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Structure-Activity Relationships of Triterpenoid Derivatives Extracted From *Gymnema inodorum* Leaves on Glucose Absorption

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ABSTRACT—The leaves of *Gymnema inodorum* (GI) have been known to be effective for some diseases including diabetes mellitus, rheumatic arthritis and gout. The crude saponin mixtures extracted from GI leaves inhibited glucose absorption in the isolated intestinal tract and suppressed the increased blood glucose in rats. In this study, we examined the relationship between chemical structure and pharmacological activity of the four components from GI leave extracts (GiA-1, GiA-2, GiA-5 and GiA-7). These components were the derivatives of (3β,4α,16β)-16,23,28-trihydroxyolean-12-en-3-yl-β-D-glucopyranosiduroic acid. GiA-2, GiA-5 and GiA-7 that have suppressive effects on the high K^+-induced contraction, an increase in ΔPD and the increased blood glucose level in the glucose tolerance test have –H at the 21st position and $-CH_2OH$ at 4β of aglycon. On the other hand, GiA-1 that does not have any effects on the three parameters mentioned above has –H at the 21st position and $-CH_3$ at 4β of aglycon. In conclusion, it is suggested that the inhibitory effect of triterpenoids in *Gymnema* leaves on glucose absorption from the intestinal tract relies on $-CH_2OH$ at 4β.

Keywords: *Gymnema inodorum*, Triterpenoid, Structure, Glucose absorption, Blood glucose

The leaves of *Gymnema sylvestre* (GS) and *Gymnema inodorum* (GI), both of which belong to the *Asclepiadacecae* Family, have been known to be effective for some diseases including diabetes mellitus, rheumatic arthritis and gout. GS is also known to suppress sweet taste and intestinal glucose absorption (1 – 4). GI suppresses the intestinal glucose absorption, but it dose not suppress the sweet taste (M. Atsuchi et al., unpublished data). On the other hand, the contraction of intestinal smooth muscle induced by high K^+ is known to be inhibited by several factors including metabolism inhibitors, hypoxia and removal of extracellular glucose (11 – 14). The smooth muscle contraction by high K^+ was also inhibited by removal of Na^+ from the medium, and reduced contraction was recovered by an application of pyruvate, which is directly utilized as an energy substrate. Therefore, one of the relaxing factors of the muscle contraction is involved to an inhibition of a glucose uptake by Na^+ depletion (14, 15 – 17). We have already demonstrated that high K^+-induced contraction of the guinea pig ileal longitudinal muscle was inhibited by the GS or GI fraction, and the inhibitory effects are caused by the inhibition of glucose uptake (6, 7). Therefore, the high K^+-induced contraction serves as an indicator of pharmacological effects on the glucose absorption in this experiment.

In this study, we evaluated the pharmacological properties of the four components (GiA-1, GiA-2, GiA-5 and GiA-7), which were purified by their single peaks on HPLC, in experiments on the high K^+-induced contraction of ileal longitudinal muscle of guinea pig, measurement of glucose evoked transmural potential difference in an inverted intestine isolated from guinea pig, and the glucose tolerance test by oral administration of glucose in rats. The chemical structures of these components were derivatives of (3β,4α,16β)-16,23,28-dihydroxyolean-12-en-3-yl-β-D-glucopyranosiduroic acid. Then we estimated the active position of these components in the inhibition of glucose absorption.

MATERIALS AND METHODS

Extraction and refining from GI leaves

Dried and crushed *Gymnema inodorum* (GI) leaves were treated with acetic acid solution and extracts were obtained with 75% ethanol. The concentrated extract under vacuum was further treated with *n*-butanol and H_2O (1:1), and then the layer of *n*-butanol was concentrated under vacuum. The

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residue was washed with petroleum ether to remove fatty components, and was extracted with methanol. After filtration, the methanol solution was concentrated under vacuum, giving a crude saponin mixture. The mixtures were separated by HPLC into four components (I, II, III and IV). The yield of each component from the dried leaves of GI was 4.06, 6.75, 1.11 and 0.33 g/kg, respectively. Components II and III were further separated by HPLC. By this process, eight single peaks were refined (GiA-1, GiA-2. GiA-3, GiA-4, GiA-5, GiA-6, GiA-7 and GiA-8). The process of extraction and refining from GI leaves is illustrated in Table 1. Among the eight single peaks, four components (GiA-1, GiA-2, GiA-5 and GiA-7) were used in this study (Fig. 1).

