

Effects of Ellagic Acid and 2-(2,3,6-Trihydroxy-4-carboxyphenyl)ellagic Acid on Sorbitol Accumulation *in Vitro* and *in Vivo*

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Caesalpinia ferrea MART. (Leguminosae) called as Jucá is one of the medicinal plants in Brazil used for diabetes. From the fruits of this plant, ellagic acid (EA) and 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid (TEA) have been recently isolated as aldose reductase (AR) inhibitors. In this study, we examined to prove the inhibitory activity against AR of EA and TEA *in vitro*, and EA *in vivo* by measurement of the accumulation of sorbitol, which is the product of glucose reduction catalyzed by AR. TEA was not examined *in vivo* because of its shortage of yield from the fruits. EA and TEA significantly and dose-dependently inhibited sorbitol accumulation in erythrocytes, lens and sciatic nerve under incubating with glucose *in vitro*. EA at a dose of 75 mg/kg/d showed the most potent inhibition of sorbitol accumulation in erythrocytes, lens and sciatic nerve at 50, 75 and 100 mg/kg/d *in vivo*. These results suggest that the inhibitory activity of EA against AR causes to inhibit sorbitol accumulation by *in vitro* and *in vivo* experiments. EA is distributed in fruits and vegetables, so that taking them might be able to relieve diabetic complications.

Key words aldose reductase inhibitor; sorbitol accumulation; ellagic acid; 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid; diabetes; *Caesalpinia ferrea*

Aldose reductase (AR) is an enzyme to catalyze the reduction of glucose to sorbitol in the polyol pathway. AR is more active in diabetes than in normal condition. And then the polyol pathway in diabetes accelerates the formation of sorbitol in insulin-insensitive tissues such as nerve, lens, retina and kidney, so that diabetic complications, such as neuropathy, cataract, retinopathy and nephropathy are induced.¹⁾ In fact, sorbitol accumulation is observed in the crystalline lens of experimental diabetic rats, resulting in induction of osmotic stress followed by tissue damage.²⁾ A number of AR inhibitors have been developed to treat for these complications, however, none of them has achieved worldwide use because of limited efficacy or undesirable side effects.³⁾

Ellagic acid (EA, Fig. 1) has been found as an AR inhibitor from whole plants of *Phyllanthus niuri* L. (Euphorbiaceae),⁴⁾ leaves of *Potentilla candicans* FISCH. ex LEHM. (Rosaceae),⁵⁾ roots of *Chrysanthemum morifolium* HEMSL. (Compositae),⁶⁾ flowers of *Ipomoea batatas* POIRET (Convolvulaceae),⁶⁾ fruits of *Caesalpinia ferrea* MART. (Leguminosae),⁷⁾ and leaves of *Myrciaria dubia* H. B. & K. (Myrtaceae).⁸⁾ The inhibitory activity of EA against AR was ten times higher than that of quercetin.⁷⁾ It is known that derivatives of EA such as 3,3',4-tri-*O*-methyl ellagic acid 4'-sulfate potassium salt, 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid (TEA, Fig. 1) and 4-(α -rhamnopyranosyl)ellagic acid showed about three times higher activities than EA.^{5,7,8)} Although EA and its derivatives have been reported to be AR inhibitors, their effects on sorbitol accumulation *in vitro* and *in vivo* have not been previously reported.

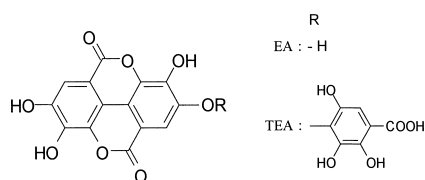


Fig. 1. Structures of Ellagic Acid (EA) and 2-(2,3,6-Trihydroxy-4-carboxyphenyl)ellagic Acid (TEA)

In this report, the effects of EA and TEA on sorbitol accumulation in erythrocytes, lens and sciatic nerve incubated with glucose *in vitro*, and the effect of EA on sorbitol accumulation in erythrocytes, lens and sciatic nerve of diabetic rats *in vivo* are discussed.

