STUDIES ON POLYPHENOLS IN UNRIPE-SOYBEAN (ÆDAMAME, *GLYCINE MAX*. L. MERRILL. 'JINDAI') LEAVES AND THEIR ANTI-DIABETIC AND ANTI-OBESE FUNCTIONS

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枝豆「神代豆」葉ポリフェノールとその抗糖尿病・抗肥満機能に関する研究

2011.9

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CONTENTS

CHAPTER 1 PREFACE 1

1.1 Soybean

1.1.1 Soybean products

1.1.2 Physiological functions of Soybean

1.2 Edamame

1.3 Polyphenols

1.4 Flavonoids

1.5 Biological activities of flavonoids

1.6 Aim of the present study

CHAPTER 2 ISOLATION AND CHRACTERIZATION OF THE POLYPHENOLS OF JIN-DAI-SOY LEAVES 10

2.1 Introduction

2.2 Material and Methods

2.3 Equipment

2.4 Results and Discussion

2.5 Summary

CHAPTER 3 ANTI-DIABETIC EFFECT OF KAEMPFEROL AND KAEMPFEROL GLYCOSIDES FROM JINDAI-SOY LEAVES ON KK-A^y MICE 28

3.1 Introduction

3.2 Materials and Methods

3.3 Results

3.4 Discussion

3.5 Summary

CHAPTER 4 ANTI-OBESE AND ANTI-DIABETIC EFFECTS OF KAEMPFEROL GLYCOSIDES FROM JINDAI-SOY LEAVES ON C57BL MICE 46

4.1 Introduction
4.2 Materials and Methods
4.3 Results
4.4 Discussion
4.5 Summary

CHAPTER 5 ANTI-OBESE AND ANTI-DIABETIC EFFECTS OF ISOFLAVONE MIXTURE (DAIDZIN + GLYCITIN) FROM JINDAI-SOY LEAVES ON C57BL MICE 64

5.1 Introduction
5.2 Materials and Methods
5.3 Results
5.4 Discussion
5.5 Summary

REFERENCES 88
SUMMARY 101
ACKNOWLEDGEMENTS 105

CHAPTER 1

PREFACE

1.1 Soybean

Soybean (*Glycine max* L.) is one of the major legume crops native to East Asia. Research on soybean is driven by its importance as a food crop worldwide. Soybean contains proteins, oils, mineral nutrients, and natural products such as isoflavonoids that improve human health and nutrition. Its products are widely used as a protein source and edible vegetable oil for human consumption, and high-protein feed supplements for the chicken and pork industries (*1-3*). Soybean is also one of the major producers of secondary metabolites, which possess health-promoting properties, thereby enjoy popular use in industrial and pharmaceutical applications (*4*). Recently, soybean has also emerged as a resource for production of biodiesel (*5*).

1.1.1 Soybean products

Fat-free soybean meal is a primary, low-cost, source of protein for animal feeds and most prepackaged meals; soy oil is another valuable product of processing the soybean crop. For example, soybean products such as textured vegetable protein (TVP) are important ingredients in many meat and dairy products (*6*) . Soybeans produce significantly more protein per acre than most other uses of land.

Traditional non fermented food uses of soybeans include soy milk, and from the latter tofu and tofu skin. Fermented foods include soy sauce, fermented bean paste, "natto", and "tempeh", among others. The oil is used in many industrial applications. The beans contain significant amounts of phytic acid, alpha-linolenic acid, and the isoflavones.

1.1.2 Physiological functions of Soybean

Soybean has long been embraced by Asian people as a source of high quality protein. It

contains also high fat and little carbohydrate. Although high in several vitamins and minerals and an assortment of phytochemical, two components of soybeans are thought to be primarily responsible for the hypothesized health benefits: soy protein and isoflavones.

Renal function

Renal problems are a main complication of diabetes, and they are markedly increasing throughout the world. One comprehensive analysis of the relationship between protein intake and kidney function concluded that low-protein diets reduce the risk of death that due to kidney failure in diabetic patients (*7*). Fortunately, it appears that not all proteins have similar effects on the kidney. Results from a study conducted by the Kentucky University showed that serum cholesterol and urinary protein excretion were reduced on the soy protein diet; the latter measure a direct indicator of improved kidney function (*8*). Lower serum cholesterol may be an additional advantage of soy protein since elevated cholesterol adversely affects renal function.

Urinary calcium excretion

Soy protein has been shown to decrease urinary calcium excretion when substituted for a similar amount of animal protein, such as meat and milk protein (*9, 10*). Factors that increase calcium excretion will likely adversely affect bone health because net calcium absorption may be no more than 10% (*11*). Consequently, excretion of an extra 50 mg of calcium/day may require consuming as much as an additional 500 mg of calcium to compensate for this loss. Calcium bioavailability from soy is quite good and is similar to the absorption from dairy milk (*12*).

Breast cancer

Low breast cancer mortality rates among soy food-consuming people have been reported (*13*). Greater lifelong exposure to estrogen is known to increase breast cancer risk. This is why earlier age at menses, later age at menopause, and hormone replacement therapy are considered to be risk factors for breast cancer (*14, 15*). Several studies have shown that isoflavones isolated from soy, especially genistein possessed antiestrogenic activity and can inhibit the effects of estrogen under certain experimental conditions (*16*).

