0014-4754/96/060564-04$1.50 + 0.20

Inhibition of aldose reductase by dihydroflavonols in *Engelhardtia chrysolepis* and effects on other enzymes

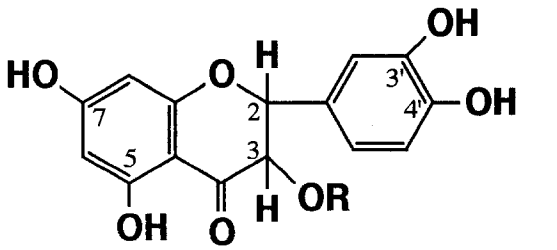
H. Haraguchi[a,*], I. Ohmi[a], H. Masuda[b], Y. Tamura[b], K. Mizutani[b], O. Tanaka[b] and W.-H. Chou[c]

[a]*Faculty of Engineering, Fukuyama University, Gakuen-cho, Fukuyama 729-02 (Japan), Fax +81 849 36 2023*
[b]*Biology and Chemistry Laboratory, Maruzen Pharmaceutical Co. Ltd., Sanada Takanishi-cho, Fukuyama 729-01 (Japan)*
[c]*South China Institute of Botany, The Academy of Science of China, Guangzhou (China)*
Received 27 June 1995; accepted 29 August 1995

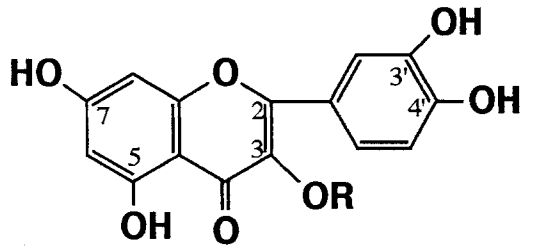
Abstract. Astilbin and neoastilbin, dihydroflavonol rhamnosides from *Engelhardtia chrysolepis*, showed potent inhibition of lens aldose reductase. Kinetic analysis showed astilbin exhibited uncompetitive inhibition against both *dl*-glyceraldehyde and NADPH. These taxifolin glycosides were selective inhibitors of aldose reductase with no inhibition of NADH oxidase.
Key words. *Engelhardtia chrysolepis*; astilbin; neoastilbin; taxifolin; aldose reductase; aldehyde reductase; NADH oxidase.

Aldose reductase (EC 1.1.1.21), a member of the NADPH-dependent aldo-keto reductase family, is a key enzyme in the polyol pathway[1]. The polyol pathway has been found in tissues, such as the lens, retina, nerve, and kidney, in which diabetic complications appear[2]. This enzyme catalyzes the reduction of various aldehydes, including the aldehyde form of glucose, to the corresponding sugar alcohol, sorbitol. Reduction of glucose to sorbitol provides a common link in the onset of diabetic complications that result in tissue and functional changes in the cornea, lens, retina, iris, peripheral nerves and kidney[3,4]. It has been reported that accumulation of sorbitol in the lens contributes to the formation of cataracts in diabetics[5,6]. The intracellular accumulation of sorbitol leads to locally hyperosmotic conditions, which appear to be responsible for the loss of clarity of the lens[7]. Therefore, the inhibition of aldose reductase may be effective in preventing cataract formation in diabetes.

Our preliminary search for inhibitors of aldose reductase from tea extracts revealed that the ethanol extract of *Engelhardtia chrysolepis* leaves showed potent inhibition of porcine lens aldose reductase. *E. chrysolepis* is a subtropical tree grown in Guangdong, Guangxi and Fujian, China. The dried leaves of this plant are drunk as a sweet tea to prevent obesity and used as a folk medicine against fever and pain. From the leaves of this plant, 3-*O*-α-L-rhamnosyl-(2*S*,3*S*)-taxifolin (neoastilbin, **3**) was isolated as a sweet principle together with its non-sweet isomers, astilbin (**2**), isoastilbin (**4**) and neoisoastilbin (**5**)[8,9]. The fresh leaves of *E. chrysolepis* contain 2.8–4.6% of astilbin (**2**) as a major component; other dihydroflavonols (**3**, **4**, **5**) were present at a concentration of 0.2–0.5%. The preliminary ethanol extract also contains astilbin as a main component (13.1–20.1%). From the leaves of *E. chrysolepis*, quercitrin (**7**), a common quercetin rhamnoside, was also isolated[8],