MATERIALS AND METHODS

Materials Ellagic acid (EA), sorbitol and sorbitol dehydrogenase (SDH) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). β -Nicotineamide adenine dinucleotide (NAD) and streptozotocin (STZ) were obtained from Wako Chemical Co. (Osaka, Japan). Epalrestat was kindly supplied by Ono Pharmaceutical Co. (Osaka, Japan). 2-(2,3,6-Trihydroxy-4-carboxyphenyl)ellagic acid (TEA) was obtained from *Caesalpinia ferrea* MART.⁷⁾

Animals Male Wistar rats were purchased from Japan SLC Inc. (Hamamatsu, Japan).

***In Vitro* Experiments** Under ether anesthesia, blood was collected into a heparinized tube from the abdominal aorta of the rats, and then lenses and sciatic nerve were quickly removed. Erythrocytes were obtained by centrifugation at $1500\times g$ and washed with cold saline twice, the content of hemoglobin in erythrocytes was measured with a kit (Hemoglobin B-test Wako; Wako Chemical Co.), 1 ml of erythrocytes was added into 3 ml of Krebs-Ringer bicarbonate buffer containing 28 mM glucose, and each compound dissolved in 25 μ l of dimethyl sulfoxide (DMSO) to prepare from 0 to 1.0×10^{-4} M, and was incubated under the circumstances equilibrated with 95% air and 5% CO₂ at 37 °C for 3 h.

The removed lens and sciatic nerve were separately put into 5 ml of Krebs-Ringer bicarbonate buffer containing 50 mM glucose, and each compound dissolved in 25 μ l of DMSO to prepare from 0 to 4.0×10^{-4} M, and were incubated under the circumstances equilibrated with 95% air and 5% CO₂ at 37 °C for 4 and 6 h, respectively.

The sorbitol contents were measured by the method of

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Terashima *et al.*,⁹⁾ and others,^{10–12)} using a Hitachi F-2000 fluorescence spectrophotometer under the excitation and emission at 366 and 452 nm, respectively.

In Vivo Experiments The rats (6 weeks of age) were housed in individual cages and had free access to food (normal rat chow) and water, and were maintained on 12 h dark/light cycle in a room with controlled temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) for one week. After fasted overnight, the diabetic rats were induced by a single intravenous injection of STZ (60 mg/kg). STZ solution was prepared as follows, 24 mg of STZ was dissolved in 1 ml of 5 mM citrate buffer (pH 4.5) before the injection, and the volume of 2.5 ml/kg was administered into rat. Normal rats were injected with the vehicle only. Six days after STZ injection, blood samples were collected from the tail vein, and the induction of diabetes was confirmed by the measurement of blood glucose using a kit (Glucose B-test Wako; Wako Chemical Co.). In this manner the rats with plasma glucose levels of greater than 300 mg/dl were regarded as diabetic rats.

One week after STZ injection, EA (50, 75, 100 mg/kg) and epalrestat (50 mg/kg)¹³⁾ were orally administered once daily for 2 weeks. These compounds were suspended in 0.5% carboxy methyl cellulose (CMC) solution, and the volume of 10 ml/kg was given into rat. Normal and untreated diabetic rats were administered with 0.5% CMC solution alone. The rats were then sacrificed by collecting blood from the abdominal aorta under ether anesthesia. The collected blood was immediately used for the measurement of glucose levels and sorbitol accumulation in plasma and erythrocytes, respectively. The lenses and sciatic nerve were removed quickly and frozen at -80°C until used. The measurement of sorbitol content was followed by *in vitro* experiments without incubation.

Statistical Analysis The data were shown as the mean \pm standard error (S.E.). Significant difference was calculated by Student's *t*-test, *p* values of less than 0.05 were considered significant. Linear regression was analyzed by the

least-squares method.