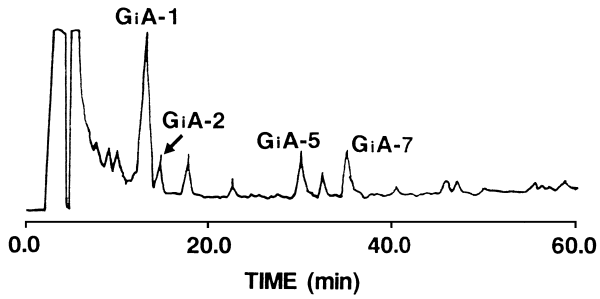
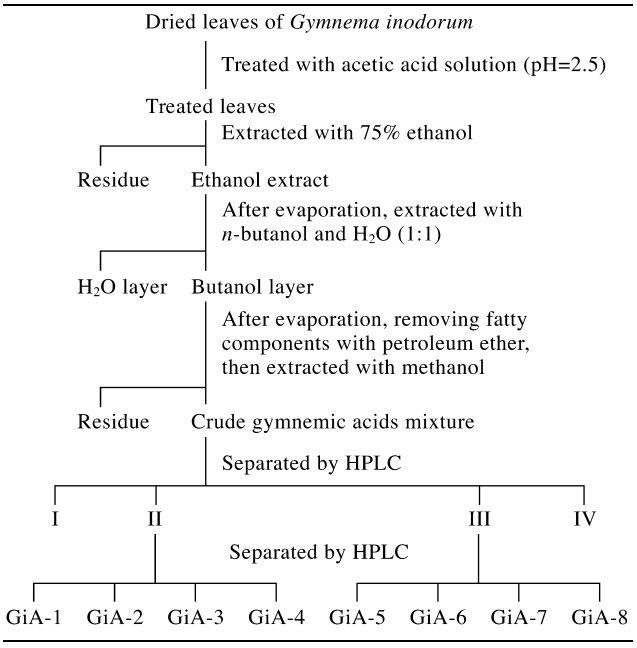


Fig. 1. HPLC behaviors of GiA-1, GiA-2, GiA-5 and GiA-7. Column, TSKgel ODS-80Tm (4.6 mm IDX 2.5 cm, 5 μm); Eluent, $CH_3CN/H_2O/AcOH = 50/50/0.1$; Flow rate, 1.0 ml/min; Detection, RI; Column temperature 40°C.

Chemical structures of four components

The chemical structures of GiA-1, GiA-2, GiA-5 and GiA-7 were identified mainly using several techniques. The following procedures were adopted. Melting points were measured with a micromelting point apparatus (MP-500D; Yanagimoto, Kyoto) and are uncorrected. Infrared (IR) spectra were taken on a Shimadzu IR-400 (Kyoto). Nuclear magnetic resonance (NMR) spectra were recorded on a JNM-GSX500 spectrometer (JEOL, Tokyo) (500.2 MHz for ^{1}H-NMR and 125.8 MHz for ^{13}C-NMR) in CD_3OD solution using tetramethylsilane (TMS) as an internal standard, and the chemical shifts are given in δ values. ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY were obtained with the usual pulse sequence. The fast atom bombardment spectrum (FAB-MS) (Xe gum, 8 kV, diethanol amine as the matrix) were measured on a ZAB-HF mass spectrometer (VG Co., UK).

Table 1. Isolation diagram of triterpenoid extracted from *Gymnema inodorum* leaves



Experiment on muscle tension

Hartley male guinea pigs weighing between 350 and 450 g (Funabashi Farm, Funabashi) were killed by a blow on the head and bled to death. After exsanguination, the abdomen was opened, and the ileum was removed. The lower part of the ileum was placed in incubation medium. The ileal longitudinal muscle strip, with a length of 10 mm and width of 5 mm, were prepared by the ordinary methods. A modified Tyrode solution with the following compositions was used: 136.8 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11.9 mM $NaHCO_3$ and 5.5 mM glucose. The bathing solution was bubbled with a gas mixture of 95% O_2 and 5% CO_2, and it had a pH of 7.2 at 37°C. The muscle strip was tied to a glass holder with silk thread at one end, while the other end was connected to a force transducer (TB-611T; Nihon Kohden, Tokyo). Then the isometric change in the tension was monitored with a recorder (RJG-4004, Nihon Kohden). The effects of four components were examined on a sustained contraction evoked by hyperosmotically added 65 mM KCl (H-65K$^+$) in the muscle strip.