		R	
1	*2R, 3R*	H	(taxifolin)
2	*2R, 3R*	Rha	(astilbin)
3	*2S, 3S*	Rha	(neoastilbin)
4	*2S, 3R*	Rha	(isoastilbin)
5	*2R, 3S*	Rha	(neoisoastilbin)



	R	
6	H	(quercetin)
7	Rha	(quercitrin)

Figure 1. Constituents isolated from *Engelhardtia chrysolepis*.

* Corresponding author.

and its content was 2.9% in alcohol extract. This paper deals with the inhibitory activities of the constituents of *E. chrysolepis* on aldose reductase and the effects on other related enzymes.

Materials and methods

Astilbin (**2**), neoastilbin (**3**) and quercitrin (**7**), isolated from the leaves of *E. chrysolepis*, were obtained from Maruzen Pharmaceutical Co. Ltd. Taxifolin (**1**) was obtained as a hydrolysis product of **2**. These compounds were identified by $[\alpha]_D$, ^1H- and ^{13}C-NMR spectra. Quercetin (**6**) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Porcine eyeball and kidney and bovine liver were obtained from Nippon Ham Co. Ltd. (Osaka, Japan).

Preparation of lens aldose reductase. Lenses were removed from porcine eyes and homogenized in 3 volumes of 135 mM phosphate buffer (pH 7.0) containing 10 mM β-mercaptoethanol[10]. The homogenate was centrifuged at 10,000 × *g* for 15 min. The supernatant fluid was saturated with 75% $(NH_4)_2SO_4$. The precipitate obtained by centrifugation was dissolved in the same buffer and used as an enzyme preparation[11].

Preparation of kidney aldehyde reductase. The porcine kidneys were dissected into cortical and medullary regions, and the cortices were homogenized in 3 volumes of 20 mM phosphate buffer (pH 7.5) containing 0.25 M sucrose, 0.5 mM EDTA and 1 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000 × *g* for 20 min. The supernatant fluid was saturated with 60% $(NH_4)_2SO_4$. The precipitate obtained by centrifugation was dissolved in 10 mM Tris buffer (pH 7.4) containing 0.5 mM EDTA and 1 mM β-mercaptoethanol, and passed through a Sephadex G-25 column equilibrated with the same buffer. The enzyme eluate was chromatographed on a DEAE-cellulose column equilibrated with the same Tris buffer and developed with a 0–0.4 M linear gradient of NaCl. This partially purified aldehyde reductase was free of aldose reductase[12].

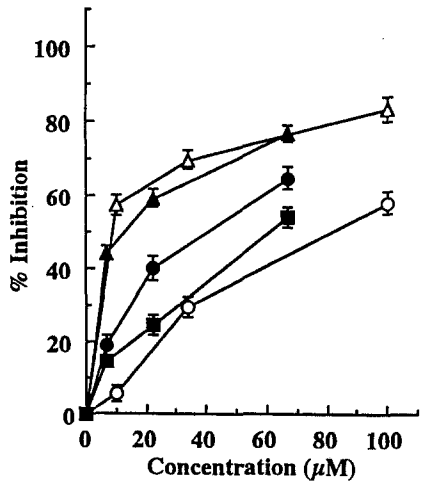


Figure 2. Effect of flavonoids isolated from *E. chrysolepis* on porcine lens aldose reductase. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ○: **1**, ●: **2**, ■: **3**, △: **6**, ▲: **7**.

Preparation of liver mitochondria. The bovine liver was homogenized in 10 volumes of 0.25 M sucrose containing 0.1 mM EDTA (pH 7.4). The homogenate was centrifuged at 600 × *g* for 10 min. The supernatant fluid was decanted and centrifuged at 15,000 × *g* for 10 min[13]. The mitochondrial pellet was washed twice with the same solution and finally suspended in 3 mM Tris buffer (pH 7.4) containing 0.07 M sucrose, 0.21 M mannitol and 0.1 mM EDTA.

