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Iridoid glycosides from Gardeniae Fructus for treatment of ankle sprain

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ABSTRACT

The iridoid glycosides, genipin 1-*O*-β-D-isomaltoside (**1**) and genipin 1,10-di-*O*-β-D-glucopyranoside (**2**), together with six known iridoid glycosides, genipin 1-*O*-β-D-gentiobioside (**3**), geniposide (**4**), scandoside methyl ester (**5**), deacetylasperulosidic acid methyl ester (**6**), 6-*O*-methyldeacetylasperulosidic acid methyl ester (**7**), and gardenoside (**8**) were isolated from an EtOH extract of Gardeniae Fructus. The structures and relative stereochemistries of the metabolites were elucidated on the basis of 1D- and 2D-NMR spectroscopic techniques, high-resolution mass spectrometry, and chemical evidence. Geniposide (**4**), one of the main compounds of Gardeniae Fructus, was tested for treatment of ankle sprain using an ankle sprain model in rats. From the second to fifth day, the geniposide (**4**) (100 mg/ml) treated group exhibited significant differences ($p < 0.01$) with ~21–34% reduction in swelling ratio compared with those of the vehicle treated control group. This indicated the potential effect of geniposide (**4**) for the treatment of disorders such as ankle sprain.

1. Introduction

Ankle sprain is a partial or complete tear of ligaments that support the ankle (Bosien et al., 1955), with symptoms like pain, swelling, and bruising around the ankle in the acute stage. They may be caused by sudden twisting of the ankle, such as stepping on an uneven surface or in a hole, by taking an awkward step when running, jumping, or stepping up or down, or by inversion of the foot, which causes ankle to "roll over" when playing sports or exercising. Ankle sprain is one of the most common injuries in athletes, particularly in sports such as basketball, soccer, football, and volleyball (Kofotolis et al., 2007; Liu and Nguyen, 1999). Non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, ibuprofen, naproxen, and piroxicam, have been used immediately post-injury and considered to be the best drug treatments due to their analgesic and anti-inflammatory effects (Buckwalter, 1995; Elder et al., 2001). However, taking such NSAIDs causes adverse effects, and is somewhat controversial regarding long-term healing (Stanley and Weaver, 1998; Stovitz and Johnson, 2003). For the treatment of soft tissue injuries, traditional drugs, such as Gardeniae Fructus (Guo et al., 1997a), Notoginseng Panax (Guo et al., 1997b), and Carthami Flos (Guo et al., 1997c) etc., have also been used for hundreds of years in Korea, Japan, and China. However, their clinical efficacy and pharmacological effect need to be further studied.

Gardeniae Fructus, the dried ripe fruit of *Gardenia jasminoides* Ellis (Rubiaceae), is widely used in traditional medicine for its cholagogue, sedative, diuretic, antiphlogistic, and antipyretic effects (Guo et al., 1997a). Additionally, it is an externally used drug with a long heritage and tradition in the treatment of sprain (Yao et al., 1991). Many iridoid glycosides were isolated from Gardeniae Fructus in previous investigations (Junko et al., 1991). Some of them, e.g. geniposide (**4**) (see Fig. 1), possess diverse biological activities such as anti-inflammatory, antithrombotic, neuritogenic, and cholagogue activities (Koo et al., 2006; Masaki et al., 1980; Suzuki et al., 2001; Yamazaki et al., 1996). However, there are few chemical and pharmacological studies related to Gardeniae Fructus for treating sprain (Yao et al., 1991). The specific aim of the present study was to investigate both the chemical constituents of Gardeniae Fructus and their potential for treatment of ankle sprain in a rat model.

2. Results and discussion

Two new iridoid glycosides **1** and **2** were isolated from the *n*-BuOH-soluble fraction of the EtOH extract of Gardeniae Fructus, together with six known iridoid glycosides (Fig. 1). The known