Prostate cancer

Though Japanese men develop prostate cancer, they rarely die from it (*17*). Soy intake may be the main reason for this as International Prostate Health Council recently concluded that isoflavones prevented latent prostate cancer from progressing to the more advanced forms of the disease (*18*). Some *in vitro* data supports the relation between soy intake and prostate cancer. Genistein inhibits the growth of hormone-dependent and independent prostate cancer cells (*19*) and independent of growth effects, inhibits the metastatic potential of prostate cancer cells (*20*).

Coronary heart disease

Soy may have effects on coronary heart disease risk independent of the cholesterol lowering properties of soy protein. Preliminary data suggest that isoflavones, like estrogen, may exert cardioprotective effects via direct effects on coronary vessels and other physiological processes involved in the etiology of heart disease. Several studies indicate isoflavones enhance endothelial function, arterial relaxation, and arterial compliance (*21, 22*). In addition, some other studies indicate that soy food consumption reduces the extent

to which low-density lipoprotein cholesterol is oxidized. Based on comparisons between isoflavone-rich and isoflavone-poor soy protein, isoflavone-rich are responsible for this effect both in human and animal studies (*23, 24*).

1.2 Edamame

Edamame is a special type of green soybean (Glycine max (L.) Merr.). It is harvested as a vegetable when the seeds are immature and have expanded to fill 80 to 90 percent of the pod width. Like field-dried soybeans, the seeds of Edamame varieties are rich in protein and highly nutritious (*25*). Worldwide, it is a minor crop, but it is quite popular in East Asia. More than 200 species of Edamame exist in Japan . Though in Japan soybeans were introduced from China at an early date, the first recorded use of Edamame is the description of "aomame" in the Engishiki (927 A.D.), a guide to trade in agricultural commodities. It describes the offering of fresh, podded soybean stems at Buddhist temples (*26*).

Edamame is consumed mainly as a snack, but also as a vegetable, an addition to soups, or processed into sweets. As a snack, the pods are lightly cooked in salted boiling water and then the seeds are pushed directly from the pods into the mouth with the fingers. At harvest, Edamame has lower trypsin-inhibitor levels, fewer indigestible oligosaccharides, and more vitamins than field-dried soybeans (*27*). Edamame protein levels were thought to be slightly higher than soybeans.

For Edamame, the two most important components of flavor are sweet and savory. Its sweet taste is determined by sucrose content and its savory taste probably by amino acids like glutamic acid (*28*). Beany flavor increases with maturity and can be divided into two components: beany and bitter (*29*). The beany taste may come from linolenic acid

oxidized by lipoxygenase and the bitter taste may be the lipoxygenase itself.

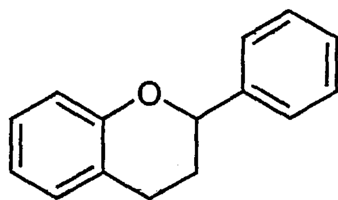
1.3 Polyphenols

Polyphenols are the most abundant antioxidants in human diet and are widespread constituent of fruits and vegetables. Despite their wide distribution, the healthy effects of dietary polyphenols have come to the attention of nutritionists in the recent years. One of the main factors responsible for delayed research on polyphenols is the diversity and the complexity of their chemical structure which influences the antioxidant effect. As antioxidant, polyphenols may protect cell constituents against oxidative damage. Therefore, they can limit the risk of various degenerative diseases associated with oxidative stress, such as cardiovascular diseases, type 2 diabetes and cancer (*30-32*).

1.4 Flavonoids

The flavonoids are almost universal pigments of plants and polyphenolic compounds. They are responsible for the colouring of fruits, flowers and sometimes of the seeds (*33*). The flavonoids are subdivided in three main groups:

- The 1,3-diphenylpropanes (**1**), which are the most widespread flavonoids.
- The isoflavonoids whose basic skeleton is that of 1,2-diphenylpropane (**2**).



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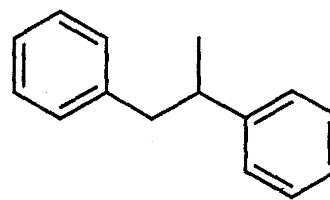

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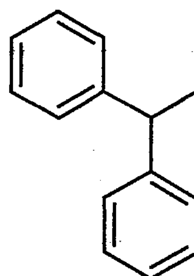
- The neo-flavonoids whose basic skeleton are that of the 1,1-diphenylpropane **3**.



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Over 5,000 flavonoids have been identified to date, and many of them occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health (*31, 34, 35*).

1.5 Biological activities of the flavonoids

The principal activity attributed to the flavonoids is summarized as "vitamin P". They are potentially vein-active by decreasing the permeability of the blood capillaries and reinforcing resistance . The flavonoids have anti-inflammatory (*36, 37*), anti-allergic, hepatoprotective, cytotoxic, anti-tumour (*38, 39*), anti spasmodic, hypocholesterolic, diuretic, antibacterial, and antiviral properties; a few of them exhibited also *in vitro* cytostatic activity (*40-42*).

In traditional medicine, flavonoid-containing drugs are used in Africa and Asia for the treatment of gastro-intestinal infections (intestinal diarrhoea, dysentery and parasites), chronic bronchitis, other respiratory problems (Asthma, bronchitis) and of conjunctivitis (*43*). They are also used in the treatment of the generalized oedemas, the lymphatic circulation, tuberculosis and joints (*44*).

The drugs containing flavonoids are used in the treatment of cramps, of capillary brittleness in the skin and mucous membranes. They are also related to contraception by intra-uterine devices.