Assay of reductase activity. Aldose reductase and aldehyde reductase were assayed spectrophotometrically on a Shimadzu MPS-2000 spectrophotometer equipped with a TCC temperature controller. The reaction mixture in a total volume of 3 ml contained 50 mM Na-phosphate buffer (pH 6.2), 0.125 mM NADPH, 400 mM Li_2SO_4, enzyme solution, and 3 mM *dl*-glyceraldehyde as a substrate[14]. The reaction was initiated by the addition of NADPH. The reaction rate was determined by following the decrease in the absorption of

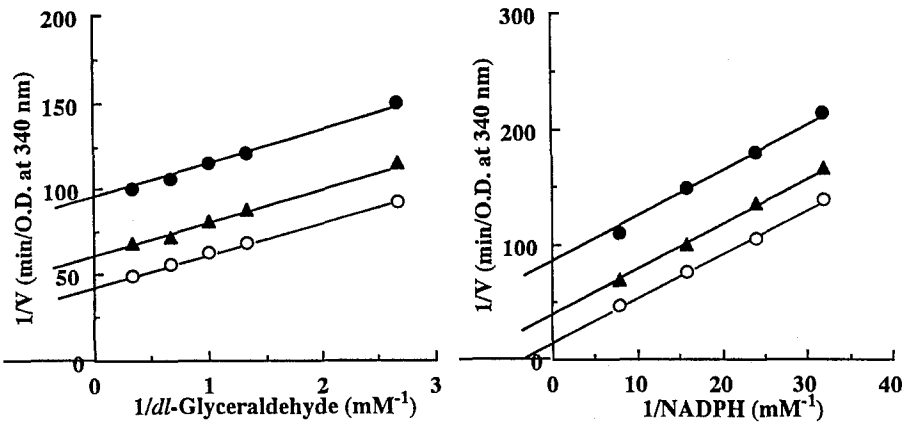


Figure 3. Inhibitory effect of astilbin (**2**) on porcine lens aldose reductase. Lineweaver-Burk plots in the absence (○) and in the presence (●, 22 μM; ▲, 6.7 μM) of astilbin are shown.

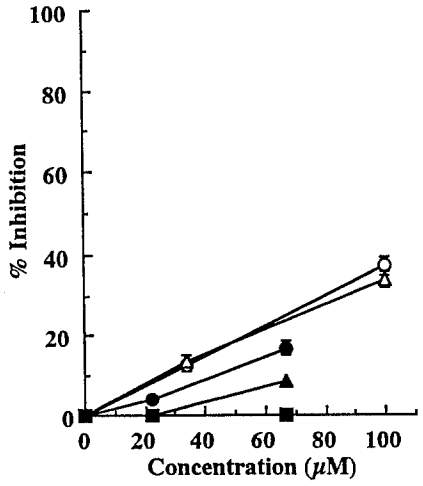


Figure 4. Effect of flavonoids isolated from *E. chrysolepis* on porcine kidney aldehyde reductase. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ○: **1**, ●: **2**, ■: **3**, △: **6**, ▲: **7**.

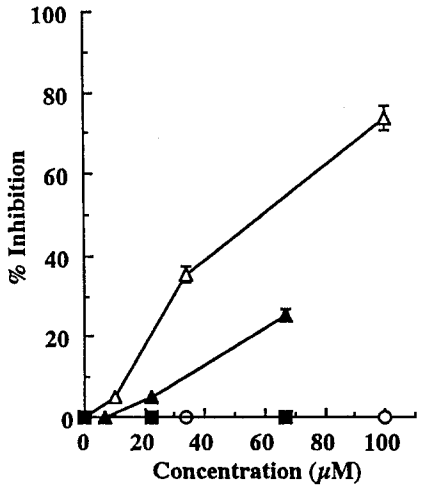


Figure 5. Effect of flavonoids isolated from *E. chrysolepis* on bovine liver mitochondrial NADH oxidase. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ○: **1**, ●: **2**, ■: **3**, △: **6**, ▲: **7**.

NADPH at 340 nm on a Shimadzu Graphic Printer PR-3.