Measurement of glucose-evoked transmural potential difference on an inverted intestine isolated from guinea pig

As described previously (6, 7), the excised ileum was cut by 4 cm and the adipose and connective tissued were carefully removed. Then it was inverted in the medium in a Petri dish. The inverted ileum was soaked in modified Kreb's solution (27.4 mM NaCl, 100 mM mannitol, 24.0 mM Tris, 5.4 mM KCl and 1.0 mM $CaCl_2$), in which the pH was maintained at 7.2 by adding 0.1 N HCl, and both the serosal and mucosal sides were carefully washed. One end of the inverted ileum tract was tied to create an ileal sac, while the other end was connected to a polyethylene tube

with an external diameter of 6 mm and length of 7 cm. This inverted ileal sac was used in the following experiment. The inverted sac was placed in a 30 ml Magnus tube in the Kreb's solution and the inside (serosal lumen) of the sac was also filled with the same solution. Two agarose bridge electrodes were placed on both the serosal and mucosal sides. These electrodes were connected to a direct current amplifier (AVH-9, Nihon Kohden) and the potential difference between the internal and external lumen was recorded on a recorder (R-52; Rika Denki, Tokyo). The Kreb's solution was continuously bubbled with the mixed gas of 95% O_2 and 5% CO_2 and its temperature was kept at 36 ± 1°C. In some experiments, NaCl (27.4 mM) in the medium was replaced with choline of an equal millimole.

Glucose tolerance test by oral administration of glucose in rat

Male SD rats, weighing 300 to 500 g (Japan SLC, Hamamatsu), were used after l8-h fasting for the experiment. An appropriate volume of 10% glucose solution was administered to the control rat group using a gastric tube in l g/kg body weight. The treated group was given the same volume of glucose solution plus each component extracted from GI leaves. The blood glucose level was measured before the treatment, and at 15, 30, 60 and 120 min after the treatment, in which the blood samples were drawn from the tail veins. The blood glucose level was analyzed by automatic glucose measurement equipment (GA-1120; Kyoto Dai-ichi Kagaku, Kyoto) by using whole blood.

Statistical analyses

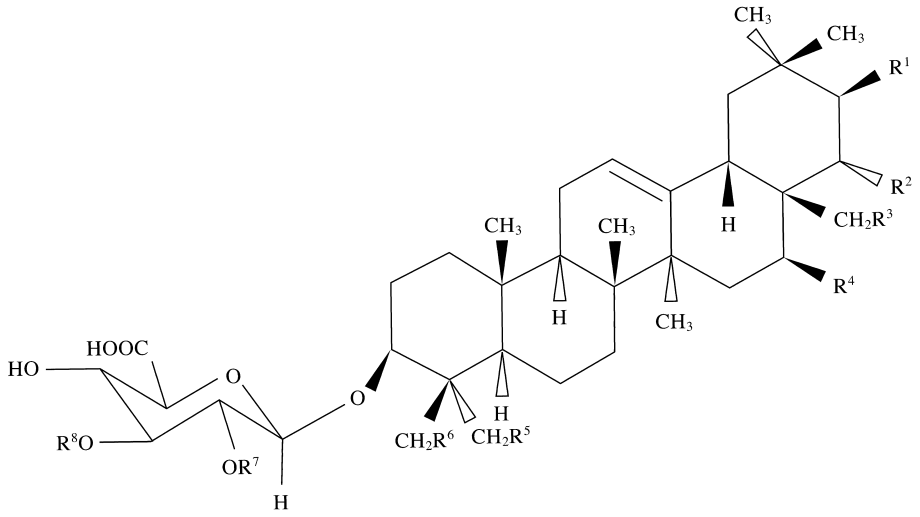
The results were expressed by the mean ± S.E.M. The statistical difference was determined by Dunnett's multiple test using SAS (statistical analysis system).

