Doctoral Thesis

Studies on the morphological, phylogenetic, and phytochemical differences between *Rosa rugosa* and Mei-gui, and the biological activities of their petals

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Sarengaowa

Contents

Chapter 1 General Introduction

1.1 *Rosa rugosa* Thunb.

Rosa rugosa Thunb., deciduous small shrubs, belongs to a species of subgenus *Rosa* of genus *Rosa*, and is widely distributed from temperate regions of eastern Asia that include Japan, Korea and China, to the subarctic zone of Kamchatka and Okhotsk as is shown in Fig. 1[1].

In Japan, *R. rugosa* is mainly growing in Hokkaido, the northern-most island of Japan, but its southern limit of distribution is Tottori prefecture along the coast of the Japan Sea, and Ibaraki prefecture along the Pacific Ocean.

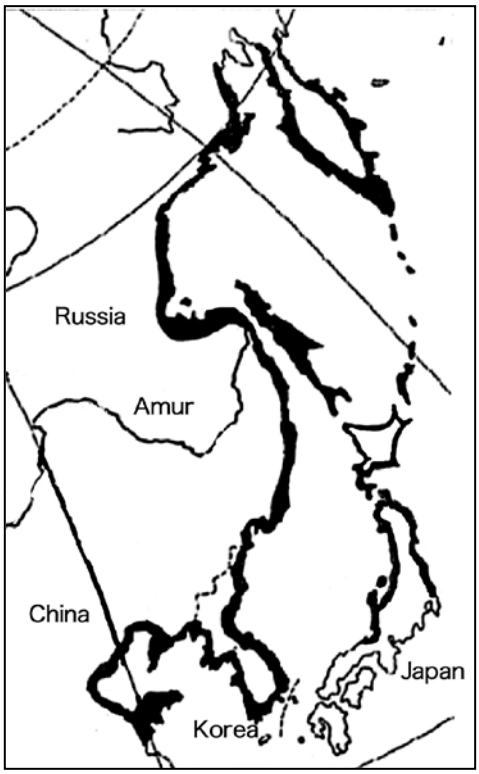

Fig.1. Distribution of *R. rugosa* quoted from *"The Roses"*, p269 Modified by author adding distribution in Honshu of Japan.

R. rugosa is called as "Hamanasu" (means "beach eggplant") in Japanese, and is called as "Japanese rose" all over the world. The scientific name of this plant was assigned by Thunberg in 1755 as *Rosa rugosa*, and it was introduced to Europe as a mother strain of hybrid roses, because it has hardy characteristics.

Besides horticultural use, the petal of *R. rugosa* has been used as a raw

material for fragrance extraction, and fruits are used for making jam rich in vitamin C.

In Japan, flower or petal of *R. rugosa* was assigned as the substitution of Mei-gui Hua, a crude drug made of bud or petal of Chinese roses. But its use in Japanese traditional medicine was limited. However, there was an old documents written by doctor on Ezo island (the old name of Hokkaido) at the end of Edo period, and he recorded that the Ainu people, former inhabitants in the northern part of Japan, had been used the dried petals of *R. rugosa* as an herbal tea to prevent scurvy, probably for supplement of vitamin C in winter [2].

In Korea, *R. rugosa* is called as “Hae Dang wha” (means “sea apple blossom”), and its petals and fruits have been used as herbal drugs in the traditional Korean medicine for treatment of vomiting, diarrhea and diabetes [3].

1.2 Mei-gui and Mei-gui Hua

Mei-gui is one of the common names of roses in China. Wild and cultivated roses in China are divided into three groups such as “Mei-gui”, “Yue-ji” and “Qian-wei”. Among these classifications, Mei-gui is recognized as a kind of rose used for medicine and herbal tea.

The wild species of Mei-gui is distributed along the coast of southeast Liaoning Province and Tumen River in Jilin Province, and it is designated an endangered species. However, Mei-gui is widely cultivated for medicinal use and for herbal tea all around China. Mei-gui cultivated for

medicinal use has double flower with characteristic fragrance, and is cultivated mainly in Shandong, Gansu, Xinjiang, Shanxi, Jiangsu Province and Beijing in China [4].

According to the authoritative medicinal plants documents, the scientific name of the wild species of Mei-gui was assigned as *Rosa rugosa* Thunb., [5] which is the same as wild Japanese rose. At present, Mei-gui widely cultivated in China for medicinal use is also recognized as *R. rugosa* in the "*Chinese Materia Medica*" and the "*Dictionary of Chinese Medicines*".

Mei-gui Hua, which is dried petals or buds of Mei-gui, have been used as a crude drug and as herbal tea. It mainly be used for treatment of haematemesis (vomit blood), diarrhea, hemoptysis (spit blood), stomachache, apoplexy, hypermenorrhea (profuse menstruation) [6-8], bruise [9] in traditional Chinese medicine.

In Xinjiang Province, the westernmost part of China, Uygur people named Mei-gui Hua as "Kizil gul", and they have been used Mei-gui Hua as a crude drug in traditional Uygur medicine [10] for treatment of

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玫瑰花 méi guī huā

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【基原】バラ科の植物，玫瑰(マイカイ)和名ハマナスの咲いたばかりの花。

【原植物】ハマナス *Rosa rugosa* Thunb.([群芳譜]に玫瑰とある) 直立低木，高さ

ハマナス

Fig.2. Description of Mei-gui Hua quoted from *"Dictionary of Chinese Medicines"*, p2440-2441.

diabetes [11]. They also have a custom to take tea containing Mei-gui.

1.3 Background of this study

We have been studying the petal of *R. rugosa* collected in Hokkaido, Japan to develop new functional foods. Our research group has clarified many biological activities such as anti-oxidative potential, supplementation of vitamin C, improvement of body odor of aged people and prebiotic effect against intestinal bacteria to improve intestinal disorder [12]. Moreover, our research group demonstrated that the petal showed anti-diabetes effects on disease-model mouse for type II diabetes, such as decrease of weight gain and fat accumulation in liver.

In China, Mei-gui Hua has been used for a crude drug and for ingredient of herbal tea. Our research on the utilization of Mei-gui Hua in China revealed that Uygur people have a custom to take Mei-gui tea and they also used it for treatment of diabetes. Therefore, our studies were focused on the anti-diabetic activities of Mei-gui Hua, and at first we investigated the inhibitory activities of extracts of the petals against α-amylase and α-glucosidase. These activities were compared with those of acarbose, an anti-diabetic drug used for treating type II diabetes. And we tried to isolate the active principles from Mei-gui.

In the course of our studies on *R. rugosa* and Mei-gui Hua, there appeared a fundamental problems on the scientific name of these plants. As mentioned above, there is confusion on the scientific name of *Rosa rugosa*. The scientific name was originally assigned for Japanese rose

"Hamanasu", but the same scientific name has been used for Mei-gui cultivated in China. There are many types of Mei-gui in China, and their morphological characteristic and botanical ecology is different from those of *R. rugosa*. In 1957, Hara reported that Mei-gui introduced from China and cultivated in Japan was different from *R. rugosa*, and he named it as *Rosa maikwai* [13]. However, this scientific name must not be applied for all type of Mei-gui cultivated in China. Therefore, it must be important to clarify the original plant of Mei-gui, especially cultivated Mei-gui.

To clear up these confusions, the original plants of Mei-gui Hua were investigated by comparing morphological characteristics of Mei-gui cultivated in Xinjiang, Shandong and other cultivation areas with those of *R. rugosa* collected in Japan and Korea. Moreover, we carry out phylogenic studies on the samples of Mei-gui and *R. rugosa*, along with phytochemical studies on flavonol glycosides in the petals.

1.4 References

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Chapter 2 Biological activities

2.1 Introduction

In Xinjiang province, the westernmost part of China, Uygur people have a custom to take herbal tea whose Uygur name is "*Kizil gul*", made from the blossom or petal of Mei-gui for the treatment of diabetes [1]. Distilled rose water has also been used in traditional Uygur medicine. The utilization of Mei-gui in traditional Uygur medicine must be influenced by Arabian medicine, where Mei-gui has been used traditionally for medicine and aromatic water. The Mei-gui was cultivated in the oasis cities around Tarim basin (Taklamakan Desert). However, there was no scientific report on their medicinal utilization and constituents.

Our research group has been studying the petal of *R. rugosa* collected in Hokkaido, Japan to clarify their biological activities on mouse models of type 2 diabetes. The petals showed the inhibitory effects on weight gain and fatty accumulation in liver. Also we clarified that hydrolysable tannins in the petals were active compounds for fatty accumulation.

In order to clarify the potency of Mei-gui and *R. rugosa* for the treatment of diabetes, we investigated the inhibitory activities of extracts of its petals against α-amylase and α-glucosidase, and these activities were compared with those of acarbose, an anti-diabetic drug used for treating type II diabetes.

2.2 Materials and methods

2.2.1 Materials and chemicals

The samples of Mei-gui were collected in Xinjiang Province, Pingying rose research institute in Shandong Province, and northeastern parts of China. Wild and cultivated *Rosa rugosa* were collected in Hokkaido of Japan and Korea. *R. maikwai* was obtained from Medicinal plants garden of Hokkaido University. The information of samples is showed in Table 2.1.

The digestive enzymes: α-amylase from *Bacillus* sp., porcine pancreatic and human saliva, and α-glucosidase from *Bacillus* sp. and rat intestinal acetone powder were purchased from Sigma-Aldrich Japan Co. (Japan). α-Glucosidase from *Saccharomyces* sp. was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2.2 Preparation of samples

The fresh petals were separated and air dried, then the pulverized petals (5 g) were extracted with 50% aqueous ethanol (250 ml) at room temperature for 24 h three times. After removal of ethanol, the combined extract was lyophilized for studying biological activities.

Table 2.1 Information of samples

No.	Species / Used name	Cultivated or collected place	Notes*
CX1	Mei-gui（Kizil gul）	Keriya (Tarim basin), Xinjiang Prov., China	
CX2	Mei-gui（Kizil gul）	Houtan (Tarim basin), Xinjiang Prov., China	
CX3	Mei-gui（Kizil gul）	Niya (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX4	Mei-gui（Kizil gul））	Kashugar (Tarim basin), Xinjiang Prov., China	
CX5	Mei-gui（Kizil gul）	Kumuru, Xinjiang Prov., China	
CX7	Mei-gui（Kizil gul）	Urumqi, Xinjiang Prov., China	
CS1	Mei-gui	Pingying rose research institute,	Cultivated, DF
CS3	Mei-gui	Shandong Prov., China	Cultivated, SF
CB	Mei-gui	Beijing botanical garden, Beijing,China	Cultivated, DF
MAI	*Rosa maikwai* Hara.	Hokkaido Univ. Medicinal plants garden, Japan	Cultivated, DF
K1	*Rosa rugosa* Thunb.	Hwajipo Gangwong-do, Korea	Wild, SF
K2	*Rosa rugosa* Thunb.	Hwasong Songsan, Gyeonggi-do,Korea	
K3	*Rosa rugosa* Thunb.	Yangyang, Gangwon-do, Korea	Cultivated, SF
K4	*Rosa rugosa* Thunb.	Samcheok Meangbang, Gangwon-do, Korea	
K5	*Rosa rugosa* Thunb.	Yeong Gwang, Jeollanam-do, Korea	
J1	*Rosa rugosa* Thunb.	Monbetsu, Hokkaido, Japan	
J2	*Rosa rugosa* Thunb.	Ishikari, Hokkaido, Japan	Wild, SF
J3	*Rosa rugosa* Thunb.	Wakkanai, Hokkaido, Japan	
J4	*Rosa rugosa cv. Plena*.	Kitami, Hokkaido, Japan	Cultivated, DF

*DF: Double flower; SF: single flower.

2.2.3 Assessment of inhibitory activity against α-amylase

Three kinds of α-amylase from *Bacillus* sp. (BSA, 20 unit/mg of protein), porcine pancreatic (PPA, 1370 unit/mg of protein) and human saliva (1920 unit/mg of protein) were used, and enzyme activity was measured as follows. Phosphate buffer (0.25 M, pH 7.0, 0.5 ml) containing 0.4 mg/ml soluble starch and test sample was incubated at 37 ˚C for 5 min. The test samples were dissolved in H_2O. After addition of enzyme (200 unit/ml, 10 μl), the reaction mixture was incubated at 37 ˚C

for 10 min. Then the enzyme reaction was stopped at 0 ˚C, and iodine solution (5 mM, 0.5 ml) was added to the reaction mixture. The amount of residual starch was determined by measuring absorption at 660 nm. The IC_{50} values were estimated graphically by plotting percentage inhibition versus the log of test sample concentrations.

2.2.4 Assessment of inhibitory activity against α-glucosidase

Two kinds of α-glucosidase from *Saccharomyces* sp. (SSG, 163 unit/mg of protein), and *Bacillus* sp. (BSG, 103 unit/mg of protein) were used. Crude rat intestinal α-glucosidase was prepared from rat intestinal acetone powder, and showed sucrose (0.16 unit/mg of protein) activities which were measured by using sucrose (20 mM) as a substrate [2]. Their activities were evaluated as follows. The test samples (10 μl, in DMSO) were added to the mixture of phosphate buffer (415 μl, 10 mM, pH 7.0) and 25 μl of 1 M sucrose, and the mixture was incubated at 37˚C for 5 min. Then 50 μl of enzyme solution was added to the mixture and was incubated at 37˚C for 20 min. The enzyme solution (10 μg/ml) was prepared by dissolving α-glucosidase in 10 mM phosphate buffer (pH 7.0) containing 0.2% BSA (albumin from bovine serum). The reaction was stopped by addition of 10 μl of 10 mM deoxynojirimycin. The residual glucose concentrations in the reaction mixture were measured using a glucose oxidase (GOD) method, (glucose CII-test, Wako Pure Chemical Industries Ltd.). The IC_{50} values were determined graphically by plotting percentage inhibition versus the log of test sample concentrations.

2.3 Result and discussion

2.3.1 Inhibitory effects of the petals of Mei-gui against α-amylase and α-glucosidase

The inhibitory activities of three Mei-gui collected at Houtan, Niya and Keriya in Xinjiang province, China, were investigated against α-amylase and α-glucosidase. Known inhibitors of α-amylase and α-glucosidase show different inhibitory activities against enzymes from different origins [2-3]. Therefore, three kinds of α-amylase from different origins were used in our study: bacterial α-amylase from *Bacillus stearothermophilus* (BSA), mammalian α-amylase from porcine pancreas (PPA) and human α-amylase from human saliva (HSA). Similarly, the following three kinds were used: yeast α-glucosidase from *Bacillus stearothermophilus* (BSG), *Saccharomyces* sp. (SSG) and mammalian α-glucosidase from rat small intestines (RIG).

Table 2.2 Inhibitory effects of the extracts of petals of Mei-gui against α-amylase and α-glucosidase from different origins

Sample`s Code No.	IC_{50} (μg/ml)[a]					
	α-amylase			α-glucosidase		
	BSA	PPA	HSA	BSG	SSG	RIG
CX1	46	34	68	27	14	121
CX2	25	20	60	14	14	99
CX3	41	26	63	11	8	94
Acarbose	4	38	73	1	38	5

[a] Sample concentration required for 50% inhibition.

The inhibitory effects of the 50% aqueous ethanol extracts of petals of Mei-gui are shown in Table 2.2. Compared their inhibitory effects with those of acarbose, the extracts exhibited strong inhibitory effects against α-amylase from PPA and HSA and α-glucosidase from SSG. However the inhibitory effects were different depending on the origin of the enzymes.

The IC_{50} values of three samples were 25, 41 and 46 μg/ml against BSA, respectively, and the IC_{50} values were nearly ten times higher than that of acarbose. The IC_{50} values of the extracts against HSA were higher than those against BSA, but the IC_{50} values of the extracts against PPA were lower than those against BSA. In the case of PPA, the IC_{50} values were lower than that of acarbose, but those against HSA were almost the same as that of acarbose.

The IC_{50} values of the extracts against α-glucosidase were in the range of 11 - 27 μg/ml for BSG, 8 - 14 μg/ml for SSG and 99 - 121 μg/ml for RIG. In the case of BSG and RIG, the IC_{50} values of acarbose (1 and 5 μg/ml, respectively) were less than one tenth of the extracts' values, but that for SSG was higher than those of the extract.

To identify the active constituents in the petals of Mei-gui, the 50% aq. ethanol extracts were partitioned between ethyl acetate and water, and both inhibitory activities against all the kinds of α-amylase and α-glucosidase were compared, and the data are shown in Table 2.3. The ethyl acetate soluble fractions showed lower IC_{50} values compared with those of original extracts. The IC_{50} values of ethyl acetate fractions of the three samples against α-amylase were lower than those of the extracts, in the range of 7 – 18 μg/ml, but those of the aqueous fractions were higher, in the range of 41

– 164 μg/ml. The IC_{50} values of ethyl acetate-soluble fractions against α-glucosidase were also lower than those of the extracts, in the range of 6 – 7 μg/ml, and those of the aqueous fractions were higher than those of ethyl acetate-soluble fractions, in the range of 12 – 27μg/ml. Therefore, the constituents in the ethyl acetate-soluble fractions were further investigated in Chapter 3.

Table 2.3 Inhibitory effects of ethyl acetate fractions and aqueous fractions of the extracts of petals of Mei-gui against α-amylase and α-glucosidase from different origins

		IC_{50} (μg/ml)					
Code No.		CX1		CX2		CX3	
fraction layer		EtOAc	H_2O	EtOAc	H_2O	EtOAc	H_2O
Extraction yield(%)		33.49	66.45	20.33	80.63	32.25	68.56
α-amylase	*bacillus* sp.	18	63	18	126	13	64
	porcine pancreas.	10	41	7	42	7	42
	human saliva.	10	119	9	164	3	17
α-glucosidase	*bacillus* sp.	7	24	6	25	6	8
	saccharomyces sp.	7	12	8	27	5	7

2.3.2 Comparison of inhibitory effects of the petals between *R. rugosa* and Mei-gui against α-amylase and α-glucosidase

The inhibitory activities of Mei-gui collected in China and *R. rugosa* collected in Japan and Korea were investigated against α-amylase and α-glucosidase. The α-amylase from *Bacillus* sp. and α-glucosidase from *Saccharomyces* sp. were used in this study.

The inhibitory effects of the 50% aq. ethanol extracts of petals of Mei-gui and *R. rugosa* against α-amylase were shown in Table 2.4.

Compared their inhibitory effect with those of acarbose, the extracts exhibited weakly inhibitory effects against α-amylase from *Bacillus* sp (BSA).

Table 2.4 Inhibitory effects of the extracts of petals of Mei-gui and *R. rugosa* against α-amylase

Mei-gui	Inhibition[a,b] (%)	IC_{50}[a,c] (μg/ml)	*Rosa rugosa*	Inhibition[a,b] (%)	IC_{50}[a,b] (μg/ml)
CX1	60	46	J1	49	49
CX2	55	25	J2	51	53
CX3	58	41	J3	37	90
CX4	58	27	J4	42	52
CX5	24	121	MAI	28	104
CX7	44	22	K1	56	40
CS1	69	25	K2	34	88
CS3	35	712	K3	55	40
CB	56	48	K4	52	37
Acarbose	82[d]	4	K5	5	186

[a]The value was an average of three determinations.

[b]Inhibition at the concentration of 50μg/ml.

[c]concentration required for 50% inhibition of the enzyme activity.

[d]inhibition rate of acarbose at 25μg/ml.

The IC_{50} values of the 50% aq. ethanol extracts of Mei-gui were in the range of 22 - 48 μg/ml against BSA except two sample collected at Kumuru and Pingying, and the IC_{50} values were nearly 5 - 12 times higher than that of acarbose. The IC_{50} values of the 50% aq. ethanol extracts of *R .rugosa* were in the range of 37 - 90 μg/ml against BSA except a sample cultivated at Yeonggang in Korea, and the IC_{50} values were nearly 7 - 23 times higher than that of acarbose. The IC_{50} values of the 50% aq.

ethanol extracts of *R. maikwai* (MAI) was 104 μg/ml against BSA, the IC_{50} value was more than 20 times higher that acarbose. According to the IC_{50} values, the Mei-gui showed stronger inhibitory effects compared with those of *R. rugosa*.

The inhibitory effects of the 50% aq. ethanol extracts of petals of Mei-gui and *R. rugosa* against α- glucosidase were shown in Table 2.5.

Table 2.5 Inhibitory effects of the extracts of petals of Mei-gui and *R. rugosa* against α-glucosidase

Mei-gui	Inhibition[a,b] (%)	IC_{50}[a,c] (μg/ml)	*Rosa rugosa*	Inhibition[a,b] (%)	IC_{50}[a,b] (μg/ml)
CX1	76	30	J1	55	59
CX2	73	32	J2	56	40
CX3	88	14	J3	44	54
CX5	41	59	J4	56	27
CB	80	24	MAI	30	68
CS1	67	30	K2	76	22
CS3	35	86	K3	52	41
Acarbose	49	55	K4	72	16

[a]The value was an average of three determinations.

[b]Inhibition at the concentration of 50μg/ml.

[c]concentration required for 50% inhibition of the enzyme activity.

[d]inhibition rate of acarbose at 25μg/ml.

Comparison with the inhibitory effect of acarbose, the extracts exhibited strong or the nearest inhibitory effects against α- glucosidase from *Saccharomyces* SP (SSG). The IC_{50} values of the 50% aq. ethanol extracts of Mei-gui were in the range of 14 - 59 μg/ml against SSG except a sample collected at Pingying, and the IC_{50} values were stronger or nearest than that of acarbose. The IC_{50} values of the 50% aq. ethanol extracts of

R .rugosa were in the range of 16 - 59 μg/ml against SSG, and the IC_{50} values were stronger or nearest than that of acarbose. The IC_{50} values of the 50% aq. ethanol extracts of *R. maikwai* (MAI) was 68μg/ml against BSA, showed weakly inhibitory effect than acarbose.

2.4 Conclusion

Compared with inhibitory effect of acarbose, the extracts of petals of Mei-gui and *R. rugosa* exhibited weakly inhibitory effects against α-amylase from *Bacillus* sp. However, the extracts exhibited stronger or the nearest inhibitory effects against α-glucosidase from *Saccharomyces* sp. than those of acarbose. These results indicated that the petals of Mei-gui and *R. rugosa* must be effective for treatment of diabetes.

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Chapter 3 Bioactive compounds studies

3.1 Isolation of hydrolysable tannins from Mei-gui

3.1.1 Materials and chemicals

Cultivated Mei-gui was collected at Houtan in Xinjiang Province, China, in May 1st through 3rd, 2007. The petals were separated and air dried, then kept at room temperature in sealed package.

The chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

3.1.2 General procedures

^{1}H and ^{13}C NMR spectra were measured with a JEOL α-500 spectrometer in acetone-d_6 or DMSO-d_6 at 30ºC. Chemical shifts were determined using acetone (δ_H: 2.04 ppm, δ_C: 29.80 ppm) or DMSO (δ_H: 2.49 ppm, δ_C: 39.50 ppm) as the internal reference. HR-ESI-TOF-MS spectra were recorded on a JEOL JMS-T100LC spectrometer. Sephadex LH-20 (GE Health care, Sweden) was used for column chromatography. Preparative HPLC were operated using a FMI pump and a C_{18} column (3.0 i.d. × 30 cm, GL Science, Tokyo, Japan), or a Shimadzu LC-8 pump, a Hitachi L-4250 UV-VIS detector with 2 mm cell and a Inertsil ODS-3 (20 i.d. × 250 mm, GL Science).

3.1.3 Isolation of hydrolysable tannins

Pulverized petals of Mei-gui (100 g), China, were extracted with 50% aq. ethanol (1 l) three times. The extract was evaporated to remove ethanol, and the aqueous fraction was extracted with ethyl acetate 3 times. The combined ethyl acetate fraction was evaporated to dryness to obtain ethyl acetate-soluble fraction (8.4 g). The ethyl acetate-soluble fraction (5 g) was dissolved in ethanol and applied to a column of Sephadex LH-20 (5 i.d. × 18 cm). The column was eluted with a solvent system of ethanol-H_2O-acetone to obtain fractions (Fr. 1-10) containing hydrolysable tannins with different degrees of galloylation [1]. These fractions were further purified by reversed-phase chromatography and preparative HPLC. Compound **6** (10 mg), **7** (8 mg) and **8** (15 mg) were separated from Fr. 4 eluted with ethanol/water (70:30). Compound **1** (47 mg) was isolated from Fr. 5 eluted with ethanol/H_2O (60:40). Compound **2** (15 mg), **4** (12 mg) and **5** (20 mg) were isolated from Fr. 6 eluted with ethanol/water/ acetone (54:36:10). Compound **3** (27 mg) was isolated from Fr. 8 eluted with ethanol/ water/ acetone (42:28:30).

3.1.4 Characterization of the isolated hydrolysable tannins

The compounds **1** - **8** were characterized as hydrolysable tannins by their ^{1}H- and ^{13}C-NMR and HR-ESI-TOF mass spectra. The structures were elucidated to be tellimagrandin I (**1**), tellimagrandin II (**2**), rugosin A (**3**), rugosin D (**4**), casuarictin (**5**), strictinin (**6**), isostrictinin (**7**) and

valoneoic acid (**8**), respectively, by comparison of their spectroscopic data with those in the literatures [2-7]. These compounds had been isolated from the petals of *Rosa rugosa* [2-7], but this is the first time they are isolated from Mei-gui cultivated in Tarim Basin of Xinjiang. The chemical structures are shown in Fig. 3.1.1.