Assay of NADH oxidase. Mitochondrial NADH oxidase was assayed by measuring the decrease in absorbance at 340 nm[15]. The reaction mixture in a total volume of 3 ml consisted of 50 mM phosphate buffer (pH 7.4), 0.1 mM NADH, and bovine liver mitochondrial protein.

Results and discussion

Astilbin (**2**), a major component of *E. chrysolepis*, at a concentration of 67 μM, showed 65% inhibition against porcine lens aldose reductase. A sweet dihydroflavonol glycoside, neoastilbin (**3**), also inhibited aldose reductase. Figure 2 shows the effect of varying concentrations of flavonoids in *E. chrysolepis* on inhibition of porcine lens aldose reductase. Taxifolin (**1**), the aglycone of astilbin and neoastilbin, has been already reported to be an inhibitor of aldose reductase[16], which showed less activity than its rhamnosides. This result was in agreement with monoglycosides having a higher activity than the corresponding flavonoid aglycones[17]. Quercitrin (**7**), also one of major constituents of *E. chrysolepis*, and the aglycone quercetin (**6**), strongly inhibited lens aldose reductase, which confirms earlier findings[16,18].

Kinetic analysis for aldose reductase inhibition by astilbin using Lineweaver-Burk plots is shown in figure 3. When the concentration of substrates *dl*-glyceraldehyde was changed, the slopes obtained from the uninhibited enzyme and from the two different concentrations of **2** were parallel. The reciprocal plot curves of enzyme activities and concentration of cofactor NADPH also showed uncompetitive inhibition. These illustrations indicate that **2** inhibits lens aldose reductase uncompetitively with respect to both *dl*-glyceraldehyde and NADPH.

Many aldose reductase inhibitors have been reported also to inhibit aldehyde reductase (EC. 1.1.1.20), known as hexonate dehydrogenase (EC. 1.1.1.19)[19–22]. Aldehyde reductase has been suggested to be an important site of action of anticonvulsant drugs. Similarities including the monomeric nature of these enzymes, their overlapping substrate specificities, and similar utilization of NADPH, suggests that both aldose reductase and aldehyde reductase possess certain structural similarities necessary for the binding of inhibitors[23]. Quercetin (**6**) is reported to have selectivity for aldose reductase inhibition, and the substitution of a 3-rhamnosyl moiety to **6** to form quercitrin (**7**) increased selectivity for aldose reductase[23]. This was confirmed in the present experiments, and this selectivity was similar to that observed for dihydroflavonols. Figure 4 shows the effect of these components of *E. chrysolepis* on porcine kidney aldehyde reductase. Astilbin (**2**) was a selective inhibitor with selectivity for aldose reductase inhibition 4.5-fold greater than for aldehyde reductase. Neoastilbin (**3**) was the most selective inhibitor; no inhibition was observed against aldehyde reductase even at 67 μM.

Many flavonoids are known to be potent inhibitors of lens aldose reductase[16–18,24]. Aldose reductase is an NADPH-dependent oxidoreductase, therefore the effects on adenine nucleotide-requiring enzymes would be an apprehension[25]. NADH oxidase is one of the most important adenine nucleotide-linked oxidation systems, producing metabolic energy in mitochondria as the terminal oxidase of substrates. Therefore, the inhibition of this enzyme system leads to an interference with normal glucose metabolism. Some flavonoids have been reported to inhibit NADH oxidase in liver mitochondria[26–28], or to stimulate the oxidase resulting in genera-

tion of toxic oxygen radicals[27–29]. For example, quercetin (**6**), an inhibitor of aldose reductase, inhibited bovine liver mitochondrial NADH oxidase; the 50% inhibition was observed at 65 μM (fig. 5). Quercitrin (**7**) also affected NADH oxidase. On the other hand, dihydroflavonols (**1, 2, 3**) in *E. chrysolepis* showed no effect on NADH oxidase even at 100 μM.

The dihydroflavonol taxifolin and its rhamnosides showed potent inhibitory activity against lens aldose reductase. Little effect was observed on aldehyde reductase, and NADH oxidase in mitochondria was not affected by these dihydroflavonols. Recently, taxifolin has been reported to decrease the serum cholesterol level in rats[30]. The present results and other biological activities of *E. chrysolepis* are of considerable interest to the food industry and in preventive medicine. The effects of these dihydroflavonols on sorbitol accumulation in vivo are under investigation.

