *mandarinorum* (Nos. 14, 15) showed very strong activity (IC_{50}, 27.9—33.1 μg/ml). In addition, AcOEt-soluble fractions of *S. miltiorhiza* (Nos. 7—9), *S. miltiorhiza* var. *miltiorhiza* f. *alba* (No. 10), *S. przewalskii* (No. 13), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15), and *S. trijuga* (No. 17) and AcOEt-insoluble fractions of *S. deserta* (No. 4), *S. przewalskii* (No. 13), and *S. przewalskii* var. *mandarinorum* (Nos. 14, 15) showed strong activity (IC_{50}, 7.2—19.5 μg/ml). Thus, as active constituents, *S. miltiorhiza* var. *miltiorhiza* f. *alba* and *S. trijuga* would contain less-polar compounds (*e.g.*, "tanshinones")[13] as *S. miltiorhiza*, while *S. przewalskii*[14] and *S. przewalskii* var. *mandarinorum*[15] would contain both the less-polar and polar compounds.

Though the activity of water extracts was weaker than that of MeOH extracts, water extracts of five species [*S. bulleyana* (No. 3), *S. deserta* (No. 4), *S. przewalskii* (No. 13), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15), *S. trijuga* (No. 17)] showed AR inhibitory activity comparable to that of MeOH extracts. Among the five, three (*S. deserta*, *S. przewalskii*, *S. przewalskii* var. *mandari-*

