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Inhibitory effect on α-glucosidase by the fruits of *Terminalia chebula* Retz.

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Abstract

Mammalian α-glucosidase inhibitory activity by *Terminalia chebula* Retz. fruits was investigated. The aqueous methanolic extract was found to have potent rat intestinal maltase inhibitory activity, whereas neither intestinal sucrase nor isomaltase activity was inhibited by this extract. Using bioassay-guided separation, three active ellagitannins were identified as chebulanin (**1**), chebulagic acid (**2**) and chebulinic acid (**3**) and were shown to possess potent intestinal maltase inhibitory activity, with the IC_{50} values of 690 μM, 97 μM and 36 μM, respectively. The intestinal maltase inhibitory activities of **2** and **3** were even higher than that of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) (**4**, IC_{50}=140 μM), which is a known potent α-glucosidase inhibitor. Comparison of the activities of **1**–**4**, 1,2,3-*O*-trigalloyl-β-D-glucose (**5**), neochebulagic acid (**6**) and corilagin (**7**) suggested that the positions of chebulloyl and galloyl groups mostly affected the potency. Kinetic studies revealed that **2**, **3**, and **4** inhibited maltose-hydrolyzing activity of intestinal α-glucosidase, noncompetitively. This is the first report on mammalian α-glucosidase inhibition by **1**, **2** and **3** isolated from *T. chebula* fruits. These results suggest a use of the extract of *T. chebula* fruits for managing Type 2 diabetes.

Keywords: α-Glucosidase inhibitor; Intestinal maltase; *Terminalia chebula*; Ellagitannins

1. Introduction

Mammalian α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), located in the brush-border surface membrane of intestinal cells, is the key enzyme which catalyzes the final step in the digestive process of carbohydrates. Hence, α-glucosidase inhibitors can retard the liberation of D-glucose from complex dietary carbohydrates and delay glucose absorption, reducing plasma glucose levels and suppressing postprandial hyperglycemia (Lebovitz, 1997). Consequently, α-glucosidase inhibitors, such as acarbose (Balfour & McTavish, 1993) and miglitol (Pogano et al., 1995) have been approved for clinical use in the management of Type 2 diabetes, as well as the treatment of obesity.

From this point of view, many efforts have been made to search for other effective and safe α-glucosidase inhibitors from natural materials in order to develop a physiological functional food or lead compounds for antidiabetes treatment (Matsui et al., 2001; Mcdougall et al., 2005; Niwa, Doi, & Osawa, 2003; Yoshikawa, Morikawa, Matsuda, Tanabe, & Muraoka, 2002). In a series of our studies on rat intestinal α-glucosidase-inhibiting principles from plant sources, we previously reported that baicalein (5,6,7-trihydroxyflavone) from *Scutellaria baicalensis,* and the related 6-hydroxyflavones from *Origanum majorana*, constituted a new class of α-glucosidase inhibitors (Kawabata et al., 2003; Nishioka, Kawabata, & Aoyama, 1998). As a continued study, various mechanistic and structural development studies have been reported (Gao & Kawabata, 2004; Gao, Nishioka, Kawabata, & Kasai, 2004; Gao & Kawabata, 2005).

Terminalia chebula Retz., indigenous to Pakistan and India, is a member of the Combretaceae family. The dried

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fruit of *T. chebula* Retz. (Chebulae Fructus), commonly known as black myrobalan in English and Xi-Qin-Ge or Zhang-Qin-Ge in China, is a popular folk medicine. In China, it is applied as a carminative, deobstruent, astringent and expectorant reagent, and also as a remedy for salivating and heartburn. *T. chebula* has been studied for its antioxidant (Chen, Lin, Yu, Yang, & Lin, 2003), antimicrobial (Burapadaja & Bunchoo, 1995), and anticancer (Saleem, Husheem, Harkonen, & Pihlaja, 2002) activities. Recently, it was reported that oral administration of the extracts form *T. chebula* reduced the blood glucose level in normal and in alloxan-diabetic rats (Sabu & Kuttan, 2002). In the course of our ongoing programme on identifying α-glucosidase-inhibiting principles from natural medicines in China, we found that the aqueous methanolic extract of the fruits of *T. chebula* showed high maltase inhibitory activity, while this extract did not show sucrase or isomaltase inhibitory activity. Since this is the first observation on the inhibitory effect of *T. chebula* on α-glucosidase activity, this finding prompted us to undertake the isolation and structural elucidation of active compounds from the fruits of *T. chebula* as well as to study their inhibitory properties and mechanisms of action.