RESULTS

Effects of EA and TEA on Sorbitol Accumulation in Isolated Erythrocytes, Lens and Sciatic Nerve Incubated with Glucose *in Vitro*

Sorbitol was accumulated in erythrocytes, lens and sciatic nerve when they were separately incubated in the medium containing glucose (Figs. 2–4). EA and TEA inhibited sorbitol accumulation in each of them. IC_{50} values of EA, TEA and epalrestat as a positive control were calculated to be 2.4×10^{-6} , 3.9×10^{-5} and 4.2×10^{-6} M, respectively, when the accumulated sorbitol concentration in erythrocytes incubated without inhibitor was assumed to be 100% (Fig. 2). The inhibitory activities of sorbitol accumulations in lens and sciatic nerve by each compound were shown in Figs. 3 and 4, respectively. TEA inhibited the sorbitol accumulation in lens and sciatic nerve more than EA. The more concentrations of EA and TEA in incubated lens and sciatic nerve were, the more inhibitory activities of them on sorbitol accumulation were. At the concentration of 4.0×10^{-4} M, in lens, EA and TEA inhibited significantly the sorbitol accumulation by 13.5 and 44.6%, respectively (Fig. 3), and in sciatic nerve, EA and TEA inhibited significantly the sorbitol accumulation by 31.6 and 39.7%, respectively (Fig. 4), however, IC_{50} was not obtained because of their poor solubility over 8.0×10^{-4} M in the medium.

Effects of EA on Body Weight and Plasma Glucose Levels in Diabetic Rats *in Vivo*

Three weeks after STZ injection, the body weight of the diabetic rats was about 60% less than the ones of the normal rats (Table 1). The plasma glucose levels in the diabetic rats increased to 3 times more than in the normal rats. The body weight and the plasma glucose levels of the diabetic rats treated with EA or epalrestat for 2 weeks were not significantly different from the untreated diabetic rats (Table 1).

Effects of EA on Sorbitol Accumulation in Erythrocytes, Lens and Sciatic Nerve of Diabetic Rats *in Vivo*

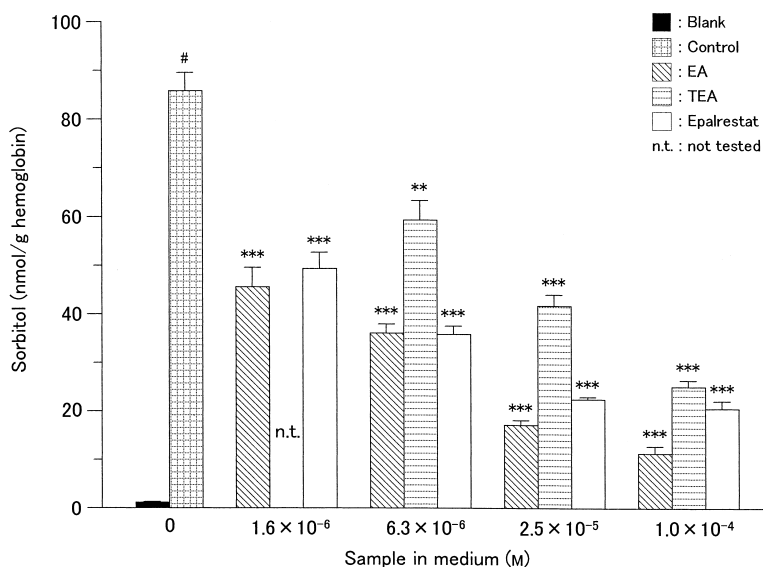


Fig. 2. Effects of EA, TEA and Epalrestat on Sorbitol Accumulation in Erythrocytes *in Vitro*

One milliliter of erythrocytes into 3 ml of Krebs-Ringer bicarbonate buffer containing 28 mM glucose, and each compound (0 to 1.0×10^{-4} M) was incubated under the circumstances equilibrated with 95% air and 5% CO_2 at 37°C for 3 h. Each column and bar represents mean \pm S.E. Statistical significance: #*p* < 0.001 vs. blank; ***p* < 0.01, ****p* < 0.001 vs. control.

The sorbitol accumulation in erythrocytes, lens and sciatic nerve were significantly increased in the diabetic rats compared with the normal rats (Table 2). In erythrocytes, EA at 75 and 100 mg/kg/d significantly reduced the elevated sorbitol accumulation. And in lens and sciatic nerve, EA significantly reduced the elevated sorbitol accumulation at a dose of 50, 75 and 100 mg/kg/d (Table 2). A dose of 75 mg/kg/d showed more potent inhibition of sorbitol accumulation in erythrocytes, lens and sciatic nerve than a dose of 100 mg/kg/d. Inhibitory activity of EA at 75 mg/kg/d on sorbitol accumulation was the almost same as epalrestat as a positive control at 50 mg/kg/d.