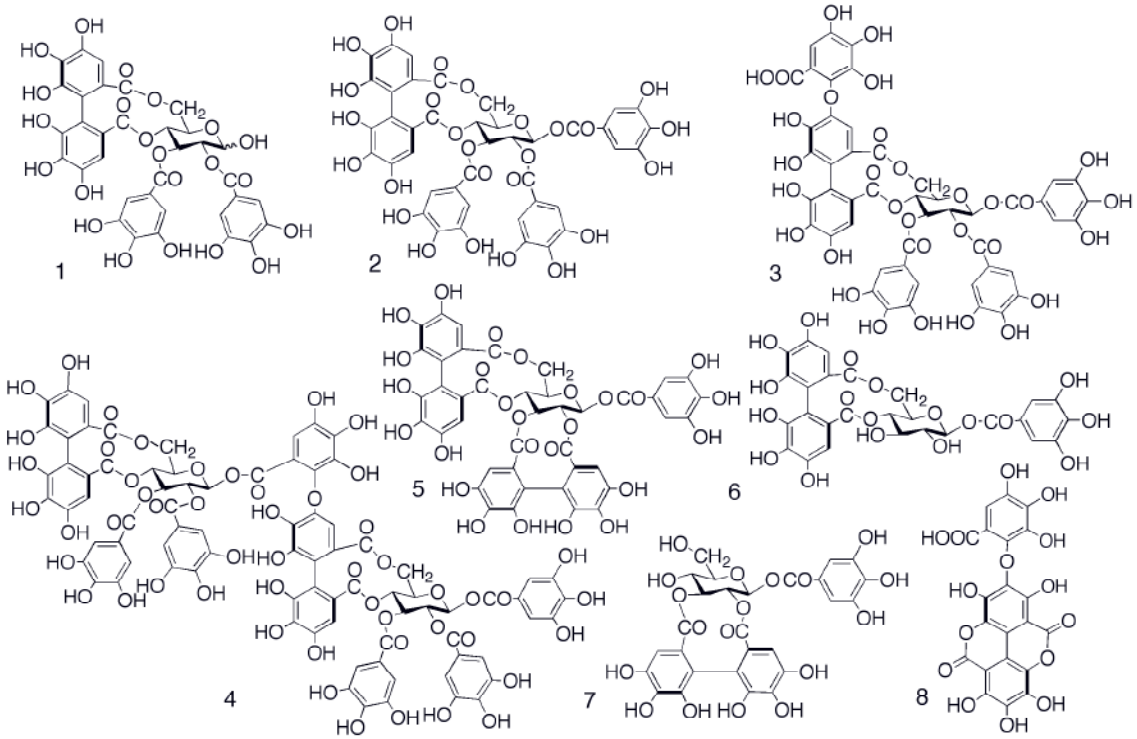


Fig.3.1.1 Structures of hydrolysable tannins isolated from petal of Mei-gui collected in Houtan of Xinjiang, China

1: Tellimagrandin I, **2**: Tellimagrandin II, **3**: Rugosin A, **4**: Rugosin D, **5**: Casuarictin, **6**: Strictinin, **7**: Isostrictinin, **8**: Valoneoic acid

Compound **1** (tellimagrandin I): light yellow powder; $[\alpha]^{26}_{D}$ +140.7 (*c* 0.1, acetone); UV λ_{max} (MeOH) nm (log ε): 218 (4.13), 227 (4.04), 252 (4.37), 275 (4.56) nm; ^{1}H NMR (500 MHz, acetone-d_6) δ: 7.07, 7.00, 7.06, 6.98 (each 2H, s); 6.67, 6.49, 6.66, 6.48 (each 1H, s), 5.57 (1H, d, *J* = 3.9 Hz, H -1a), 5.08 (1H, d, *J* = 8.3 Hz, H-1b), 5.12 (1H, dd, *J* = 3.9, 9.8 Hz, H-2a), 5.28 (1H, d, *J* = 8.3, 9.8 Hz, H-2b), 5.88 (1H, t, *J* = 9.8 Hz, H-3a), 5.61 (1H, t, *J* = 9.8 Hz, H-3b), 5.12 (1H, t, *J* = 9.8 Hz, H-4a), 5.08 (1H, t, *J* = 9.8 Hz, H-4b), 4.68 (1H, dd, *J* = 6.3, 9.8 Hz, H-5a), 4.27 (1H, dd, *J* = 6.3, 9.8 Hz, H-5b), 5.30 (1H, dd, *J* = 6.3, 13.2 Hz, H-6a), 5.27 (1H, dd, *J* = 6.3, 12.7 Hz, H-6b), 3.86 (1H, d, *J* = 13.2 Hz, H-6′a), 3.79 (1H, dd, *J* = 1.5, 12.7 Hz, H-6'b); HR-ESI-TOF MS *m/z* 809.0813 $[M + Na]^{+}$ (calcd. for $C_{34}H_{26}O_{22}Na$, 809.0812).

Compound **2** (tellimagrandin II): light yellow powder; $[\alpha]^{27}_{D}$ +56.4 (*c* 0.05, acetone); UV λ_{max} (MeOH) nm (log ε): 229 (4.02), 245 (3.99), 278 (4.55) nm; ^{1}H NMR (500 MHz, acetone-d_6) δ: 7.12, 7.01, 6.98 (each 2H, s), 6.66, 6.48 (each 1H, s), 6.20 (1H, d, *J* = 8.3 Hz, H-1), 5.60 (1H, dd, *J* = 8.3, 9.8 Hz, H-2), 5.84 (1H, t, *J* = 9.8 Hz, H-3), 5.23 (1H, t, *J* = 9.8 Hz, H-4), 4.56 (1H, dd, *J* = 6.3, 9.8 Hz, H-5), 5.37 (1H, dd, *J* = 6.3, 13.7 Hz, H-6), 3.89 (1H, d, *J* = 13.7 Hz, H-6′); HR-ESI-TOF MS *m/z* 961.0923 $[M + Na]^{+}$ (calcd. for $C_{41}H_{30}O_{26}Na$, 961.0906).

Compound **3** (rugosin A): light brown powder; $[\alpha]^{26}_{D}$ +91.6 (*c* 0.1, acetone); UV λ_{max} (MeOH) nm (log ε): 215 (4.03), 243 (4.18), 276 (4.32) nm; ^{1}H NMR (500 MHz, acetone-d_6) δ: 7.10, 6.99, 6.98 (each 2H, s), 7.14, 6.48, 6.34 (each 1H, s), 6.16 (1H, d, *J* = 8.4 Hz, H-1), 5.57 (1H, dd, *J* = 8.4, 9.8 Hz, H-2), 5.81 (1H, t, *J* = 9.8 Hz, H-3), 5.16 (1H, t, *J* = 9.8 Hz, H-4),

4.51 (1H, dd, *J* = 6.6, 9.8 Hz, H-5), 5.29 (1H, dd, *J* = 6.6, 13.7 Hz, H-6), 3.79 (1H, d, *J* = 13.7 Hz, H-6′); HR-ESI-TOF MS *m/z* 1129.0982 [M + Na]$^{+}$, (calcd. for $C_{48}H_{34}O_{31}Na$, 1129.0992).

Compound **4** (rugosin D): light brown powder; $[\alpha]^{27}_{D}$ +93.2 (*c* 0.1, methanol); UV λ_{max} (MeOH) nm (log ε): 239 (4.75), 278 (5.30) nm; ^{1}H NMR (500MHz, acetone-d_6) δ: 7.13, 7.02, 7.01, 7.01, 6.98 (each 2H, s), 6.66, 6.49, 7.13, 6.48, 6.25 (each 1H, s), glucose core right: δ 6.18 (1H, d, *J* = 8.8 Hz, H-1), 5.61 (1H, dd, *J* = 8.8, 10.0 Hz, H-2), 5.83 (1H, t, *J* = 10.0 Hz, H-3), 5.17 (1H, t, *J* = 10.0 Hz, H-4), 4.51 (1H, dd, *J* = 6.7, 10.0 Hz, H-5), 5.32 (1H, dd, *J* = 6.7, 13.4 Hz, H-6), 3.78 (1H, d, *J* = 13.4 Hz, H-6`), glucose core left: 6.13 (1H, d, *J* = 8.3 Hz, H-1), 5.53 (1H, dd, *J* = 8.3, 10.0 Hz, H-2), 5.78 (1H, t, *J* = 10.0 Hz, H-3), 5.17 (1H, t, *J* = 10.0 Hz, H-4), 4.48 (1H, dd, *J* = 6.3, 10.0 Hz, H-5), 5.32 (1H, dd, *J* = 6.3, 13.4 Hz, H-6), 3.82 (1H, d, *J* = 13.4 Hz, H-6′); HR-ESI-TOF MS *m/z* 1897.1792 [M + Na]$^{+}$, (calcd. for $C_{82}H_{58}O_{52}Na$, 1897.1743).

Compound **5** (casuarictin): light yellow powder; $[\alpha]^{27}_{D}$ +16.2 (*c* 0.1, methanol); UV λ_{max} (MeOH) nm (log ε): 204 (4.57), 212 (4.56), 229 (4.09), 246 (4.51), 258 (4.71), 275 (4.32) nm; ^{1}H NMR (500 MHz, acetone-d_6) δ: 7.17 (2H, s), 6.67, 6.51, 6.45, 6.36 (each 1H, s), 6.20 (1H, d, *J* = 8.8 Hz, H-1), 5.18 (1H, t, *J* = 8.8 Hz, H-2), 5.43 (1H, t, *J* = 8.8, 10.0 Hz, H-3), 5.15 (1H, t, *J* = 10.0 Hz, H-4), 4.49 (1H, dd, *J* = 6.8, 10.0 Hz, H-5), 5.35 (1H, dd, *J* = 6.8, 13.2 Hz, H-6), 3.87 (1H, d, *J* = 13.2 Hz, H-6′); HR-ESI-TOF MS *m/z* 935.0790 [M + H]$^{-}$ (calcd. for $C_{41}H_{27}O_{26}$, 935.0924).

Compound **6** (strictinin): light yellow powder; UV λ_{max} (MeOH) nm (log ε): 217 (4.53), 233 (4.74), 249 (4.65), 270 (4.66) nm; ^{1}H NMR (500

MHz, acetone-d_6) δ: 7.18 (2H, s), 6.69, 6.58 (each 1H, s), 5.72 (1H, d, *J* = 7.8 Hz, H-1), 3.69 (1H, dd, *J* = 7.8, 9.3 Hz, H-2), 3.75 (1H, t, *J* = 9.3 Hz, H-3), 4.89 (1H, t, *J* = 9.8 Hz, H-4), 4.09 (1H, dd, *J* = 6.3, 9.8 Hz, H-5), 5.19 (1H, dd, *J* = 6.3, 13.2 Hz, H-6), 3.81 (1H, d, *J* = 13.2 Hz, H-6′); HR-ESI-MS *m/z* 657.0704 $[M+Na]^+$, (calcd. for $C_{27}H_{22}O_{18}Na$, 657.0743).

Compound **7** (isostrictinin): light yellow powder; UV λ_{max} (MeOH) nm (log ε): 204 (4.67), 211 (4.57), 244 (4.54), 271 (4.50) nm; ^{1}H NMR (500 MHz, acetone-d_6) δ: 7.14 (2H, s), 6.69, 6.41 (each 1H, s), 6.09 (1H, d, *J* = 9.3 Hz, H-1), 5.01 (1H, dd, *J* = 9.3, 9.8 Hz, H-2), 5.19 (1H, t, *J* = 9.3 Hz, H-3), 3.72 - 3.91 (4H, m, H-4, H-5, H-6, H-6′); HR-ESI-TOF MS *m/z* 657.0704 $[M+Na]^+$ (calcd. for $C_{27}H_{22}O_{18}Na$, 657.0737).

Compound **8** (valoneoic acid): white powder; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.47, 7.02, 6.93 (each 1H, s), ^{13}C NMR (125 MHz, DMSO-d_6) δ: 165.8, 159.09, 159.01, 149.8, 149.4, 148.5, 142.9, 140.6 (br), 139.50, 139.46, 139.0, 136.6, 136.1, 135.1, 114.6, 113.8, 112.1, 110.4 (br), 108.4 (br), 108.3 (br), 108.0; HR-ESI-TOF MS *m/z* 471.0199 $[M+H]^+$ (calcd. for $C_{21}H_{11}O_{13,}$ 471.0205).

3.2 Inhibitory effects of hydrolysable tannins against α-amylase and α-glucosidase

3.2.1 Materials and chemicals

The inhibitory effects of hydrolysable tannins against α-amylase and α-glucosidase were tested, using tellimagrandin I (**1**), tellimagrandin II (**2**),

rugosin A (**3**), rugosin D (**4**), casuarictin (**5**), strictinin (**6**) and isostrictinin (**7**) isolated from Mei-gui. Also valoneoic acid (**8**) isolated from Mei-gui and gallic acid was tested. Gallic acid, ellagic acid and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

The digestive enzymes: α-amylase from *Bacillus* sp. was purchased from Sigma-Aldrich Japan Co. (Japan). α-Glucosidase from *Saccharomyces* sp. was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

3.2.2 Assessment of inhibitory activity against α-amylase

α-Amylase from *Bacillus* sp. (BSA, 20 unit/mg of protein), was used, and enzyme activity was measured as follows. Phosphate buffer (0.25 M, pH 7.0, 0.5 ml) containing 0.4 mg/ml soluble starch and test sample was incubated at 37˚C for 5 min. The test samples were dissolved in H_2O. After addition of enzyme (200 unit/ml, 10 μl), the reaction mixture was incubated at 37˚C for 10 min. Then the enzyme reaction was stopped at 0˚C, and iodine solution (5 mM) was added to the reaction mixture. The amount of residual starch was determined by measuring absorption at 660 nm. The IC_{50} values were estimated graphically by plotting percentage inhibition versus the log of test sample concentrations.

3.2.3 Assessment of inhibitory activity against α-glucosidase

α-Glucosidase from *Saccharomyces* sp. (SSG, 163 unit/mg of protein)

was used, and their activity was evaluated as follows. The test samples (10 μl, in DMSO) were added to the mixture of phosphate buffer (415 μl, 10 mM, pH 7.0) and 25 μl of 1 M sucrose, and the mixture was incubated at 37˚C for 5 min. Then 50 μl of enzyme solution was added to the mixture and was incubated at 37˚C for 20 min. The enzyme solution (10 μg/ml) was prepared by dissolving α-glucosidase in 10 mM phosphate buffer (pH 7.0) containing 0.2% BSA (albumin from bovine serum). The reaction was stopped by addition of 10 μl of 10 mM deoxynojirimycin. The residual glucose concentrations in the reaction mixture were measured using a glucose oxidase (GOD) method, (glucose CII-test, Wako Pure Chemical Industries Ltd.). The IC_{50} values were determined graphically by plotting percentage inhibition versus the log of test sample concentrations.

3.2.4 Result and discussion

The inhibitory activities of seven hydrolysable tannins against α-amylase and α-glucosidase were tested along with those of gallic acid and ellagic acid. As shown in Table 3.2.1, gallic acid, ellagic acid and valoneoic acid showed almost no inhibition against α-amylase from *Bacillus* sp. (BSA). Strictinin and isostrictinin, hydrolysable tannins with a hexahydroxydiphenoyl (HHDP) group and a galloyl group also showed no activities. The IC_{50} value of tellimagrandin I, hydrolysable tannin with a HHDP group and two galloyl groups, was 235 μM. However, the IC_{50} value of tellimagranidin II, hydrolysable tannin with an HHDP group and

three galloyl groups, was 54 μM. The IC_{50} values of casuarictin, rugosin A and rugosin D were 18, 25 and 7 μM, respectively. The IC_{50} value of rugosin D (7 μM) was comparable to that of acarbose (6 μM). These results indicated that bulky hydrolysable tannins showed strong inhibitory activities against α-amylase. The inhibitory activity of tannic acid against α-amylase was studied in 2004[8]; however, this is the first time that this hydrolysable tannin has potent inhibitory activity against α-amylase.

Table 3.2.1 Inhibitory effects of hydrolysable tannins isolated from Mei-gui against α-amylase and α-glucosidase

compounds	IC_{50} [a]			
	α-amylase[b]		α-glucosidase[b]	
	μM	μg/ml	μM	μg/ml
Acarbose	6	4	59	38
Rugosin D	7	13	2	3
Rugosin A	23	25	8	9
Casuarictin	18	17	22	21
Tellimagrandin I	235	185	13	10
Tellimagrandin II	54	51	6	6
Strictinin	>158	>100	181	115
Isostrictinin	>158	>100	199	126
Gallic acid	>5882	>1000	841	143
Ellagic acid	>166	>50	1847	558
Valoneoic acid	>106	>50	642	302

[a] Sample concentration required for 50% inhibition.

[b] α-Amylase from *Bacillus* sp., α-glucosidase from *Saccharomyces* sp.

In the case of α-glucosidase from *Saccharomyces* sp. (SSG), the IC_{50} values of rugosin D, tellimagrandin II, rugosin A, tellimagrandin I and casuarictin were 2, 6, 8, 13 and 22 μM, respectively, and these values were

lower than that of acarbose (59 μM). The IC_{50} values of strictinin and isostrictinin were also higher than that of acarbose, while gallic acid, ellagic acid and valoneoic acid showed almost no inhibition. The increase of number of galloyl groups on glucose core of hydrolysable tannins enhanced their inhibitory activities against α-glucosidase, and one dimeric hydrolysable tannin was shown to be potent inhibitor of α-glucosidase from SSG.

The inhibitory effects of hydrolysable tannins against rat intestinal α-glucosidase have been reported, and an increase in the number of galloyl groups in the hydrolysable tannins enhanced thier inhibitory activity against α-glucosidase [9-10]. This trend was the same in the case of α-glucosidase from *Saccharomyces* sp., and rugosin D, a dimeric hydrolysable tannin, that showed the strongest activity, which was almost thirty times stronger than that of acarbose.

3.3 Quantitative determination of the hydrolysable tannins

3.3.1 General procedures

The Waters ACQUITY UPLC system consisted of following apparatus: binary solvent manager, sample manager, column heater, and PDA (photodiode array) detector. Acquity UPLC BEH phenyl (2.1 i.d. × 100 mm, 1.7μm), Acquity UPLC BEH C_{18} (2.1 i.d. × 100 mm, 1.7μm), Acquity UPLC HSS C_{18} (2.1 i.d. × 100 mm, 1.8 μm) and Acquity UPLC HSS T3 (2.1 i.d. × 100 mm, 1.8 μm) analytical column were used. The

column temperature was range from 10ºC to 50ºC and the injection volume was 1μl. The mobile phase was binary eluent of (A) mix of 2.5% (v/v) acetonitrile and 0.1% (v/v) formic acid and (B) acetonitrile with 1% formic acid under gradient conditions (0-4min: 0%B, 10min: 10%B, 24min: 15%B, 28min: 20%B, 29min: 40%B, 30min: 100%B). The flow rate was 0.2 ml/min. The detector wavelength was set from 200nm to 500nm and max absorption wavelengths of standard compounds were selected for samples detection. The chromatographic data were collected and processed by Empower software (Waters).

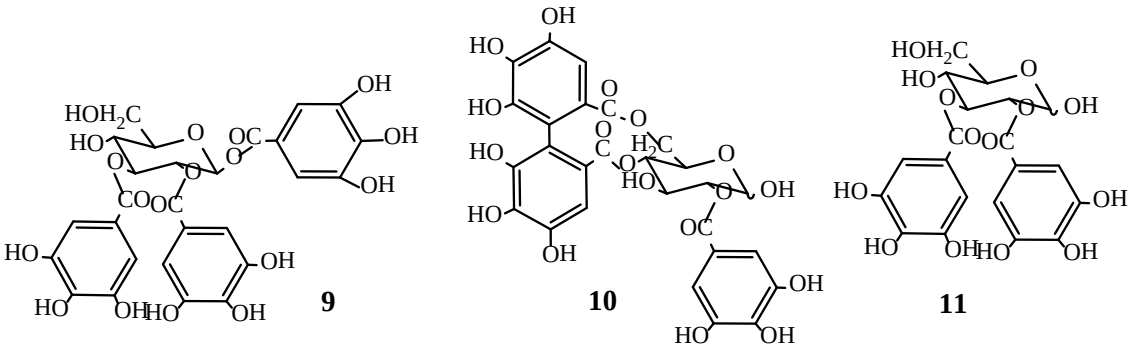
3.3.2 Plant materials and standards

The petals of *R. rugosa* collected from Hokkaido and Niigata of Japan and Korea. 9 samples of Mei-gui cultivated in northern parts of China were used for quantitative determination. The petals were air-dried and kept at room temperature in sealed package.

The compounds **1-7** isolated from the petals of Mei-gui, and compounds **9** – **11**(Chemical structures shown in Fig.3.3.1) isolated from *R. rugosa* were used as standard samples to optimize determination condition of hydrolysable tannins. Among the hydrolysable tannins, five compounds **1-5** were selected for quantitative determination. Gallic acid, ellagic acid and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Water purified with a Milli-Q Labo system (Millipore, Bedford, MA) was used to this study.

The stock solutions of standards were prepared with analytical accuracy dissolving 1 mg of standard substance in 1 ml of methanol. The

working solutions of the standards were prepared by diluting the stock solutions by a factor of 10, using methanol.



9 **10** **11**

Fig.3.3.1 Structures of Compounds **9**, **10** and **11**

9: 1, 2, 3-*O* -tri-galloy - β-D-glucose **10**: 3-*O*-galloyl-4, 6-*O*-HHDP-D-glucose **11**: 2,3-*O*-di-galloyl-D-glucose

3.3.3 Preparation of samples

The pulverized petals (200 mg, under than 200 μm in diameter) were extracted with 50% aqueous (aq.) ethanol (30 ml) in an ultrasonic bath for 30 min three times. The supernatants were collected by centrifugation (2500 rpm, 20 min), and the combined extracts were concentrated. Then the extracts were dissolved with 50% aq. ethanol to 50 ml. The sample solutions were filtrated through a 0.45 μm PTFE Millipore filter unit (Advantec, Japan) for UPLC analysis.

3.3.4 Results and discussion

3.3.4.1 Evaluation of sample extraction conditions

For the selection of effective extraction solvent, extraction efficiency of water, ethanol, ethanol-water, acetone-water, acetone and methanol were tested. Total contents of five hydrolysable tannins; tellimagrandin I(**1**), tellimagrandin II(**2**), rugosin A(**3**), rugosin D(**4**) and casuarictin(**5**) were determined in the extracts of petals of *R. rugosa* at room temperature. As shown in Table 3.3.1, sample extracted with mixtures solvents of water and ethanol or acetone showed higher content of hydrolysable tannins than sample extracted with 100% water, ethanol, methanol and acetone. Moreover, sample extracted with 50% aq. ethanol showed higher total contents of five hydrolysable tannins than those of 20%, 70% aq. ethanol and 20%, 50%, 70% aq. acetone. Therefore, 50% aq. ethanol was selected as an optimum solvent for extraction.

Table 3.3.1 Selection of extraction solvent for sample

Contents of hydrolysable tannins in the petals of *R. rugosa* (mg/g dried petal)										
Analyte	Ethanol-H_2O				Acetone-H_2O				Methanol	Water
	20%	50%	70%	100%	20%	50%	70%	100%	100%	100%
TellimagrandinI	26.75	27.32	27.17	12.20	28.50	26.69	27.25	2.00	24.65	22.94
Tellimagrandin II	9.70	10.73	10.82	7.01	9.81	10.52	10.60	1.19	9.77	3.42
Rugosin A	6.56	6.54	6.01	3.98	6.19	6.19	6.04	0.56	5.36	4.15
Rugosin D	6.72	10.63	10.9	7.06	8.11	9.29	10.72	0.78	7.46	0
Casuarictin	2.65	2.60	2.60	1.54	2.32	2.35	2.31	0.18	2.21	1.84
Total	52.38	57.82	57.50	31.70	55.00	55.04	56.92	4.71	49.45	32.35

In order to extracted hydrolysable tannins completely and rapid, three extraction methods were studied. The samples collected in Otoshibe were

extracted with 50% aq. ethanol by the ultrasonic bath 10 min, 30 min and 60 min. According to the total content of hydrolysable tannins were shown in Table 3.3.2, the contents extracted by ultrasonic bath for 10 min almost equal to extracted by ultrasonic bath for 60 min, and the both of contents higher than those of extracted by ultrasonic bath for 10 min and other both methods, such as shaking extraction 180 min and keeping at room temperature 24 hours. Therefore, the ultrasonic bath extraction method was selected as an optimum extraction method for hydrolysable tannins, and extraction time was 30 min.

Table 3.3.2 Selection of extraction method for sample

Analyte	contents of hydrolysable tannins mg/g dried petal				
	Ultrasonic extraction			Shaking	Keep at R.T.
	10min	30min	60min	180min	1440min
Tellimagrandin I	27.46	27.32	27.33	26.69	26.14
Tellimagrandin II	10.28	10.82	10.67	10.53	9.54
Rugosin A	6.28	6.01	6.68	6.41	5.78
Rugosin D	10.29	10.9	10.26	10.16	8.84
Casuarictin	2.40	2.60	2.40	2.31	2.21
Total	56.71	57.65	57.34	56.1	52.51

We also examined contents of hydrolysable tannins from sample with 50% ethanol by the ultrasonic bath for 30 min four times. From the results in Table 3.3.3, the total hydrolysable tannins were mostly (content of 94.6%) extracted by first time. The content of 4.9% is extracted by second times, and 0.4% and 0.2% were extracted by third time and forth times. The content of 99.9% extracted from sample by three times, therefore, three times extraction selected to use in this study.