2. Materials and methods

2.1. Materials

The dried fruit of *T. chebula* was purchased from a local herbal market in Chengdu, People's Republic of China. A voucher specimen was deposited in the department of Public Health, Hua Xi Medical Center of Sichuan University. All chemicals used were of reagent grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan) unless otherwise stated. All solvents were distilled before use.

2.2. General procedure

NMR spectra were recorded with a Bruker AMX500 instrument (^{1}H, 500 MHz). Electron spray ionization (ESI) and ESI-high resolution (HR) MS were obtained with a Jeol JMS-700TZ instrument. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. Melting points were measured with a hot-stage apparatus and are uncorrected. Column chromatography was performed with Diaion HP-20 (Mitsubishi Kasei Co., Ltd.) and Toyopearl HW-40 (fine grade, Tosoh Corp.). Preparative HPLC was conducted with an Inertsil PREP-ODS-3 column (20.0 × 250 mm, GL-Science). Detailed analytical conditions are mentioned in each section.

2.3. Extraction and isolation of chebulanin (*1*), chebulagic acid (*2*) and chebulinic acid (*3*)

The dried fruit of *T. chebula* (20 g) was crushed into powder with a mixer, followed by extraction (three times) with 70% methanol (2 l) in total for 24 h, with continuous stirring at room temperature, and filteration. Evaporation of the solvent under reduced pressure yielded the 70% methanol extract (6.0 g). This extract was suspended in water (150 ml) and re-extracted with hexane (300 ml × 3) and then with chloroform (300 ml × 3). Each fraction was evaporated at reduced pressure to give hexane (0.6 g), chloroform (0.5 g) and water (4.9 g) fractions. The water fraction (4.9 g) was applied on a Diaion HP-20 column (4.2 × 60 cm), with a water–methanol gradient, to give seven fractions. The eluted solvent volume and yield of each fraction were as follows: fr. 1: water (2000 ml) (1.22 g), fr. 2: 10% methanol in water (2000 ml) (0.1 g), fr. 3: 20% methanol in water (3000 ml) (0.55 g), fr. 4: 30% methanol in water (3000 ml) (0.64 g), fr. 5: 40% methanol in water (4000 ml) (1.5 g), fr. 6: 50% methanol in water (3000 ml) (0.5 g) and fr. 7: 100% methanol (2000 ml) (0.3 g). Fraction 4 (0.64 g) was subjected to the Toyopearl HW-40 column [4.2 × 36 cm, water–methanol (70:30)] and the first eluted (1 l) fraction (0.21 g) was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (40:60:0.1); flow rate, 5 ml/min; t_R, 17.4 min; detection, 280 nm) to give chebulanin (**1**, 80 mg). Fraction 5 (1.5 g) was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (40:60:0.1); flow rate, 5 ml/min; t_R, 20.8 min; detection, 280 nm) to give chebulagic acid (**2**, 900 mg). Fraction 6 (0.5 g) was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (50:50:0.1); flow rate, 5 ml/min; t_R, 16.7 min; detection, 280 nm) to give chebulinic acid (**3**, 210 mg).

Chebulanin (**1**): an off-white powder, $[\alpha]_D^{23}$ +13.5° (MeOH; *c* 0.5); mp 217–219 °C; HR-ESIMS (negative), *m/z* 651.0834 $[M–H]^-$; calcd for $C_{27}H_{23}O_{19}$, 651.0832; ^{1}H NMR δ (acetone-d_6) ppm (*J* in Hz): 2.18 (2H, m, che-5′-H), 3.89 (1H, m, che-4′-H), 4.00 (1H, dd, *J* = 11.3, 5.7, glc-6-H), 4.13 (1H, dd, *J* = 11.3, 6.7, glc-6-H), 4.30 (1H, t, *J* = 6.4, glc-5-H), 4.82 (1H, br s, glc-3-H), 4.88 (1H, m, glc-4-H), 4.92 (1H, d, *J* = 7.1, che-2′-H), 5.17 (1H, dd, *J* = 7.1, 1.5, che-3′-H), 5.23 (1H, dd, *J* = 2.7, 1.5, glc-2-H), 6.35 (1H, d, *J* = 2.7, glc-1-H), 7.19 (2H, s, galloyl-H), 7.49 (1H, s, che-3″-H).