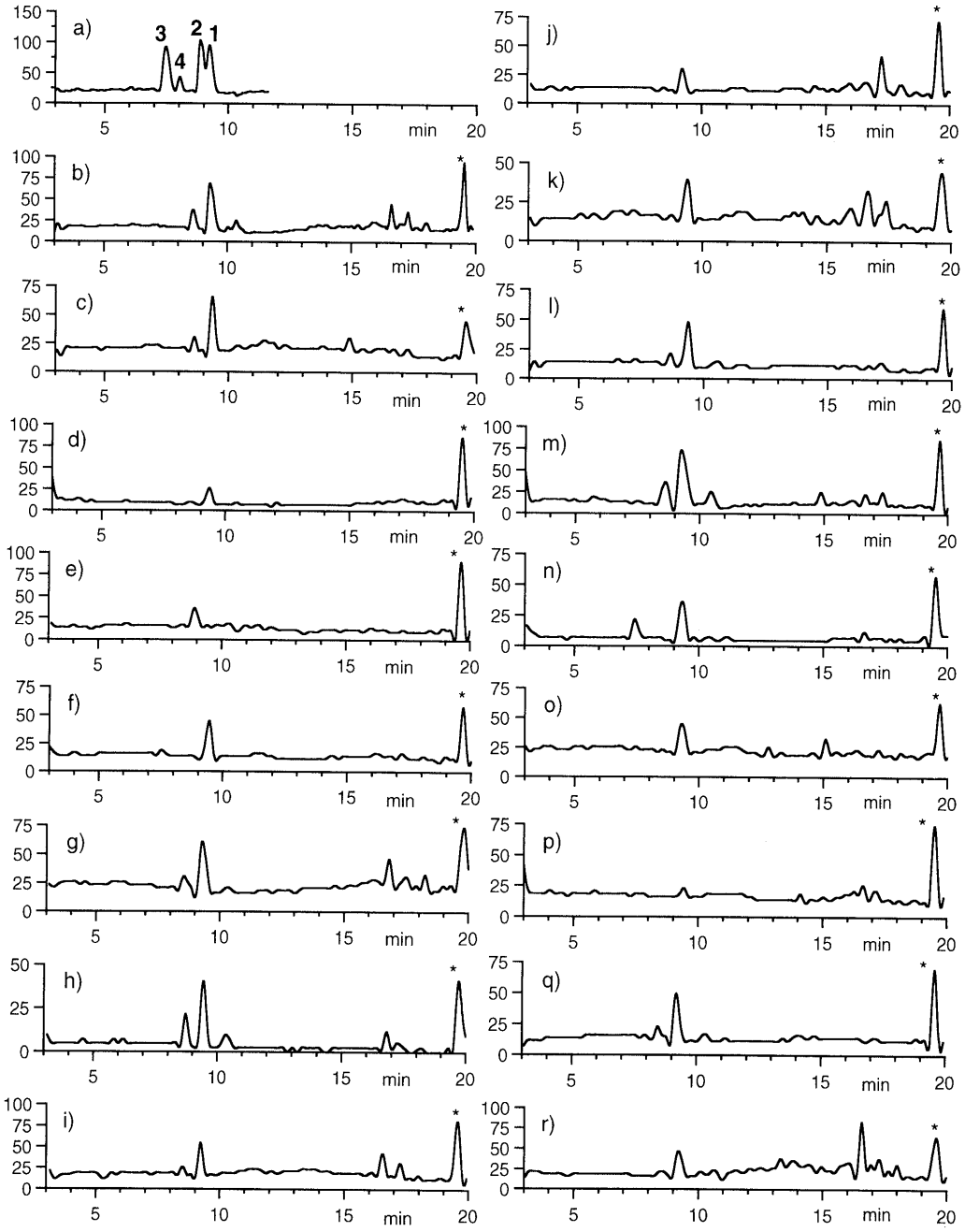

Fig. 1. Total Ion Chromatogram of Water Extracts of Seventeen *Salvia* Plants

a) A mixture of **1**—**4**. *b*—*r*) Sample No. 1—No. 17. * This is a solvent-derived peak, because an injection of solvent also revealed only this peak.

Table 3. Amounts (μg/mg) of Compounds **1**—**3** in Water Extracts by LC-MS Analysis

Sample No.	Plant name	**1**	**2**	**3**	**1**/(**1**+**2**+**3**) (%)
1	*S. bowleyana*	210.3	0.40	1.7	99.0
2	*S. bowleyana*	141.1	0.10	0.6	99.5
3	*S. bulleyana*	15.9	0.36	0.5	94.9
4	*S. deserta*	0.3	29.28	2.5	0.94
5	*S. flava*	7.3	0.03	9.7	42.9
6	*S. meiliensis*	118.1	0.18	0.8	99.2
7	*S. miltiorhiza*	145.4	0.10	0.2	99.8
8	*S. miltiorhiza* (cultivated)	127.1	0.05	0.1	99.9
9	*S. miltiorhiza*	39.0	0.06	0.1	99.6
10	*S. miltiorhiza* var. *miltiorhiza* f. *alba* (cultivated)	116.5	0.14	0.3	99.6
11	*S. paramiltiorhiza*	130.0	0.02	0.4	99.7
12	*S. paramiltiorhiza* f. *purpureo-rubra*	258.3	0.04	0.6	99.8
13	*S. przewalskii*	6.9	0.02	5.0	57.9
14	*S. przewalskii* var. *mandarinorum*	19.0	0.19	5.6	76.6
15	*S. przewalskii* var. *mandarinorum*	6.2	0.01	1.5	80.4
16	*S. sinica* f. *purpurea*	125.8	0.11	0.4	99.6
17	*S. trijuga*	77.5	0.04	1.2	98.4

norum) were the species in which AcOEt-insoluble fraction showed stronger AR inhibitory activity than the corresponding AcOEt-soluble fraction.

These results suggest that, with regard to the AR inhibitory activity, there are three types in the genus *Salvia*: the first type containing less-polar active compounds (*S. miltiorhiza*, *S. miltiorhiza* var. *miltiorhiza* f. *alba*), the next type containing polar active compounds (*S. bulleyana*, *S. deserta*), the third type containing both active compounds (*S. przewalskii*, *S. przewalskii* var. *mandarinorum*, *S. trijuga*). It is noteworthy in that the yields and activities of the same species [*S. bowleyana* (Nos. 1, 2), *S. miltiorhiza* (Nos. 7—9), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15)] were almost the same and that *S. miltiorhiza* var. *miltiorhiza* f. *alba* (No. 10), *S. paramiltiorhiza* f. *purpureo-rubra* (No. 12), and *S. przewalskii* var. *mandarinorum* (No. 14, 15) showed similar inhibitory activities to their corresponding species [*S. miltiorhiza* (Nos. 7—9), *S. paramiltiorhiza* (No. 11), and *S. przewalskii* (No. 13), respectively].

LC-MS Analysis of Water Extracts of Seventeen *Salvia* Plants The polar compounds **1**—**4** obtained from *S. deserta*[7] showed a slightly overlapped total ion chromatogram (TIC) but they were well separated on a mass chromatogram at the respective protonated molecular ion (**1**, *m/z* 719.2, t_R 9.20 min; **2**, *m/z* 557.3, t_R 8.82 min; **3**, *m/z* 523.3, t_R 7.48 min; **4**, *m/z* 361.3, t_R 8.01 min). We thus calculated their amounts from the mass chromatogram except for that of **4** (Table 3), because the ion strength of **4** did not show linearity against the amount. The amount of **1** was large (100—260 μg/mg) as usual, but it was small in *S. bulleyana* (No. 3; 15.9 μg/mg), *S. deserta* (No. 4; 0.3 μg/mg), *S. flava* (No. 5; 7.3 μg/mg), *S. przewalskii* (No. 13; 39.0 μg/mg), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15; 19.0 and 6.2 μg/mg), and *S. trijuga* (No. 17; 77.5 μg/mg). On the other hand, the ratio of **1** against the total amount of **1**—**3** was high (>90%) as usual, but that of *S. deserta*, *S. flava*, *S. przewalskii*, and *S. przewalskii* var. *mandarinorum* was low (0.94— 80.4%).