Antioxidant activity

Flavonoids are the most commonly known compounds for their antioxidant activity. Additionally, at high experimental concentrations that would not exist *in vivo*, the antioxidant abilities of flavonoids in vitro are stronger than those of vitamin C and E (*45*). Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their putative role in prevention of cancers and cardiovascular diseases (*46*). Physiological evidence of flavonoids effects are not yet established, though the beneficial effects of fruits, vegetables, tea, and red wine have been attributed to flavonoids compounds. Flavonoids may protect against cancer through inhibition of oxidative damage (*47*). They have been shown to have both antioxidant and pro-oxidant activities *in vitro* and in animal models, and have been labeled as "high level" natural antioxidant on the basis of their abilities to scavenge free radicals and active oxygen species (*48, 49*). They contain conjugated ring structures and hydroxyl groups that have the potential to function as antioxidants *in vitro* or cell free systems by scavenging superoxide anion, singlet oxygen, lipid peroxyradicals, and stabilizing free radicals involved in oxidative process through hydrogenation or complexing with oxidizing species (*48*). Flavonoids including naringenin, hesperetin, and apigenin were also found to form pro-oxidant metabolites that oxidized NADH and glutathione upon oxidation by peroxidase / hydrogen peroxide (*50*). Flavonoids have been reported to chelate iron and copper and this may partly explain their antioxidant effects (*51*).

Metabolism and clinical effects

Flavonoids are absorbed by the gastrointestinal tracts of humans and animals, and are excreted either unchanged or as their metabolites in the urine and feces. Colonic bacteria split the heterocyclic ring and degrade flavonoids to phenyl acids, which may be absorbed, conjugated, excreted, or metabolized further by the bacteria (*52*). Some flavonoids glycosides are rapidly deglycosylated by enzymes in human tissues, whereas others may remain unchanged. The rate and extent of deglycosylation depends on the structure of the flavonoid and the position/nature of the sugar substitutions. Measurement of plasma and urine antioxidant power after ingestion of green tea has shown that absorption of antioxidants is rapid (*53*).

Anti-cancer effects

Physiological process of unwanted flavonoid compounds induces so-called Phase II enzymes that also help to eliminate mutagens and carcinogens, and therefore may be of value in cancer prevention. Flavonoids could also induce mechanism that may kill cancer cells and inhibit tumor invasion. In preliminary studies, cancer researchers have proposed that smokers who ate food containing certain flavonoids, such as catechins found in strawberries and green/black teas; kaempferol from brussel sprouts and apples; and quercetin from beans, onions and apples, may have reduced risk of lung cancer (*54*).

1.6 Aim of the present study

Edamane is a preparation of immature soybeans in the pod commonly found in Japan, China, Hawaii, and Korea. Soybeans (Glycine max (L.) Merrill) is an important source of

protein, which is part of the worldwide diet. The physiological functions of soybeans have been studied, but no interest has been paid to the utilization of soybeans leaves, especially for leaves of unripe soybean (Edamame, Glycine max (L.) Merrill. 'Jindai'). Jindai-soybean is a local variety, cultivated in Shonai area, Yamagata Prefecture, Japan. However, leaves of Jindai-soybean at the unripe stage are discarded without being used. Therefore, the use of the leaves and their extracts as raw food materials and food ingredients is very important from the standpoint of the efficient use of bio-resources. Until now, it is known that some leaves of soybeans contain some kaempferol glycosides which are not found in soybeans (*55, 56*), but it has not been examined what flavonoids are contained in the leaves of unripe Jindai-soybean and their physiological functions. In this study, we isolated and identified some polyphenols, and examined the anti-diabetic and anti-obese effect of some major polyphenols from Jindai-soybean leaves. The following steps of investigation have been taken under consideration:

1. Isolation and identification of the polyphenols from Jindai-soybean leaves.
2. Investigation of the anti-diabetic effect of the kaempferol glycosides-rich fraction from Jindai-soybean leaves by applying it on type 2 diabetes mice KK-A^y.
3. Investigation of the anti-obese effect of the kaempferol glycosides-rich fraction from Jindai-soybean leaves by applying it on C57BL mice.
4. Investigation of the anti-obese effect of the isoflavone mixture from Jindai-soybean leaves by applying it on C57BL mice.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF THE POLYPHENOLS OF JIN-DAI-SOY LEAVES

2.1 Introduction

Until now, it is known that the leaves of some soybeans contain some flavonols which are not found in soybeans(*56*), but it has not been examined what flavonoids are contained in the leaves of unripe Jindai-soybean. In this chapter, isolation and identification of the major flavonoids which contained in the leaves of Jindai-soybean (*Glycine max.* L. Merr. 'Jindai') were carried out. In addition, minor components such as coumestan were identified by HPLC, comparing with authentic or previously isolated compounds.

2.2 Material and Methods

Material

The leaves of Jindai-soybean (*Glycine max.* L. Merr. 'Jindai') were collected in September 2008 at the Mogami area of Yamagata Prefecture, Japan. They were then washed with water and dried in the shady open air. The dried leaves were used for the extraction of the polyphenolic compounds.

Extraction Procedure

The dried leaf material was extracted twice with 70% methanol (MeOH) at 60-70 °C under reflux for 3 h. The evaporated extract was dissolved with the mixture of $CHCl_3$/ MeOH/H_2O (1:1:2). The upper phase of the mixture after removing chlorophyll, was concentrated to remove solvent and then applied to Diaion-exchange resin (Diaion, HP-20: Mitsubish Co. Ltd., Japan) or Silica gel (Kanto Chemical Co., Tokyo, Japan) column chromatography. A linear gradient of 0-100% of solvent B (40% CH_3CN) and solvent A (5% CH_3CN in 1% acetic acid) over the course of 180 min at a flow rate of 0.8 ml/min; detection at 330 nm was used to manage the HPLC (Develosil C-30-UG-5: 4.6 i.d. ×250

mm, Nomura Chemical Co., Aichi, Japan) to detect polyphenols. The chromatogram of the polyphenolic fraction was shown in Fig. 2.1.