RESULTS

Structures of GiA-1, GiA-2, GiA-5 and GiA-7

The four compounds had the following characteristics: GiA-1, colorless or yellowish powder, mp 212 – 215°C, $[\alpha]_D^{25}$ +2.4° (C = 0.08, MeOH), $C_{42}H_{68}O_{14}$ revealed a quasi-molecular peak $[M\text{-}H]^-$ at m/z 795 in the negative FAB-MS; GiA-2, colorless or yellowish powder, mp 203 – 206°C, $[\alpha]_D^{25}$ +5.3° (C = 0.09, MeOH), $C_{36}H_{58}O_{10}$ based on the elemental analysis; GiA-5, colorless or yellowish powder, mp 218 – 221°C, $[\alpha]_D^{25}$ –4.1° (C = 0.10, MeOH), has the molecular formula $C_{50}H_{75}O_{17}N$ based on the elemental analysis; GiA-7, colorless or yellowish powder, mp 213 – 216°C, $[\alpha]_D^{25}$ –7.5° (C = 0.10, MeOH), $C_{44}H_{65}O_{12}N$ revealed a quasi-molecular peak at m/z 799 in the negative FAB-MS. The structural formulas of the four components are illustrated in Table 2. Based on the established structure of GiA-1, GiA-2, GiA-5 and GiA-7, the ^{13}C-NMR signals of the GiA-1, GiA-2, GiA-5 and GiA-7 were assigned as shown in Table 3. The four components were all derivatives of (3β,4α,16β)-16,23,28-trihydroxyolean-12-en-3-

Table 2. Structural formula of the triterpenoids in *Gymnema inodorum*



Component	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
GiA-1	H	H	OH	OH	H	H	β-Glu	H
GiA-2	H	H	OH	OH	OH	H	H	H
GiA-5	H	O – NMAt	OH	OH	OH	H	H	β-Glu
GiA-7	H	O – NMAt	OH	OH	OH	H	H	H

β-Glu: – β-glucopyranosyl; O – NMAt: – O – *N*-methylanthraniloxy.

Table 3. ^{13}C-NMR data for GiA-1, GiA-2, GiA-5 and GiA-7 (in CD_3OD, 125.8 MHz, δ-values)

C-No.	GiA-1	GiA-2	GiA-5	GiA-7
1	40.0	39.6	39.7	39.7
2	27.1	26.3	26.3	26.2
3	91.6	83.3	82.6	82.3
4	40.4	43.9	44.0	43.9
5	57.0	48.1	48.1	48.1
6	19.4	18.8	18.9	18.8
7	33.9	33.3	33.3	33.2
8	41.2	41.1	41.2	41.2
9	48.2	48.2	48.2	48.2
10	37.8	37.5	37.5	37.5
11	24.7	24.7	24.8	24.8
12	123.9	223.9	124.9	124.9
13	144.3	144.3	142.8	142.8
14	44.7	44.7	44.0	43.9
15	36.7	36.7	37.0	37.0
16	67.9	67.9	66.9	66.8
17	41.6	41.6	46.5	46.5
18	45.2	45.1	44.9	44.9
19	48.0	47.9	47.1	47.1
20	31.7	31.7	33.0	33.0
21	34.8	34.8	39.9	39.9
22	26.2	26.1	74.4	74.3
23	28.6	64.8	64.9	64.8
24	17.0	13.4	13.4	13.4
25	16.2	16.6	16.7	16.7
26	17.5	17.4	17.5	17.5
27	27.5	27.5	28.0	28.0
28	69.2	69.1	61.3	61.2
29	33.7	33.7	33.5	33.5
30	24.3	24.3	25.6	25.6
β-Glu				
1'	105.6	105.9	105.1	105.3
2'	80.9	75.2	74.4	75.0
3'	77.9	77.8	87.1	78.0
4'	73.0	73.2	71.6	73.5
5'	76.4	76.5	75.7	76.6
6'	172.4	172.5		
Glu				
1"	104.7		105.0	
2"	76.4		74.0	
3"	77.9		78.0	
4"	72.0		71.6	
5"	78.3		78.2	
6"	63.2		62.6	
O-NMAt				
A1			169.6	169.6
A2			112.1	112.1
A3			153.0	153.0
A4			111.9	111.9
A5			135.6	135.6
A6			115.3	115.3
A7			133.0	133.0
A8			29.7	29.7

yl-β-D-glucopyranosiduroic acid. GiA-1 has a –H at the 21st position and $-CH_3$ at 4β of aglycon. GiA-2, GiA-5 and GiA-7 commonly have a –H at the 21st position and $-CH_2OH$ at 4β of aglycon.