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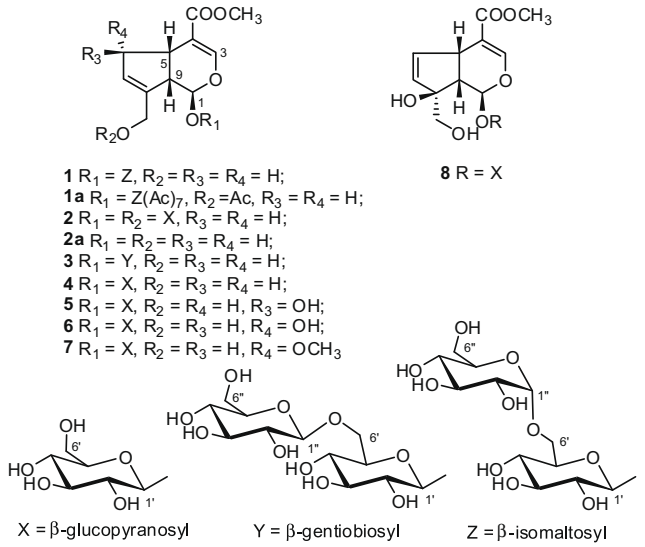

Fig. 1. Chemical structures of isolated compounds from Gardeniae Fructus.

compounds were identified as genipin 1-*O*-β-D-gentiobioside (**3**), geniposide (**4**) (Endo and Taguchi, 1973), scandoside methyl ester (**5**) (Guvenalp et al., 2006), deacetylasperulosidic acid methyl ester (**6**) (Damtoft et al., 1981; Kim et al., 2005), 6-*O*-methyldeacetylasperulosidic acid methyl ester (**7**) (Machida et al., 2003), and gardenoside (**8**) (Farid et al., 2002) by comparing their physical and spectroscopic data with those of published literature.

Compound **1** was obtained as a white amorphous powder. The HR–FAB–MS spectrum of **1** showed a quasi-molecular ion at *m*/*z* 573.1757 $[M+Na]^+$, with a molecular formula of $C_{23}H_{34}O_{15}$. Its UV spectrum exhibited an absorption maximum similar to that of geniposide (**4**) with a maximum at 239 nm, suggesting the presence of α,β-unsaturated ester carbonyl groups. The IR spectrum indicated the presence of hydroxyl (3360 cm^{-1}) and ester (1700 cm^{-1}) groups. The ^1H and ^{13}C NMR spectroscopic data of **1** were almost identical to those of genipin 1-*O*-β-D-gentiobioside (**3**) (Table 1). Sugar unit analyses using the GC method for the hydrolysis product of **1** established the presence of D-glucose (Hara et al., 1987). However, careful examination of the ^1H and ^{13}C NMR spectra of both compounds showed considerable differences in the signals of one of the sugar units. In the HMQC spectrum, a correlation between the proton resonance at δ 4.81 (1H, *d*, *J* = 3.6 Hz, H-1″) and the carbon signal at δ 100.2 (C-1″) suggested that H-1″ was an anomeric proton of a sugar moiety. However, H-1″ displayed a more downfield shift and a smaller coupling constant than that of **3** at δ 4.37 (1H, *d*, *J* = 7.5 Hz, H-1″). In the COSY spectrum, the proton resonance at δ 3.36 (1H, *dd*, *J* = 9.6, 3.6 Hz) showed a cross-peak with H-1″, indicating that the proton signal of H-2″ appeared at δ 3.36. Similarly, H-3″ at δ 3.59 (1H, *dd*, *J* = 9.6, 9.0 Hz), H-4″ at δ 3.26 (1H, *t*, *J* = 9.0 Hz), H-5″ at δ 3.61 (1H, *m*), H-6″a at δ 3.81 (1H, *dd*, *J* = 11.4, 1.8 Hz), and H-6″b at δ 3.63 (1H, *dd*, *J* = 11.4, 5.4 Hz) could be assigned by analysis of the COSY spectrum (Fig. 2). The coupling constant of $J_{H-1'',H-2''}$ 3.6 Hz in the ^1H NMR spectrum of **1** suggested the presence of an axial-equatorial form between H-1″ and H-2″, while the large coupling constant of $J_{H-2'',H-3''}$ 9.6 Hz indicated that the H-2″ was axial according to the vicinal Karplus correlation (Karplus, 1959). Therefore, H-1″ was a proton in the equatorial position. The above data thus suggested that the sugar unit was an α-D-glucosyl moiety, and this was supported further by the ^1H–^1H ROESY correlation peak between the anomeric proton H-1″ and that of H-2″. There was, however, no correlation observed between either H-1″ and H-3″, or between H-1″ and H-5″ (Fig. 2). The ^1H NMR spectroscopic data of an octaacetyl derivative (**1a**) (Table 1), peracetylated from **1**, provided better resolution and this enabled facile measurement of coupling constants for the proton signals of the sugar units which were hitherto partly overlapping, i.e., δ 3.24 (H-2′), δ 3.26 (H-4″), δ 3.32 (H-4′), δ 3.36 (H-2″), δ 3.59 (H-3″), and δ 3.61 (H-5″). Carbon signals at δ 73.81, 75.3, 72.0, 73.85, and 62.9 were assigned to C-2″, C-3″, C-4″, C-5″, and C-6″ and this was further confirmed by analysis of the HMQC spectrum. The HMBC long-range correlations from H-6′ [δ 3.76 (1H, *dd*, *J* = 10.8, 1.8 Hz) and δ 3.86 (1H, *dd*, *J* = 10.8, 6.0 Hz)] to C-1″ (δ 100.2), and H-1″ to C-6′ (δ 68.2) suggested the connection of an α-glucose moiety with a β-glucose at the 6′-OH position (Fig. 2). Therefore, the sugar moiety was a disaccharide, and identified as β-isomaltosyl. The aglycone 'genipin' was identified following hydrolysis of **1** and comparison of its spectroscopic data with that previously reported (Drewes et al., 1996). The anomeric carbon (δ 100.7, C-1′) of the β-isomaltosyl group showed an HMBC correlation with δ 5.17 (1H, *d*, *J* = 7.8 Hz, H-1) as well as that of δ 98.8 (C-1) with the anomeric proton δ 4.81 (1H, *d*, *J* = 7.8 Hz, H-1′). This established the connectivity of the glycosylation at the 1-OH position of genipin. Thus, compound **1** was genipin 1-*O*-β-D-isomaltoside.