Chebulagic acid (**2**): a white powder, $[\alpha]_D^{23}$ −47.7° (EtOH; *c* 0.8); mp 252–253 °C; HR-ESIMS (negative), *m/z* 953.0905 $[M–H]^-$; calcd. for $C_{41}H_{29}O_{27}$, 953.0894; ^{1}H NMR δ (acetone-d_6) ppm (*J* in Hz): 2.18 (2H, m, che-5′-H), 3.87 (1H, m, che-4′-H), 4.39 (1H, dd, *J* = 8.1, 5.4, glc-6-H), 4.76 (1H, d, *J* = 9.4, glc-6-H), 4.79 (1H, d, *J* = 5.2, glc-5-H), 4.95 (1H, d, *J* = 7.1, che-2′-H), 5.11 (1H, dd, *J* = 7.1, 1.5, che-3′-H), 5.21 (1H, d, *J* = 3.7, glc-4-H), 5.50 (1H, br s, glc-2-H), 5.94 (1H, br s, glc-3-H), 6.49 (1H, br s, glc-1-H), 6.64 (1H, s, HHDP-H), 7.07 (1H, s, HHDP-H), 7.18 (2H, s, galloyl-H), 7.51 (1H, s, che-3″-H).

Chebulinic acid (**3**): a white powder, $[\alpha]_D^{23}$ +61.4° (EtOH; *c* 0.8); mp 272–274 °C; HR-ESIMS (negative), *m/z* 955.0886 $[M–H]^-$; calcd. for $C_{41}H_{31}O_{27}$, 955.1053; ^{1}H NMR δ (acetone-d_6) ppm (*J* in Hz): 2.18 (2H, m, che-5′-H), 3.94 (1H, m, che-4′-H), 4.71 (1H, m, glc-5-H), 4.73

(1H, m, glc-6-H), 4.85 (1H, dd, $J = 11.1$, 5.2, glc-6-H), 4.96 (1H, d, $J = 7.1$, che-2′-H), 5.08 (1H, d, $J = 3.5$, glc-4-H), 5.17 (1H, dd, $J = 7.1$, 3.7, che-3′-H), 5.47 (1H, br s, glc-2-H), 6.34 (1H, s, glc-3-H), 6.51 (1H, br s, glc-1-H), 7.05 (2H, s, galloyl-H), 7.22 (2H, s, galloyl-H), 7.28 (2H, s, galloyl-H), 7.55 (1H, s, che-3″-H).

1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**4**): compound **4** was prepared from β-D-glucose and 3,4,5-tri(benzyloxy)benzoic acid, using dicyclohexylcarbodiimide (DCC) as the coupling reagent, followed by deprotection according to the method described in the literature (Khanbabaee & Lotzerich, 1997; Ren, Himmeldirk, & Chen, 2006). Purification by preparative HPLC (mobile phase, water–methanol–formic acid (40:60:0.1); flow rate, 5 ml/min; t_R, 27.3 min; detection, 280 nm) gave **4** as a pale yellow powder, $[\alpha]_D^{23}$ +15.0° (MeOH; *c* 0.5); mp 265–267 °C; HR-ESIMS (negative), *m/z* 939.1096 [M–H]$^-$; calcd. for $C_{41}H_{31}O_{26}$, 939.1101; ^{1}H NMR δ (acetone-d_6) ppm (*J* in Hz): 4.40 (1H, dd, $J = 12.8$, 4.7, glc-6-H), 4.52 (1H, dd, $J = 12.8$, 2.2, glc-6-H), 4.56 (1H, m, glc-5-H), 5.60 (1H, dd, $J = 9.9$, 8.4, glc-2-H), 5.64 (1H, t, $J = 9.8$, glc-4-H), 5.99 (1H, t, $J = 9.8$, glc-3-H), 6.32 (1H, d, $J = 8.1$, glc-1-H), 6.96, 7.00, 7.05, 7.10 and 7.17 (each 2H, s, galloyl-H).

Neochebulagic acid (**6**) and corilagin (**7**): compounds **6** and **7** were prepared according to the method described in the literature (Tanaka, Kouno, & Nonaka, 1996) with a slight modification. A solution of 40 mg of **2** in 10 ml of water was heated at 90 °C for 10 h, and subjected to column chromatography on Sephadex LH-20 (3 × 20 cm) with a mixture of methanol–water (8:2) to give **6** (6.4 mg) and **7** (5 mg). Compound **6**: an off-white solid, HR-ESIMS (negative), *m/z* 971.0995 [M–H]$^-$; calcd. for $C_{41}H_{31}O_{28}$, 971.0999; ^{1}H NMR δ (DMSO-d_6) ppm (*J* in Hz): 2.11 (1H, m, che-2′-H), 2.78 (1H, m, che-2′-H), 3.02 (1H, m, che-3′-H), 3.69 (1H, d, $J = 9.1$, che-4′-H), 3.97 (1H, d, $J = 6.4$, glc-2-H), 4.11 (1H, dd, $J = 11.8$, 5.4, glc-6-H), 4.32 (1H, m, glc-5-H), 4.42 (1H, dd, $J = 11.8$, 5.4, glc-6-H), 4.70 (1H, d, $J = 3.2$, glc-3-H), 5.16 (1H, br s, che-2″-H), 5.47 (1H, d, $J = 3.2$, glc-4-H), 5.92 (1H, d, $J = 6.4$, glc-1-H), 6.50, 6.60 (each 1H, s, HHDP-H), 6.88 (1H, s,