Table 3.3.3 Extraction efficiency of extraction times

Contents of hydrolysable tannins(mg/g petal)				
Analyte	1st	2nd	3rd	4th
Tellimagrandin I	27.52	1.57	0.10	0.04
Tellimagrandin II	10.73	0.52	0.06	0.04
Rugosin A	6.65	0.29	0.02	0.01
Rugosin D	10.59	0.55	0.06	0.02
Casuarictin	2.56	0.06	0.00	0.00
Total	58.05	2.99	0.24	0.11
Yield*	94.6%	4.9%	0.4%	0.2%

*100× (content of each extraction) / (total content of extraction by four times)

3.3.4.2 Optimization of chromatography conditions

Two types of columns with different particles were tried in this study. The one type is used 1.7 μm Ethylene Bridged Hybrid (BEH) particle. This type is including to two kinds of column with different chemical structure. One kind of column is Acquity UPLC BEH phenyl column which is utilized a trifunctional C_6 alkyl tether between the phenyl ring and the silyl functionality. Another is Acquity UPLC BEH C_{18} column utilized C_{18} alkyl phase bonded to the silyl functionality. The second type is used 1.8 μm High Strength Silica (HSS) particle. This type used 100% silica particles with C_{18} alkyl phase, and has two kinds of column: Acquity UPLC HSS C_{18} column and Acquity UPLC HSS T3 column. HSS T3 column is utilizing T3 bonding which is utilizes a trifunctional C_{18} alkyl phase bonded at a ligand density that promotes polar compound retention and aqueous mobile phase compatibility. The chemical modification of column is shown in Fig. 3.3.2.

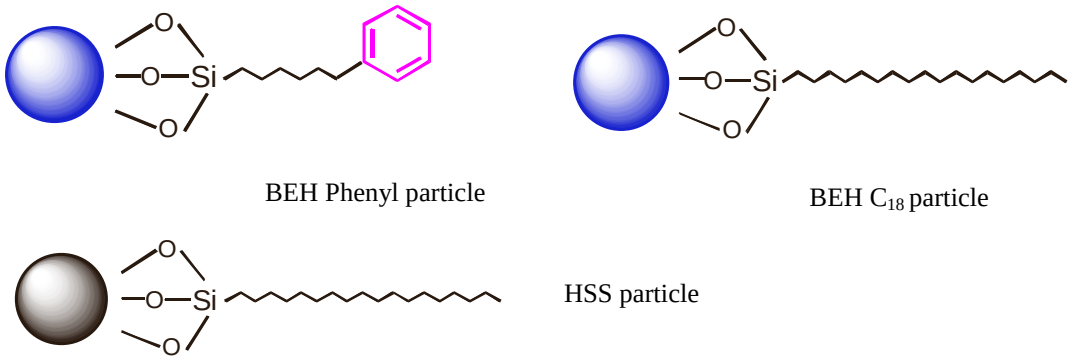


Fig. 3.3.2 Chemical modification of column used in this study

The 50% aq. ethanol extracts of *R. rugosa* were injected to four kinds of column in the same chromatography condition mentioned above at the same column temperature (25ºC). Their chromatograms are shown in Fig. 3.3.3.

The hydrolysable tannins and related compounds (**1**-**5**, **12**, **13**) were more strongly retained on the HSS T3 column than on the other, while the retention capacity of the BEH C_{18} column was the weakest. The peaks of tellimagrandin II and rugosin A were not separated completely on the HSS T3 column, HSS C_{18} column and BEH C_{18} column; moreover, the peak of ellagic acid was overlapped with either peak on these columns.

The peaks of tellimagrandin II, rugosin A and ellagic acid were completely separated on the BEH phenyl column, and the peaks of hydrolysable tannins and related compounds appeared in over a wide range on the chromatogram.

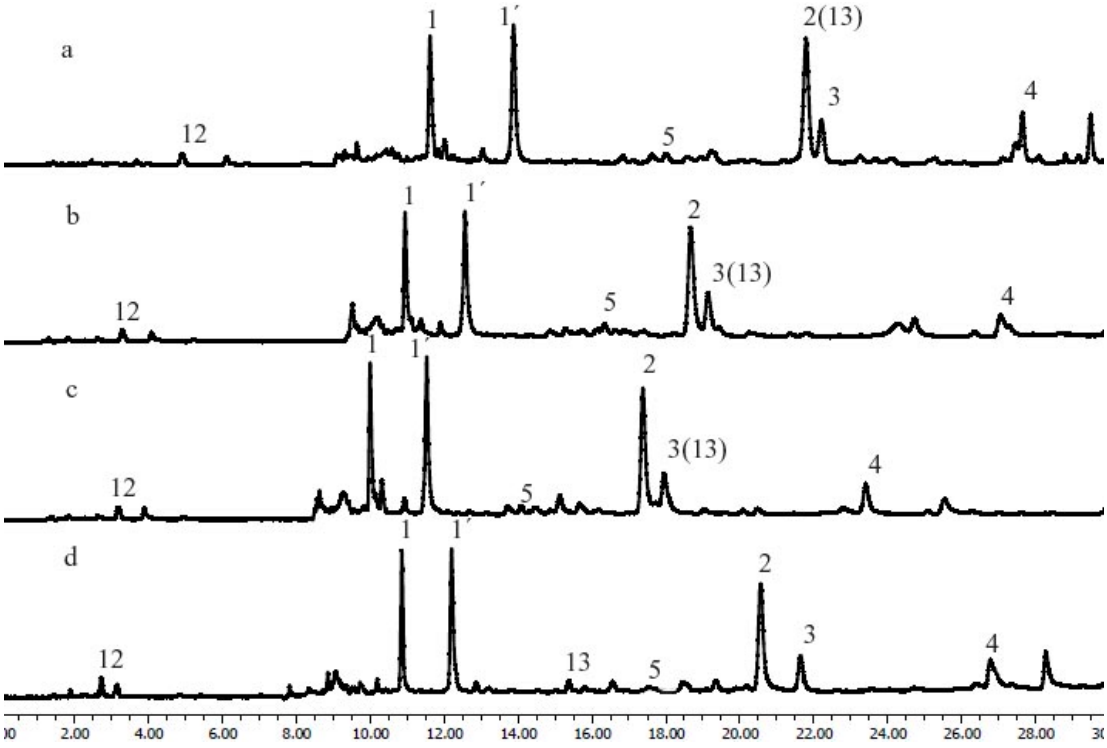


Fig. 3.3.3 UPL chromatograms of the 50% ethanol extracts of *R. rugosa* in the four kinds of column;

a: HSS T3 column b: HSS C_{18} column c: BEH C_{18} column d: BEH phenyl column;

1, 1′: tellimagrandin I, 2: tellimagrandin II, 3: rugosin A, 4: rugosin D, 5: casuarictin, 12: gallic acid, 13: ellagic acid

The BEH phenyl column and the BEH C_{18} column are based on the same BEH technology, but both is difference in their chemical structures. The BEH phenyl column is more suitable separate to hydrolysable tannins than BEH C_{18} column. As there were remarkable differences in retention times of hydrolysable tannins between the BEH C_{18} column and the BEH phenyl column, the retention times of 11 compounds (**1**-**7**, **9**-**11**) on BEH phenyl column were compared with those on the BEH C_{18} column. The differential retention times of the 11 compounds were calculated by changing column temperature (10 - 50°C), and the compounds were

classified into three groups (group 1 - 3) as shown in Table 3.3.4 - 3.3.6 according to the temperature dependence of differential retention times.

Table 3.3.4 Temperature dependency on the differential retention time between the BEH phenyl column and the BEH C_{18} column (Group 1)

CT[1]	Retention time (min)								
	Gallic acid			Ellagic acid			TGG[5]		
(°C)	Phenyl[2]	C_{18}[3]	ΔRt[4]	Phenyl	C18	ΔRt	Phenyl	C18	ΔRt
10	4.05	6.44	-2.39	20.77	24.55	-3.78	20.74	24.46	-3.72
15	3.54	5.02	-1.48	18.62	20.85	-2.23	18.68	21.20	-2.52
20	3.05	4.05	-1.00	16.34	18.05	-1.72	16.29	18.14	-1.85
25	2.93	3.64	-0.71	15.70	16.70	-1.01	15.73	16.75	-1.02
30	2.76	3.31	-0.55	14.89	15.68	-0.79	14.91	15.69	-0.79
35	2.61	3.02	-0.41	14.25	14.65	-0.40	14.14	14.73	-0.59
40	2.49	2.79	-0.30	13.71	13.79	-0.08	13.48	13.80	-0.31
45	2.39	2.60	-0.21	13.13	13.01	0.12	12.93	13.01	-0.09
50	2.24	2.41	-0.18	12.32	12.33	-0.01	12.29	12.33	-0.05

[1] CT: column temperature [2] Phenyl: BEH phenyl column [3] C_{18}: BEH C_{18} column
[4] ΔRt= (retention time on the BEH phenyl column) – (retention time on the BEH C_{18} column) [5] TGG: 1, 2, 3-*O* -tri -galloyl -*β*-D-glucose

In Group 1, the differences of retention time (ΔRt) of compounds between in the BEH phenyl column and the BEH C_{18} column were minus (ΔRt < 0), as shown in Fig 3.3.4. Gourp 1 was composed of gallic acid (**11**), ellagic acid (**12**) and 1,2,3-tri-*O*-galloyl-*β*-D-glucose (**8**). Those hydrolysable tannins belong to the gallotannins which have similar structures containing a core glucose which is surrounded by several galloyl groups. And the molecule motion of those hydrolysable tannins is easily. the retention times of these compounds were almost the same on these two columns at high temperature (40 - 50°C), but gallic acid and ellagic acid were retained stronger on the BEH C_{18} column than on the BEH Phenyl column at low temperature(10 - 20°C). 1,2,3-Tri-*O*-galloyl-*β*-D-glucose

showed the same tendency, and gallotannins with free hydroxyl groups on the glucose core may show the same behaviour as phenolic carboxylic acids.

Table 3.3.5 Temperature dependency on the differential retention time between the BEH phenyl column and the BEH C_{18} column (Group 2)

CT[1]	Retention time (min)											
	Isostrictinin			Strictinin			Tellimagrandin I			GHG[5]		
(°C)	Phenyl[2]	C_{18}[3]	ΔRt[4]	Phenyl	C_{18}	ΔRt	Phenyl	C_{18}	ΔRt	Phenyl	C_{18}	ΔRt
10	9.89	9.77	0.12	13.57	12.87	0.70	15.76	15.19	0.57	8.57	8.37	0.20
15	9.28	9.43	-0.14	12.12	11.49	0.64	13.91	13.01	0.91	8.39	8.37	0.02
20	8.93	9.03	-0.10	11.16	10.44	0.72	12.12	11.49	0.63	8.07	8.31	-0.24
25	8.82	8.55	0.27	10.73	9.75	0.98	11.64	10.87	0.78	7.21	7.50	-0.30
30	8.70	8.30	0.39	10.34	9.40	0.95	11.11	10.37	0.74	6.15	6.06	0.10
35	8.47	8.00	0.47	9.91	9.06	0.85	10.77	9.89	0.88	5.51	5.28	0.23
40	8.27	7.89	0.38	9.58	8.86	0.71	10.28	9.57	0.72	4.99	4.71	0.28
45	8.28	7.60	0.68	9.39	8.64	0.75	9.96	9.24	0.72	4.50	4.21	0.30
50	7.84	7.40	0.44	9.03	8.46	0.57	9.47	8.99	0.48	4.11	3.78	0.34

[1] CT: column temperature [2] Phenyl: BEH phenyl column [3] C_{18}: BEH C_{18} column
[4] ΔRt= (retention time on the BEH phenyl column) – (retention time on the BEH C_{18} column) [5] GHG: 3-*O*-galloyl-4, 6-*O*-HHDP-D-glucose

In Group 2, the ΔRt of four hydrolysable tannins between in the BEH phenyl column and the BEH C_{18} column were lower than 1 ($0 < \Delta Rt < 1$) as shown in Fig 3.3.4. Group 2 was composed of tellimagrandin I (**1**), 3-*O*-galloyl-4,6-*O*-HHDP-D-glucose (**9**), strictinin (**6**) and isostrictinin (**7**). These compounds were ellagitannins with a free hydroxyl group on the core glucose, also have a common characteristic of their structure; a core glucose which is have a hexahydroxydiphenoyl (HHDP) group and 1 - 2 galloyl groups to bonded through a ester (depside) bond. The retention times of these compounds were almost the same on these two columns, and were not affected by the column temperature.

Table 3.3.6 Temperature dependency on the differential retention time between the BEH phenyl column and the BEH C_{18} column (Group 3)

CT[1]	Retention time (min)											
	Casuarictin			Tellimagrandin II			Rugosin A			Rugosin D		
(°C)	Phenyl[2]	C_{18}[3]	ΔRt[4]	Phenyl	C_{18}	ΔRt	Phenyl	C_{18}	ΔRt	Phenyl	C_{18}	ΔRt
10	24.13	21.74	2.39	27.13	24.91	2.22	27.89	25.69	2.20	30.55	30.07	0.48
15	21.02	18.26	2.76	24.19	21.20	2.99	25.22	21.82	3.40	29.62	27.07	2.56
20	18.66	15.42	3.24	21.68	18.01	3.68	21.51	18.55	2.96	26.59	23.23	3.35
25	17.26	13.98	3.28	20.18	16.39	3.79	20.52	16.89	3.62	25.71	22.25	3.46
30	16.04	13.02	3.02	18.80	15.23	3.58	19.11	15.68	3.43	23.98	20.77	3.22
35	14.80	12.11	2.69	17.35	14.10	3.25	17.79	14.65	3.14	22.36	19.27	3.09
40	13.83	11.40	2.43	16.12	13.16	2.96	16.60	13.51	3.10	21.38	17.89	3.49
45	12.98	10.78	2.20	15.04	12.34	2.70	15.69	12.62	3.07	20.11	16.65	3.46
50	12.12	10.26	1.86	13.99	11.63	2.36	14.35	11.84	2.51	18.38	15.49	2.89

[1] CT: column temperature [2] Phenyl: BEH phenyl column [3] C_{18}: BEH C_{18} column
[4] ΔRt= (retention time on the BEH phenyl column) – (retention time on the BEH C_{18} column)

In Group 3, the ΔRt of four hydrolysable tannins between in the BEH phenyl column and the BEH C_{18} column were higher than 1 and lower than 4 ($1 < \Delta Rt < 4$) as shown in Fig.3.3.4. Group 3 was composed of casuarictin (**5**), tellimagradin II (**2**) and rugosin A (**3**) and D (**4**), and these compounds were retained on the BEH phenyl column stronger than on the BEH C_{18} column. These hydrolysable tannins have a higher molecular weight, respectively. Their structures include core glucose, a valoneoyl group, 1 or 2 HHDP groups and 1 - 5 galloyl groups and all the hydroxyl groups of core glucose were esterified through a depside bond. The column temperature did not affect the retention capacities of these compounds on these two columns, except for rugosin D.

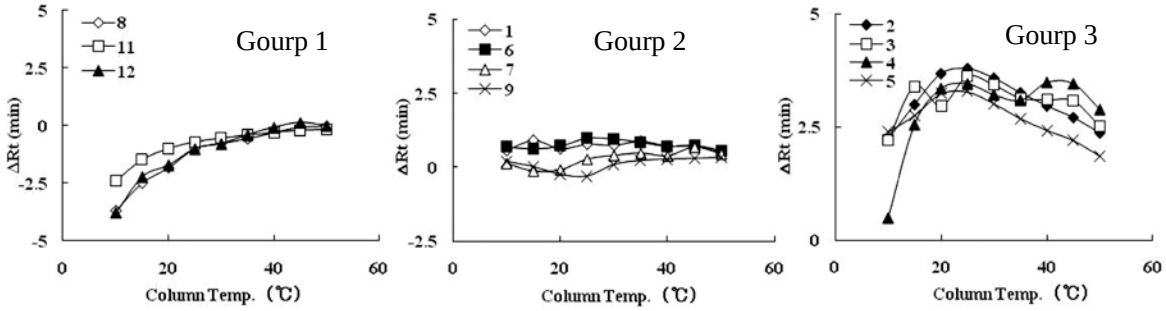


Fig. 3.3.4 The difference of retention time of BEH phenyl column and BEH C_{18} column
8: 1,2,3-*O*-tri-galloy-*β*-D-glucose 11: Gallic acid 12: Ellagic acid
1: Tellimagrandin I 6: Strictinin 7: Isostrictinin 9: 3-*O*-galloyl-4,6-*O*-HHDP-D-glucose
2: Tellimagrandin II 3: Rugosin A 4: Rugosin D 5: Casuarictin
ΔRt= (retention time on the BEH phenyl column) – (retention time on the BEH C_{18} column)

The retention of the hydrolysable tannins on the BEH phenyl column depended on the presence or absence of a free hydroxyl group on the glucose core; the absence of a free hydroxyl group enhances the retention on the BEH phenyl column. The orientation of acyl groups and/or flexibility of the molecule must be responsible for the difference in the retention capacity.

As the retention capacities of the hydrolysable tannins and related compounds were dependent on their structural characteristics, the BEH phenyl column was shown to be suitable for simultaneous determination of hydrolysable tannins in the petal of *R. rugosa* and Mei-gui.

The retention time changes of those hydrolysable tannins were checked by different column temperature range from the 10ºC to 50ºC in the BEH phenyl column. The higher temperature can shorting the retention time but not separate hydrolysable tannins completely. The

peaks of the hydrolysable tannins with free hydroxyl group on the anomeric carbon were broader at high column temperature as shown in Fig. 3.3.5; therefore the column temperature was chosen as 25°C.

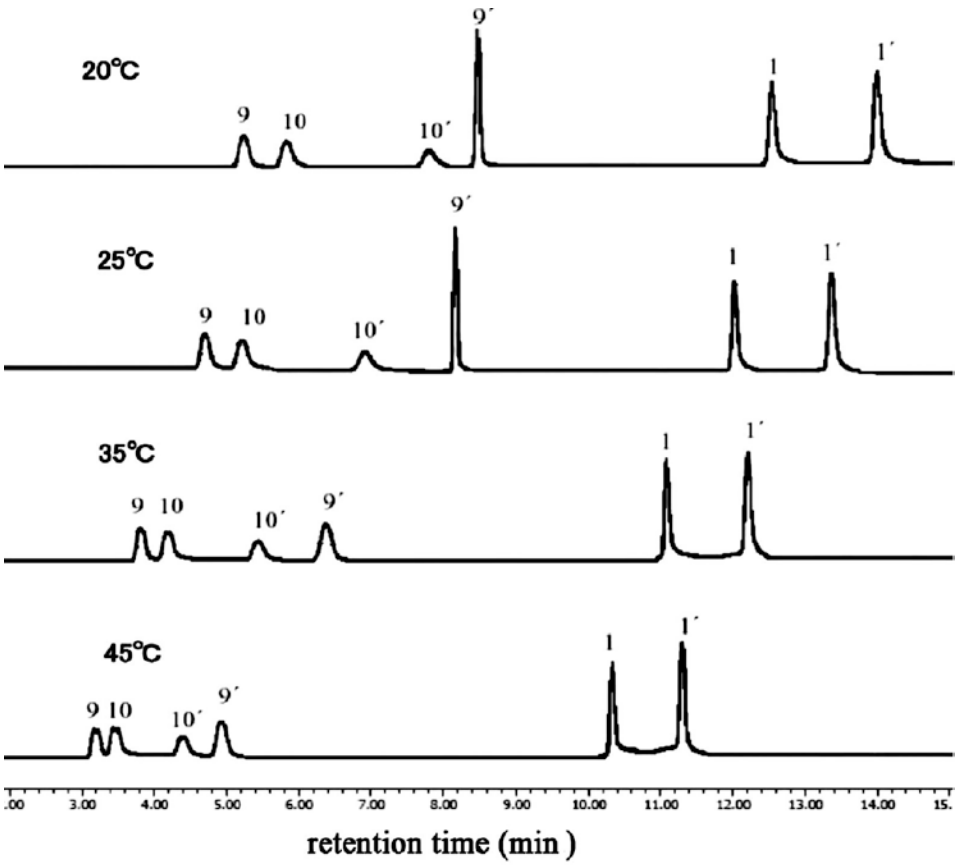


Fig. 3.3.5 Chromatograms of hydrolysable tannins with free anomeric hydroxyl group on the BEH phenyl column at different temperature (280 nm)
1,**1´**: Tellimagrandin I **9**,**9´**: 3-*O*-galloyl-4,6-*O*-HHDP-D-glucose **10**,**10´**: 2,3-*O*-di-galloyl-D-glucose

4.2.4.3 Determination of hydrolysable tannins

The five hydrolysable tannins, tellimagrandin I (**1**), tellimagrandin II (**2**), rugosin A (**3**), rugosin D (**4**) and casuarictin (**5**) in the dried petals of 18 samples were determined by the optimized method. The calibration curve

of these hydrolysable tannins showed excellent linearity in the range of 20 - 600 ng, with correlation coefficients over 0.999. Detection limits of compound **1** - **5** were all 0.015 mg/g, and almost all coefficients of variation of the determination values were less than 3%.

The nine samples were Mei-gui collected in China, five samples were *R. rugosa* from Hokkaido and Niigata of Japan, and four samples were from Korea. Their UPL chromatograms were shown in Fig.3.3.6. The five hydrolysable tannins were detected all the samples except a sample collected in Pingying.

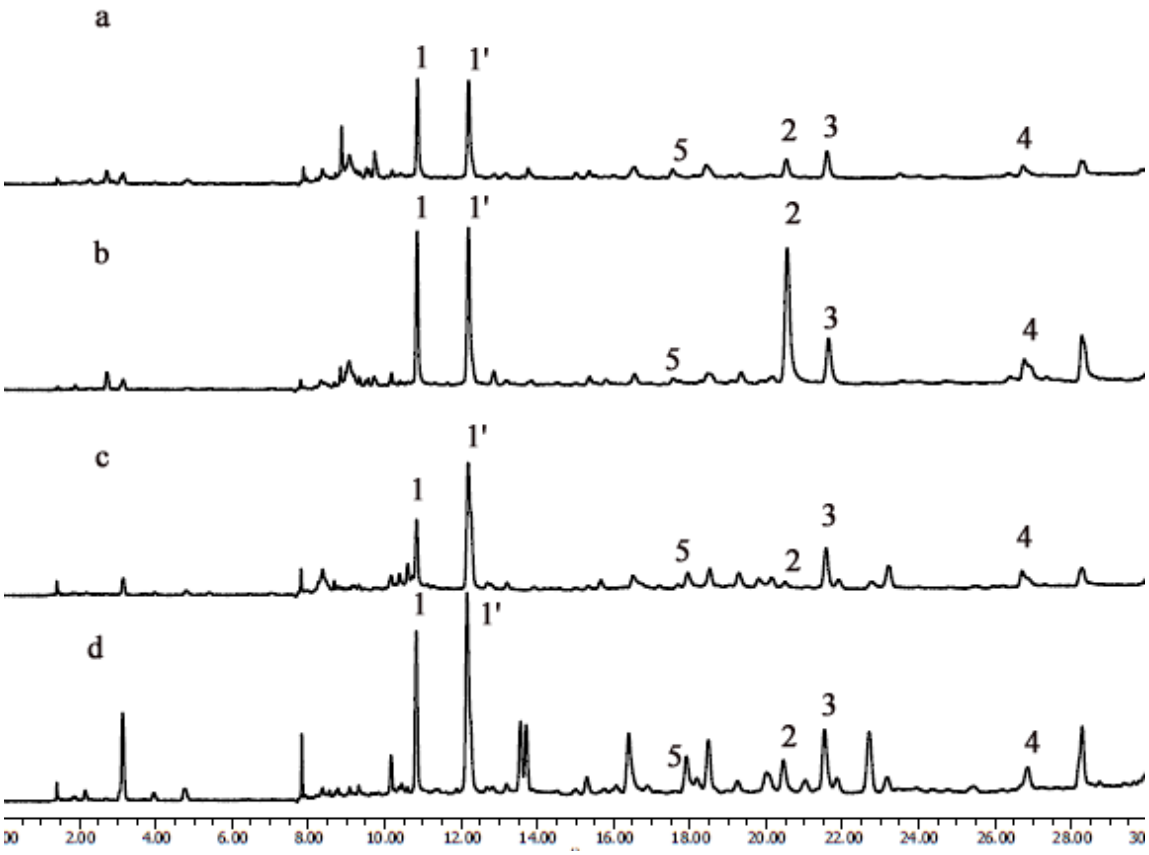


Fig.3.3.6 UPLC chromatograms of the petals extract of *Rosa maikwai* (a), *R. Rugosa* (b), Mei-gui cultivated at Pingying(c) and Mei-gui cultivated at Tarim basin (d) in 280nm. 1:Tellimagradinin I, 2:Tellimagradinin II, 3: Rugosin A, 4: Rugosin D, 5: Casuarictin

The contents of these hydrolysable tannins were shown in Table 3.3.7. Total contents of the hydrolysable tannins in the petal of Mei-gui were in the range of 14.0 - 67.9 mg/g. The total contents of hydrolysable tannins were high in *R. rugosa* collected in Japan and Korea except cultivated samples in Yeonggwang and were in the range of 51.6 - 101.8 mg/g. The total content of hydrolysable tannin in *R. maikwai* was 24.8 mg/g.

Table 3.3.7 Contents of hydrolysable tannins in the dried petals of *R. rugosa* and its related plants

Location		Code No.	Hydrolysable tannins(mg/g dry weight)					
			Tellimagrandin I	Tellimagrandin II	Rugosin A	Rugosin D	Casuarictin	Total
China	Keriya	CX1	25.4	3.4	3.7	3.1	0.4	36.0
	Houtan	CX2	39.6	2.7	6.3	2.9	0.1	51.6
	Niya	CX3	33.1	3.7	5.8	3.7	0.5	46.8
	Kashugar	CX4	14.5	2.4	0.6	1.3	2.1	20.9
	Kumuru	CX5	39.9	6.8	8.6	8.2	4.4	67.9
	Urumqi	CX7	39.6	11.8	8.9	2.9	0.7	63.9
	Pingying1	CS1	36.5	2.3	6.5	4.1	1.2	50.6
	Pingying2	CS3	8.3	-	1.2	3.8	0.7	14.0
	Beijing	CB1	19.0	3.1	3.1	5.9	2.0	33.1
Japan	Komuke	J1	41.7	28.9	6.4	4.0	1.6	82.6
	Kitami	J3	30.3	4.4	6.4	8.5	2.0	51.6
	Wakkani	J6	41.6	22.8	5.7	5.0	1.7	76.8
	Sado	J7	26.8	6.9	7.6	48.0	3.2	92.5
	Sapporo	M	15.0	3.2	2.7	2.2	1.7	24.8
Korea	Songsan	K2	41.6	35.3	6.7	16.8	1.4	101.8
	Yangyang	K3	21.1	18.7	1.9	9.1	0.8	51.6
	Samcheok	K4	33.1	22.8	2.6	12.1	1.0	71.6
	Yeonggwang	K5	14.1	9.6	1.1	2.5	0.8	28.1

The total contents in the cultivated samples in China and *R. maikwai* were lower than those of *R. rugosa* collected in Japan and Korea. This difference in the contents of hydrolysable tannins may be due to the difference in growing conditions and/or the difference in plant sources; the

samples in Japan and Korea were *R. rugosa* with single flowers, but those in China were Mei-gui with double flowers.