Compound **2** was purified as a white amorphous powder. The HR–FAB–MS spectrum of **2** showed a quasi-molecular ion at *m*/*z* 573.1812 $[M+Na]^+$, corresponding to a molecular formula of $C_{23}H_{34}O_{15}$. The structure of the compound was established from analyzing its ^1H and ^{13}C NMR spectroscopic data (Table 1) which were compared to those of **3**, as well as the 2D-NMR spectra including HMQC, HMBC, COSY, and ROESY. Compound **2** showed similar UV, IR, and ^1H and ^{13}C NMR spectra to **3**. Two glucopyranosyl signals were shown in the ^1H and ^{13}C NMR spectra of **2**. A significant difference was observed in the ^1H NMR spectrum, namely that the signals of H-10 [δ 4.60 (1H, *d*, *J* = 12.6 Hz) and δ 4.27 (1H, *d*, *J* = 12.6 Hz)] of **2** were more downfield than those of **3** [δ 4.32 (1H, *d*, *J* = 14.7 Hz) and δ 4.19 (1H, *d*, *J* = 14.7 Hz)]. This suggested that one glucose moiety was connected to the 10-OH group of the aglycone. The HMBC experiment further supported this assignment as evidenced by the long-range correlations from H-10 to that of the anomeric carbon [δ 104.7 (C-1″)] of one glucose, and the anomeric proton H-1″ [δ 4.34 (1H, *d*, *J* = 7.8 Hz)] to δ 69.1 (C-10). In addition, the proton signal at δ 5.26 (1H, *d*, *J* = 7.8 Hz, H-1) showed a long-range correlation with the anomeric carbon of the other glucose at δ 100.3 (C-1′); this indicated linkage between the glucose and the 1-OH group of the aglycone, with the same pattern of **4** (Fig. 2). The configurations of the glycosidic linkage for two glucopyranosyl units were determined to be β on the basis of examination of the $J_{1,2}$ values for the anomeric protons at 7.5 Hz (H-1') and 7.8 Hz (H-1″). The aglycone was also identified as genipin by analysis of the spectroscopic data of its hydrolysis product as well as from a ROESY experiment (Fig. 2). Therefore, compound **2** was determined to be genipin 1,10-di-*O*-β-D-glucopyranoside.

Gardeniae Fructus, as a traditional medicine, is often externally used for the treatment of soft tissue injuries. Geniposide (**4**) is one of the main components for Gardeniae Fructus. Because it is a naturally occurring, biodegradable molecule with low cytotoxicity, it has recently been used in many biological investigations (Huang et al., 1998). In this study, the EtOH extract of Gardeniae Fructus and geniposide (**4**) isolated from the *n*-BuOH-soluble fraction of the EtOH extract were investigated for treatment of ankle sprain using a rat model. This model was first established in the research of acupuncture analgesia for ankle sprain (Koo et al., 2002), and lateral ankle sprain is also a common source of persistent pain in humans. To model this condition, the rat's right hind ankle was bent repeatedly, overextending lateral ligaments, for 4 min under ethyl ether anesthesia. The rat subsequently showed swelling of the ankle for the next several days. To estimate the degree of edema

Table 1
NMR spectroscopic data of compounds **1**–**3**.