Isolation of the individual polyphenols on Diaion-exchange resin

After pre-washing the Diaion column with H_2O, the dark-green residue (extracts) was applied to the top of the column, followed by washing with H_2O, and eluted with aqueous 20%, 50% and 100% MeOH solution, successively. Solvent was removed under reduced pressure and further chromatography (Silica gel, Sephadex LH-20 and polyamide column) as well as the preparative HPLC to isolated active compounds (Fig. 2.2. Fig. 2.3). UV, mass spectrometry, ^{1}H and ^{13}C NMR spetra were used for the identification of the compounds. A part of these extracts was used for the animal experiments.

2.3 Equipment

UV spectra were recorded with a Shimzdzu UV-VIS spectrometer (UV -1200). High-resolution electrospray ionization Tof-mass spectroscopy (HRESI-Tof-MS) in the negative mode was performed on Xevo QTof MS with UPLC (Waters, Ltd, Milfoed, MA, USA) using the column (BEH C-18, 0.5 × 50 mm, 1.7 μm, Waters. USA) and solvent system composed of 0.1% HCOOH in water (solvent A) and 0.1 % HCOOH in acetonitril (solvent B). ^{1}H-, ^{13}C-, ^{1}H-^{1}H COSY-, ^{1}H-^{13}C COSY-, DEPT-, HMQC-, and HMBC-NMR spectra were measured with a JEOL-Gx-400 spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts are given in δ ppm relative to DMSO-d6. The HPLC analysis was performed on a Develosil ODS-UG-5 & ODS-HG-5 (4.6 × 250 mm) packed column (Nomura Chemical Co., Ltd. Japan) with a Hitachi L- 7405 UV-Vis & L-7455 Diode Array detector, a Hitachi L-7120 pump and a Hitachi column oven L-7300. Sugars were

identified by silica gel TLC using solvent system composed of.

2.4 Results and Discussion

Treatment of 20% MeOH fraction

The 20% MeOH fraction was subjected to Silica gel column chromatography, eluted with ethyl acetate:methanol 9:1, 8:2, 7:3, and finally 5:5. The eluted fractions (17ml each), were combined together according to the TLC behavior. The fraction No.412-441 were chromatographed on polyamide column eluted with solvent system of EtOAC:$CHCl_3$:HCO_2H:H_2O at (19:1:1:1). Tube No.194 was purified and identified as kaempferol 3-*O*-β-D-(2,6-di-*O*-α-L-rhamnopyranosyl) galactopyronoside (compound 4) by UV, ^{1}H & ^{13}C NMR and mass spectrophotometry. Fraction No.197-212 was further fractionated by Sephadex LH-20 column chromatography using solvent system of 50% MeOH to obtain fraction No.42-58. The sub-fraction No.1 was purified by preparative HPLC and kaempferol 3-*O*-β-D-glucopyranosy(1→2)-*O*-[α-L-rhamnopyranosyl (1→6)]-β-D-galactopyranoside (compound 1) was identified using UV, ^{1}H & ^{13}C NMR and mass spectrometry. The sub-fraction No.2-4 were collected and purified by preparative HPLC, to get Kaempferol 3-*O*-β-D-glucopyranosyl (1→2) -*O*-[α-L-rhamnopyranosyl (1→6)]-β-D-glucopyranoside (compound 2) and kaempferol 3-*O*-β-D-(2-*O*-β-D-glucopyranosy) galactopyranoside (compound 3) (Fig. 2.2).

Treatment of 50% MeOH fraction

The 50% MeOH fraction was first subjected to sephadex LH-20 column chromatography using solvent system of 50% MeOH to obtain fraction No.32-35 and 36-37. The fraction No.32-35 was then fractionated by preparative HPLC and purified to give narigenin

7-*O*-glucose (11). The silica gel fractions No. 36-37 was purified by preparative HPLC too, to give apigenin 7-*O*-glucose (12) (Fig. 2.2).

Treatment of 100% MeOH fraction

The 100% MeOH fraction was first subjected to Silica gel column chromatography and elution was made with the solvent system ethyl acetate:methanol (9:1, 8:2,7:3, and 5:5). The sub-fraction No.141-190 and No.191-242 were combined according to the TLC. Using Sephadex LH-20 and preparative TLC, fraction No.141-190 was purified to give genistin (9) and kaempferol 3-*O*-glucose (13). The fraction No. 191-242 was purified too by Sephadex LH-20 and preparative HPLC to give genistein 7-*O*-glucose (8) (Fig. 2.2).

Polyphenols isolated from Silica gel column chromatography

The fraction containing polyphenols was first subjected to Silica gel column chromatography, eluted with ethyl acetate:methanol (9:1, 8:2, 7:3, and 5:5). The sub-fractions were passed through several sephadex LH-20 and preparative HPLC columns, to purify and to identify compounds 3,4-dihydroxycinnamic acid (5), daidzin (6), glycitin (7), 1-hydeoxy coumesterol (16), and 8-hydoxy coumesterol (17) (Fig. 2.3).