Among the five hydrolysable tannins, tellimagrandin I (8.3–41.7 mg/g) was the predominant component of hydrolysable tannins in the petal of *R. rugosa* and Mei-gui, and it was the major hydrolysable tannin in all the samples. The contents of tellimagrandin II (2.3–35.3 mg/g) were also major hydrolysable tannin next to tellimagrandin I in samples of *R. rugosa*. The rugosin D was abundant in some Japanese sample showing higher amounts of 48.0 mg/g dried petals, and it was also the major hydrolysable tannins in samples of *R. rugosa*. However, the contents of rugosin A and casuarictin were less than 10 mg/g in all the samples, and no large peak was observed in the chromatograms other than for these five hydrolysable tannins. As the amounts of constituents must depend on climate, growing conditions and other factors, our results demonstrated that the predominant hydrolysable tannins in the petal of *R. rugosa* are tellimagrandin I, tellimagrandin II and rugosin D. However, tellimagrandin I, tellimagrandin II and rugosin A were the predominant hydrolysable tannins in the petal of Mei-gui.

3.4 References

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Chapter 4 Morphological survey

4.1 *Rosa rugosa* collected in Japan

R. rugosa widely distributed in Hokkaido, Japan, and was designated as "the symbol flower of Hokkaido". It is naturally growing mainly in coast or beaches. We investigated the morphological characteristic of wild and cultivated *R. rugosa* in Hokkaido, Akita and Niigata of Japan in 2007 - 2009. The locations of investigation and collection places are shown in Fig. 4.1.

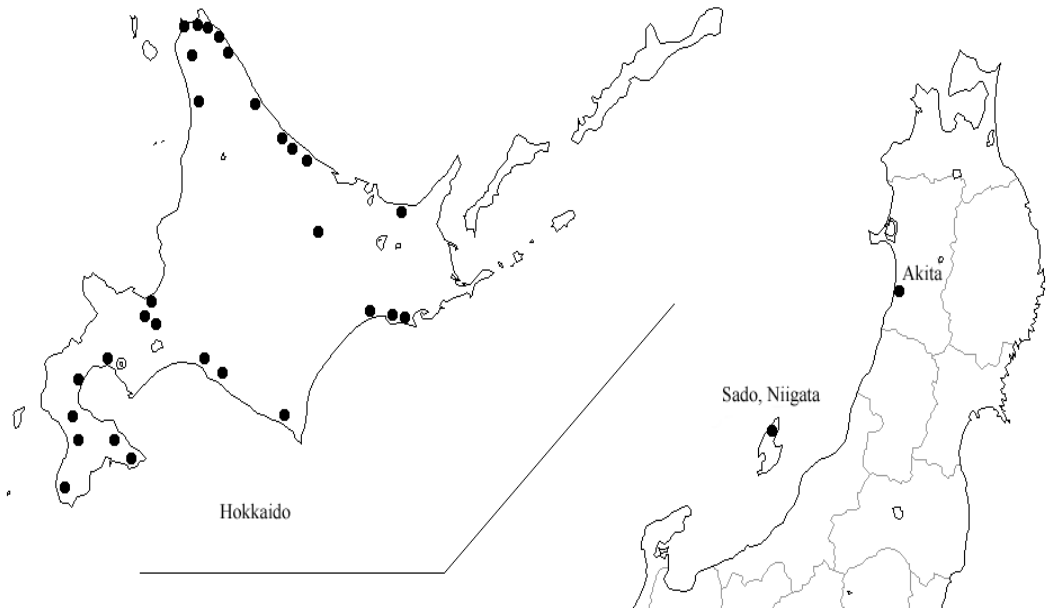


Fig.4.1. The investigation and collection places of *R. rugosa* in Hokkaido

Fig.4.2. *R. rugosa* collected in Hokkaido

As shown in Fig.4.2, *R. rugosa* growing in Hokkaido has following characteristics; a deciduous shrub, growing to 1 - 1.5 m tall. Stems are erect, and have dense prickles. Its branchlets are tomentose. Leaves are pinnate, compound with 5 - 9 leaflets, usually 7 and have a large stipule. There are 5 sepals, lanceolate, apex and slightly acuminate or slightly caudate. Stipules are mostly adnate to petiole and the free parts ovate and large. The style is not exserted to sepals and is free. Flowers are solitary or several and have a bracteole. Leaflets are ovate, deeply rugose and their length and width are about 2.2 - 5 cm × 1.2 - 2 cm. There are 5 petals, purplish-pink, or occasionally white, obovate and the apex is emarginated. Hip is depressed-globose, their length and width are about 1.5 - 2 cm × 2 - 2.5 cm, with a distinct neck below the sepals, red, and most often smooth. The flowering time is from June to September; the fruit

bearing time is from August to October and overlapped with flowering time.

4.2 *Rosa rugosa* collected in Korea

We investigated the wild and cultivated *R. rugosa* in Korea in 2007 - 2008. The locations of investigation and collection places are shown in Fig. 4.3.

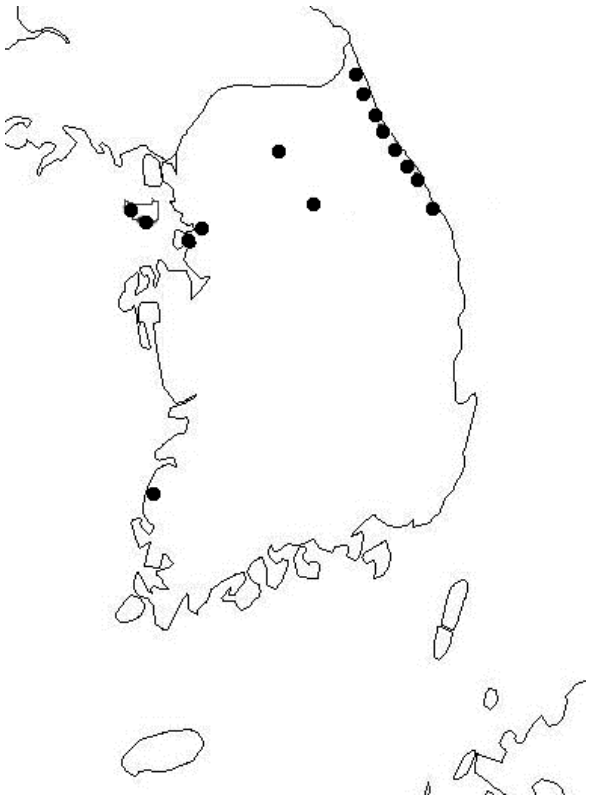


Fig.4.3. The investigation and collection places of *R. rugosa* in Korea

As shown in Fig.4.4, *R. rugosa* collected in Korea is a prickly shrub, growing to 1 - 2 m tall. Stems are dense prickly and bristly. Its

branchlets are tomentose. Leaves are pinnate, compound with 5 - 9 leaflets usually 7 and have a large stipule. The leaflets are elliptic and 2 - 5 cm long. Stipules most adnate to petioles and the petioles are bristly. Flowers are solitary and purplish-red. Petals are 5, purplish-pink, obovate and the apex is emarginated. Hip is depressed-globose, their length and width are about 1.5 - 2 cm × 2 - 2.5 cm.

The morphological characteristics of *R. rugosa* collected in Korea were the same to those of *R. rugosa* growing in Hokkaido, Japan.

Fig. 4.4 *R. rugosa* (Hae dang wha) collected in Korea

4.3 Mei-gui (玫瑰) cultivated in China

Mei-gui is cultivated all around China, the main cultivation areas are Shandong, Gansu, Xinjiang, Shanxi and Jiangsu Province, and Beijing in China.

We investigated Mei-gui collected in Xinjiang Province in May 2007, Pingying rose research institute in Shandong Province, and northeastern parts of China in May 2008. The locations of investigation and collection places are shown in Fig. 4.5.

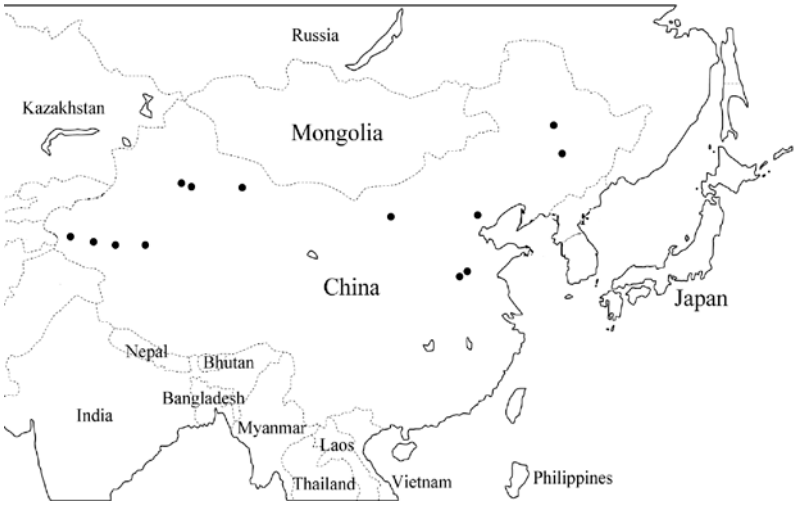


Fig. 4.5 The investigation and collection places of Mei-gui

4.3.1 Mei-gui cultivated in Xinjiang Province, China

In Xinjiang Province, the western-most part of China, Uygur people named Mei-gui “Kizil gul” in Uygur language and widely cultivated in all around Xinjiang Province.

We have mainly surveyed the cultivation places around of Tarim Basin; Kashigar (Ke-shen), Houtan (He-tian), Keriya (Yu-tian) and Niya (Min-feng), along with western parts of Xinjiang Province; Urumch

(Urumuqi) and Kumuru (Ha-mi) in 2006 - 2008. In Xinjiang, the area around Tarim basin (79°50'20"-79°56'40"E, 36°59'50"-37°14'23"N) is the major cultivation area of Mei-gui, include Houtan (Hetian), Niya(Yutian), Kariya(Minfeng), Luopu, Pishan and Cele prefecture. The Mei-gui collected in Kashigar, Houtan, Keriya and Niya have the same morphological characteristics (Fig.4.6).

Fig. 4.6 Mei-gui collected in Niya, Kariya and Houtan of Tarim basin

As shown in Fig.4.7, the plant is an erect shrub, growing to 2 m tall. Leaves are pinnate, compound with 3 - 5 leaflets and have a stipule. There are 5 sepals, ovate-lanceolate and stipitate glandular. Stipules are mostly adnate to petiole and the free part is ovate and thin. The style is not exserted to sepals and is free. Flower solitary or several and rarely have a bracteole. Leaflets are rugose, not shiny and their length and width are about 3.4 - 4.8 cm and 1.5 - 3.0 cm, respectively, and have acutely

serrate margin. There are 30 - 40 petals, double, dark pink, obovate and the apex is emarginated. Hip is depressed-globose and red. The flowering time is from end of April or the beginning of May to June. And in Urumqi (86°37'33" - 88°58'24"E, 42°45'32" - 44°08'00"N), Mei-gui blossoms in the end of May.

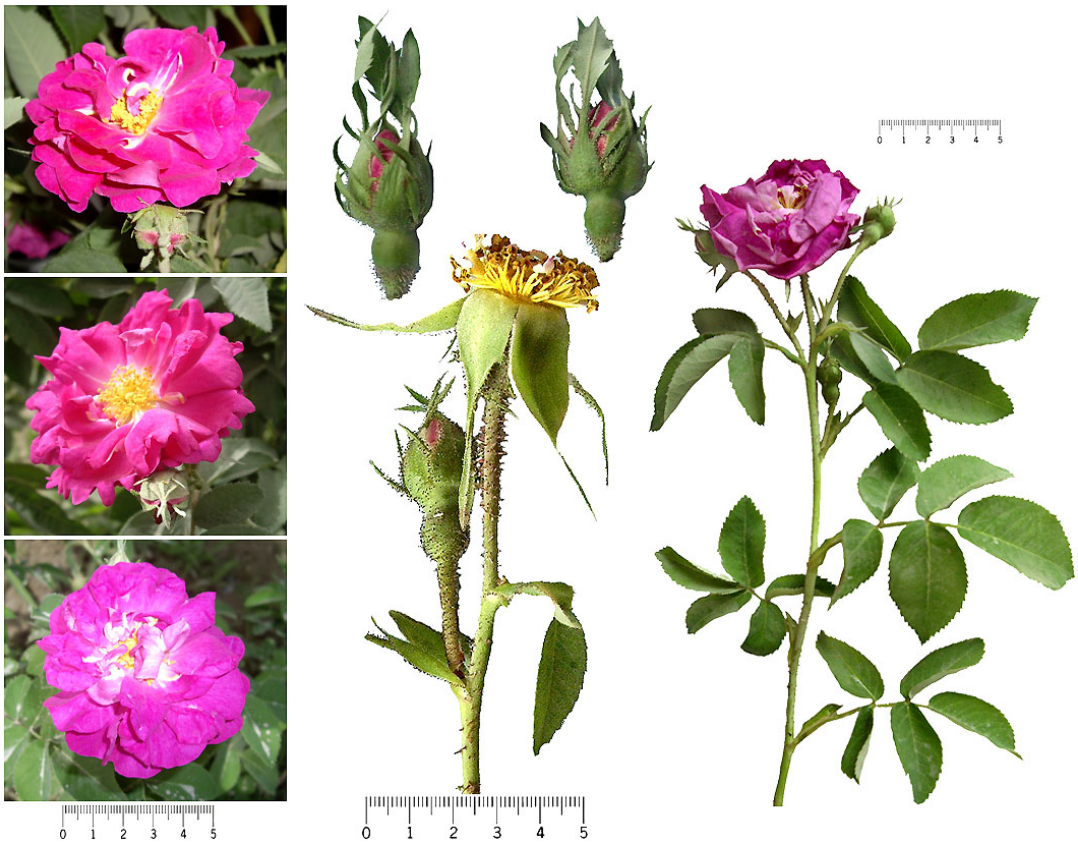


Fig. 4.7 The morphological characteristics of Mei-gui collected in Tarim Basin

4.3.2 Mei-gui cultivated in northern parts of China

Shandong, Gansu Province and Beijing are also main cultivation areas of Mei-gui in China. The Mei-gui, called as “traditional Mei-gui” is widely cultivated in Shangdong Province to produce many hybrid species

as a mother strain. Its hybrid also used as raw materials of herbal tea and drug, and the scientific name is assigned as *R.rugosa* cv. Plena.

The Mei-gui cultivated in Beijing is the same species as that cultivated in Shangdong Province, *R. rugosa* cv. Plena. However, the Mei-gui cultivated in Gansu Province was identified as the hybrid species of *R. rugosa* and *R. sertata* by prof. Yu dejun (Li Wanying et al. 1983) [1]. In this study, we investigated the Mei-gui cultivated in Shangdong Province.

There is two species of Mei-gui in Shangdong Province; one is with single flower and bearing many hips. Other is with double flower and few or no hips.

The morphological characteristics of former (Fig.4.8) are very similar to those of Japanese wild species, *R. rugosa*.

Fig. 4.8 Mei-gui with single flower cultivated in Shangdong Province

The morphological characteristics of the latter are different from Japanese wild species and Mei-gui cultivated in Xinjiang. The most Mei-gui cultivated in northern parts of China is showed similar morphological characteristics to those of the Mei-gui cultivated in Shangdong with double flower and few or no hips.

Fig. 4.9 Mei-gui with double flower cultivated in Shangdong Province

As shown in Fig.4.9, the Mei-gui is shrub erect, growing to 1.5 - 2 m tall. Prickle of stem has sparsely, scatter, and curve. Its branchlet is

subglabrous. Leaves are pinnate, compound with leaflets 5 - 9 and have a stipule. Sepal is 5, lanceolate, apex slightly acuminate or slightly and caudate. Stipules mostly adnate to petiole and free part ovate and thin. Style is not exserted to sepals and free. Flower solitary or several and have a racteole. Leaflets length and width are about 2.2 - 4 cm and 0.8 – 1.9 cm, ovate, deeply rugose. Petals are 13 - 40, double, pink, obovate and apex is emarginated.

4.4 *Rosa maikwai* cultivated in Japan

Cultivated *R. maikwai* was obtained from Medicinal plants Garden of Hokkaido University.

Fig. 4.10 *R. maikwai* cultivated in Hokkaido, Japan

As shown in Fig.4.10, the plants are shrub erect, to 1 – 3 m tall. Prickle of stem has sparsely, scatter, curve and branchlet is subglabrous. Leaves are pinnate and compound with leaflets 5 - 9 and have a stipule. Sepal is 5, lanceolate, apex slightly acuminate or slightly and caudate. Stipules mostly adnate to petiole and free part ovate and thin. Style is not exserted to sepals and free. Flower solitary or several and have a racteole. Leaflets length and width are about 1.8 - 3.3 cm and 0.9 - 1.3 cm, ovate, deeply rugose. Petals are 30 - 49, double, pink, obovate and apex is emarginated. Petiole is reddish.

4.5 Comparison of morphological characteristics between *Rosa rugosa* and Mei-gui

Through our spot-investigation, the Mei-gui cultivated in China, *R. rugosa* collected in Hokkaido, Japan and Korea and *R. maikwai* had common characteristics of deciduous shrub, leaves are compound and pinnate, sepals are lanceolate and tomentose or glabrous, stipule are mostly adnate to petiole, and style are free and slightly exserted. However, morphological characteristics of plant tall stem, leave and flower were different each other as shown in Table 4.1and Fig. 4.11.

According to these differences in morphological characteristics, the samples were classified into four types (Type 1 - 4). Type 1 was composed of Mei-gui cultivated around Tarim Basin in Xinjiang Province in China, Type 2 of Mei-gui cultivated in other places of Xinjiang Province and northeastern parts of China, Type 3 of *R. rugosa* collected in Japan and

Korea and Mei-gui cultivated in Shangdong Province in China with single flower. Type 4 was *R. maikwai*.

The plants of Type 1 had a solitary flower and rarely have a bract. However, those of Type 2 - 4 have a solitary or several flowers, and have a bract. The plants of Type 1 have 3 - 5 leaflets (usually 5), while those of Type 2 - 4 have 5 - 9 leaflets (usually 7). The stems of the plants in Type 1 had a few small prickles, while those of Type 3 have dense prickles. The stipules of the plants of Type 1 were mostly adnate to petiole but thinner than those of Type 3.

Morphological characteristics of the plants in Type 2 resembled those of Type 4 (*R. maikwai*); double flower, curved and scattered prickles, shape of bract and sepal. However, there were differences in the color of petiole, and the shape and degree of rugose in leaflet. The morphological characteristics of cultivated Mei-gui with single flower in Shandong Province were classified into the same type of *R. rugosa* collected in Japan and Korea, and Japanese cultivated samples with double flower were classified into Type 3.

These morphological characteristics of Type 2 and 4 were closely similar to those of *R. rugosa* (Type 3). However, those of Type 1 were different form *R. rugosa* and were similar to those of *R. gallica* according to nomenclature lists (Fig. 4.12) which is classified sections of genus *Rosa* by morphological characteristics [2].

Table 4.1. Morphological characteristics of Mei-gui, *Rosa rugosa* and *Rosa maikwai*.

	Type 1	Type 2	Type 3	Type 4
Code no.	CX1-4	CX5-9, CS1-2,CI, CB1, CH, CJ	CS3-4, J1-5, K1-5	M
Plant tall	1.5 - 2 m	1.5 - 2 m	1 - 1.5 m	1 - 3 m
Stem Prickle	sparsely, scatter , strongly, curve	sparsely, scatter , curve	dense, bristly, erect	sparsely, scatter , curve
Branchlet	subglabrous	subglabrous	tomentose	subglabrous
Leaflets	3 - 5; ovate, slightly rugose, 2.8 - 5.7 cm × 1.6 - 3.6 cm	5 - 9; ovate, deeply rugose, 2.2 - 4 cm × 0.8 - 1.9 cm	5 - 9; ovate, deeply rugose, 2.2 - 5 cm × 1.2 - 2 cm	5 - 9; elliptic, slightly rugose, 1.8 - 3.3 cm × 0.9 - 1.3 cm
Stipule	thin	thin	large	thin
petiole	green	light green	light green	reddish
Flower	solitary	solitary or several	solitary or several	solitary or several
Petal	25 - 40, double	13 - 40, semidouble or double	5, single (wild) or double (cultivated)	30 - 49, double
Bract	Absent or rarely	bracteate, ovate	bracteate, ovate	bracteate, ovate
Sepal	twisted ovate-lanceolate, pinnate tear and apex caudate	lanceolate apex slightly acuminate or slightly caudate	lanceolate apex slightly acuminate or slightly caudate	Lanceolate apex acuminate or slightly caudate

Fig. 4.11. Photographs of Mei-gui, *Rosa rugosa* and *Rosa maikwai*

1: *Rosa rugosa* (J1, Hokkaido, Japan), 2: Mei-gui (CS1, Pingying rose research institute, Shandong, China), 3: Mei-gui (CX2, Houtan, Xinjiang, China), 4: Mei-gui (CS3, Pingying rose research institute, Shandong, China), 5: *Rosa Maikwai* (M, Medicinal plants garden, Hokkaido University, Japan)

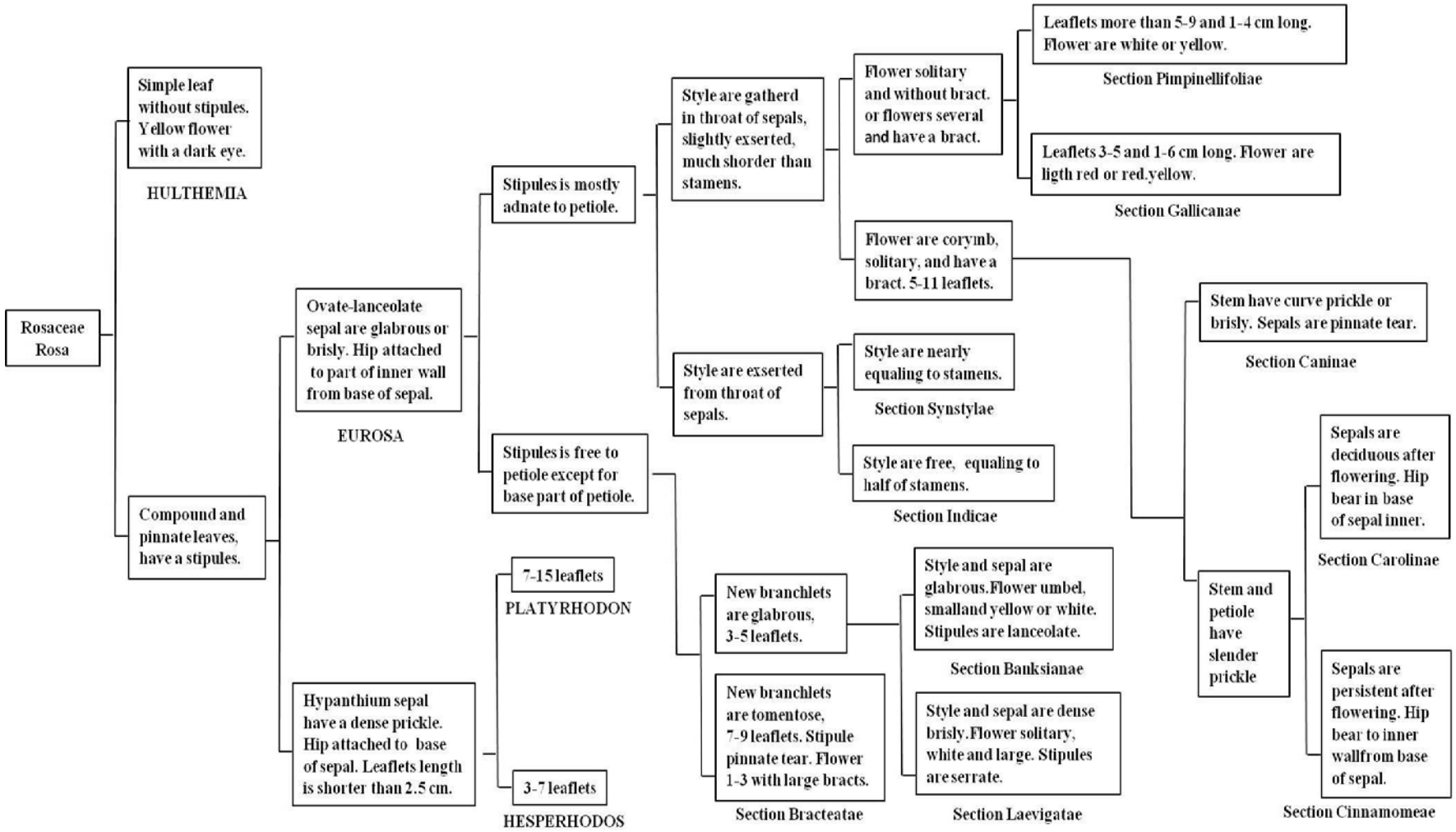


Fig. 4.12 Nomenclature list of morpholpgical characteristic of genus *Rosa*

4.6 References

[1]李万英,王文中,我国玫瑰资源初探, 园艺学, 1983,8,10(3):211-215

[2]Seizo Suzuki (1996) The world`s best roses. Shogakukan,Tokyo, pp 16-17.