No.	**1**			**1a**	**2**			**3**	
	δC, mult.	δH (*J* in Hz)[a]	HMBC	δH (*J* in Hz)[b]	δC, mult.	δH (*J* in Hz)[a]	HMBC	δC, mult.	δH (*J* in Hz)[c]
1	98.8, *d*	5.13, *d* (7.8)	3, 5, 8, 9, 1′	5.16, *d* (7.5)	97.8, *d*	5.26, *d* (7.8)	3, 5, 8, 9, 1′	98.9, *d*	5.15, *d* (7.8)
3	153.4, *d*	7.51, *d* (0.9)	1, 4, 5, 11	7.41, *s*	153.4, *d*	7.50, *d* (0.9)	1, 4, 5, 11	153.5, *d*	7.51, *d* (1.2)
4	112.6, *s*				112.8, *s*			112.5, *s*	
5	36.7, *d*	3.18, *br dd* (8.4, 7.8)	1, 3, 4, 6, 9	3.20, *q* (7.0)	36.1, *d*	3.18, *br dd* (8.4, 7.5)	1, 3, 4, 6, 7, 8, 9	36.8, *d*	3.17, *br dd* (8.4, 7.8)
6	40.0, *t*	2.81, *br dd* (8.4, 8.4)	7, 8	2.87, *m*	39.8, *t*	2.83, *br dd* (8.4, 8.4)	4, 7, 8	39.8, *t*	2.83, *br dd* (8.4, 8.4)
		2.14, *ddt* (8.4, 8.4, 2.1)	7, 8	2.27, *m*		2.12, *ddt* (8.4, 8.4, 2.1)	4, 7, 8		2.17, *ddt* (8.4, 8.4, 2.1)
7	129.2, *d*	5.85, *br s*	5, 6, 9, 10	5.86, *br s*	130.5, *d*	5.87, *br s*	5, 6, 9, 10	129.1, *d*	5.85, *br s*
8	144.7, *s*				141.6, *s*			144.9, *s*	
9	47.4, *d*	2.70, *br t* (7.8)	1, 5, 6, 7, 8	2.82, *t* (7.5)	47.1, *d*	2.95, *br t* (7.5)	1, 5, 6, 7, 8	47.1, *d*	2.71, *br t* (7.8)
10	61.5, *t*	4.32, *br d* (14.4)	7, 8	4.75, *br d* (14.0)	69.1, *t*	4.60, *br d* (12.6)	7, 8, 9, 1″	61.6, *t*	4.32, *br d* (14.7)
		4.17, *br d* (14.4)	7, 8	4.69, *br d* (14.0)		4.27, *br d* (12.6)	1″		4.19, *br d* (14.7)
11	169.7, *s*				169.7, *s*			169.7, *s*	
1′	100.7, *d*	4.71, *d* (7.8)	1, 2′, 3′	4.87, *d* (8.0)	100.3, *d*	4.71, *d* (7.5)	1, 2′, 3′, 5′	100.7, *d*	4.71, *d* (7.8)
2′	74.9, *d*	3.24, *dd* (9.0, 7.8)	1′, 3′	4.95, *dd* (9.0, 8.0)	75.0, *d*	3.24, *m*	1′, 3′	74.9, *d*	3.16, *m*
3′	78.0, *d*	3.39, *t* (9.0)	2′, 4′	5.24, *t* (9.0)	78.13, *d*	3.37, *m*	2′, 4′, 5′	78.1, *d*	3.40, *m*
4′	71.9, *d*	3.32, *m*	3′, 6′	5.04, *t* (9.0)	71.8, *d*	3.28, *m*	3′, 5′, 6′	71.7, *d*	3.37, *m*
5′	76.9, *d*	3.52, *ddd* (9.0, 6.0, 1.8)	1′, 3′, 4′, 6′	3.75, *m*	78.6, *d*	3.29, *m*	3′, 4′, 6′	77.9, *d*	3.51, *m*
6′	68.2, *t*	3.76, *dd* (10.8, 1.8)	4′, 1″	3.76, *m*	62.92, *t*	3.86, *br d* (12.0)	4′, 5′	69.9, *t*	3.87, *br d* (12.0)
		3.86, *dd* (10.8, 6.0)	4′, 5′, 1″	4.05, *m*		3.65, *dd* (12.0, 5.4)	4′, 5′		3.65, *dd* (12.0, 5.4)
1″	100.2, *d*	4.81, *d* (3.6)	6′, 2″, 3″, 5″	5.13, *d* (3.0)	104.7, *d*	4.34, *d* (7.8)	10, 3″, 5″	104.9, *d*	4.37, *d* (7.5)
2″	73.81, *d*	3.36, *dd* (9.6, 3.6)	3″	4.80, *dd* (10.0, 3.0)	75.4, *d*	3.20, *m*	1″, 3″	75.3, *d*	3.23, *m*
3″	75.3, *d*	3.59, *dd* (9.6, 9.0)	4″, 5″	5.43, *dd* (10.0, 9.5)	78.0, *d*	3.37, *m*	2″, 4″, 5″	78.0, *d*	3.39, *m*
4″	72.0, *d*	3.26, *t* (9.0)	3″, 5″, 6″	5.02, *t* (9.5)	71.7, *d*	3.29, *m*	3″, 5″, 6″	71.8, *d*	3.31, *m*
5″	73.85, *d*	3.61, *m*	4″	3.75, *m*	78.10, *d*	3.28, *m*	3″, 4″, 6″	77.8, *d*	3.27, *m*
6″	62.9, *t*	3.81, *dd* (11.4, 1.8)	4″,	4.06, *br d* (12.0)	62.90, *t*	3.87, *br d* (12.0)	4″, 5″	62.7, *t*	3.87, *br d* (11.4)
		3.63, *dd* (11.4, 5.4)	4″, 5″	4.22, *dd* (12.0, 4.5)		3.69, *dd* (12.0, 5.4)	4″, 5″		3.65, *dd* (11.4, 5.4)
–OCH_3	51.8, *q*	3.71, *s*	11	3.72, *s*	51.8, *q*	3.71, *s*	11	51.8, *q*	3.71, *s*
Ac				1.99, 2.01, 2.02, 2.03, 2.07, 2.09, 2.10, 2.11					