1 Kador, P. F., Kinoshita, J. H., and Sharpless, N. E., J. med. Chem. *28* (1985) 841.
2 Gabbay, K. H., A. Rev. Med. *26* (1975) 521.
3 Kador, P. F., Med. Res. Rev. *8* (1988) 325.
4 Kador, P. F., Akagi, Y., Terubayashi, H., Wyman, M., and Kinoshita, J. H., Archs Ophthal. *106* (1988) 1099.
5 Kinoshita, J. H., Invest. Ophthal. *13* (1974) 713.
6 Kinoshita, J. H., Fukushi, S., Kador, P., and Merola, L. O., Metabolism *28* (1979) 462.
7 Chylack, L. T., and Kinoshita, J. H., Invest. Ophthal. *8* (1969) 401.
8 Kasai, R., Hirono, S., Chou, W.-H., Tanaka, O., and Chen, F.-H., Chem. Pharm. Bull. *36* (1988) 4167.
9 Kasai, R., Hirono, S., Chou, W.-H., Tanaka, O., and Chen, F.-H., Chem. Pharm. Bull. *39* (1991) 1871.
10 Aida, K., Shindo, H., Tawata, M., and Onaya, T., Planta med. *53* (1987) 131.
11 Hayman, S., and Kinoshita, J. H., J. biol. Chem. *240* (1965) 877.
12 Sato, S., and Kador, P. F., Biochem. Pharmac. *40* (1990) 1033.
13 Johnson, D., and Lardy, H., in: Methods in Enzymology, vol. 10, p. 94. Eds R. W. Estabrook and M. E. Pullman. Academic Press, New York 1967.
14 Nishikawa, M., Tsurumi, Y., Namiki, T., Toshida, K., and Okuhara, M., J. Antibiot. *40* (1987) 1394.
15 Triphathi, R. K., and Gottlieb, D., J. Bact. *100* (1969) 310.
16 Varma, S. D., and Kinoshita, J. H., Biochem. Pharmac. *25* (1976) 2505.
17 Shimizu, M., Ito, T., Terashima, S., Hayashi, T., Arisawa, M., Morita, N., Kurokawa, S., Ito, K., and Hashimoto, Y., Phytochemistry *23* (1984) 1885.
18 Varma, S. D., Mikuni, I., and Kinoshita, J. H., Science *188* (1975) 1215.
19 O'Brien, M. M., Schofield, P. J., and Edwards, M. R., J. Neurochem. *39* (1982) 810.
20 Srivasstava, S. K., Petrash, J. M., Sadana, J. J., Ansari, N. H., and Partridge, C. A., Curr. Eye Res. *2* (1982) 407.
21 DeJong, K. S., Schofield, P. J., and Edwards, M. R., Biochem. J. *242* (1987) 143.
22 Poulsom, R., Biochem. Pharmac. *35* (1986) 2955.
23 Sato, S., and Kador, P. F., Biochem. Pharmac. *40* (1990) 1033.
24 Varma, S. D., in: Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships, p. 343. Eds Y. Cody, E. Middleton and J. B. Harborne. Alan R. Liss, Inc., New York 1986.
25 Inagaki, K., Miwa, K., Yashiro, T., and Okuda, J., Chem. Pharm. Bull. *30* (1982) 3244.
26 Bohmont, C., Aaronson, L. M., Mann, K., and Pardini, R. S., J. nat. Produc. *50* (1987) 427.
27 Hodnick, W. F., Kung, F. S., Roettger, W. J., Bohmont, C. W., and Pardini, R. S., Biochem. Pharmac. *35* (1986) 2345.
28 Hodnick, W. F., Roettger, W. J., Kung, F. S., Bohmont, C. W., and Pardini, R. S., in: Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships, p. 249. Eds V. Cody, E. Middleton, and J. B. Harborne. Alan R. Liss, Inc., New York 1986.
29 Hodnick, W. F., Duval, D. L., and Pardini, R. S., Biochem. Pharmac. *47* (1994) 573.
30 Itaya, S., and Igarashi, K., Biosci. Biotech. Biochem. *56* (1992) 1492.