[3] Gerd Krussmann (1982) Roses. B T Batsford Ltd., London, pp 73-74, 255-256

Chapter 5 Phylogenetic analysis

5.1 Plant materials

The samples of Mei-gui were collected in Xinjiang Province in May 2007, Pingying rose research institute in Shandong Province, and northeastern parts of China in May 2008. Wild and cultivated *R. rugosa* were collected in Hokkaido, Japan and Korea in 2007 - 2008. *R. maikwai* was obtained from Medicinal Plants Garden of Hokkaido University. Information of these samples is shown in Table 5.1, and the location of collection places are shown in Fig. 5.1.

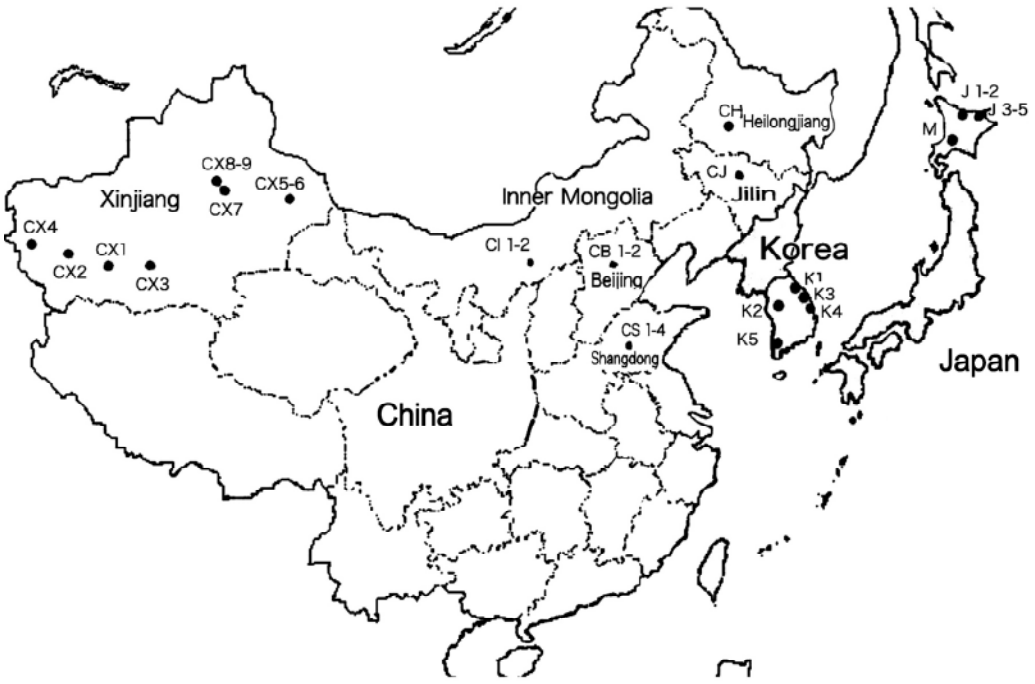


Fig. 5.1 Collection places of Mei-gui, *R. rugosa* and *R. maikwai*

Table 5.1 Information of samples

No.	Species / Used name	Cultivated or collected place	Notes*
CX1	Mei-gui（Kizil gul）	Keriya (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX2	Mei-gui（Kizil gul）	Houtan (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX3	Mei-gui（Kizil gul）	Niya (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX4	Mei-gui（Kizil gul））	Kashugar (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX5	Mei-gui（Kizil gul）	Hami, Xinjiang Prov., China	
CX6	Mei-gui（Kizil gul）		Cultivated, DF
CX7	Mei-gui（Kizil gul）		
CX8	Mei-gui（Kizil gul）	Urumqi, Xinjiang Prov., China	Cultivated, DF
CX9	Mei-gui（Kizil gul）		
CS1	Mei-gui		
CS2	Mei-gui	Pingying rose research institute,	Cultivated, DF
CS3	Mei-gui	Shandong Prov., China	
CS4	Mei-gui		Cultivated, SF
CI1	Mei-gui	Huhhot, Inner Mongolia, China	Cultivated,DF
CI2	Mei-gui		
CB1	Mei-gui	Beijing botanical garden, Beijing,China	Cultivated, DF
CB2	Mei-gui		Cultivated, SF
CH	Mei-gui	Daqing, Heilongjiang Prov. China	Cultivated, DF
CJ	Mei-gui	Zuojia agriculture research institute, Jilin Prov., China	Cultivated, DF
M	*Rosa maikwai* Hara.	Hokkaido Univ. Medicinal plants garden, Japan	Cultivated, DF
K1	*Rosa rugosa* Thunb.	Hwajipo Gangwong-do, Korea	
K2	*Rosa rugosa* Thunb.	Hwasong Songsan, Gyeonggi-do,Korea	
K3	*Rosa rugosa* Thunb.	Yangyang, Gangwon-do, Korea	Wild, SF
K4	*Rosa rugosa* Thunb.	Samcheok Meangbang, Gangwon-do, Korea	
K5	*Rosa rugosa* Thunb.	Yeong Gwang, Jeollanam-do, Korea	Cultivated, SF
J1	*Rosa rugosa* Thunb.	Monbetsu, Hokkaido, Japan	
J2	*Rosa rugosa* Thunb.	Ishikari, Hokkaido, Japan	Wild, SF
J3	*Rosa rugosa cv. Plena.*		
J4	*Rosa rugosa cv. Plena.*	Kitami, Hokkaido, Japan	Cultivated, DF
J5	*Rosa rugosa cv. Plena.*		

*DF: Double flower; SF: single flower.

5.2 Experimental

5.2.1 Total DNA extraction

The fresh young leaves of the plants were frozen and stored at -80 ºC for genetic analysis. Total DNA was extracted from young leaf tissue (100mg) by modified CTAB method (DNeasy Plant Mini Kit, Qiagen, Germany) [1].

Then, the extracted total DNA was diluted 100 times by TE solution (10 mM tris-hydrochloric acid buffer and 1 mM EDTA) and the concentration was determined by Bio UV (Biochrom Ltd., Japan). The extracted total DNA which its radio (Absorption 260 nm /Absorption 280 nm) was range from 1.6 - 1.8, was used in PCR amplification.

5.2.2 PCR amplification and sequencing

Chloroplast *trn*H - *psb*A, *trn*L(intron) and *trn*L(3' exon) - *trn*F were amplified by polymerase chain reaction (PCR). Primers for amplifying and sequencing three intergenetic spacers were shown in Table 5.2[2].

PCR amplification was performed in 50μl reaction solutions containing 34.75μl of sterilized distill water, 5 μl of 10 × Ex *Taq* polymerase reaction buffer, 4 μl of 2.5 mM dNTP mixture, 3 μl of 25 mM $MgCl_{2,}$ 0.25 μl of *rTaq* DNA polymerase, 0.5 μl of 20 μM each primer (Takara Bio Inc, Japan) and 2 μl of template DNA (10 ng/μl) [3]. The solution was first denatured for 5 min at 95°C, followed by 30 cycles of amplification which

consisted of denaturation at 95°C for 1 min, annealing of primers at 50°C for 1 min, and polymerization at 72°C for 1.5 min by a Gene Amp, PCR system 9700 (Applied Biosystems).

Table 5.2 Primer designed to amplify and sequence three intergenetic spacers in genus *Rosa*

Primers		5' Sequences 3'	Posotion
*psb*A - trnH			
Forward	psbA-F	GTTATGCATGAACGTAATGTCTC	608 - 587
Reverse	trnH-R	CGCGCATGGTGGATTCACAAATC	28 - 49
*trn*L (intron)			
Forward	trnL-intronF	CGAAATCGGTAGACGCTACG	49306 - 49325
Reverse	trnL-intronR	GGGGATAGAGGGACTTGAAC	49863 - 49882
*trn*L (3 exon) - *trn*F			
Forward	trnL-3exonF	GGTTCAAGTCCCTCTATCCC	49862 - 49881
Reverse	trnF-R	ATTTGAACTGGTGACACGAG	50270 -50299

*Positions of primers correspond to nucleotide positions of cp DNA of tabaco.

After amplification, DNA sequences were aligned manually by sequential pairwise comparison with the Clustal W program (data is shown in Table 3.3). Boundaries of the intergenetic region were determined by BLAST analysis. Phylogenetic analyses were conducted by MEGA4. All positions containing gaps and missing data were eliminated from the dataset. The evolutionary history was inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Fig.5.2).

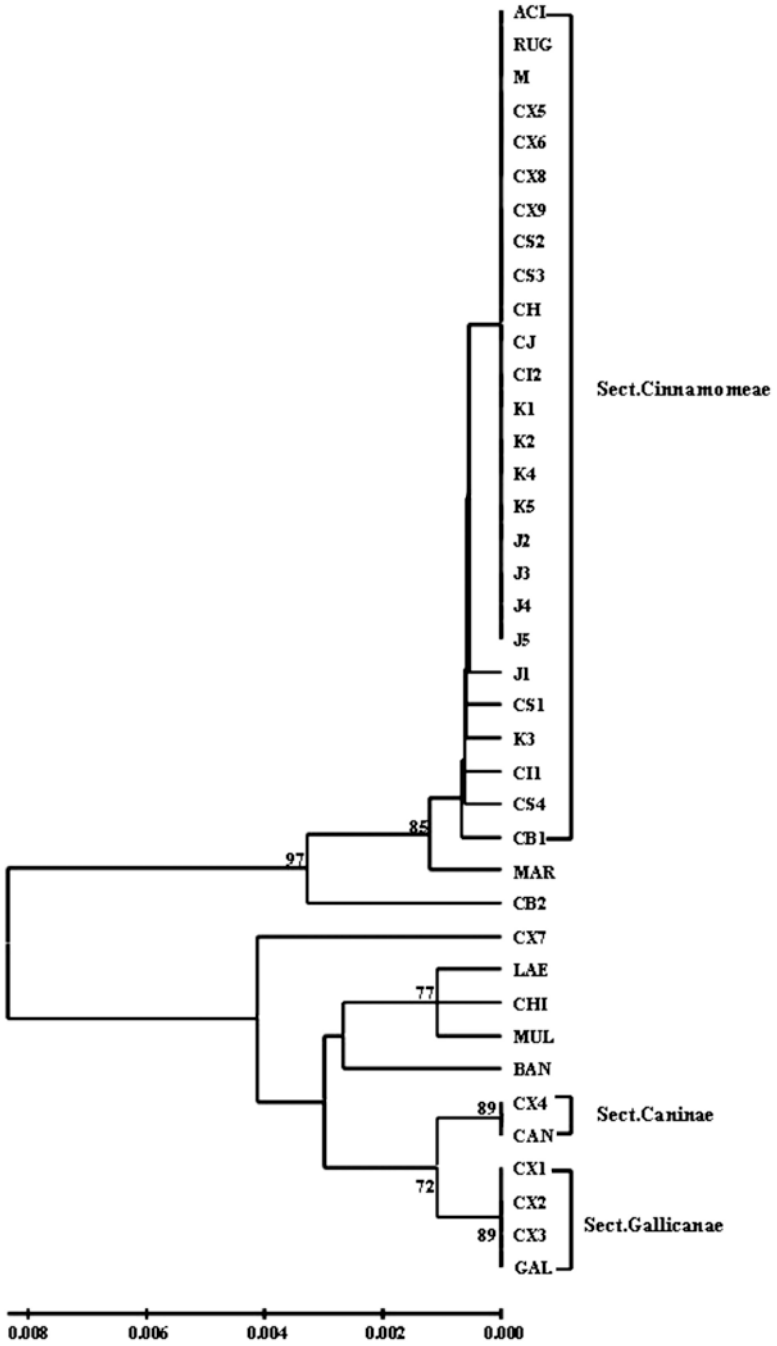


Fig.5.2 Phylogenetic analysis of the genus *Rosa* using intergentic spacers in choroplast DNA

Numbers below branches indicate bootstrap values (>50%).

ACI: *Rosa acicularis* var. *nipponensis*, RUG: *Rosa rugosa*, MAR: *Rosa marretii*, LAE: *Rosa laevigata*, CHI: *Rosa chinensis* var. *spontanea*, MUL: *Rosa multiflora*, BAN*: Rosa banksiae* var. *normalis*, CAN: *Rosa canina*, GAL: *Rosa gallica*

5.3 Results

A total of 946 nucleotides of three intergenetic spacers in chloroplast DNA of the samples, *trn*H - *psb*A, *trn*L (intron) and *trn*L (3' exon) - *trn*F were compared with those of 9 species of genus *Rosa* in DDBJ (data shown is Table 3.3- 3.6), and three clades (Clade 1 - 3) were observed as shown is Fig. 5.2.

Clade 1 was composed of three samples of cultivated Mei-gui around Tarim Basin of Xinjiang Province in China (CX1 - 3) and *R. gallica*, and another sample (CX4) was in the Clade 2 with *R. canina*. Clade 3 was composed of 13 samples of Mei-gui cultivated in China including Xinjiang Province, cultivated and wild samples of Japan and Korea and *R. maikwai* along with *R. acicularis* var. *nipponica*, and *R. rugosa*. Two samples of Mei-gui cultivated in Beijing (CB2) and Urumqi (CX7) were shown to be no relation with those three clades mentioned above.

5.4 Discussion

These assumptions were supported by phylogenetic analysis. Three samples (CX1 - 3) cultivated around Tarim Basin were in the clade of *R. gallica* (sect. *Gallicanae*), but one (CX4) was in the clade of *R. canina* (sect. *Caninae*). The morphological characteristics of CX4 did not correspond to those of *R. canina* since its flowers were solitary and rarely have a bract. The chloroplast DNA is known to be inherited from its

mother strain. Therefore, CX1 - 3 was *R. gallica* or closely related species as the mother strain(s), and CX4 was *R. canina* or closely related species.

Twenty-three samples of Mei-gui were in the same clade of *R. rugosa* and *R. acicular*is (sect. *Cinnamomeae*). These Mei-gui had the same mother strain with *R. rugosa* and/or *R. acicular*is. However, two samples of Mei-gui cultivated in Beijing and Urumqi were phylogenetically isolated from other samples, and these Mei-gui had mother strain of other species in genus *Rosa*.

5.5 References

[1] Doyle, J. J. and Doyle J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19: 11-15

[2] Takeuchi S, Nomura K, Uchiyama H, Yoneda K (2000) Phylogenetic relationship in the genus *Rosa* based on the restriction enzyme analysis of the chloroplast DNA. J Japan Soc Hort Sci 69(5):598–604

[3] Wu S, Ueda Y, Nishihara S, Matsumoto S (2001) Phylogenetic analysis of Japanese *Rosa* species using DNA sequences of nuclear ribosomal internal transcribed spacers (ITS). Journal of horticultural science and biotechnology 76(2):127–132

Table 5.3 The DNA sequences in the intergenetic spacers in chloroplast of some samples

	trnL(3exon) - trnF										trnL (intron)			psb A-trnH																
	9	52	93	188	204	272	278	295	299	352	173	305	308	7	25	40	62	77	81	110	165	174	177	205	248	251	263	297	304	308	312
R. rugosa	A	G	T	C	C	A	A	C	T	T	A	G	A	G	T	A	T	A	A	A	A	G	T	C	T	T	T	T	T	T	T
R. acicularris	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
R. marretti	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
R. cannina	·	·	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	·	·	·	A	·	·	C	·	·	·	G
R. gallica	·	·	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	T	C	·	A	·	·	C	·	·	·	G
R. bancsiae	G	·	G	A	C	·	C	·	G	A	G	T	C	A	·	·	A	·	G	T	·	·	·	A	·	·	·	·	·	·	·
R. multiflora	·	T	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	·	·	·	A	·	·	·	C	·	·	·
R. laevigata	·	T	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	·	·	·	A	·	·	·	·	C	·	·
R. chinensis	·	T	G	A	T	·	C	T	G	A	G	T	C	·	·	·	A	·	G	T	·	·	·	A	·	·	·	·	·	·	·
CX7	·	·	·	·	·	·	·	·	·	A	G	T	C	·	·	·	A	·	G	T	T	C	·	A	·	·	C	·	·	·	G
CB2	·	·	·	A	·	C	C	·	·	·	·	T	C	·	·	·	·	·	·	·	·	·	C		·	·	·	·	·	·	·
CX1	·	·	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	T	C	·	A	·	·	C	·	·	·	G
CX2	·	·	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	T	C	·	A	·	·	C	·	·	·	G
CX3	·	·	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	T	C	·	A	·	·	C	·	·	·	G
CX4	·	·	G	A	T	·	C	·	G	A	G	T	C	·	·	·	A	·	G	T	·	·	·	A	·	·	C	·	·	·	G
J1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CI1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·
K2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·
CB1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·
CS1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·

Table 5.4 DNA sequence in the chloroplast *trn*H-*psb*A

Table 5.5 DNA suquence in the chloroplast *trn*L(3 exon)-*trn*F

Table 5.6 DNA suquence in the chloroplast *trn*L(intron)

Chapter 6 Phytochemical studies

6.1 Isolation of flavonol glycosides from *R. rugosa*

6.1.1 General procedures

^{1}H- and ^{13}C-NMR spectra were measured with a JEOL α-500 spectrometer in CD_3OD at 30ºC. Chemical shifts were determined by using CD_3OD (δ_H: 3.30 ppm, δ_C: 49.0 ppm) as the internal reference. High-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESI-TOF-MS) spectra were recorded on a JEOL JMS-T100LC spectrometer. GC-MS system was consisting of a GC-17A Gas chromatograph and a QP-5000 mass spectrometer (Shimadzu, Japan). The system was controlled by a Class 5000. Helium was used as carrier gas and the applied column was InertCap5 (0.25 mm i.d. × 30 m, GL Sciences Inc., Japan). Optical rotation was measured on a Jasco P-2200 polarimeter. UV/Vis spectrum was measured Ultrospec 3300 pro. (Amersham Pharmacia Biotech.,Japan). Sephadex LH-20 (GE Healthcare, Sweden) and Wakosil 40 C_{18} (Wako Pure Chemical Industries, Ltd., Japan) were used for open column chromatography. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

6.2.2 Extraction and isolation

Air-dried and pulverized petals of *R. rugosa* (100 g) collected at Monbetsu in Hokkaido were extracted with 50% aqueous ethanol (1 l) three times. The extract was evaporated to remove ethanol, and the aqueous solution was lyophilized to obtain the extract (50.7 g). The extract was used for the isolation of flavonal glycoside, and the isolation scheme was shown in Fig 6.1.1.

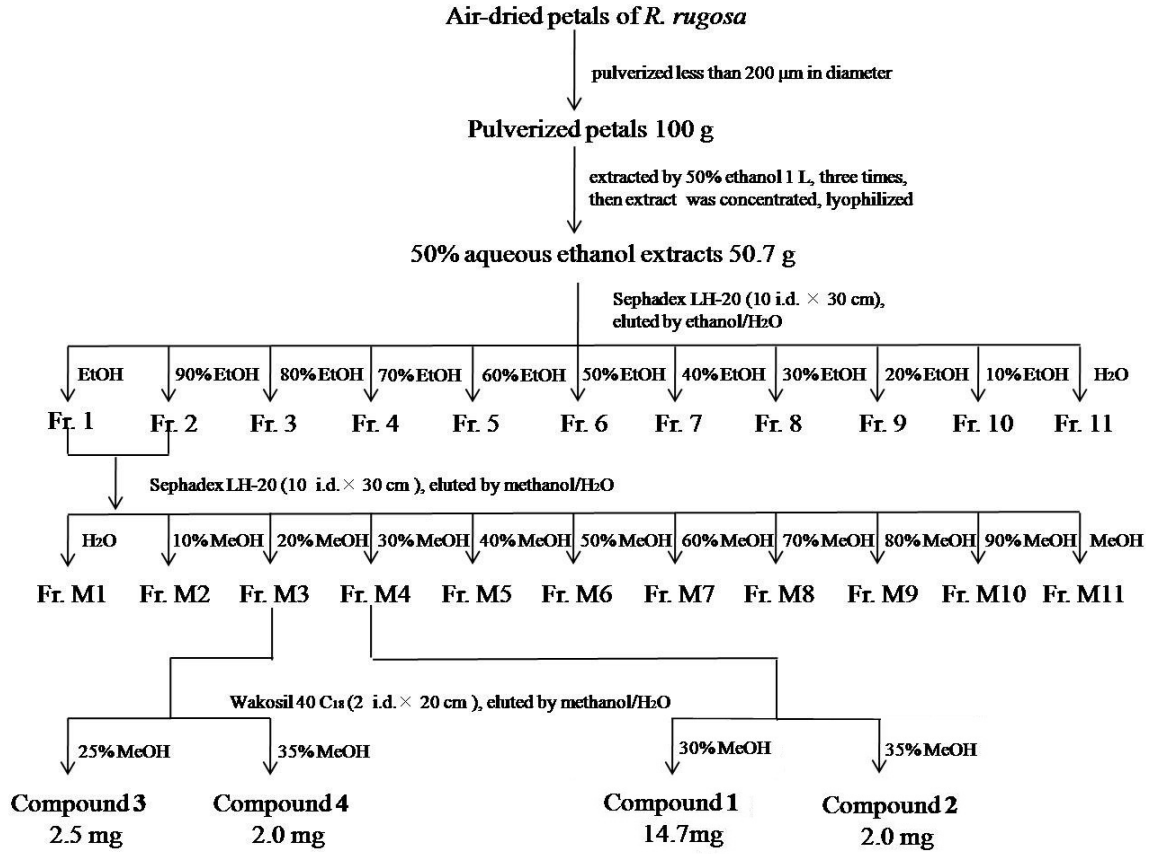


Fig. 6.1.1 Isolation scheme of compound **1** – **4** from an extract of *R. rugosa* petals

The extract applied to a column of Sephadex LH-20 (10 i.d. × 30 cm). The column was eluted with a solvent system comprising ethanol/H_2O to obtain fractions (Fr. 1-11). Fractions 1-2 were containing flavonol glycosides. The fractions (21.3 g) obtained from elute with

100% - 90% ethanol were combined, and were applied to a column of Sephadex LH-20 (10 i.d. × 30 cm), and the column was eluted with a solvent system of methanol/H_2O. The fractions containing flavonol glycoside were further purified by reversed-phase chromatography using Wakosil 40 C_{18} (2 i.d. × 20 cm) and elution were a solvent system of methanol/H_2O. Compound **3** (2.5 mg) were isolated from the fraction eluted with Methanol/H_2O (25:75), compound **1** (14.7 mg) from that with 30:70, compound **2** (2.0 mg) from that with 35:65, compound **4** (2.0 mg) from that with 35:65.

6.2.3 Characterization of structures

The structures of compounds **1** - **4** were characterized by their physical and ^{1}H- and ^{13}C-NMR, ESI-TOF-Mass spectra and GC-MS [1-9].

Compound **1** was obtained as a brown powder with a negative specific rotation $[\alpha]_D^{21}$ –38.7° (c 0.25 in methanol). Its molecular formula was determined as $C_{26}H_{28}O_{16}$ by HR-ESI-TOF-MS (Reserpine, $C_{33}H_{40}N_2O_9$ was used as internal standard), while a quasi-molecular ion peak at *m/z* 619.27 $[M+Na]^+$ was detected in the positive-ion APCI-MS. The UV spectrum showed characteristic flavonol absorption at 255.6 nm and 352.5 nm. The ^{1}H-NMR spectrum of compound **1** (Fig. 6.1.2) showed the presence of a quercetin skeleton , such as ring A: δ_H 6.38 and 6.19 (each 1H, d, *J* = 1.5 Hz, H-6,8, respectively); ring B: δ_H 6.89 (1H, d, *J* = 8.3Hz, H-5′), 7.50 (1H, dd, *J* = 2.0, 8.3 Hz, H-6′) and 7.59 (1H, d, *J* = 2.0 Hz,

H-2′) and the spectrum shown as Fig. 6.1.2.a.