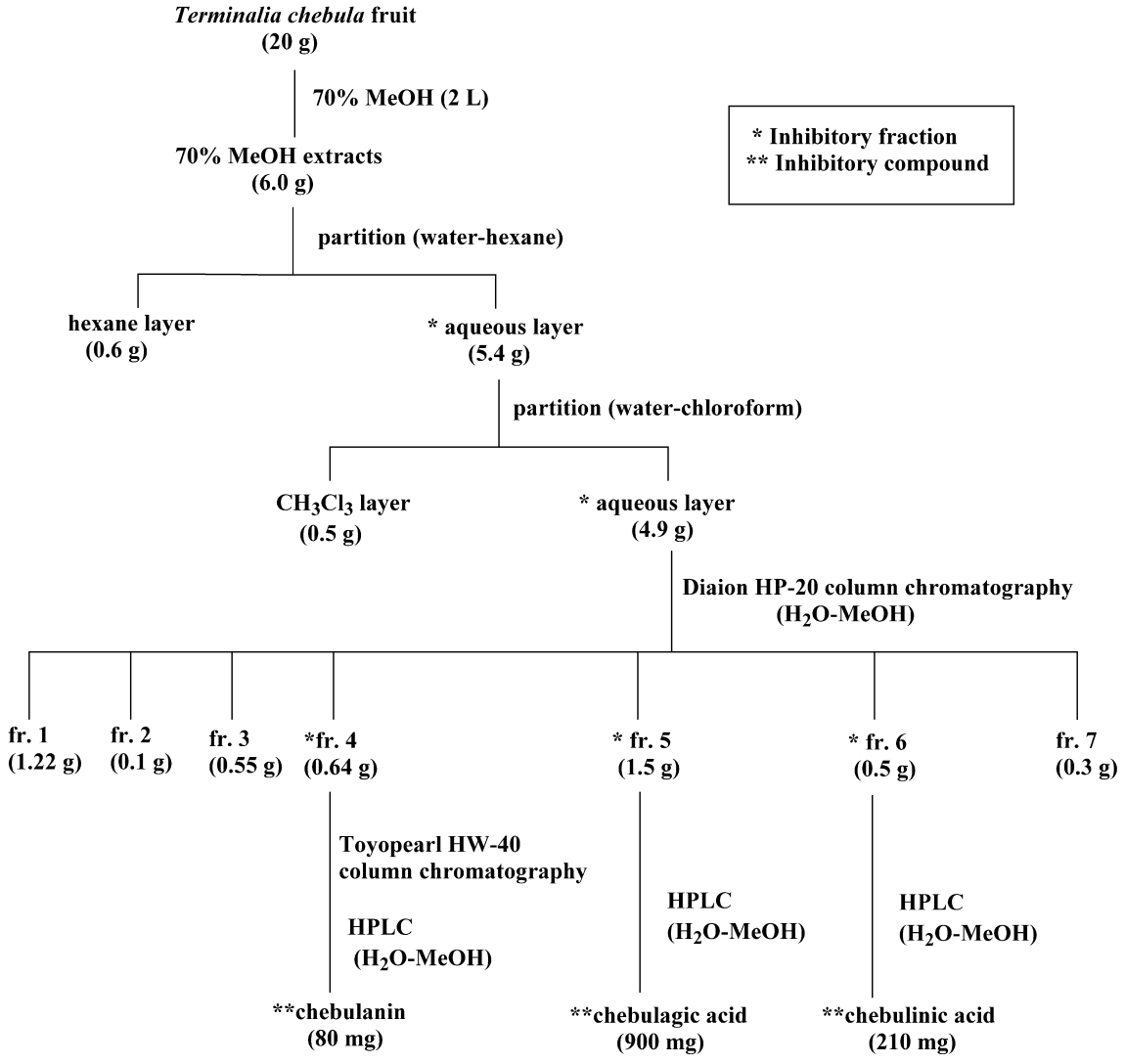

Fig. 1. Isolation scheme of the maltase inhibitory compounds from *Terminalia chebula* fruit.