Thus, with regard to the amount of **1**, there were three types in the genus *Salvia*: the first containing a large amount of **1** (*S. bowleyana*, *S. meiliensis*, *S. miltiorhiza*, *S. miltiorhiza* var. *miltiorhiza* f. *alba*, *S. paramiltiorhiza*, *S. paramiltiorhiza* f. *purpureo-rubra*, *S. sinica* f. *purpurea*), the second containing a small amount but a high ratio of **1** (*S. bulleyana*, *S. trijuga*), and a third containing a small amount and a low ratio of **1** (*S. deserta*, *S. flava*, *S. przewalskii*, *S. przewalskii* var. *mandarinorum*). Although the precise relationship between the amount of **1** and the AR inhibitory activity is not clear and more study is needed, the fact that five of the six species of the second and the last types showed relatively strong inhibitory activity could suggest that there is a negative correlation between the amount of **1** and the AR inhibitory activity. It should be noted that the plants of the same species [*S. bowleyana* (Nos. 1, 2), *S. miltiorhiza* (Nos. 7—9), *S. przewalskii* var. *mandarinorum* (Nos. 14, 15)] were not same in their content of **1**—**3**, though they belong to the same group.

Conclusion

We examined the AR inhibitory activity of water and MeOH extracts of seventeen *Salvia* plants, including ten species of Danshen resources, and their water extracts were also analyzed by LC-MS. The results indicated that there were four types with regard to the AR inhibitory activity and three types with regard to the amount of lithospermic acid B (**1**). Ten species used as Danshen resources showed the AR inhibitory activity with varied intensity, and contained **1** in varying amounts. These facts suggested that the ten species were not the same, although their AR inhibitory activity was well correlated with the morphological classification. Thus, their use as a Danshen resource should be based on their activity and/or active constituents.

Experimental

Plant Materials The roots of fifteen *Salvia* plants (sample Nos. 1—3, 5—8, 10—17), which had been identified by an expert and were preserved, were supplied from the Department of Medicinal Botany, China Pharmaceutical University. Those of *S. deserta* (sample No. 4) and *S. miltiorhiza* (sample No. 9) were supplied by Alps Pharmaceutical Co.,

Ltd., Furukawa, Japan and identified by an expert, and the voucher samples are preserved in the Museum of Materia Medica, Analytical Research Center for Ethnomedicines, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University.

Preparation of Extracts and Fractions Roots of each plant were chopped into small pieces and extracted with water (15 ml/g, 80 °C, 3 h, × 3). The extracts were combined and lyophilized to give each water extract. The residue was then extracted with MeOH (10 ml/g, reflux, 3 h, × 3) and the MeOH solution was evaporated to give MeOH extract. A part of this extract was suspended in water (5 ml/mg) and extracted with AcOEt (5 ml/mg × 3). The AcOEt extract was evaporated to give an AcOEt-soluble fraction, while the water layer was liophilized to yield an AcOEt-insoluble fraction.

Assay Method of AR Inhibitory Activity The crude AR was isolated from the eye lens of ether anesthetized male Wistar rats (eight-weeks-old, 260 g) following the procedure of Shimizu *et al.*[16] and the AR inhibition assay was performed by the method of Kador and Sharpless,[17] with slight modification. Details were described in our previous paper.[5] The activity was compared with those of positive controls, epalrestat[11] (IC_{50}, 0.04 μM) and quercetin[12] (IC_{50}, 5.20 μM).

LC-MS Measurements of Water Extracts The solution of a water extract (10 μg/ml) in HPLC grade MeOH was filtered using a Millipore SJLG 250 filter, and 10 μl of the filtrate was directly subjected to LC-MS analysis. This analysis was performed on a Finnigan-Mat LCQ system, equipped with a Shimadzu LC10A HPLC system, by electrospray ionization (ESI) mode [capillary temperature, 180 °C; ion injection time, 0.1 s; column, Waters Symmetry C_{18} (150 mm × 4.6 mm i.d.); column temperature, 40 °C; mobile phase, linear gradient from 0.1% trifluoroacetic acid–MeOH (60 : 40, v/v) to MeOH for 20 min; flow rate, 0.8 ml/min].

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