Effects of the components from GI leaves on a high K^+-induced contraction of guinea pig ileal longitudinal muscle

GiA-2, GiA-5 and GiA-7 gradually inhibited a sustained contraction that was induced by hyperosmotically added 65 mM KCl (H-65K^+), and their inhibition increased along the time course. In 30 min of application, GiA-2, GiA-5 and GiA-7 at a concentration of 0.1 mg/ml inhibited the contraction by approximately 60%, 20% and 25%, respectively. GiA-2, GiA-5 and GiA-7 seems to inhibit the H-65K^+-induced contraction for a relatively longer time (60 min). On the other hand, GiA-1 showed the smallest effect on H-65K^+-induced contraction, which is different from the other three components (Fig. 2).

Effects on a glucose-evoked transmural potential difference (ΔPD) in the inverted intestinal sac

After stabilizing the potential difference across the wall of the inverted intestinal sac, an addition of 5.5 mM of glucose to the medium increased ΔPD by 3 to 4 mV, and then ΔPD stabilized at the increased level. The increased ΔPD level returned to the baseline level by removing glucose. As illustrated in Fig. 3, 0.1 mg/ml of GiA-1 had almost no effect on the increased ΔPD level. The same dose of GiA-2, GiA-5 and GiA-7 suppressed the ΔPD

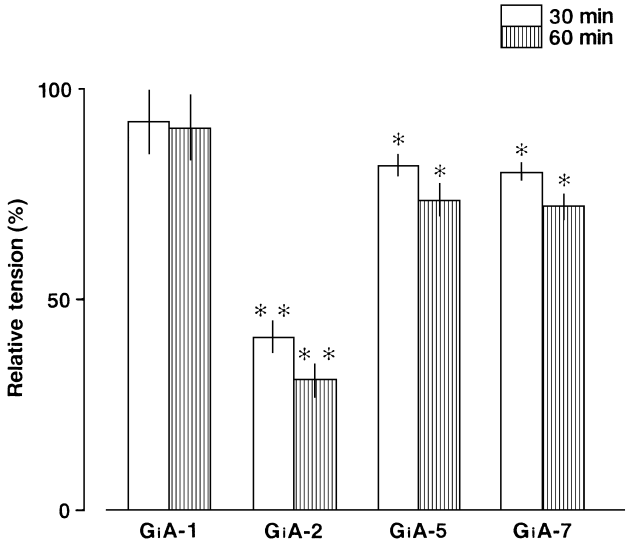


Fig. 2. Inhibitory effects of GiA-1, GiA-2. GiA-5 and GiA-7 (0.1 mg/ml) on the H-65K^+-induced contraction in guinea pig ileal smooth muscle. A steady level of hyperosmotically added 65 mM KCl (H-65K^+)-induced contraction was taken as 100%. Significantly different from the control value at 30 or 60 min, respectively: *P<0.05 or **P<0.01.