che-3″-H), 7.01 (2H, s, galloyl-H). Compound **7**: an off-white solid, HR-ESIMS (negative), *m/z* 633.0727 [M–H]$^-$; calcd. for $C_{27}H_{21}O_{18}$, 633.0726; ^{1}H NMR δ (DMSO-d_6) ppm (J in Hz): 3.83 (1H, br s, glc-2-H), 3.93 (1H, d, $J = 10.6$, glc-6-H), 4.20 (1H, br s, glc-4-H), 4.23 (1H, d, $J = 10.6$, glc-6-H), 4.34 (1H, t, $J = 7.1$, glc-5-H), 4.58 (1H, br s, glc-3-H), 6.19 (1H, d, $J = 7.1$, glc-1-H), 6.48, 6.55 (each 1H, s, HHDP-H), 7.00 (2H, s, galloyl-H).

2.4. Intestinal α-glucosidase inhibitory activity determination

The α-glucosidase inhibitory activity was measured as described previously (Gao & Kawabata, 2005). The crude enzyme solution prepared from rat intestinal acetone powder (Sigma Aldrich Japan Co. Tokyo, Japan) was used as a source of the small intestinal α-glucosidases, sucrase, maltase and isomaltase, with specific activities of 0.38 U/ml, 1.85 U/ml, and 1.56 U/ml, respectively. The reaction mixture consisted of crude enzyme solution (as sucrase, 0.2 ml; as maltase, 0.05 ml; as isomaltase, 0.2 ml), substrate (sucrose: 56 mM, 0.2 ml; maltose: 3.5 mM, 0.35 ml; isomaltose: 7 mM, 0.2 ml, respectively) in 0.1 M potassium phosphate buffer (pH 6.3), and the test compound in 25% aqueous dimethyl sulfoxide (DMSO, 0.1 ml) in a final reaction volume of 0.5 ml. After incubation for 15 min at 37 °C, the reaction was stopped by adding 0.75 ml of 2 M Tris HCl buffer (pH 7.0). The reaction mixture was passed through a short column of basic alumina (ICN Alumina B, grade I, ICN Biomedical GmbH, Eschwege, Germany) to remove phenolic compounds which might interfere with glucose quantification. The amount of liberated glucose was measured by the glucose oxidase method using a commercial test kit (Glucose B test Wako, Wako Pure Chem. Co., Osaka, Japan). The concentration of inhibitors required for inhibiting 50% of the α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