DISCUSSION

During the search of AR inhibitors from the Amazonian plants, EA and its derivatives such as TEA, 4-*O*-methyl-ellagic acid and 4-(α -rhamnopyranosyl)ellagic acid have been isolated as active compounds in fruits of *Caesalpinia ferrea*⁷⁾ and leaves of *Myrciaria dubia*.⁸⁾ EA has been reported to be an AR inhibitor from other plants.^{4–6)} EA is widely distributed in many plants including fruits and vegetables.^{14–19)} Therefore, if EA inhibits sorbitol accumulation *in vitro* and

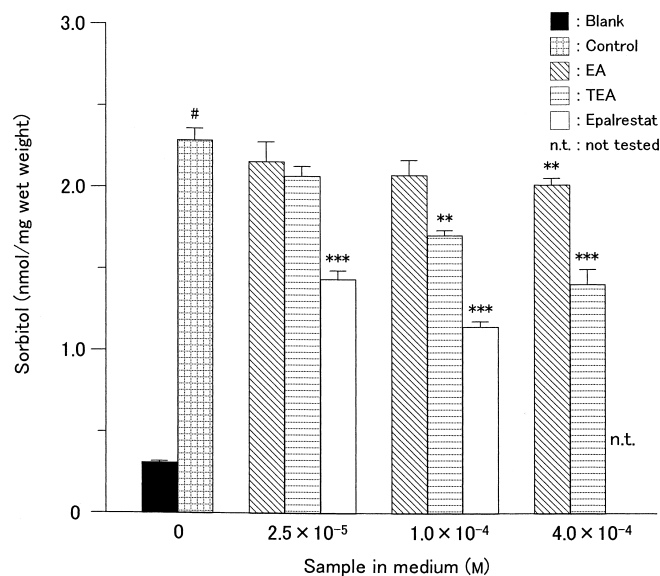


Fig. 3. Effects of EA, TEA and Epalrestat on Sorbitol Accumulation in Lens *in Vitro*

The removed lens into 5 ml of Krebs-Ringer bicarbonate buffer containing 50 mM glucose, and each compound (0 to 4.0×10^{-4} M) was incubated under the circumstances equilibrated with 95% air and 5% CO₂ at 37°C for 4 h. Each column and bar represents mean \pm S.E. Statistical significance: # $p < 0.001$ vs. blank; ** $p < 0.01$, *** $p < 0.001$ vs. control.

Table 2. Effects of EA and Epalrestat on Sorbitol Accumulation in Erythrocytes, Lens and Sciatic Nerve of STZ-Induced Diabetic Rats *in Vivo*

Group	Dose (mg/kg/d)	Erythrocytes (nmol/g hemoglobin)	Lens (nmol/mg wet weight)	Sciatic nerve (nmol/mg wet weight)
Normal ($n=5$)		14.4 \pm 0.3	0.2 \pm 0.0	0.09 \pm 0.00
Untreated diabetic ($n=6$)		101.5 \pm 3.8 [#]	27.4 \pm 0.9 [#]	2.31 \pm 0.07 [#]
EA-treated ($n=6$)	50	89.6 \pm 5.4	21.2 \pm 0.8 ^{***}	1.90 \pm 0.09 ^{**}
	75	70.6 \pm 4.3 ^{***}	16.7 \pm 0.5 ^{***}	1.62 \pm 0.08 ^{***}
	100	87.7 \pm 4.3 [*]	19.1 \pm 0.8 ^{***}	1.80 \pm 0.10 ^{**}
Epalrestat-treated ($n=6$)	50	61.8 \pm 4.0 ^{***}	15.7 \pm 0.7 ^{***}	1.39 \pm 0.10 ^{***}

The treatment was started 1 week after STZ injection, and EA and epalrestat were orally administered once daily for 2 weeks. Each value represents mean \pm S.E. Statistical significance: # $p < 0.001$ vs. normal group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated diabetic group.

in vivo, the fruits and vegetables which contain EA will be able to be useful for treatment of diabetic complications.