[a] Spectrum recorded at 600 MHz in CD_3OD.
[b] 500 MHz in $CDCl_3$.
[c] 300 MHz in CD_3OD.

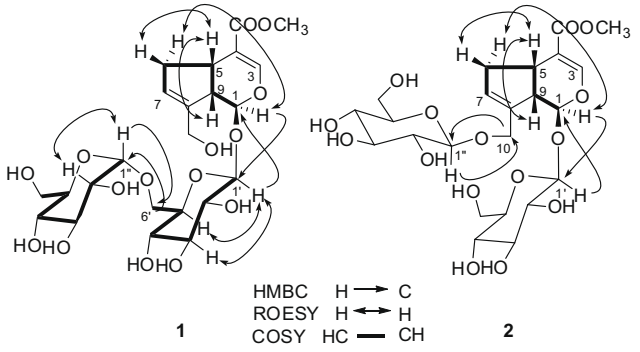

Fig. 2. Selected HMBC, ROESY, and correlations of **1** and **2**.

produced by ankle sprain, changes in the volume of the foot were measured. As shown in Fig. 3, the edema increased to peak swelling at around 12 h and then gradually recovered afterward. Additionally, the rats were greatly inactive during high swelling and then gradually recovered to normal activity as swelling lessened.

Inflammation is a necessary component of the healing process. Current evidence suggests that reducing the inflammatory response too early following injury may actually delay acute healing, slow muscle regeneration and compromise long-term healing (Almekinders, 1999; Hertel, 1997; Stovitz and Johnson, 2003). Thus, the ankle swelling in rats was treated with 200 μl of a drug every 24 h from the twelfth hour after induction of ankle sprain. Compared with those of the vehicle treated control group, a positive control (commercial diclofenac 12.5 mg/ml in gel) showed good effect with significant differences ($p < 0.01$) on the first through fifth days with ~20–38% reduction in the swelling ratio. The EtOH ex. (100 mg/ml) treated group also showed significant differences ($p < 0.05$) on the fourth and fifth days with ~13 and 18% reduction, respectively. The geniposide (**4**) (10 mg/ml) treated group showed significant differences ($p < 0.01$) on the third through fifth days with ~20–23% reduction, whereas at higher dosage (100 mg/ml), the treated group through the second and fifth days had ~21–34% reduction (Fig. 4). These findings suggest that external use of Gardeniae Fructus was helpful in the treatment of soft tissue injuries, and that geniposide, one of the main compounds of Gardeniae Fructus, might be applied as topical drug for the treatment of ankle sprain.