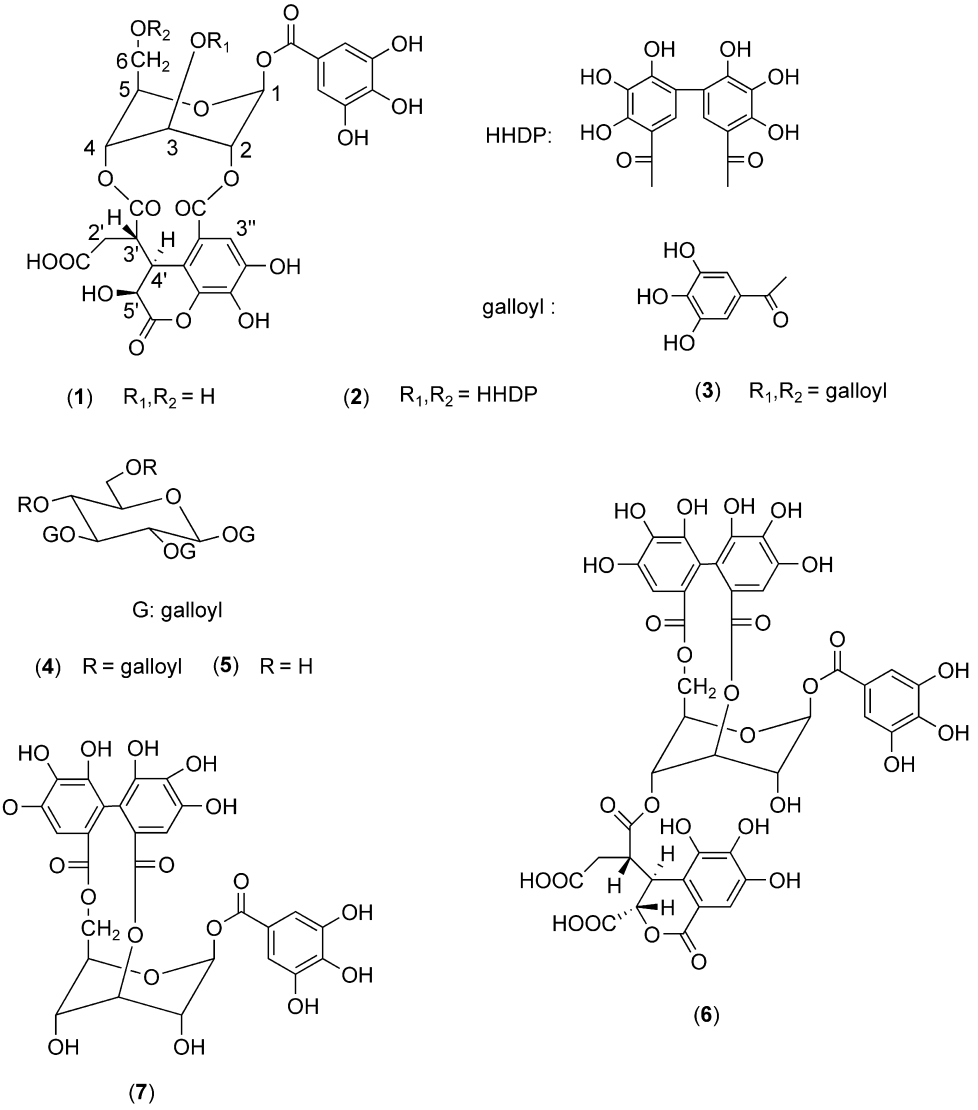

Fig. 2. Chemical structures of chebulanin (**1**), chebulagic acid (**2**), chebulinic acid (**3**), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**4**), and 1,2,3-tri-*O*-galloyl-β-D-glucopyranose (**5**), neochebulagic acid (**6**) and corilagin (**7**).

For kinetic analyses of maltase by chebulagic acid (**2**), chebulinic acid (**3**) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**4**), the enzyme and the test compounds [**2**, 125 μg/ml (131 μM); **3**, 31.3 μg/ml (33 μM) or **4**, 125 μg/ml (133 μM), respectively] were incubated with increasing concentrations of maltose. Inhibitory kinetics of **2**, **3**, and **4** for maltase were analyzed by Lineweaver–Burk plots.

3. Results and discussion

The dried *T. chebula* fruits were extracted with 70% methanol at room temperature. The aqueous methanolic extract inhibited rat intestinal maltase activity with maltose as a substrate, with an IC_{50} value of 100 μg/ml. The aqueous methanolic extract showed no inhibitory effect on rat intestinal sucrase or isomaltase activity with respect to sucrose or isomaltose as a substrate tested up to a high concentration of 500 μg/ml (data not shown). To isolate maltase inhibitors from the fruits of *T. chebula*, the aqueous methanolic extract was fractionated and fractions monitored for α-glucosidase activity using a bioassay based on maltase inhibitory activity against rat small intestinal enzyme (Fig. 1). The aqueous methanolic extract was first evaporated, and the resulting residue was successively extracted with hexane and chloroform. The hexane and chloroform fractions were inactive, while the water fraction was active (62% inhibition at 500 μg/ml), and this fraction was subjected to Diaion HP-20 column chromatography to yield seven fractions (fr. 1–7). The active fraction 4 (52% inhibition at 500 μg/ml), eluted with water–methanol (7:3), was then subjected to Toyopearl HW-40 column chromatography and finally preparative HPLC to give chebulanin (**1**, 80 mg). And also, the active fractions 5 (63% inhibition at 500 μg/ml) and 6 (74% inhibition at 500 μg/ml) from the Diaion column were further purified by preparative HPLC, yielding chebulagic acid (**2**, 900 mg) and chebulinic acid (**3**, 210 mg), respectively. The isolates were identified by comparison of the spectral data with those in literature (Ding, Liu, Wang, Ji, & Sheng, 2001; Klica et al., 2004; Tanaka et al., 1996). The structures of **1**, **2** and **3** are shown in Fig. 2.

Compounds **1**–**3** inhibited rat intestinal maltase activity in a dose-dependent manner (Fig. 3). At the high concentration of 500 μg/ml (768 μM), **1** inhibited 52% of the maltase activity and the IC_{50} value was estimated to be 450 μg/ml (690 μM). However, 1,2,3-tri-*O*-galloyl-D-glucose (**5**) showed no inhibitory effect on the maltase activity (Toda, Kawabata, & Kasai, 2001). Hence, this result revealed that a chebuloyl group, involving diester bridges at C-2 and C-4 of the glucose core, was an important factor for the inhibitory activity.