In vitro assay was performed to see the effects of EA and TEA on sorbitol accumulation in isolated erythrocytes, lens and sciatic nerve incubated in the medium containing glucose (Figs. 2–4). Sorbitol was accumulated in isolated erythrocytes, lens and sciatic nerve incubated in the medium

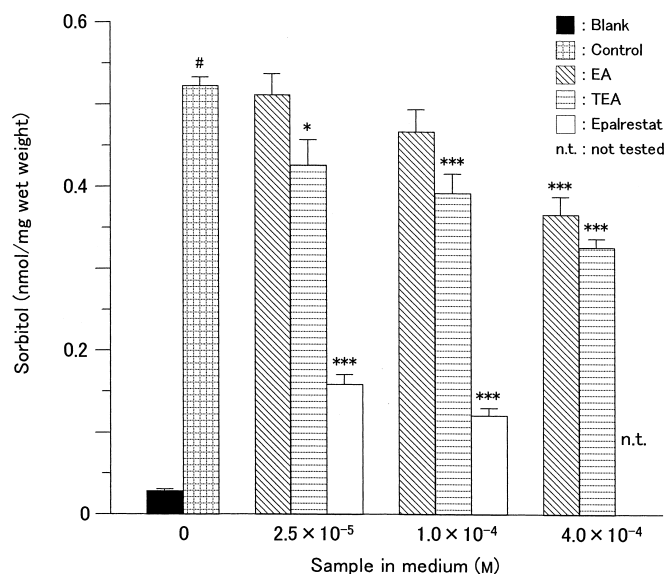


Fig. 4. Effects of EA, TEA and Epalrestat on Sorbitol Accumulation in Sciatic Nerve *in Vitro*

The removed sciatic nerve into 5 ml of Krebs-Ringer bicarbonate buffer containing 50 mM glucose, and each compound (0 to 4.0×10^{-4} M) was incubated under the circumstances equilibrated with 95% air and 5% CO₂ at 37°C for 6 h. Each column and bar represents mean \pm S.E. Statistical significance: # $p < 0.001$ vs. blank; * $p < 0.05$, *** $p < 0.001$ vs. control.

Table 1. Effects of EA and Epalrestat on Body Weight and Plasma Glucose Levels in Streptozotocin (STZ)-Induced Diabetic Rats *in Vivo*

Group	Dose (mg/kg/d)	Body weight (g)	Plasma glucose (mg/dl)
Normal ($n=5$)		247.6 \pm 5.5	187.3 \pm 2.9
Untreated diabetic ($n=6$)		154.3 \pm 7.0 [#]	548.7 \pm 16.9 [#]
EA-treated ($n=6$)	50	153.7 \pm 6.9	565.7 \pm 24.7
	75	160.0 \pm 5.6	552.6 \pm 23.6
	100	149.0 \pm 2.3	579.2 \pm 14.7
Epalrestat-treated ($n=6$)	50	150.8 \pm 3.6	535.6 \pm 24.4

The treatment was started 1 week after STZ injection, and EA and epalrestat were orally administered once daily for 2 weeks. Each value represents mean \pm S.E. Statistical significance: # $p < 0.001$ vs. normal group.

with glucose, although sorbitol was almost not found in them incubated in the medium without it. Both EA and TEA inhibited sorbitol accumulation in isolated erythrocytes, lens and sciatic nerve incubated in the medium containing glucose. These results suggest that the inhibitory activities of EA and TEA against AR cause to inhibit sorbitol accumulation in erythrocytes, lens and sciatic nerve *in vitro*. EA was more active than TEA in erythrocytes, but TEA was more active than EA in lens and sciatic nerve.

In vivo assay, EA at 50, 75 and 100 mg/kg/d, and epalrestat at 50 mg/kg/d as a positive control were administered into the STZ-induced diabetic rats for 2 weeks.²⁰⁾ The body weight and plasma glucose levels of the untreated diabetic rats were reduced and elevated, respectively, comparing with normal rats (Table 1). The body weight and plasma glucose levels of the diabetic rats treated with EA and epalrestat were not recovered to those of the normal rats (Table 1). The sorbitol was accumulated in erythrocytes, lens and sciatic nerve of the untreated diabetic rats (Table 2). The sorbitol accumulations in erythrocytes, lens and sciatic nerve of the diabetic rats treated with EA were less than those of the untreated diabetic rats (Table 2). These observations demonstrate that EA inhibits sorbitol accumulation in erythrocytes, lens and sciatic nerve of the diabetic rats by its inhibitory activity against AR. EA at a dose of 75 mg/kg/d showed the most potent inhibition of sorbitol accumulation in three dosages. Inhibitory effects of EA at 100 mg/kg/d were not more than ones of 75 mg/kg/d. That is because it could be a limiting absorbance in digestive tube at 100 mg/kg/d.