2.1. Concluding remarks

Two new genipin glycosides (**1** and **2**) were isolated from Gardeniae Fructus. In the structure of **1**, the sugar moiety was identified as β-isomaltose. To our knowledge, the structure combining with such a disaccharide is rare in plant secondary metabolites to date. The EtOH extract of Gardeniae Fructus and geniposide (**4**), one of the main components for Gardeniae Fructus, exhibited a potential effect on treating ankle sprain in rat. Although Gardeniae Fructus has been externally used for the treatment of soft tissue injuries in traditional medicine for a long time, only few related chemical and pharmacological studies have been reported (Yao et al., 1991). Our findings provided a better understanding for the traditional use, as well as will help for further insight into the pharmacological action and mechanism. Furthermore, whether the EtOH extract of Gardeniae Fructus and geniposide (**4**) affect ligament cells proliferation and collagen synthesis is under investigation in our research group.

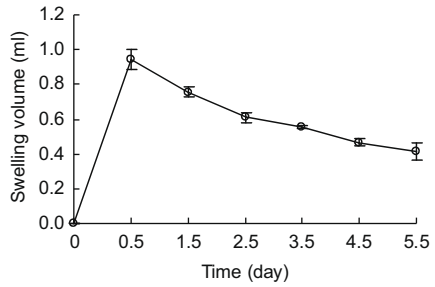

Fig. 3. Changes in foot swelling volume after ankle sprain. Swelling volumes were expressed as the differences of foot volume at various times before and after the induction of ankle sprain. Data were presented as mean ± SE.

3. Experimental

3.1. General experimental procedures

FT–IR and UV spectra were obtained using a Jasco Report-100 infrared spectrometer and a Beckman Du-650 spectrophotometer, respectively, whereas optical rotations were measured on a JASCO DIP-370 polarimeter. FT–NMR spectra were recorded on a Bruker DRX-300 spectrometer (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz) using CD_3OD as solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) were expressed in ppm with reference to TMS signals. Two-dimensional (2D) NMR (HMQC, HMBC, COSY, and ROESY) experiments were acquired on a Bruker Avance 500 spectrometer. HR–FAB–MS were measured with a JEOL JMS-700 MStation mass spectrometer. Semipreparative HPLC were conducted using a Gilson instrument with TRILUTION LC control software, Gilson 321 pumps, UV/VIS 151 detector and GX-271 liquid handler, equipped with a YMC-Pack Pro C18 column (10 μm, 250 × 20 mm, I.D.). Column chromatography (CC) was performed using silica-gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck), whereas thin layer chromatography (TLC) used Merck pre-coated Silica-gel 60 F_{254} and/or RP-18 F_{254s} plates (0.25 mm); compounds were observed under either UV 254 and 365 nm or were visualized by spraying the dried plates with 10% H_2SO_4 followed by heating at 180 °C.

3.2. Plant material

Gardeniae Fructus was purchased from the pharmacy store in Daejeon, Korea in July 2006, and identified by Prof. KiHwan Bae. A voucher specimen (CNU 1516-3) has been deposited at the Herbarium in the College of Pharmacy, Chungnam National University.

3.3. Extraction and isolation

The dried Gardeniae Fructus slice (3.0 kg) was extracted with hot EtOH (5 l × three times) for two days. The EtOH extracts were filtered, combined, and concentrated *in vacuo* to give a rufous gummy residue (980 g). To this was then added H_2O (2 l) to give a suspension that was partitioned consecutively with *n*-hexane, EtOAc, and *n*-BuOH (each 2 l), then exhaustively concentrated to yield an *n*-hexane-soluble fraction (95.0 g), an EtOAc-soluble fraction (36.5 g), and an *n*-BuOH-soluble fraction (760.0 g), respectively.