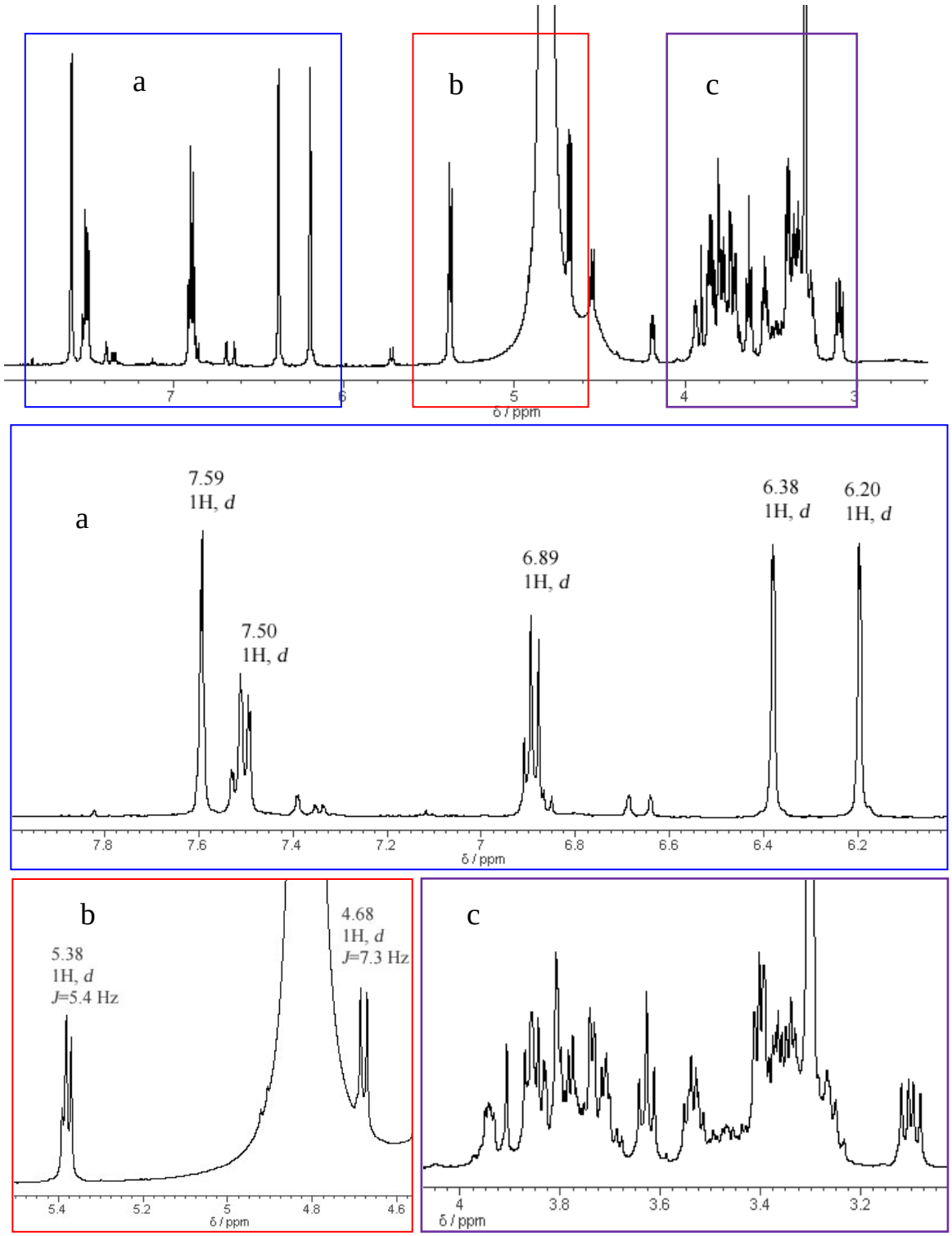


Fig. 6.1.2 ^{1}H-NMR spectrum of compound **1**

The coupling constants of the anomeric protons δ_H 5.38 (1H, d, J = 5.4

Hz, H-1") and δ_H 4.68 (1H, d, *J* = 7.3 Hz, H-1‴) on **1**(Fig. 6.1.2.b), the configuration at C-1″ and C-1‴ of the sugar units was determined to be the β-configurations. While the sugar moieties' protons were confirmed by COSY spectrum (Fig. 6.1.3).

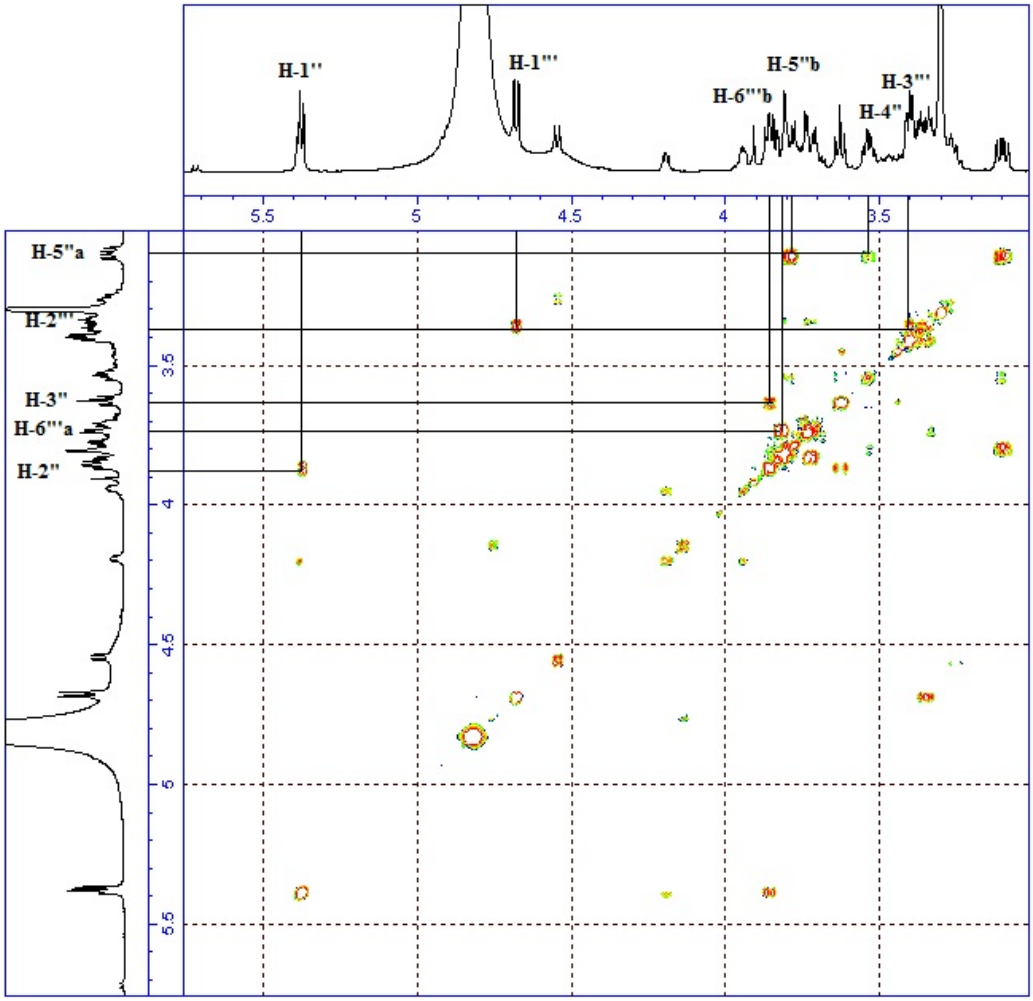


Fig. 6.1.3 COSY spectrum of sugar moieties of compound 1 in CD_3OD at 30 ºC

In the ^{13}C-NMR spectrum (Fig. 6.1.4), 26 carbon signals were observed, and 11 signals were confirmed to the sugar carbons. The signals of C-6, C-8, C-2′, C-5′ and C-6′ of aglycone were determined by HSQC spectrums (Fig. 6.1.5).

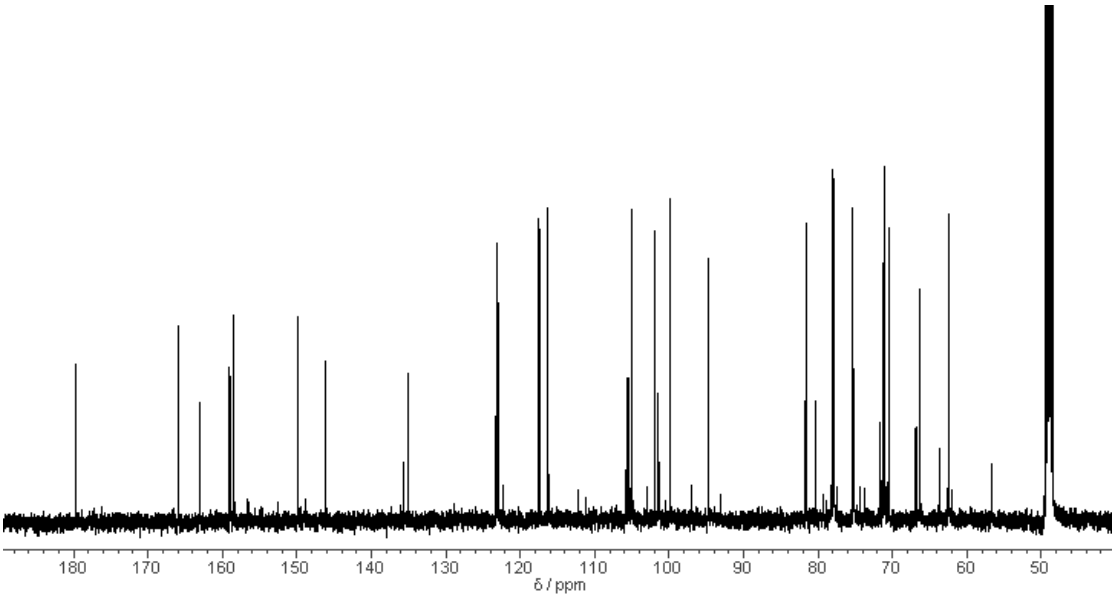


Fig. 6.1.4 ^{1}C-NMR spectrum of compound **1**

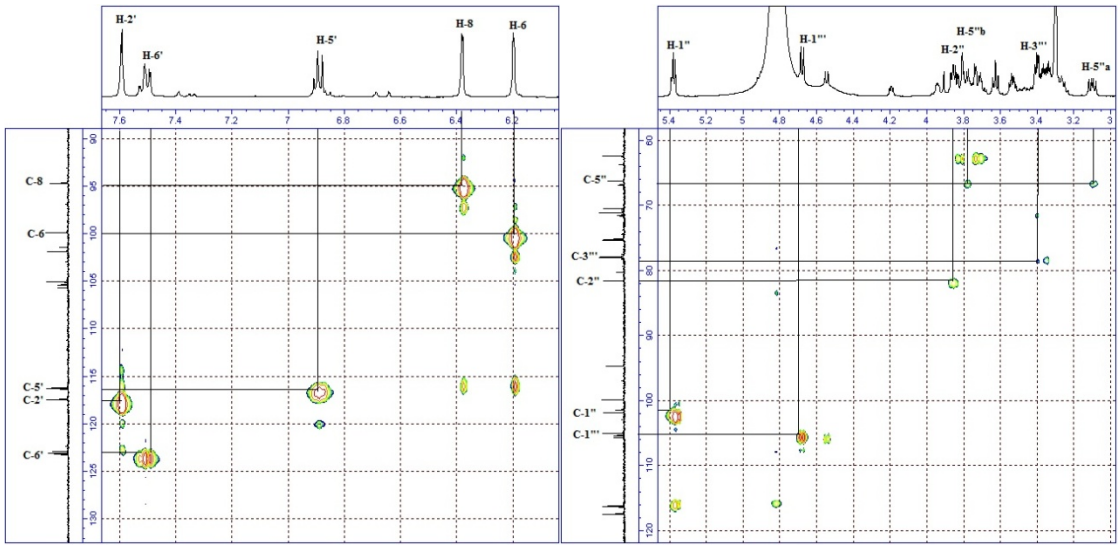


Fig. 6.1.5 HSQC spectrum of aglycone moiety and sugar moieties of compound 1 in CD_3OD at 30 ºC

In the HMBC experiment, the H-1″ signal of a pentose at δ_H 5.38 correlated with the C-3 resonance of aglycone at δ_C 135.1, suggesting that the sugar unit was located at the C-3 position. And the glucosyl H-1‴ signal at δ_H 4.68 correlated with the C-2″ resonance of xylosyl at δ_C 81.7, suggesting that the a hexose unit was connected in the C-2″ of a pentose

(Fig.6.1.6).

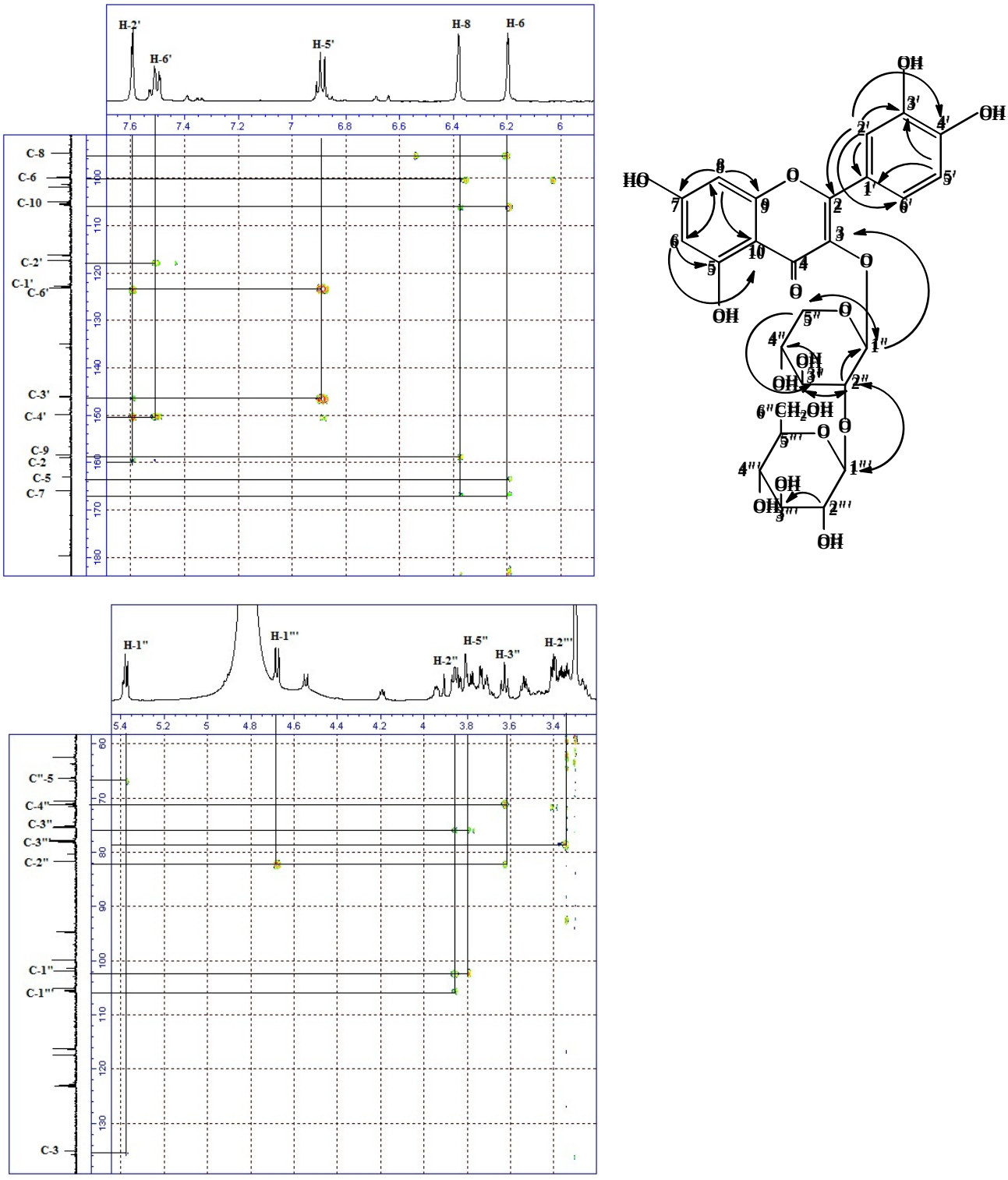


Fig. 6.1.6 HMBC spectrum of aglycone and sugar moiety of compound 1 in CD_3OD at 30 ºC

In order to characterize sugar units, acid hydrolysis of compound 1 was determined by GC-MS analysis following conversion to the trimethylsilylthiazolidine derivatives. The analysis of the coupling constants of sugar protons, the presence of xylose and glucose were implied. Therefore, D-xylose and D-glucose were used for standards. The acid hydrolysate of compound **1**, in addition to the aglycone, revealed the characteristic peaks of xylose; retention time were 7.687, 7.891, 8.270, 8.845 min, and glucose; 10.181, 10.341, 10.891, 11.169 min. These results were further confirmed that sugar units of compound **1** were D-glucose and D-xylose.

From these data, the structure of compound 1 was determined to be quercetin 3-*O*-(2″-*O*-*β*-D-glucosyl)-*β*-D-xyloside: brown powder. UV λmax (MeOH) nm (log ε): 257 (3.87), 354 (3.70). APCI-MS m/z: 619.27 $[M+Na]^+$ APCI-MS m/z: 619.27 $[M+Na]^+$. HR-ESI-MS *m/z*: 619.1254 $[M+Na]^+$ (calcd for $C_{26}H_{28}O_{16}Na$: 619.1275). $[\alpha]_D^{21}$ –38.7° (c 0.25, MeOH). The ^1H- and ^{13}C –NMR was showed in Table 6.1.1.

Table 6.1.1 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of Compound **1**

	quercetin 3- O-(2"-O-glucosyl)-D-β-xyloside(**1**)	
No.	δ_H, mult, *J*(Hz)	δc
Aglycone		
2		159.0
3		135.1
4		179.7
5		163.1
6	6.20, *d*, 1.5	99.9
7		166.0
8	6.38, *d*, 1.5	94.7
9		158.4
10		105.7
1′		122.9
2′	7.59, *d*, 2.0	117.5
3′		146.1
4′		149.9
5′	6.89, *d*, 8.3	116.4
6′	7.50, *d*, 8.3	123.3
Xylosyl		
1″	5.38, *d*, 5.4	101.9
2″	3.86, *dd*, 5.4, 7.8	81.7
3″	3.63, *t*,7.8	75.4
4″	3.53, *dd*,7.8, 4.3	70.5
5″	3.10, *dd*, 7.8, 12.2	66.3
	3.79, *dd*, 4.3, 12.2	
Glucosyl		
1‴	4.68, *d*, 7.3	105.1
2‴	3.35, *t*, 8.3	75.3
3‴	3.40, *m*	78.1
4‴	3.37, *m*	71.2
5‴	3.28, *m*	77.9
6‴	3.72, *dd*, 4.3, 11.7	62.4
	3.82, *m*	

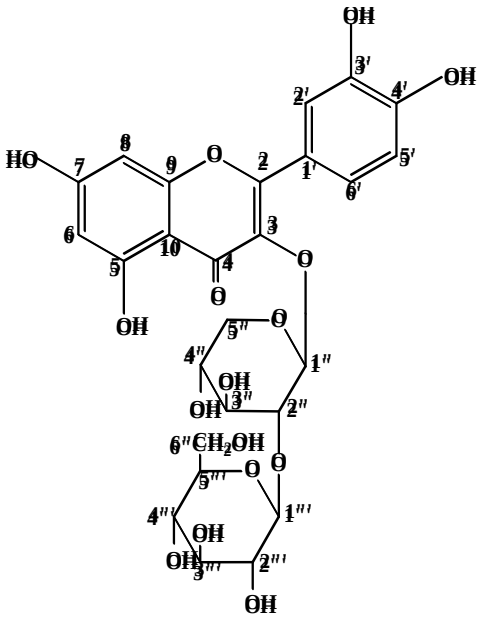


Fig.6.1.7 Structure of Compound **1**

Compound **2** (kaempferol 3-*O*-(2″-*O*-*β*-D-glucosyl)-*β*-D-xyloside) was obtained as a light brown powder with a negative specific rotation $[\alpha]_D^{21}$ –38.7° (c 0.1 in methanol). Its molecular formula was determined as $C_{26}H_{28}O_{15}$ by HR-ESI-TOF-MS (Reserpine, $C_{33}H_{40}N_2O_9$ was used as internal standard), while a quasi-molecular ion peak at *m/z* 603.27 $[M+Na]^+$ was detected in the positive-ion APCI-MS. The UV spectrum showed

characteristic flavonol absorption at 265.4 nm and 346.3 nm. The ^{1}H- and ^{13}C-NMR spectral data of **2** were closely similar to those of compound **1,** except for the presence of an aglycone signals. The ^{1}H-NMR spectrum of compound **2** showed the characteristic signals of the kaempferol nucleus, two doubles at δ_H 6.18 and 6.37 (each 1H, d, *J* = 2.0 Hz), assigned to the H-6 and H-8 protons, respectively, and a pair of phenyl protons at δ_H 6.90 (2H, d, *J* = 8.8 Hz) and 7.99 (1H, d, *J* = 8.8 Hz), assigned to the H-3′/5′ and H-2′/6′, respectively. The spectrum was shown as Fig.6.1.8. From the coupling constants of the anomeric protons δ_H 5.47 (1H, d, *J* = 5.4 Hz, H-1") and δ_H 4.67 (1H, d, *J* = 7.3 Hz, H-1"') on **2**, the configuration at C-1″ and C-1‴ of the sugar units was determined to be the β-configurations.

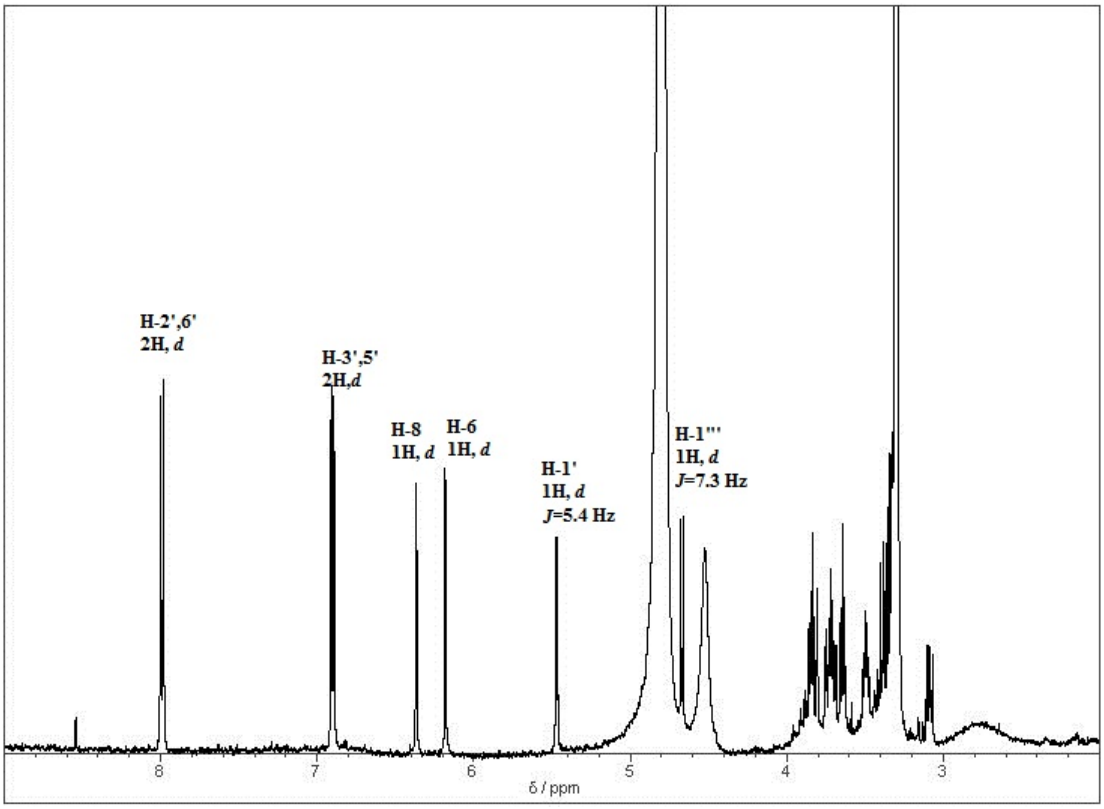


Fig. 6.1.8 ^{1}H-NMR spectrum of compound **2**

From these data of COSY, HSQC, HMBC and GC-MS of **2**, according to the same determination method of compound **1**, the structure of compound **2** was determined to be kaempferol 3-*O*-(2″-*O*-*β*-D-glucosyl)-*β*-D-xyloside: light brown powder. UV λmax (MeOH) nm (log ε): 265.4 (3.64), 346.3 (3.29). APCI-MS m/z: 603.27 $[M+Na]^+$. HR-ESI-MS *m/z*: 603.1354 $[M+Na]^+$ (calcd for $C_{26}H_{28}O_{15}Na$: 603.1326). $[\alpha]_D^{21}$ –31.2° (c 0.10, MeOH). The ^1H- and ^{13}C –NMR was showed in Table 6.1.2.

Table 6.1.2 ^1H (500 MHz) and ^{13}C (125 MHz) NMR data of Compound 2

	kaempferol 3- O-(2"-O-glucosyl)-D-β-xyloside(2)	
No.	δ_H, mult, *J*(Hz)	δc
Aglycone		
2		159.0
3		134.9
4		179.5
5		161.1
6	6.18, *d*, 2.0	100.3
7		163.1
8	6.37, *d*, 2.0	95.0
9		158.6
10		105.7
1′		122.7
2′/6′	7.99, *d*, 8.8	132.0
3′/5′	6.90, *d*, 8.8	116.0
4′		163.0
Xylosyl		
1″	5.47, d, 5.4	100.3
2″	3.85, dd, 5.4, 7.3	81.3
3″	3.65, dd, 7.3, 6.8	75.4
4″	3.48, dd, 6.8, 4.9	70.5
5″	3.09, dd, 6.8, 12.2	66.1
	3.74, dd, 12.2, 9.8	
Glucosyl		
1‴	4.67, d, 7.3	105.4
2‴	3.34, d, 6.8	74.9
3‴	3.39, m	78.2
4‴	3.37, m	71.4
5‴	3.29, m	77.9
6‴	3.70, dd, 4.8, 12.2	62.7
	3.82, dd, 2.4, 12.2	

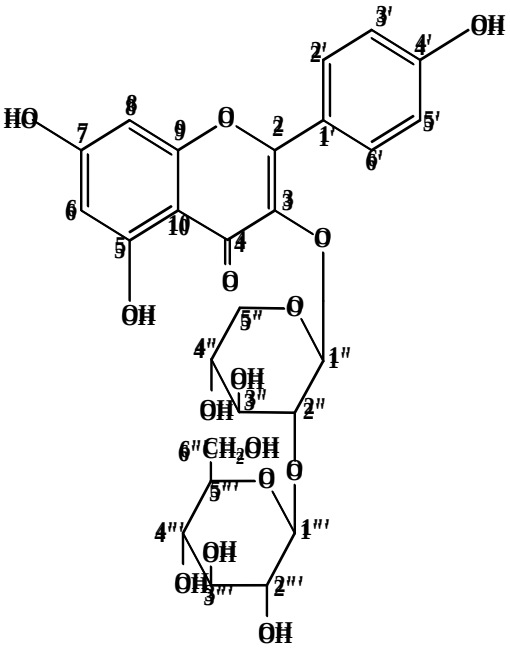


Fig.6.1.9 Stucture of Compound **2**

Compound **3** was obtained as a yellow powder with a negative specific rotation $[\alpha]_D^{21}$ –17.4° (c 0.22 in methanol). Its molecular formula was determined as $C_{27}H_{30}O_{17}$ by HR-ESI-TOF-MS (Reserpine, $C_{33}H_{40}N_2O_9$ was used as internal standard), while a quasi-molecular ion peak at *m/z* 649.27 $[M+Na]^+$ was detected in the positive-ion APCI-MS. The UV spectrum showed characteristic flavonol absorption at 256.6 nm and 357.1 nm. In the ^{1}H-NMR spectrum of compound **3,** the spectra of the aglycone moieties showed characteristic signals of quercetin, and the spectrum was closely similar to those of Compound **1**. However, the signals of sugar moieties were determined to D-glucose. Also acid hydrolysis of compound 3 yielded D-glucose by the GC-MS analysis. From the coupling constants of the anomeric protons δ_H 5.34 (1H, d, *J* = 7.8 Hz, H-1") and δ_H 4.75 (1H, d, *J* = 7.3 Hz, H-1"') on **3**, the configuration at C-1″ and C-1‴ of the D-glucose was determined to be the β-configurations.