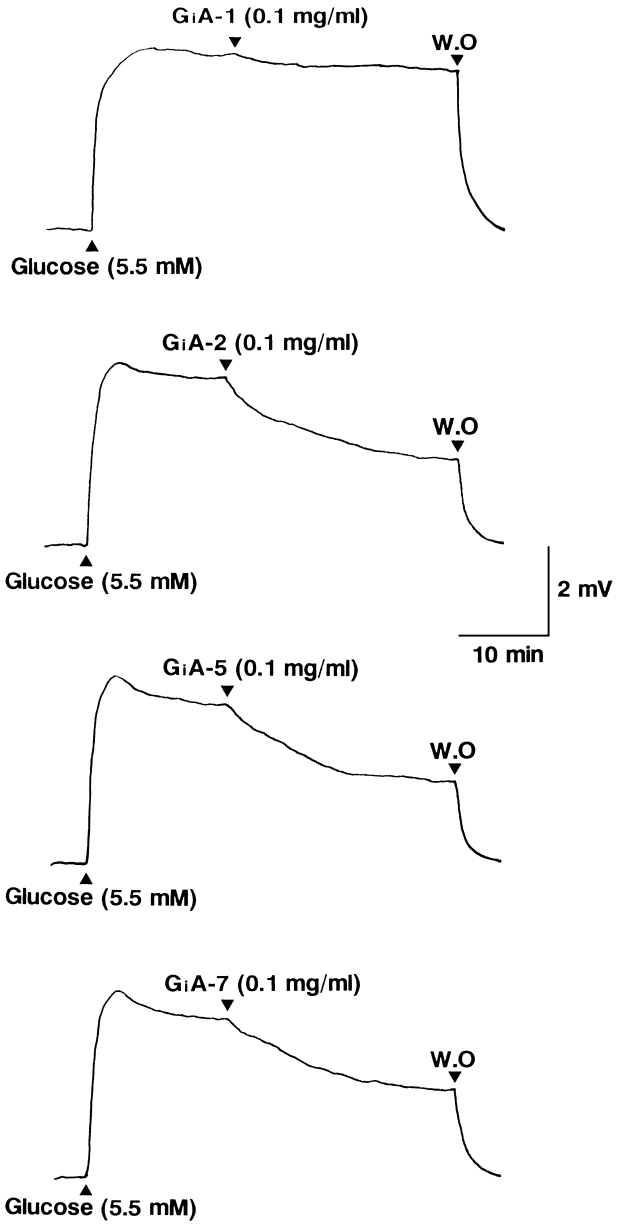


Fig. 3. Effects of GiA-1, GiA-2, GiA-5 and GiA-7 on glucose-eveoked transmural potential difference (ΔPD) by adding glucose. After the ΔPD of inverted intestine of guinea pig was stabilized in the medium adding 5.5 mM glucose, each component (0.1 mg/ml) was added. Trace of typical result in four experiments.

level, and the degree of suppression increased along the time course until 25 min after the application. The decrease in ΔPD was approximately 40% in 25 min with these three components (Fig. 3).

Effects on blood glucose level in glucose tolerance test

The 260 mg/kg of GiA-1 did not produce any difference from the control group in the glucose level at 15 and 30 min after the glucose administration. With 30 mg/kg of GiA-2, there were 30% and 32% lower blood glucose levels at 15 and 30 min, compared to the control group, respectively. At 30 mg/kg, GiA-5 also showed 21% and 35% lower blood glucose levels at 15 and 30 min, respectively. Also at 30 mg/kg, GiA-7 resulted in 19% and 16% lower blood glucose levels at 15 and 30 min, respectively (Table 4).

Table 4. Effect of each component on the glucose tolerance test in rat

Component	Dose (mg/kg)	Values of blood glucose (mg/dl)				
		0	15	30	60	120 (min)
Control	—	63 ± 1	118 ± 8	136 ± 4	112 ± 2	81 ± 3
GiA-1	260	63 ± 2	112 ± 2	135 ± 7	106 ± 4	78 ± 2
GiA-2	30	63 ± 1	88 ± 3*	104 ± 3*	112 ± 4	84 ± 1
GiA-5	30	62 ± 1	97 ± 4*	101 ± 5*	96 ± 3	82 ± 3
GiA-7	30	66 ± 1	99 ± 1*	120 ± 3*	106 ± 4	84 ± 1

The control group of rats was orally given l g/kg body weight of glucose, in which the increment in blood glucose was measured at 15, 30, 60 and l20 min. Values represent means ± S.E.M., n = 5 animals. *P<0.05: the treated groups are compared with the untreated group.

DISCUSSION

GI, which belongs to the same group as GS, has been known to have the same effects on some diseases as GS does, including diabetes mellitus, rheumatoid arthritis and gout. GS has a suppressive effect on sweet taste (1, 3, 4) and an inhibitory one on tooth decay (8), as part of its pharmacological effects. GI, however, has neither effect. Gymnemic acids which are extracts from GS are known to suppress glucose absorption from the intestinal tract, and subsequently suppress the increase in blood glucose level (5, 6). In regard to the structure of gymnemic acids, Stoecklin et al. (9) reported the structure of gymnema genin, which is an aglycon of gymnemic acids. Thereafter, other substances of gymnemic acids were isolated and their structures identified (4, 10). In this study, we obtained the extracts from GI leaves by modifying the technique of GS extraction (4, 10). The crude fractions were further purified by HPLC, and the single peaks which were demonstrated on HPLC were determined as components (GiA-1, GiA-2, GiA-5 and GiA-7).