An aliquot of the *n*-BuOH-soluble fraction (700 g) was dissolved in a minimal amount of MeOH–H_2O (1:1, v/v). This was then applied to a Diaion HP 20 column (100 × 20 cm, I.D.) eluting with a step gradient of H_2O–MeOH (1:0, 4:1, 1:1, 1:4, and 0:1, v/v) to give fractions GB1∼5. GB1 was next subjected to an ODS CC (50 × 2.5 cm, I.D.) using MeOH–H_2O (1:2, v/v) as eluent to give five sub-fractions. The second sub-fraction was then applied to a semi-preparative RP HPLC [Gilson trilution system; Optimapak OP C18 (250 × 10 mm, I.D.) column; MeOH–H_2O (2:8, v/v); UV detection, 240 nm] to afford **1** (5.9 mg), **2** (7.0 mg), **3** (164.5 mg), **5** (132.0 mg), **6** (60.0 mg), and **7** (40.0 mg). GB4 was applied to silica-gel column (100 × 10 cm, I.D.) with EtOAc–MeOH (20:1, v/v) eluent to give **4** (5.0 g) and **8** (160.0 mg).

3.4. Genipin 1-*O*-β-D-isomaltoside (*1*)

White amorphous powder, $[\alpha]_D^{22}$ +50.0 (*c* 0.3, MeOH); UV λ_{max}^{MeOH} nm (log ε): 240 (3.18); IR ν_{max}^{KBr} cm^{-1}: 3400, 2900, 1698, 1625, 1080; for ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) spectroscopic assignments, see Table 1; HR–FAB–MS *m*/*z* 573.1757 $[M+Na]^+$ (calcd. for $C_{23}H_{34}O_{15}Na$, 573.1795).

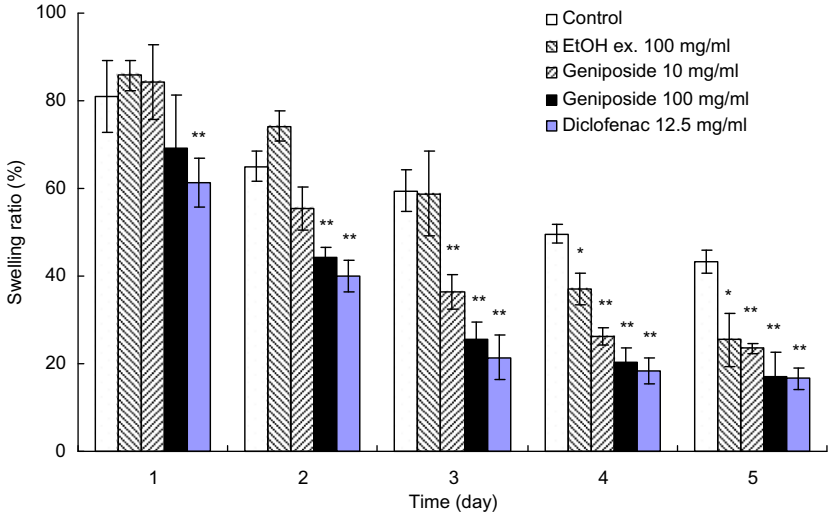

Fig. 4. Effect of geniposide and EtOH extract of Gardeniae Fructus on ankle swelling in rats with ankle sprain. A commercial diclofenac in gel was used as positive control. Swelling ratio was expressed as the ratio of swelling volume at various times before and after drug treatment. Data were presented as mean ± SE. *, $p < 0.05$; **, $p < 0.01$ compared with vehicle treated control group, one-way ANOVA, Dunnett's *post hoc* test.

3.5. Acetylation of genipin 1-O-β-D-isomaltoside

A solution of **1** (2.0 mg) in Ac_2O and anhydrous pyridine (1 ml, 1:1, v/v) was stirred for 2 h at room temperature. Evaporation of the reagents under reduced pressure afforded the octaacetyl derivative **1a**. White amorphous powder; for ^1H NMR (500 MHz, $CDCl_3$) assignments, see Table 1.

3.6. Enzymatic and acidic hydrolysis of *1*

Naringinase (100.0 mg, 300 U/g) (Sigma–Aldrich, St. Louis, MO, USA) was added to a suspension of **1** (2.0 mg) in 50 mM acetate buffer (pH 5.5) and the mixture was stirred at 37 °C for 3 h. The reaction mixture was then extracted with EtOAc (3 × 20 ml) and evaporated to dryness. The resulting residue was then dissolved in MeOH and subjected to reversed phase HPLC [Gilson trilution system; YMC-pack Pro C18 (250 × 20 mm, I.D.) column; MeOH–H_2O (3:7, v/v); UV detection, 240 nm] to give genipin (**2a**), identified by comparing physical and spectroscopic data with those of published literature data (Drewes et al., 1996). The H_2O layer was passed through a Sep-Pak C18 cartridge (Waters, Milford, MA) and concentrated to dryness. The dried residue was dissolved in 2 M HCl and dioxane (50 ml each) and heated to 100 °C for 1 h. After dilution with H_2O, the solution was passed through an Amberlite IRA-60E column. The eluate was concentrated to dryness and stirred with D-cysteine methyl ester hydrochloride, hexamethyldisilazane, and trimethylsilyl chloride in pyridine using the same procedures as in previous reports (Hara et al., 1987). After the reactions, the supernatant was analyzed by GC analysis. GC conditions: column, Supelco SPB-1 (30 m × 0.32 mm); detector, FID; detector temp, 300 °C; injector temp, 270 °C; carrier gas, He; column temperature, 190 °C; t_R 22.4 min (D-glucose), 20.6 min (L-glucose). D-Glucose was detected from **1**.