According to COSY, HSQC and HMBC spectrum, the structure of compound **3** was determined to be quercetin 3-*O*-sophoroside: yellow powder. UV λmax (MeOH) nm (log ε): 257 (3.63), 357 (3.39), 381 (3.16). APCI-MS m/z: 649.27 $[M+Na]^+$. HR-ESI-MS *m/z*: 649.1300 $[M+Na]^+$ (calcd for $C_{27}H_{30}O_{17}Na$: 649.1380). $[\alpha]_D^{22}$ –17.4° (c 0.22, MeOH). The ^{1}H- and ^{13}C –NMR was showed in Table 6.1.3.

Its ^{1}H spectrum of sugar moieties was shown in Fig. 6.1.11, and the spectrum was the same to compound **4;** therefore, determination procedure was the same to compound **4**.

Table 6.1.3 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of Compound **3**

	quercetin 3-O-sophoroside(3)	
No.	δ_H, mult, *J*(Hz)	δc
Aglycone		
2		154.8
3		135.1
4		179.7
5		158.6
6	6.18, *d*, 2.0	100.1
7		158.8
8	6.36, *d*, 2.0	94.9
9		153.4
10		105.4
1′		123.1
2′	7.66, *d*, 2.0	117.7
3′		146.0
4′		149.9
5′	6.88, *d*, 8.3	116.2
6′	7.52, *d*, 8.3	123.0
Glucosyl A		
1″	5.34, *d*, 7.8	101.2
2″	3.76, *dd*, 7.8, 9.3	82.9
3″	3.57, *t*, 9.3	77.9
4″	3.34-3.42, *m*	71.0
5″	3.17, *dd*, 2.4, 5.8	78.1
6″	3.70, *dd*, 4.9, 12.2	62.4
	3.52, *dd*, 12.2, 5.8	
Glucosyl B		
1‴	4.75, *d*, 7.3	105.0
2‴	3.37, *dd*,7.3, 8.8	75.6
3‴	3.40, *t*, 8.8	78.3
4‴	3.62, *m*	71.1
5‴	3.32, *m*	78.3
6‴	3.79, *dd*, 2.4, 12.2	62.3
	3.68, *dd*, 2.4, 12.2	

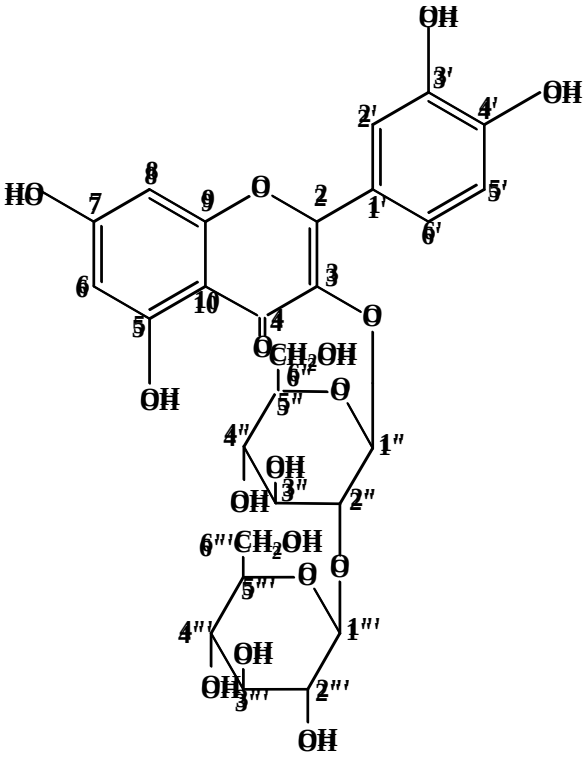


Fig.6.1.10 Stucture of Compound **3**

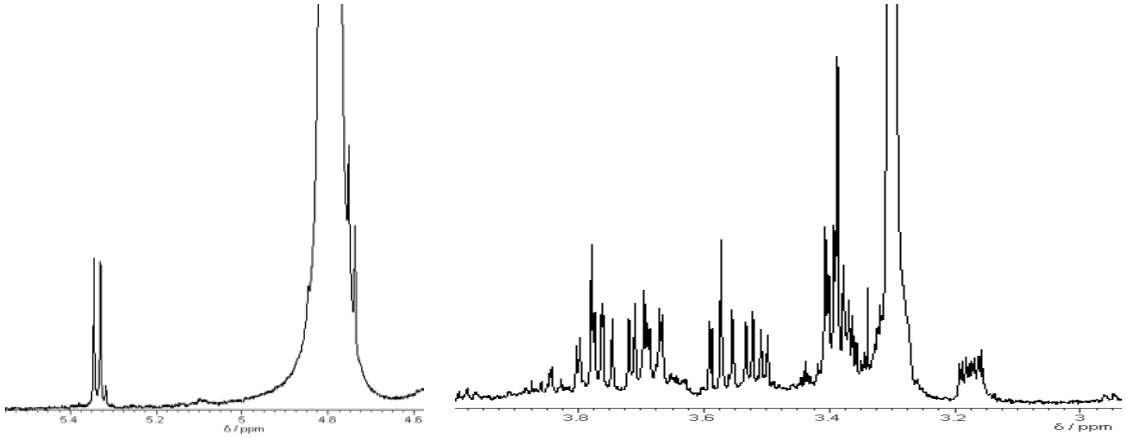


Fig. 6.1.11 ^{1}H-NMR spectrum of sugar moieties of compound **3**

Compound **4** was obtained as a yellow powder with a negative specific rotation $[\alpha]_D^{22}$ –19.9° (c 0.10 in methanol). Its molecular formula was determined as $C_{27}H_{30}O_{16}$ by HR-ESI-TOF-MS (Reserpine, $C_{33}H_{40}N_2O_9$ was used as internal standard), while a quasi-molecular ion peak at *m/z* 633.27 $[M+Na]^+$ was detected in the positive-ion APCI-MS. The UV spectrum showed characteristic flavonol absorption at 267.6 nm and 345.1 nm. In the ^{1}H-NMR spectrum of compound **4,** the spectra of the aglycone moieties showed characteristic signals of kaempferol, such as ring A: δ_H 6.19 and 6.38 (each 1H, d, *J* = 2.0 Hz, H-6,8, respectively); ring B: δ_H 6.90 (2H, d, *J* = 9.0 Hz, H-3′/5′) and 8.03 (2H, d, *J* = 9.0 Hz, H-2′/6′) and the spectrum shown as Fig.6.1.12.a.

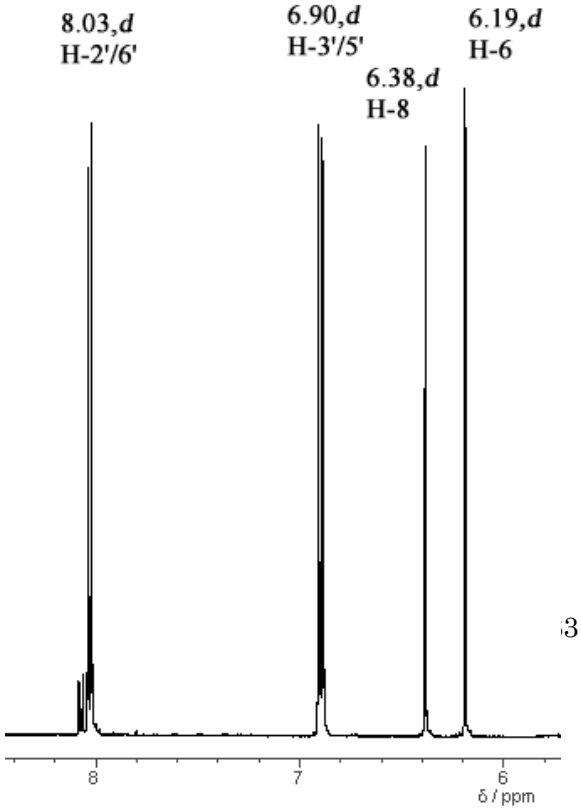


Fig. 6.1.12.a ^{1}H-NMR spectrum of aglycone moieties of compound **4**

The coupling constants of the anomeric protons δ_H 5.43 (1H, d, *J* = 7.5 Hz, H-1") and δ_H 4.75 (1H, d, *J* = 7.5 Hz, H-1"') on **4**(Fig.6.1.12.b) indicated that the configuration at C-1″ and C-1‴ of the sugar was determined to be the β-configurations. While the sugar moieties' protons were confirmed by COSY spectrum (Fig. 6.1.13).

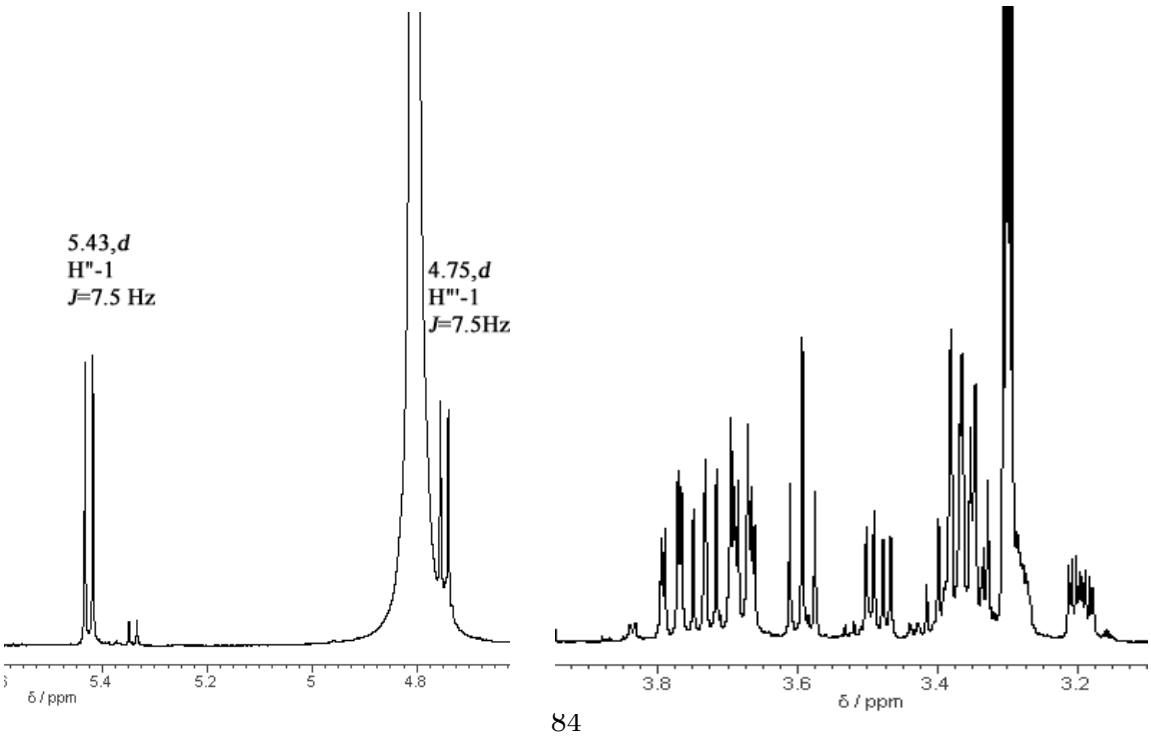


Fig. 6.1.12.b ^{1}H-NMR spectrum of sugar moieties of compound **4**

In the ^{13}C-NMR spectrum (Fig. 6.1.14), 27 carbon signals were observed, and 12 were confirmed to the sugar carbons. The signals of C-6, C-8, C-2′/6′, C-3′/5′ of aglycone and 12 carbons of sugar units were determined by HSQC spectrums (Fig. 6.1.15).

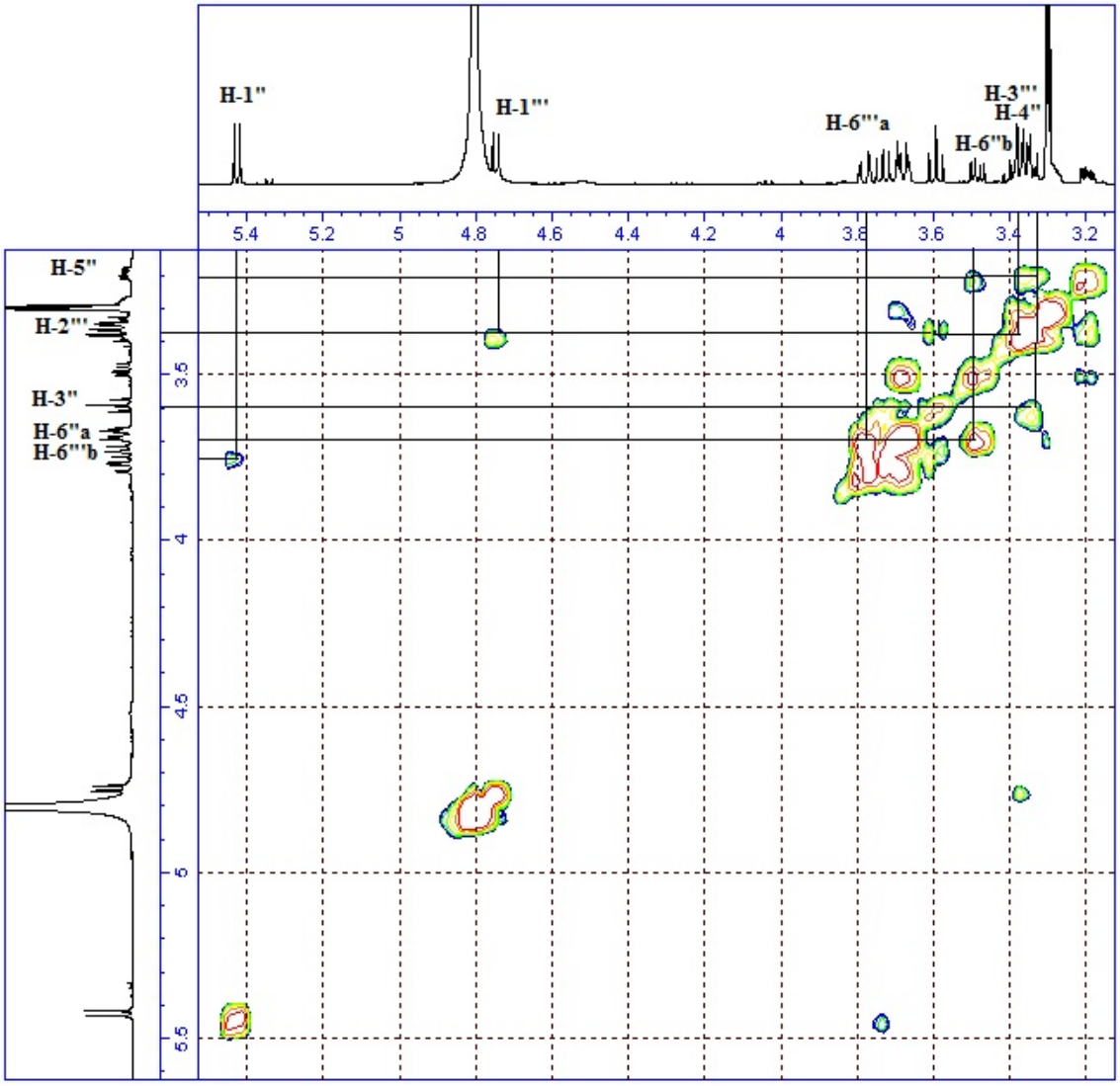


Fig. 6.1.13 COSY spectrum of sugar moieties of compound **4** in CD_3OD at 30 ºC

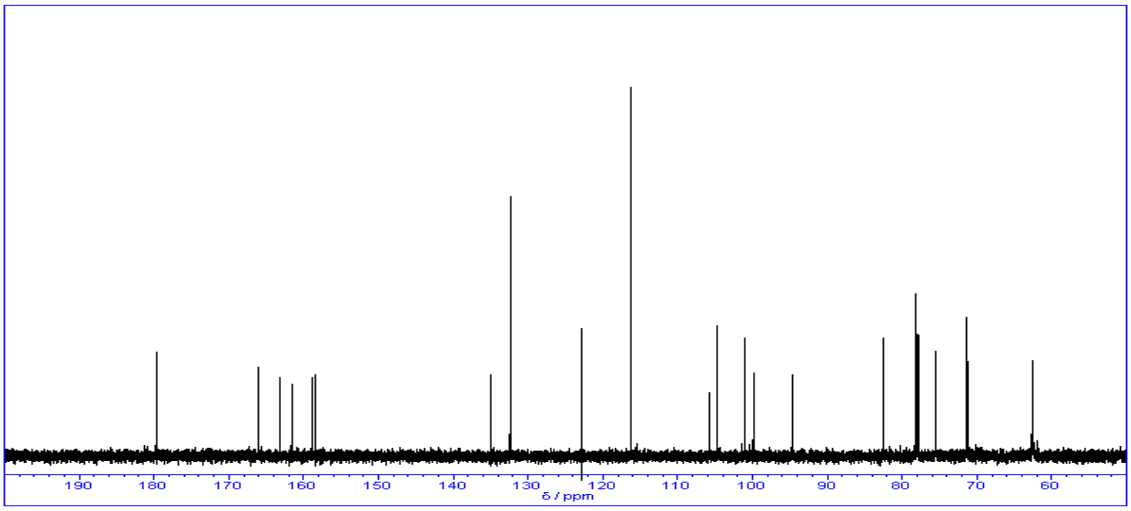


Fig. 6.1.14 ^{1}C-NMR spectrum of compound **4**

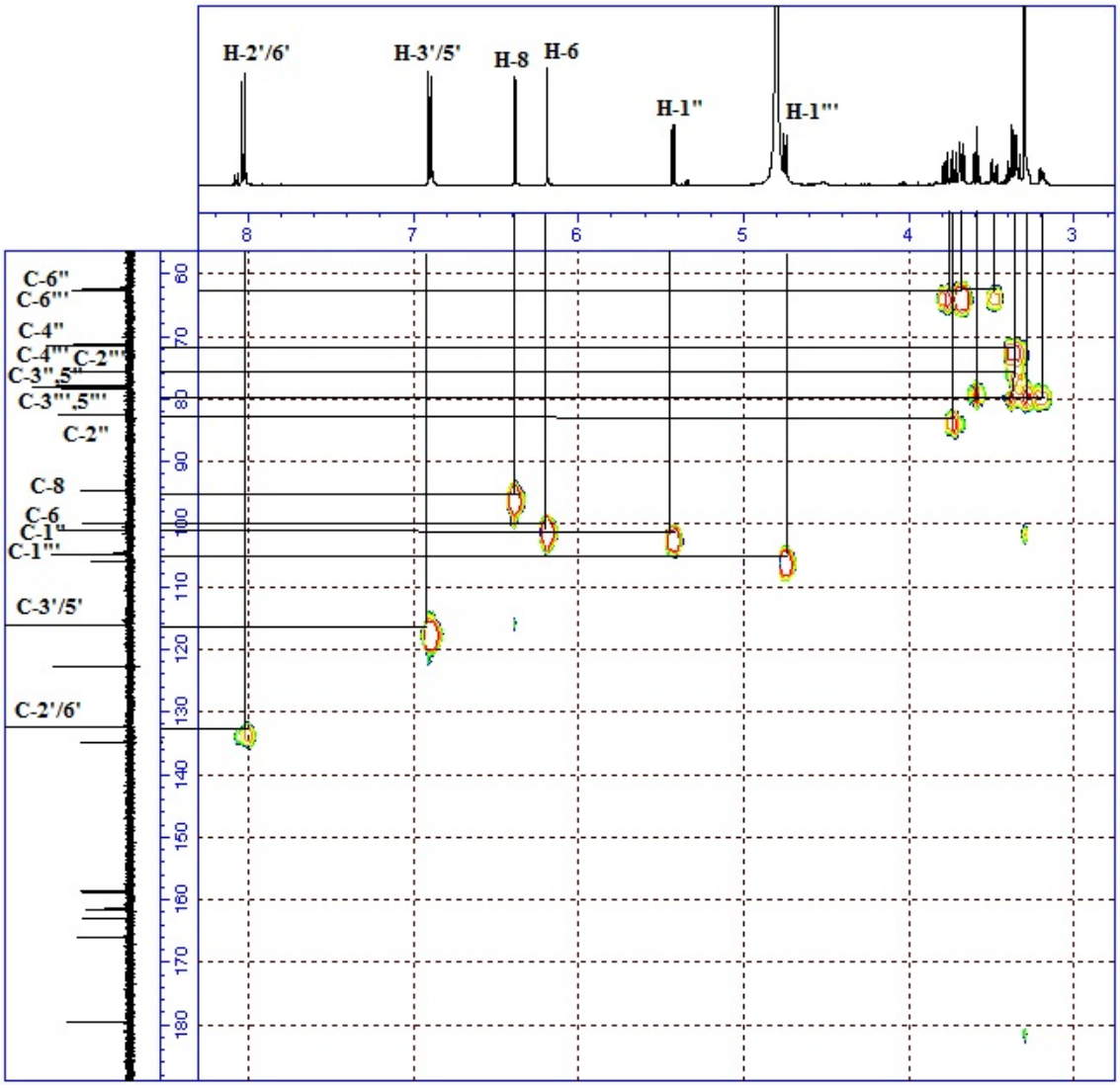
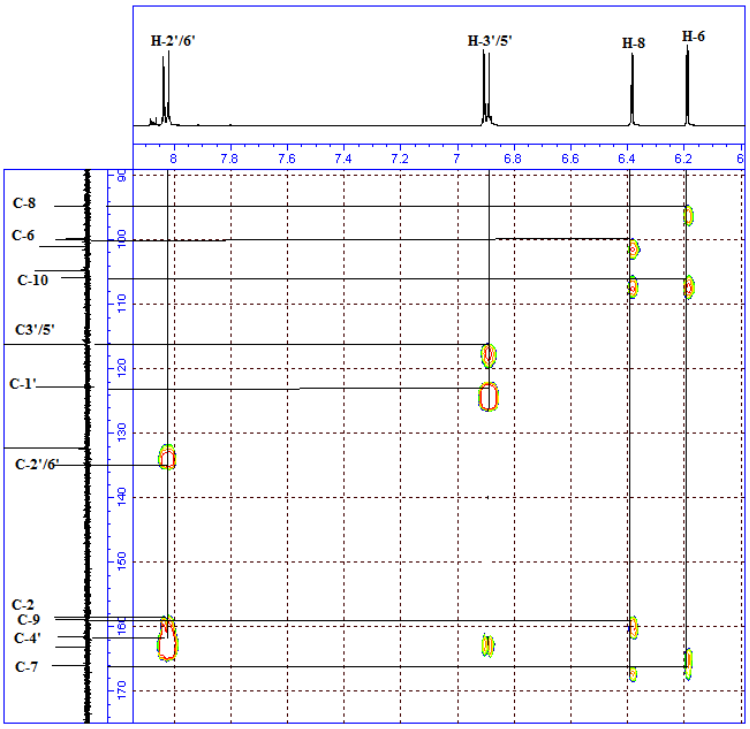
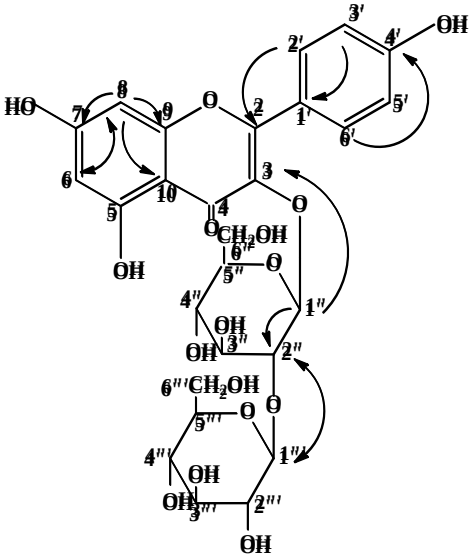


Fig. 6.1.15 HSQC spectrum of compound **4** in CD_3OD at 30 ºC

In the HMBC experiment, the aglycone H-2′/6′ signal at δ_H 8.03 correlated with C-2 and C-4′ resonance at δ_C 158.5 and 161.5, respectively. The H-3′/5′ signal atδ_H 6.90 correlated with C-1′ resonance at δ_C 122.9. The H-8 signal at δ_H 6.38 correlated with C-6, C-7, C-9 and C-10 resonance at δ_C 99.9, 166.0, 158.9 and 105.8, respectively. The H-1″ signal of a hexose at δ_H 5.43 correlated with the C-3 resonance of aglycone at δ_C 134.9, suggesting that the sugar unit was located at the C-3 position.