Identification of some major compounds

(Kaempferol 3-*O*-β-D-glucopyranosyl (1→2)-*O*-[α-L-rhamnopyranosyl (1→6)]-β-D-galactopyranoside) (1)

The HRESI-Tof-MS of the compound 1 showed *pseudo* molecular ions ([M-H]-) at m/z 755.2059 (755.2035, calculated for $C_{33}H_{39}O_{20}$), indicating their respective molecular formulas to be $C_{33}H_{40}O_{20}$. The fragment ion due to ([M-(galactose+gluctose+rhamnose)

moiety]⁻), at m/z 285.0396~285.0400 (285.0410, calculated for $C_{15}H_{10}O_6$) in compound 1 indicated that its aglycone was kaempferol. In the HMBC spectrum of compound 1 (Fig. 2.8) (in DMSO-d6), the anomeric proton at δ 5.60 (1H, d, J=7.3 Hz) showed a cross peak with the carbon signal at δ 132.78, indicating that a sugar moiety with the anomeric proton at δ 5.60 was attached to the C-3 position of kaempferol via the 1"-hydorxyl group. The other anomeric proton of the compound 1 at δ 4.57 (^{1}H, d, J=7.3 Hz) showed a cross peak with the carbon signal at δ 80.13 which corresponded to the C-2" position of the core sugar moiety, showed that an outer sugar moiety with an anomeric proton at δ 4.57 attached to the C-2" position of core sugar moiety. Anomeric protons at δ 4.57 (1H, s) of the other sugar moiety (rhamnose moiety) of the compound 3, showed a cross peak with the carbon signal at δ 64.73 which corresponds to the C-6" position of the core sugar moiety. The chemical shifts of compound 1 in the ^{13}C-NMR spectrum agreed well with the values recorded in the previous study (*57*). Taken together, compound 1 was identified as kaempferol 3 -*O* -β -D - glucopyranosy (1→2) -*O*- [α-L-rhamnopyranosyl (1→6)] -β -D-galactoyranoside. The ^{13}C-NMR spectrum of the compound 1 and chemical shift of each carbon signal are shown in Fig. 2.4 and Table 2.1, respectively.

Kaempferol 3 -*O* -β -D –glucopyranosyl (1→2)-*O*-[α-L-rhamnopyranosyl (1→6)]-β-D-glucopyranoside (2)

In the HMBC spectrum (Fig. 2.9) of compound 2, the anomeric proton at δ 4.58 (^{1}H, d, *J* =7.8 Hz) showed a cross peak with the carbon signal at δ 82.0, indicating that a sugar moiety with the anomeric proton at δ 4.58 was attached to the C-2" position of core sugar (glucose) moiety *via* the 1"'-hydorxyl group. By the irradiation of the anomeric proton at δ 5.51 (1H, bd, *J* =6.3 Hz), the weaker negative NOE (Nuclear Overhauzer Effect) was

observed on H-6' of kaempferol moiety, indicating that a sugar moiety with the anomeric proton at δ 5.51 was attached to the C-3 position of kaempferol *via* the 1"-hydorxyl group. A cross peak between an anomeric proton at δ 4.31 (1H, bs,) and C-6" at δ 66.08 of the core sugar moiety indicated that the other moiety with the anomeric proton at δ 4.31 was attached to the C-6" of the core sugar moiety *via* the 1""- hydoroxyl group. Chemical shifts due to two molecule sugar moieties (core sugar moiety + sugar moitety with the anomeric proton at δ 4.58 (^{1}H, d. J=7.8 Hz) of the compound 2 in the ^{13}C-NMR spectrum was very similar to those of kaempferol 3-*O*-sophoroside (*58*), except for the downfield shift of C-6" of the core sugar moiety, indicating that compound 2 is kaempferol 3-*O*-sophoroside attached with a sugar moiety at its C-6" position. Chemical shifts of the sugar moiety attached to C-6" was almost the same as those of compound 3 as described later, indicating that the sugar moiety is rhamnose. From these results, compound 2 was identified as kaempferol 3-*O*-β-D-glucopyranosyl(1→2)-*O*-[α-L-rhamnopyranosyl(1→6)] -β-D-glucopyranoside. The ^{13}C-NMR spectrum of the compound 1 and chemical shift of each carbon signal are shown in Fig. 2.5 and. Table 2.1, respectively.

Kaempferol 3-*O*-β-D-(2-*O*-β-D-glucopyranosyl) galactopyranoside (3)

The ^{13}C-NMR chemical shifts of compound 3 were almost the same with those of compound 1, except that the chemical shift due to the rhamnose moiety of compound 1, was not observed and that the chemical shift due to the C-6" position (core sugar moiety) shifted to a higher field. The ^{13}C NMR spectrum and chemical shift of each carbon signal in the compound 3 are shown in Fig. 2.6 and Table 2.1, respectively. From these results, compound 2 was identified to be kaempferol 3 -*O* -β -D - (2 -*O* -β -D -glucopyranosyl) galactopyranoside.

kaempferol 3-*O*-β-D- (2,6-di-*O*-α-L-rhamnopyranosyl) galactopyronoside (4)

In the HMBC spectrum (Fig. 2.10) of compound 4, the anomeric protons at δ 4.35 (^{1}H, bs) and 5.05 (^{1}H, bs) due to the two different sugar moieties (rhamnose moieties) showed cross peaks with the carbon signals at δ 65.09 and 74.81 which corresponded to the C-6" and C-2" positions, indicating that the respective rhamnose moieties were attached to C-6" and C-2" *via* their respective 1"' and 1"" hydroxyl groups. An anomeric proton at δ 5.56 (1H, d, *J* =7.8 Hz) (core sugar moiety) showed a cross peak with a carbon signal at δ 132.65, indicating that the anomeric proton at δ 5.56 was attached to the C-3 position of the kaempferol moiety. The chemical shifts of compound 4 in the ^{13}C NMR spectrum (Fig. 2.7) were very similar with those of the literature values (*56*). Taken together, compound 4 was identified as kaempferol 3-*O*-β-D-(2,6-di-*O*-α-L-rhamnopyranosyl) galactopyronoside.