To further reveal the effects of the chebuloyl group on the maltase inhibitory activity, neochebulagic acid (**6**) and corilagin (**7**) were prepared and compared with **2**. Partial hydrolysis of **2** by heating at 90 °C in water gave **6** and **7**, respectively. Compound **2** was particularly active, with $IC_{50} = 97$ μM (Fig. 3). In compound **6,** where a neochebuloyl group was present at C-4 of the glucose core instead of a chebuloyl group, a loss of activity was observed (29% inhibition at a concentration of 1000 μM). Compound **7,** lacking substituents at C-2 and C-4 of the glucose core, also showed a similar degree of loss of activity (28% inhibition at a concentration of 1000 μM). These results support the importance of the chebuloyl group in a diester bridge with C-2 and C-4 of the glucose core.

1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**4**) was prepared and compared with **3**. The maltase inhibitory activity of **3** ($IC_{50} = 36$ μM) was much stronger than that of **4** ($IC_{50} = 140$ μM), which is a known potent maltase inhibitor (Channell, Farmer, & Walker, 1988; Toda et al., 2001). Compound **3** consists of a $^1C_4$ glucopyranose core,

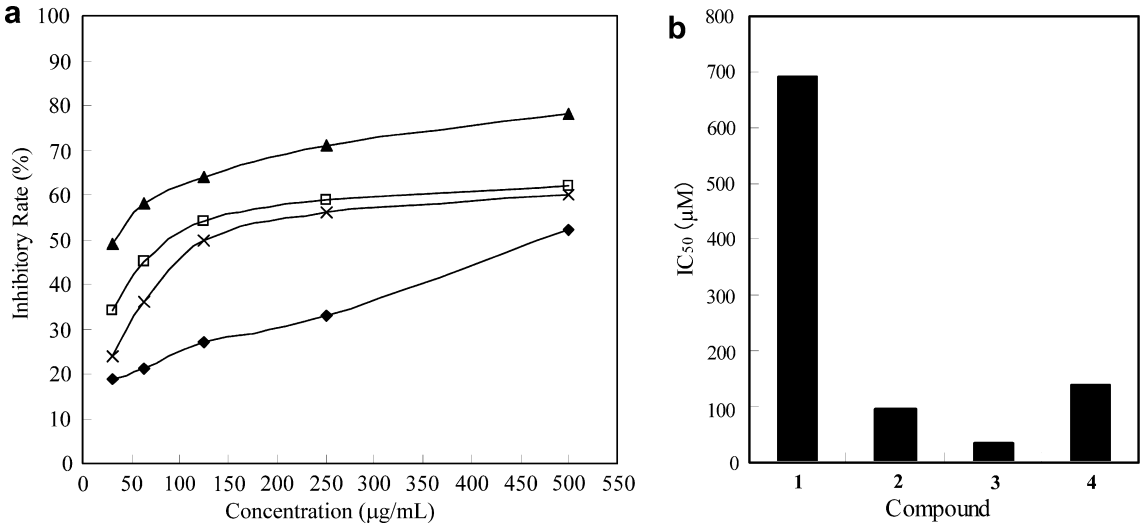

Fig. 3. (a) Inhibitory effects of chebulanin (**1**, ♦), chebulagic acid (**2**, □), chebulinic acid (**3**, ▲), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (**4**, ×), on the activity of rat small intestinal α-glucosidase for the hydrolysis of maltose. (b) IC_{50} values of compounds **1**–**4** against rat small intestinal α-glucosidase for the hydrolysis of maltose.

whereas **4** contains a $^4C_1$ glucose. This result not only supports the importance of the chebuloyl group, but also suggests that the orientation and geometry of the $^1C_4$ conformation in the molecules might affect the maltase inhibitory activity, and that the role of the chebuloyl group is to lock the glucose core in the $^1C_4$ configuration.

The comparison of **1**–**3** showed that the inhibitory values were in order of **3** > **2** > **1**, which indicates that esterification with galloyl groups at positions C-3 and C-5 produced higher maltase inhibitory activity, and that single esterification is more potent than diester bridge linkage with a HHDP group.