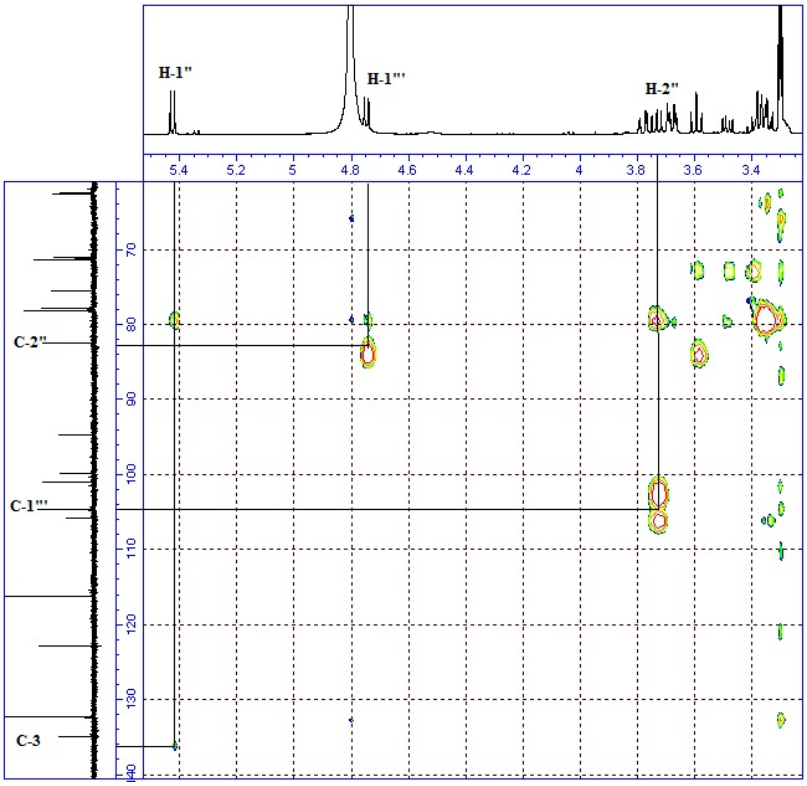


Fig. 6.1.16 HMBC spectrum of aglycone and sugar moiety of compound **4** in CD_3OD at 30 ºC

And the glucosyl H-1‴ signal at δ_H 4.75 correlated with the C-2″ resonance of glucosyl at δ_C 82.5, suggesting that the glucosly unit was connected in the C-2″ of glucosyl which connected to aglycone (Fig.6.1.16).

In order to characterize sugar units, acid hydrolysis of compound 1 was determined by GC-MS analysis following conversion to the trimethylsilylthiazolidine derivatives. The analysis of the coupling constants of sugar protons, the presence of glucose was implied. Therefore, D-glucose was used for standards. The acid hydrolysate of compound **4**, in addition to the aglycone, revealed the characteristic peaks of glucose; 10.181, 10.341, 10.891, 11.169 min. These results were

further confirmed that sugar unit of compound **4** was D-glucose.

From these data, the structure of compound **4** was determined to be kaempferol 3-*O*-sophoroside: yellow powder. UV λmax (MeOH) nm (log ε): 267 (3.44), 345 (3.11), 381 (2.80). APCI-MS m/z: 633.27 $[M+Na]^+$. HR-ESI-MS *m/z*: 633.1369 $[M+Na]^+$ (calcd for $C_{27}H_{30}O_{16}Na$: 633.1431). $[\alpha]_D^{22}$ –19.9° (c 0.10, MeOH). The ^1H- and ^{13}C –NMR was showed in Table 6.1.4.

Table 6 .1.4 ^1H (500 MHz) and ^{13}C (125 MHz) NMR data of Compound **4**

	kaempferol 3-O-sophoroside(4)	
No.	δ_H, mult, *J*(Hz)	δc
Aglycone		
2		158.5
3		134.9
4		179.7
5		163.1
6	6.19, *d*, 2.0	99.9
7		166.0
8	6.38, *d*, 2.0	94.7
9		158.9
10		105.8
1′		122.9
2′/6′	8.03, *d*, 9.0	132.3
3′/5′	6.90, *d*, 9.0	116.3
4′		161.5
Glucosyl A		
1″	5.43, *d*, 7.5	101.0
2″	3.73, *dd*, 7.5, 9.0	82.5
3″	3.59, *t*, 9.0	77.8
4″	3.33, *m*	71.1
5″	3.20, *ddd*, 2.4, 5.3, 9.7	77.9
6″	3.69, *d*, 2.4, 11.9	62.6
	3.48, *dd*, 11.9, 5.3	
Glucosyl B		
1‴	4.75, *d*, 7.5	104.7
2‴	3.35, *m*	75.6
3‴	3.37, *t*, 7.5	78.3
4‴	3.38, *t*, 7.5	71.3
5‴	3.28, *m*	78.2
6‴	3.78, *dd*, 2.4, 11.9	62.5
	3.67, *d*, 11.9	

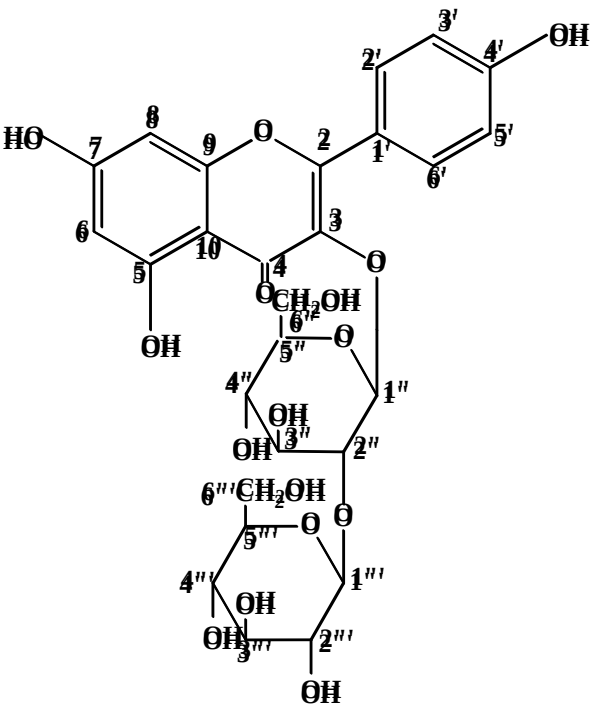


Fig.6.1.17 Stucture of Compound **4**

Fig.6.1.17 Stucture of Compound **4**

6.2 Quantitative determination of flavonol glycosides

6.2.1 General procedures

UPLC was performed using an Acquity UPLC system (Ultra Performance Liquid Chromatography; Waters, USA) consisted of a binary solvent manager, a sample manager, a column heater, a PDA (photodiode array) detector, and an Acquity UPLC BEH C_{18} (2.1 i.d. × 100mm, 1.7 μm) column. The initial mobile phase (A) was 2.5% (v/v) acetonitrile containing 0.1% (v/v) formic acid, and final mobile phase (B) was acetonitrile with 0.1 % formic acid. The gradient condition was as followed: 0 - 4min (B: 0 %), 26 min (B: 10 %), 45 min (B: 15 %), 47min (B: 20 %), 49min (B: 50 %) and 50min (B: 100 %). The flow rate was 0.2 ml/min. The detector wavelength range was set from 200nm to 500nm and the maximum absorption wavelengths of standard compounds were selected for samples detection.

6.2.2 Plant materials and chemicals

The samples of Mei-gui were collected in Xinjiang Province, Pingying rose research institute in Shandong Province. Wild and cultivated *R. rugosa and hybrids of R. rugosa* were collected in Hokkaido of Japan and Korea. *R. maikwai* was obtained from Medicinal Plants of Hokkaido University. *R. davurica and R. damascena* were obtained from Research Center for Medicinal Plants Resources, Nayoro, Hokkaido of Japan. The information of samples is showed in Table 6.2.1.

Table 6.2.1 Information of samples

No.	Species / Used name	Cultivated or collected place	Notes*
CX1	Mei-gui（Kizil gul）	Keriya (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX2	Mei-gui（Kizil gul）	Houtan (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX3	Mei-gui（Kizil gul）	Niya (Tarim basin), Xinjiang Prov., China	Cultivated, DF
CX5	Mei-gui（Kizil gul）	Hami, Xinjiang Prov., China	Cultivated, DF
CS1	Mei-gui	Pingying rose research institute,	Cultivated, DF
CS3	Mei-gui	Shandong Prov., China	Cultivated, SF
MAI	*Rosa maikwai* Hara.	Hokkaido Univ. Medicinal plants garden, Japan	Cultivated, DF
K1	*Rosa rugosa* Thunb.	Hwajipo Gangwong-do, Korea	
K2	*Rosa rugosa* Thunb.	Hwasong Songsan, Gyeonggi-do,Korea	Wild, SF
K3	*Rosa rugosa* Thunb.	Yangyang, Gangwon-do, Korea	
J1	*Rosa rugosa* Thunb.	Monbetsu, Hokkaido, Japan	
J2	*Rosa rugosa* Thunb.	Ishikari, Hokkaido, Japan	Wild, SF
J3	*Rosa rugosa cv. Plena*.	Kitami, Hokkaido, Japan	Cultivated, DF
J7	*Rosa rugosa* Thunb.	Niigata, Hokkaido, Japan	Wild, SF
HY1	Therese bugnet		
HY2	Hansa	Kimide farm, Hokkaido, Japan	
HY3	Purple pavement		Cultivated, DF
HY4	Kushui Mei-gui	Kushui, GansuProv. China	
Dav	*R. davurica* Pallas.	Nayoro, Hokkaido, Japan	

Dam *R. damascena* Miller.

*DF: Double flower; SF: single flower.

Six flavonol glycosides, compound **1**, **2**, **3**, **4** isolated from *R. rugosa* and kaempferol 3-*O*-*β*-D-glucoside and quercetin 3-*O*-*β*-D-glucoside were quantitatively determined in the dried petals of Mei-gui, *R. rugosa* and its allied plants.

Quercetin 3-*O*-*β*-D-glucoside and kaempferol 3-*O*-*β*-D-glucoside were purchased from Kanto Chemical Co. Inc. (Tokyo Japan) and ChromaDex, Japan (Tokyo Japan), respectively. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

6.2.3 Preparation of Samples

Pulverized petals (5g) of samples were extracted three times with 250 ml of 50% aq. EtOH at room temperature for 24h, respectively. After removal of solvent, the aqueous solution was lyophilized. The extracts were dissolved in 50% aq. MeOH (20 mg/ml), and filtrated through a 0.45 μm Millipore filter unit (Advantec, Japan), and then 1 μl was injected into UPLC for analysis.

6.2.4 Results and discussion

6.2.4.1 Quantitative determination of flavonal glucosides in the petals *R. rugosa* and its allied plants

To confirm characteristic flavonol glycosides of petals of *R. rugosa*, four flavonol glycosides, compound **1**, **2**, **3**, **4** isolated from *R. rugosa* were quantitatively determined in the dried petals of *R. rugosa* and its four kinds of hybrids and two allied plants.

As shown in Table 6.2.2, the 3-*O*-(2″-*O*-glycosyl)-xyloside and 3-*O*-sophoroside of kaempferol (K) and quercetin (Q) were detected in all the samples.

Table 6.2.2 Flavonol glycosides contents in the dried petals of *R. rugosa* and its hybrids

		Flavonol glycosides (mg/g dry weight)*			
		3-*O*-(2″-*O*-*β*-D-glucosyl)-*β*-D-xyloside		3-*O*-sophoroside	
	Samples	quercetin	kaempferol	quercetin	kaempferol
R.rugosa	J1	0.45	0.06	1.53	0.08
	J7	0.47	0.07	2.76	0.52
	K2	0.34	0.08	1.28	0.20
	K3	0.40	0.06	2.39	0.25
Hybrid of *R. rugosa*	HY1	1.60	7.36	13.06	18.41
	HY2	0.16	0.20	3.81	0.63
	HY3	1.01	0.52	4.48	0.99
	HY4	2.78	5.38	14.33	3.69
R. davurica	Dav	0.00	0.00	17.71	7.10
R.damascena	Dam	0.00	0.00	0.00	0.00

*Determination limits were less than 0.005 mg/g.

Among the four flavonol glucoside, Q 3-*O*-sophoroside (Q-3S) was detected as main flavonol glucoside of petals of *R. rugosa* and its hybrids in the range of 1.28 - 14.33 mg/g. Kaempferol 3-*O*-sophoroside (K-3S) was abundant to some hybrid samples showing higher amounts of 18.41

mg/g, and it were detected in the range of 0.20 - 3.69 mg/g in other samples. The content of 3-*O*-(2″-*O*-glycosyl)-xyloside of K and Q were less than 0.5 mg/g in *R. rugosa*, however, the compounds were detected higher contents than those of *R. rugosa* in the range of 0.20 - 7.36 mg/g.

We also determined the contents of four flavonol glucoside in the petals of *R. davurica* and *R. damascene* to compare with those of *R. rugosa* and its hybrids. The 3-*O*-(2″-*O*-glycosyl)-xyloside of K and Q were not detected in the petals of *R. davurica* and *R. damascene*. The 3-*O*-sophoroside of K and Q were detected in the petals of *R. davurica* of 7.10 and 17.71 mg/g. However, the 3-*O*-sophoroside of K and Q were not detected in the petals of *R. damascene*.

The 3-*O*-(2″-*O*-glycosyl)-xyloside of K and Q were isolated from *R. rugosa* for the first time, and were detected in the samples collected in Japan, and cultivated at Hokkaido as a hybrids species of *R. rugosa*. However, this compounds not detected in *R. davurica* and *R. damascene*. Therefore, these compounds can use as characteristic flavonol glycosides in *R. rugosa*.

6.2.4.2 Quantitative comparison of flavonal glucosides in the petals between *R. rugosa* and Mei-gui

The contents of glycosides of two flavonols of K and Q in the petals of Mei-gui collected in China, and *R. rugosa* collected in Japan and Korea were quantitative determination by UPLC method. The six glycosides with three patterns; 3-*O*-glucoside, 3-*O*-sophoroside and

3-*O*-(2″-*O*-glycosyl)-xyloside, were determined in the petals of *R. rugosa* and Mei-gui. The typical chromatograms in 350nm are shown in Fig. 6.2.1.

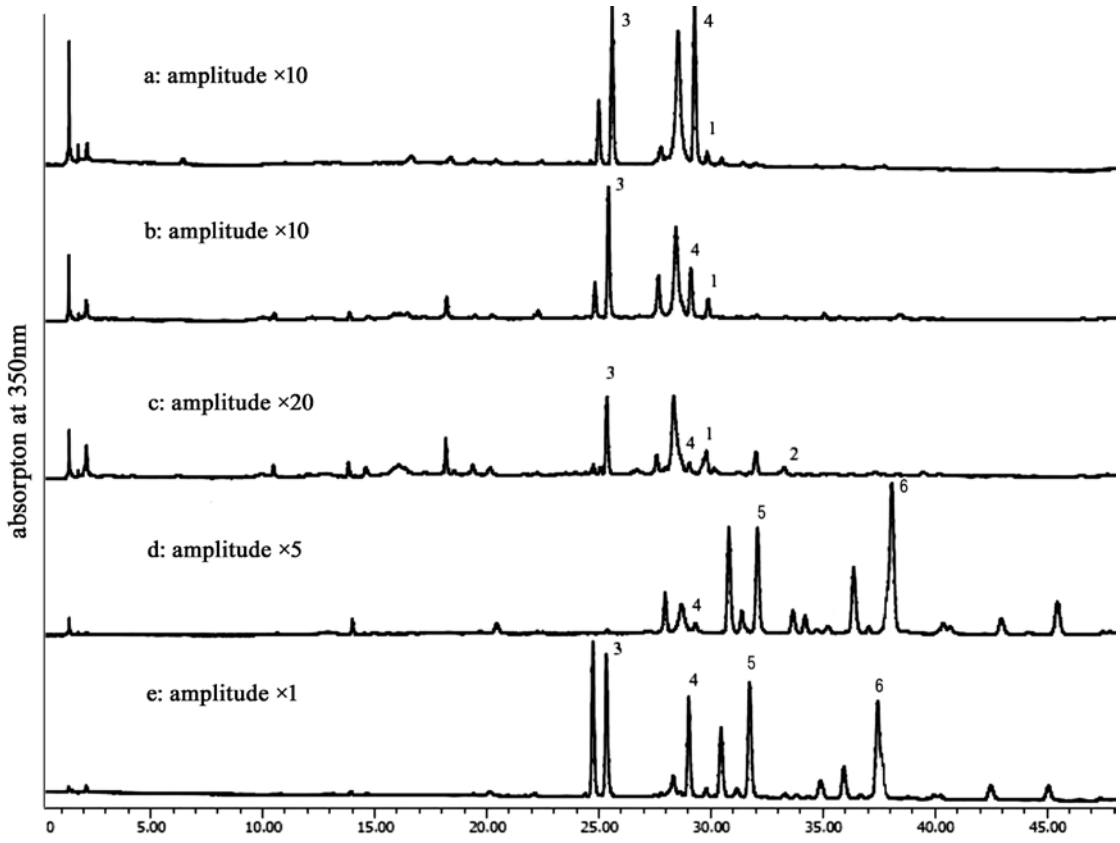


Fig.6.2.1 UPL chromatograms of the petal extracts of Mei-gui, *R. rugosa* and *R. maikwai*

(a) CS3: Mei-gui with single flower cultivated in Pingying, (b) M: *R. maikwai*, (c) J1: *R. rugosa*,

(d) CS1: Mei-gui with double flower cultivated in Pingying, (e) CX2: Mei-gui cultivated at Tarim Basin

1: quercetin 3-*O*-(2″-*O*-*β*-D-glucosyl)-*β*-D-xyloside,

2: kaempferol 3-*O*-(2″-*O*-*β*-D-glucosyl)-*β*-D-xyloside,

3: quercetin 3-*O*-sophoroside, 4: kaempferol 3-*O*-sophoroside,

5: quercetin 3-*O*-*β*-D-glucoside, 6: kaempferol 3-*O*-*β*-D-glucoside

As shown in Table 6.2.3, K 3-*O*-sophoroside (K-3S) was detected in all the samples in the range of 0.08 – 10.43 mg/g, and Q 3-*O*-sophoroside

(Q-3S) was detected ten samples in the range of 1.16 - 20.13 mg/g. K 3-*O*-glucoside (K-3G) and Q 3-*O*-glucoside (Q-3G) were detected in four samples, and K 3-*O*-(2″-*O*-glucosyl)-xyloside (K-3XG) and Q 3-*O*-(2″-*O*-glucosyl)-xyloside (Q-3XG) were detected in four and seven samples, respectively.

Table 6.2.3 Flavonol glycosides contents in the dried petals of Mei-gui, *R. maikwai* and *R. rugosa*

	Flavonol glycosides (mg/g dry weight)*					
	3-*O*-*β*-D-glucoside		3-*O*-sophoroside		3-*O*-(2″-*O*-*β*-D-glucosyl)-*β*-D-xyloside	
Code no.	kaempferol	quercetin	kaempferol	quercetin	kaempferol	quercetin
CX1	9.59	20.59	10.43	20.13	0	0
CX2	3.18	11.62	5.11	11.26	0	0
CX3	3.04	10.21	5.77	9.40	0	0
CX5	0	0	1.44	4.06	0	0.25
CS1	4.07	10.06	0.41	0	0	0
CS3	0	0	3.63	4.62	0	0.20
MAI	0	0	0.82	2.95	0	0.33
J1	0	0	0.08	1.53	0.06	0.45
J2	0	0	0.20	1.16	0.03	0.39
J3	0	0	0.66	3.19	0.05	0.25
K1	0	0	0.31	1.60	0.05	0.09
K2	0	0	0.20	1.28	0.08	0.34

*Determination limits were less than 0.005 mg/g.

According to the contents of these 6 flavonol glycosides, we classified these samples into 4 groups. Group 1 contains 3 samples cultivated around Tarim Basin in Xinjiang Province in China (CX1 - 3), in which Q-3S and Q-3G were the main flavonol glycoside along with K-3S and

K-3G. Group 2 is a sample from Shandong Province (CS1), which contained Q-3G as the main flavonol glycoside with K-3G and K-3S. Group 3 was composed of one sample from Xinjiang Province, one from Shandong Province and *R, maikwai*. These samples contained Q-3S as the main flavonol glycoside with K-3S and Q-3XG and did not contain K-3XG, K-3G and Q-3G. Group 4 was composed of *R. rugosa* collected and cultivated in Japan and Korea, which contained Q-3S as the main flavonal along with K-3S, K-3XG and Q-3XG, but K-3G and Q-3G were not detected. The contents of Q-3S and K-3S were lower than those of other samples.

Mei-gui (CX1 - 3) showed the same pattern (Group 1); Q-3S and Q-3G were the main flavonol glycosides along with K-3S and K-3G. The Mei-gui (CS1) cultivated in Shandong Province showed similar pattern, but the main flavonol glycoside was Q-3G and Q-3S was not detected.

In one sample of Mei-gui from Xinjiang Province (CX5), one from Shandong Province (CS3) and *R. maikwai*, Q-3S was the main flavonol glycoside along with K-3S and Q-3XG, but K-3G and Q-3G were not detected (Group 3). The Mei-gui (CX5 and CS3) must have close relation with *R. maikwai*. In Group 4 was composed of *R. rugosa* collected and cultivated in Japan and Korea, and K-3G and Q-3G were not detected in this group. In Group 4, the contents of K-3S and Q-3S were lower than those in Group 1 and 3.

K-3XG and Q-3XG were isolated from *R. rugosa* for the first time, and were detected in the samples (J3) cultivated in Hokkaido as hybrids species of *R. rugosa*. Therefore, these compounds can be used as

characteristic flavonol glycosides in *R. rugosa*. According to this assumption, Mei-gui in Group 3 might be hybrids of *R. rugosa*.

Mikanagi *et al*. [9, 10] studied on kaempferol (K) and quercetin (Q) glycosides, and classified genus *Rosa* into three groups. According to their study, the group where section *Gallicanae* belongs to contains 3-*O*-glucoside of K and Q as the main flavonol glycosides, as well as small an amount of 3-*O*-sophorosides. Section *Caninae* and section *Rosa* (sect. *Cinnamomaeae*) were belonged to another group, which contain 3-*O*-sophoroside of K and Q as the main flavonol glycosides as well as small an amount of 3-*O*-glucoside.

In three samples of Mei-gui cultivated around Tarim Basin, K-3G and Q-3G were detected. These samples also showed morphological characteristics of *R. gallica*.

According the pattern of flavonol glycosides contents, there are two groups of Mei-gui, and one has the same pattern as *R. maikwai*. The pattern of *R. rugosa* is different from these two groups. However, two groups of Mei-gui were detected K-3G and Q-3G as the main flavonol glycosides also detected K-3XG and Q-3XG. Therefore, the two groups of Mei-gui are considered as hybrids of *R. rugosa.*

4.5 References

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Chapter 7 Conclusion

7.1 Inhibitory effects of Mei-gui and *R. rugosa* on the digestive enzymes

The inhibitory activities of Mei-gui collected in China and *R. rugosa* collected in Japan and Korea were investigated against α-amylase and α-glucosidase. Compared with the inhibitory effect of acarbose, the extracts of petals of Mei-gui and *R. rugosa* exhibited stronger or the nearest inhibitory effects against α-glucosidase from *Saccharomyces* sp. These results indicated that the petals of Mei-gui and *R. rugosa* must be effective for treatment of diabetes. The hydrolysable tannins were isolated from Mei-gui. These hydrolysable tannins showed strong inhibitory effect against α-glucosidase, and the contents of these hydrolysable tannins were shown to be higher in the dried petals of the plant. Therefore, these hydrolysable tannins must be responsible for the activities of the plant.

7.2 Botanical origins of Mei-gui cultivated in China

There are many species of plants called as Mei-gui cultivated in China for production of Mei-gui Hua. These plants are morphologically different from *R. rugosa* and *R. maikwai*. Therefore, we investigated the

botanical origins of Mei-gui cultivated in China. As the results of morphological characterization, Mei-gui cultivated around Tarim Basin in Xinjiang Province was morphologically identical with *R. gallica* and this was supported by phylogenetic analysis and phytochemical studies on flavonol glycosides. The Me-gui cultivated in other parts of China were also morphologically different from *R. rugosa* and *R. maikwai*, but the phylogenetic analysis and phytochemical study on flavonol glycosides suggested that most of Mei-gui are considered to hybrids of species of sect. *Cinnamomeae* such as *R. rugosa* and *R. acicularis* var. *nipponica*.

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Sarengaowa

Summary

"Mei-gui" is a common type of rose in China, and its petals, Mei-gui Hua have been used as a crude drug in traditional medicine and as herbal tea in China. The scientific name of Mei-gui is known as *Rosa rugosa* thunb., which is the same to Japanese wild rose, but morphological characteristics of Mei-gui imply that it different to *R. rugosa* and it probably has many different botanical origins.

We studied the inhibitory effects of Mei-gui and *R. rugosa* on the digestive enzymes, α-amylase and α-glucosidase, and the hydrolysable tannins isolated from the petal of these plants were identified as active compounds. Moreover, in order to identify origins of Mei-gui, we have surveyed the cultivation areas in China. Also, we investigated wild or cultivated *R. rugosa* in Japan and Korea. We compared the morphological characteristics of Mei-gui and *R. rugosa* through an on-the-spot investigation. And we examined nucleotide sequences from three regions of intergenetic spacers in chloroplast DNA isolated from the fresh leaves of Mei-gui and *R. rugosa*. We also studied on the phenolic compounds of Mei-gui and *R. rugosa*. The flavonol glycosides are isolated, and compared them between *R. rugosa* and Mei-gui by quantitative determination.

In conclusion, compared with the inhibitory effect of acarbose, the extracts of petals of Mei-gui and *R. rugosa* exhibited strong inhibitory effects against α-glucosidase. The results proved that the petals of Mei-gui and *R. rugosa* are effective for treatment of diabetes.

The Mei-gui cultivated around Tarim Basin in China was morphologically identical with *R. gallica* and this assumption was supported by phylogenetic analysis and phytochemical studies on flavonol glycosides. The Mei-gui cultivated in China were also morphologically different from *R. rugosa* and *R. maikwai*, but the phylogenetic analysis and phytochemical study on flavonol glycosides suggested that most of Mei-gui cultivated in China are considered to hybrids of species of *R. rugosa*.