2.5 Summary

Some flavonols, isoflavones and coumestans were separated and identified from the polyphenolic fraction of the leaves of Jindai. Isolated compounds were daidzin, glycitin, genistein 7-*O*-glucose, genistin, narigenin 7-*O*-glucose, apigenin 7-*O*-glucose, daizein, 2-Hydeoxy coumesterol, coumesterol (Fig.2.1). The major components of Jindai-mame leaves-polyphenolics, judged from HPLC chromatogram, were kaempferol 3-*O*-β-D-glucopyranosyl (1→2) -*O*-[α-L-rhamnopyranosyl (1→6)]-β-D-galactopyranoside, kaempferol 3 -*O* -β -D -glucopyranosyl (1→2)-*O*-[α-L-rhamnopyranosyl (1→6)] -β -D-glucopyranoside, 3-*O*-β-D- (2-*O*-β-D-glucopyranosyl) -galactopyranoside and kaempferol 3-*O* -β –D - (2,6 -di -*O* -α -L -rhamnopyranosyl) galactopyronoside. And the former 3 compounds had not yet been reported on the leaves of unripe soybeans.

1. **Kaempferol 3-*O*-β-D-glucopyranosyl(1→2)-*O*-[α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside**
2. **Kaempferol 3-*O*-β-D-glucopyranosyl(1→2)-*O*-[α-L-rhamnopyranosyl(1→6)]-β-D-glucopyranoside**
3. **Kaempferol 3-*O*-β-D-(2-*O*-β-D-glucopyranosyl) galactopyranoside**
4. **kaempferol-3-*O*-β-D- (2,6-di-*O*-α-L-rhamnopyranosyl) galactopyronoside**
5. **3,4-dihydroxycinnamic acid**
6. **Daidzin**
7. **Glycitin**
8. **Genistein 7-O-glucose**
9. **Genstin**
10. **Rutin**
11. **Narigenin 7-O-glucose**
12. **Apigenin 7-O-glucose**
13. **Kaempferol 3-O-glucose**
14. **Malonyl-Genistin**
15. **Daizein**
16. **1-Hydeoxy Coumesterol**
17. **8-Hydoxy Coumesterol**