The analysis showed that all four substances were derivatives of (3β,4α,16β)-16,23,28-trihydroxyolean-l2-en-3-yl-β-D-glucopyranosiduroic acid. GiA-1 is (3β,16β,22α)-16,28-dihydroxyolean-12-en-3-yl-*O*-β-D-glucopyranosyl-β-D-glucopyranosiduronic acid; $C_{42}H_{68}O_{14}$ (MW = 795). GiA-2 is (3β,4α,16β)-16,23,28-trihydroxyolean-12-en-3-yl-β-D-glucopyranosiduronic acid; $C_{36}H_{58}O_{10}$ (MW = 650). GiA-5 is (3β,4α,16β,22α)-22-(*N*-methylanthraniloxy)-16,23,28-trihidoroxyolean-12-en-3-yl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosiduronic acid; $C_{50}H_{75}O_{17}N$ (MW = 961). GiA-7 is (3β,4α,16β,22α)-22-(*N*-methylanthranil-

oxy)-16,23,28-trihydroxyolean-12-en-3-yl-β-D-glucopyranosiduronic acid; $C_{44}H_{65}O_{12}N$ (MW = 799). The structural formula of the four components are illustrated in Table 2.

These four components were used for the experiments on guinea pig ileal smooth muscle contraction. In this study, the contractions of guinea pig ileal longitudinal muscle induced by high K^+ were inhibited by GiA-2, GiA-5 and GiA-7, but not by GiA-1. It is therefore thought that GI leaves contain the components to induce intestinal smooth muscle relaxation by inhibiting utilization of glucose, which is the main energy substrate of the smooth muscle (6, 7).

We also evaluated the effects of the four components on the glucose absorption from the intestinal tracts. ΔPD represents a transport with glucose by a glucose-carrier mediated Na^+-dependent active transport system when glucose is absorbed from the intestinal tract (18, 19). GiA-2, GiA-5 and GiA-7, which inhibited the high K^+-induced contraction of the intestinal smooth muscle, suppressed an increase of ΔPD. On the other hand, GiA-1 which have not any effect on the smooth muscle contraction did not change anything in the increased ΔPD level. From the results, GiA-2, GiA-5 and GiA-7 seem to have an inhibitory action in the Na^+-glucose co-transport system.

Yoshioka (5) reported that the fractions from GS leaves had inhibitory effects on increasing blood glucose level in the glucose tolerance test in rats and speculated that this was a result of inhibition of the Na^+-glucose co-transport system. In this study, the increased blood glucose level was observed in rats in the glucose tolerance test. When GiA-2, GiA-5 and GiA-7 were given, the degree of increasing blood glucose level was significantly suppressed at 15 and 30 min. However, GiA-1, did not show any effects on increased blood glucose level. From these results, GiA-2, GiA-5 and GiA-7 are considered to suppress the blood glucose increase by inhibiting the glucose absorption.

GiA-2, GiA-5 and GiA-7, all of which showed inhibitory effects on high K^+-induced contraction in intestinal smooth muscle, on increasing level of ΔPD in inverted intestine and on increasing level of blood glucose, and GiA-1 did not show any effect on those actions. The four components were all derivatives of (3β,4α,16β)-16,23,28-trihydroxyolean-l2-en-3-yl-β-D-glucopyranosiduroic acid. GiA-1, which has no inhibitory effects on glucose absorption, has a –H at the 21st position and $-CH_3$ at 4β of aglycon. GiA-2, GiA-5 and GiA-7, which had inhibitory effects on glucose absorption, commonly have a –H at the 21st position and $-CH_2OH$ at 4β of aglycon. It has been reported that GS components that have the inhibitory effect on the glucose absorption have $-CH_2OH$ at 4β of aglycon (4, 10); that is, $-CH_2OH$ at 4β of aglycon of GI components is probably an essential site to inhibit the glucose absorption.

The chemical structure of other gymnemic acids which show a suppression of sweet taste has already been identified (4, 10), and they have an acyloxy group such as tiglic acid or 2 methyl-acetate at the 21st position of aglycon. Deacylated substances of gymnemic acids at the 21st position, which are obtained by alkaline hydrolysis and have a –OH at the 21st position (20), lose activity for suppression of sweet taste (1) and prevention of tooth decay (21). GI components which are used in this study are not shown to suppress sweet taste (M. Atsuchi et al., unpublished data), which support indirectly the role of a acyloxy radical at position 21 to suppress sweet taste.

In conclusion, our study suggests that the inhibitory effects of triterpenoids from GI leaves on glucose absorption from the intestinal tract is associated with $-CH_2OH$ at 4β of aglycon.

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