The inhibitory activities of EA and TEA against AR were proved *in vitro*, as well as the one of EA *in vivo*. EA is distributed in many plants including medicinal plants,^{4–8)} fruits and vegetables.^{14–19)} The patients with diabetic complications might make sorbitol accumulation decreased by taking fruits and vegetables containing EA. The fruits and vegetables containing EA might be able to relieve diabetic complications.

REFERENCES AND NOTES

- 1) Haraguchi H., Kanada M., Fukuda A., Naruse K., Okamura N., Yagi A., *Planta Med.*, **64**, 68–69 (1998).
- 2) Sakai I., Izumi S., Murano T., Okuwaki S., Makino T., Suzuki T., *Jpn. J. Pharmacol.*, **85**, 322–326 (2001).
- 3) Hotta N., Toyota T., Matsuoka K., Shigeta Y., Kikkawa R., Kaneko T., Takahashi A., Sugimura K., Koike Y., Ishii J., Sakamoto N., *Diabetes Care*, **24**, 1776–1782 (2001).
- 4) Shimizu M., Horie S., Terashima S., Ueno H., Hayashi T., Arisawa M., Suzuki S., Yoshizaki M., Morita N., *Chem. Pharm. Bull.*, **37**, 2531–2532 (1989).
- 5) Terashima S., Shimizu M., Nakayama H., Ishikura M., Ueda Y., Imai K., Suzui A., Morita N., *Chem. Pharm. Bull.*, **38**, 2733–2736 (1990).
- 6) Terashima S., Shimizu M., Horie S., Morita N., *Chem. Pharm. Bull.*, **39**, 3346–3347 (1991).
- 7) Ueda H., Tachibana Y., Moriyasu M., Kawanishi K., Alves S. M., *Phytomedicine*, **8**, 377–381 (2001).
- 8) Ueda H., Kuroiwa E., Tachibana Y., Kawanishi K., Ayala F., Moriyasu M., *Phytomedicine*, in press.
- 9) Terashima H., Hama K., Yamamoto R., Tsuboshima M., Kikkawa R., Hatanaka I., Shigeta Y., *J. Pharmacol. Exp. Ther.*, **229**, 226–230 (1984).
- 10) Malone J. I., Knox G., Benford S., Tedesco T. A., *Diabetes*, **29**, 861–864 (1980).
- 11) Haraguchi H., Ohmi I., Fukuda A., Tamura Y., Mizutani K., Tanaka O., Chou W.-H., *Biosci. Biotech. Biochem.*, **61**, 651–654 (1997).
- 12) Shinohara R., Ohta Y., Yamauchi M., Ishiguro I., *Clin. Chim. Acta*, **273**, 171–184 (1998).
- 13) Yoshida T., Nishioka H., Yoshioka K., Nakano K., Kondo M., Terashima H., *Diabetes*, **36**, 6–13 (1987).
- 14) Narayanan B. A., Geoffroy O., Willingham M. C., Re G. G., Nixon D. W., *Cancer Lett.*, **136**, 215–221 (1999).
- 15) Amakura Y., Okada M., Tsuji S., Tonogai Y., *J. Chromatogr. A*, **896**, 87–93 (2000).
- 16) Cordenunsi B. R., Oliveira do Nascimento J. R., Genovese M. I., Lajolo F. M., *J. Agric. Food Chem.*, **50**, 2581–2586 (2002).
- 17) Wada L., Ou B., *J. Agric. Food Chem.*, **50**, 3495–3500 (2002).
- 18) Mullen W., Yokota T., Lean M. E. J., Crozier A., *Phytochemistry*, **64**, 617–624 (2003).
- 19) Mertens-Talcott S. U., Talcott S. T., Percival S. S., *J. Nutr.*, **133**, 2669–2674 (2003).
- 20) TEA was not tested for *in vivo* because of its shortage of yield.