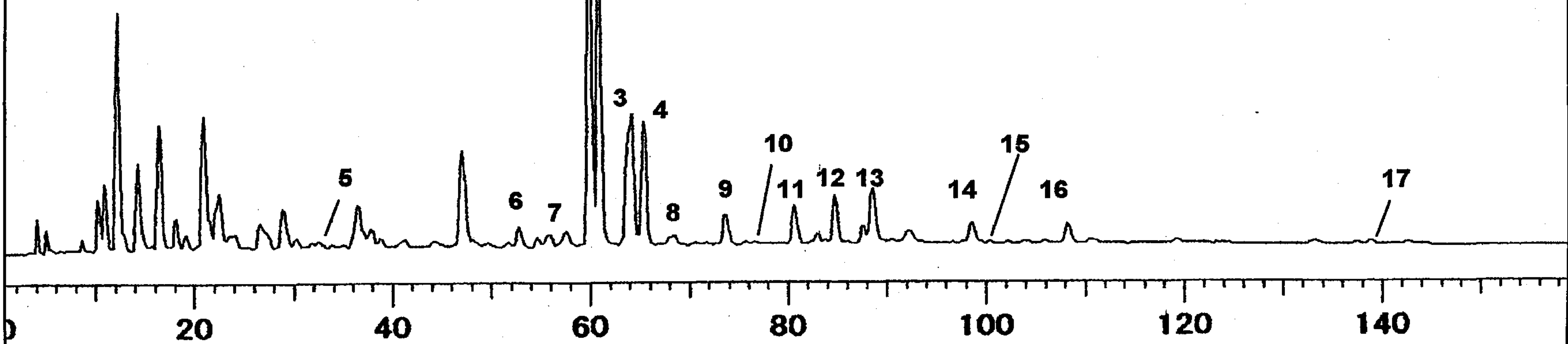


Fig. 2.1 HPLC chramatography polyphenols in Jindai-mame leaves

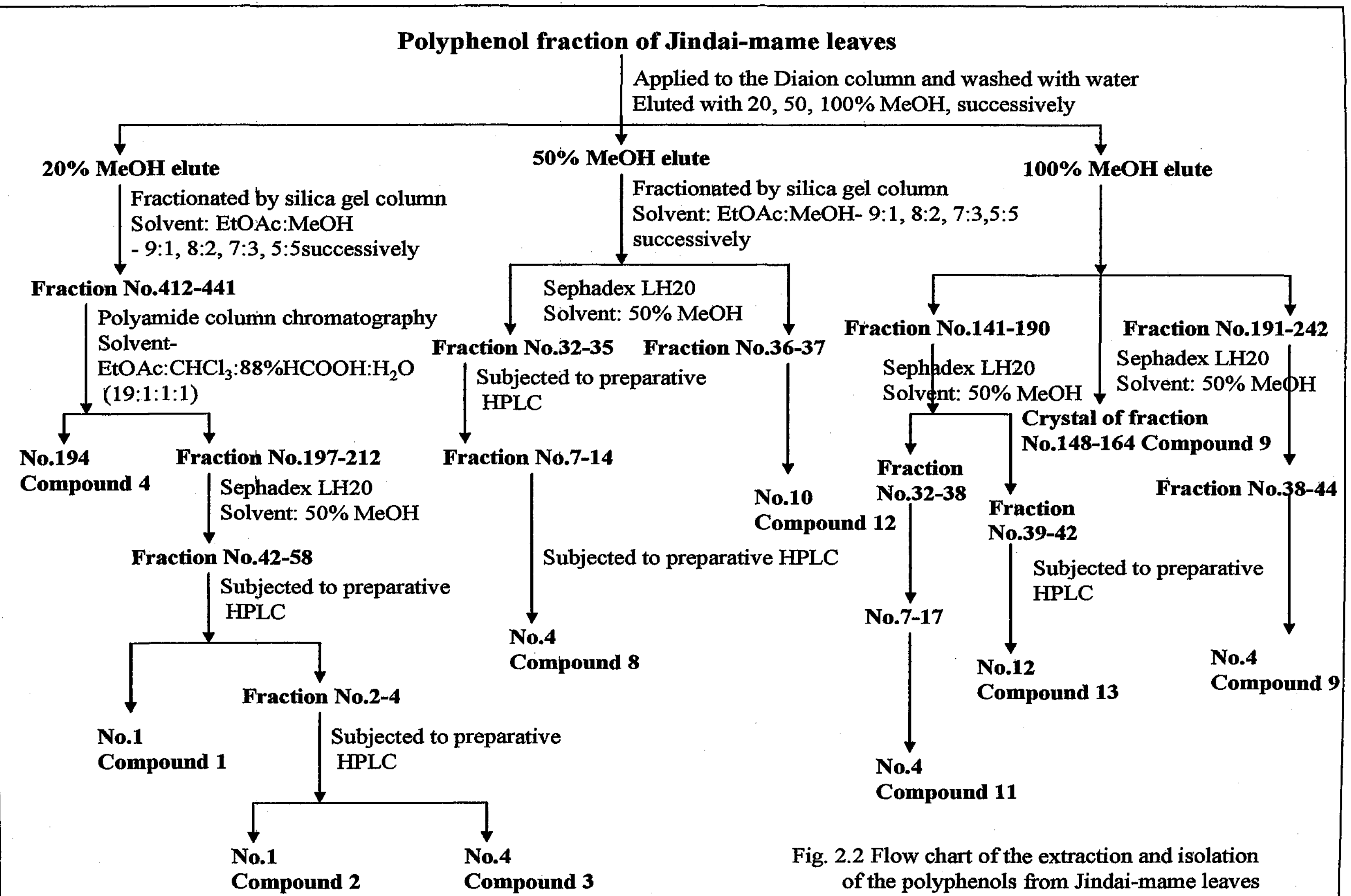


Fig. 2.2 Flow chart of the extraction and isolation of the polyphenols from Jindai-mame leaves