In order to examine the mechanism of inhibition of rat intestinal maltase by **2**, **3** and **4**, the crude rat intestinal α-glucosidase mixture were incubated with increasing concentrations of maltose, without or with a fix concentration of inhibitor. A Lineweaver–Burk plot of rat intestinal maltase kinetics is shown in Fig. 4. In all cases the kinetics observed were correlated with the classical pattern of non-competitive inhibition. Compounds **2**, **3** and **4** showed the same inhibitory type (non-competitive inhibition), in which these compounds and the maltose substrate seem to bind simultaneously to the enzyme (Ozaka, Lilley, & Haslam, 1987). The difference of K_i values (**2**, 208 μM; **3**, 24 μM; **4**, 234 μM) suggests that the maltase inhibitory activity of **3** is much higher than that of **2** or **4** due to differences in affinity for the enzyme inhibitor site.

This is the first report on the isolation of the mammalian intestinal α-glucosidase inhibitory principles, chebulanin (**1**), chebulagic acid (**2**) and chebulinic acid (**3**), from *T. chebula* in substantial yield. *T. chebula* might be useful as a medicinal food or as a source of natural α-glucosidase inhibitors for use in suppressing postprandial hyperglycemia in the management of Type 2 diabetes.

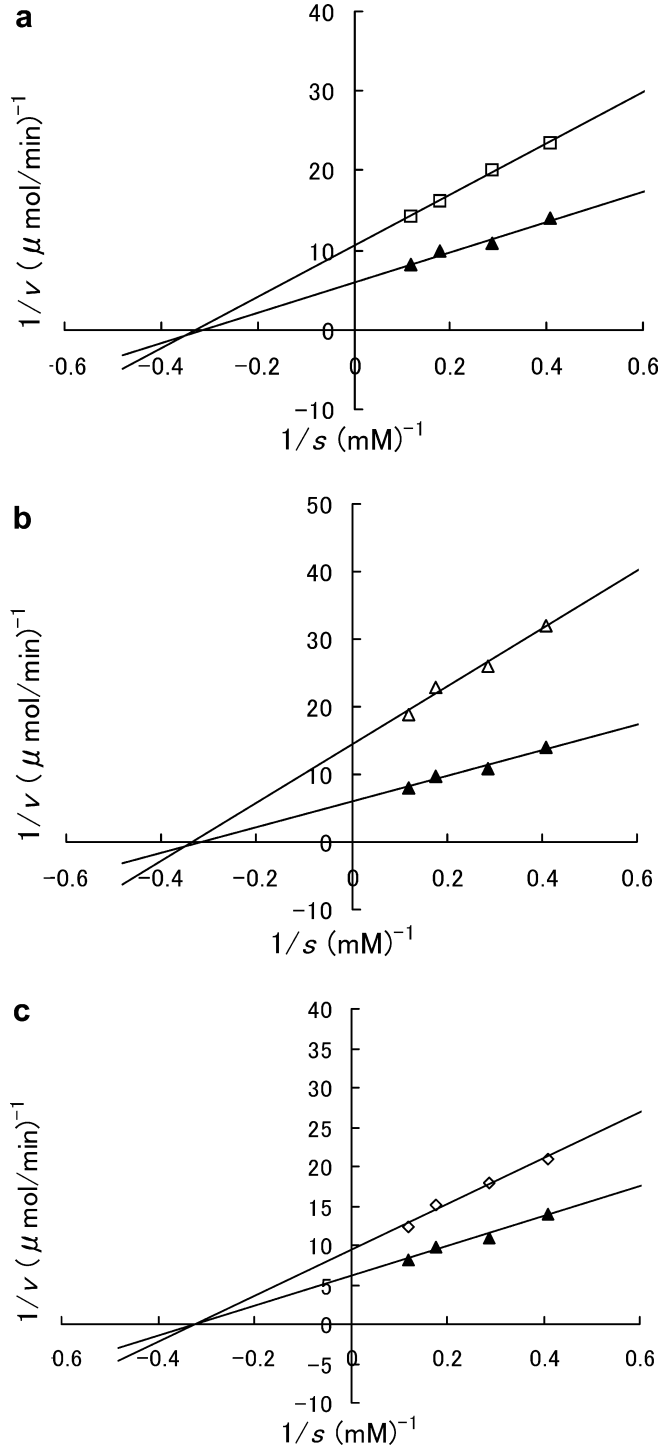

Fig. 4. Lineweaver–Burk plots analysis of inhibition kinetics of rat small intestinal α-glucosidase inhibitory effects by chebulagic acid (a) ▲, 0 μM; □, 131 μM, chebulinic acid (b) ▲, 0 μM; △, 33 μM, and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (c) ▲, 0 μM; ◇ 133 μM.

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