3.7. Genipin 1,10-di-O-β-D-glucopyranoside (*2*)

White amorphous powder, $[\alpha]_D^{22}$ +3.0 (*c* 0.3, MeOH); UV λ_{max}^{MeOH} nm (log ε): 239 (3.19); IR ν_{max}^{KBr} cm^{-1}: 3360, 2920, 1700, 1640, 1440, 1300, 1080; for ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) spectroscopic data, see Table 1; HR–FAB–MS *m*/*z* 573.1812 $[M+Na]^+$ (calcd. for $C_{23}H_{34}O_{15}Na$, 573.1795).

3.8. Enzymatic hydrolysis of *2*

Enzymatic hydrolysis of **2** used the same method as for **1** above to give genipin (**2a**), which was identified by comparing physical and spectroscopic data with those of published literature data (Drewes et al., 1996). The H_2O layer was passed through a Sep-Pak C18 cartridge (Waters, Milford, MA). The eluate was concentrated and subjected to the same procedure described above. D-Glucose was detected from **2**.

3.9. Biological assay

3.9.1. Experimental animals

Male Wistar rats, eight-week-old, weight 200–250 g, were obtained from Central Lab. Animal Inc. (Seoul, Korea). Procedures involving animals and their care were conducted in conformity with international rules on care and use of laboratory animals. All animals were acclimated for seven days before the experiment began.

3.9.2. Induction of ankle sprain in rat

Induction of ankle sprain in rat was done according to the method of previously reported rat model with minor modifications (Koo et al., 2002). In brief, the rat was anesthetized with Et_2O vaporized in air. The right hind foot was repeatedly bent for 4 min in the direction of simultaneous inversion and plantar flexion so that it eventually reached a 180° inversion. Anesthesia was discontinued and the rat recovered from anesthesia within 5–10 min.

3.9.3. Drug treatment and measurement of foot volume change

Test samples were administered cutaneous (topical administration to the skin). Swollen ankles in rats were treated with EtOH–H_2O (7:3, v/v) (control group), 100 mg/ml of EtOH extract of Gardeniae Fructus (EtOH ex. 100 mg/ml group), geniposide (**4**) (geniposide 100 mg/ml group), geniposide (**4**) (geniposide 10 mg/ml group), and Voltaren® Schmerzgel (diclofenac 12.5 mg/ml, positive control group), 200 μl per day from the twelfth hour after induction of ankle sprain.

Changes in the volume of the foot were measured before and after the sprain procedure, using the Archimedes's principle as described in the literature (Koo et al., 2002). Briefly, each rat was marked at about 5 mm proximal to the ankle in the distal part of the leg. Then, a small beaker, about half full of water, was placed on a digital scale that has the capability to weigh at a resolution of 0.01 g. The foot was immersed into the water up to the premarked line. The change in the weight of the water was then recorded. The weight increase in each gram is equated to 1 ml of foot volume.

3.10. Statistical analysis

The parameters included in the statistical evaluation were defined as follow:

$$\text{Swelling volume } (Sv_i) = V_i - V$$
$$\text{Swelling ratio } (\%) = (Sv_i/Sv_0) \times 100$$

where V_i was the foot volume at various times after induction of ankle sprain (*i* from 0 to 5 according to days of administration), and *V* was the foot volume before induction of ankle sprain. Foot volumes were tested before administration and measured as ml. Data were expressed as the mean ± standard error of mean (SE) for the number (n = 3–6) in each group. The statistical analysis accomplished using the SPSS 13.0 statistical software (SPSS ins., Chicago, IL, USA). Analysis of variance was performed using ANOVA procedures followed by the Dunnett's *post hoc* test. A p value <0.05 was considered to be statistically significant.

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