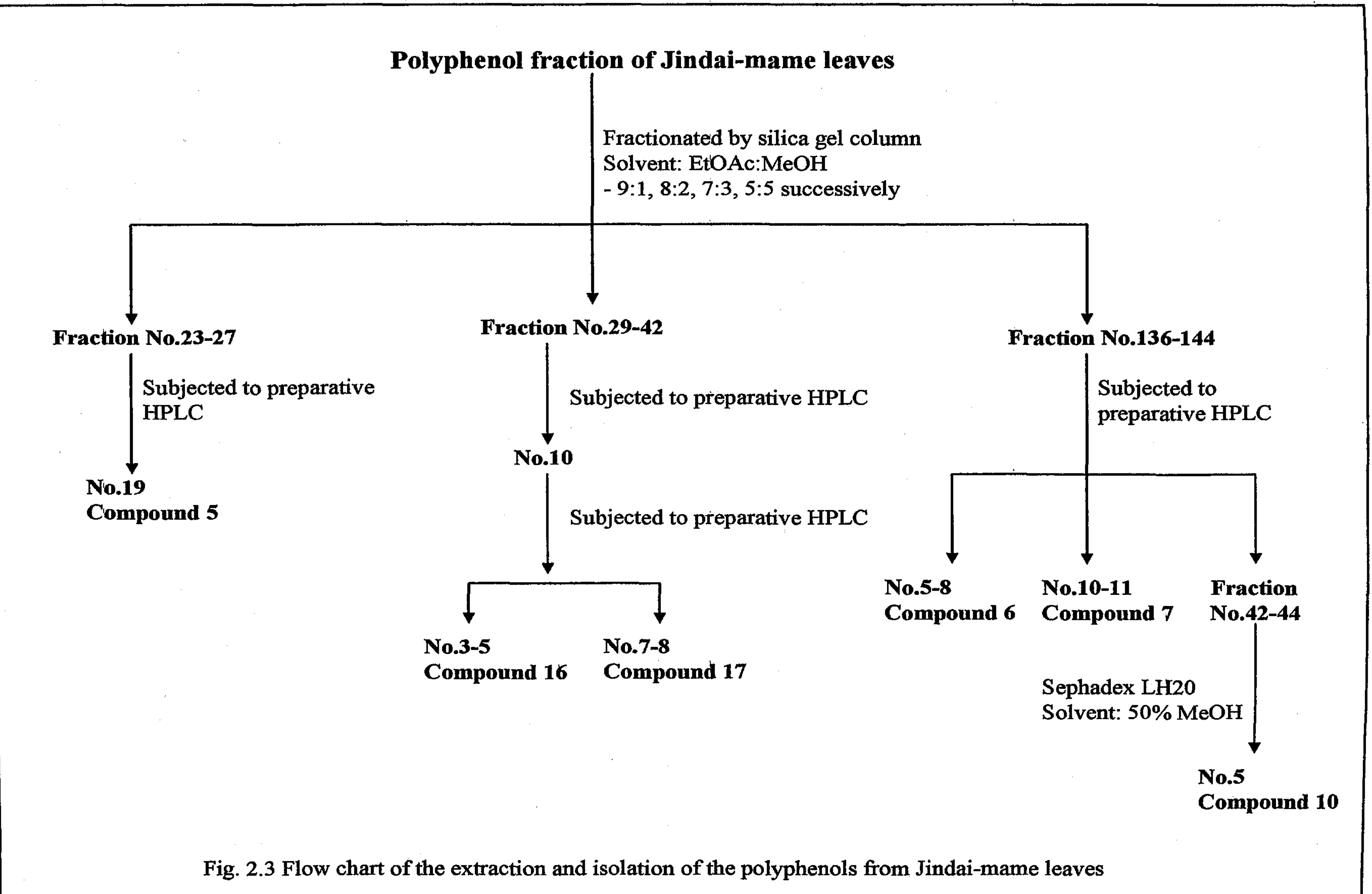


Fig. 2.3 Flow chart of the extraction and isolation of the polyphenols from Jindai-mame leaves

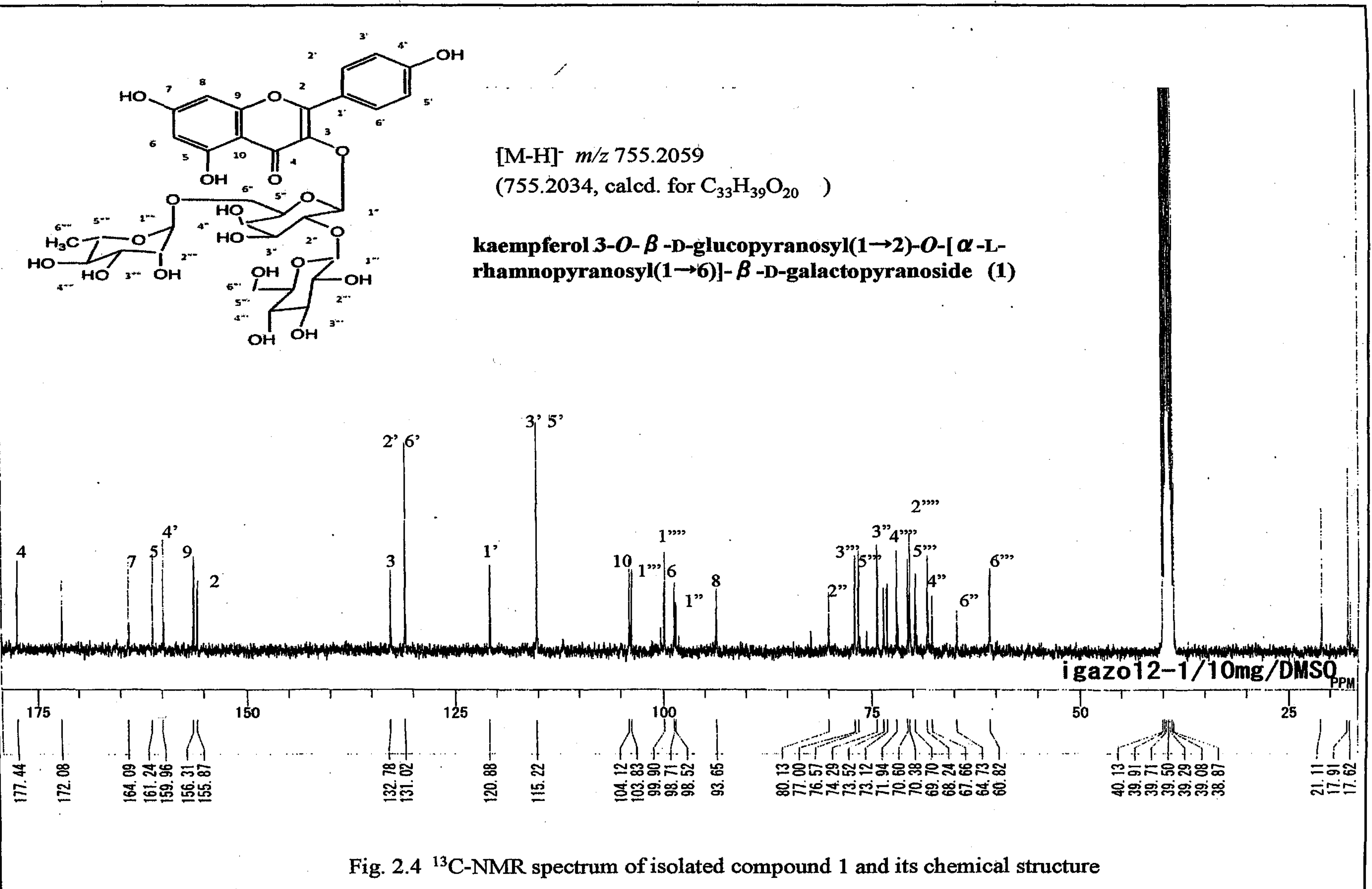


Fig. 2.4 ^{13}C-NMR spectrum of isolated compound 1 and its chemical structure

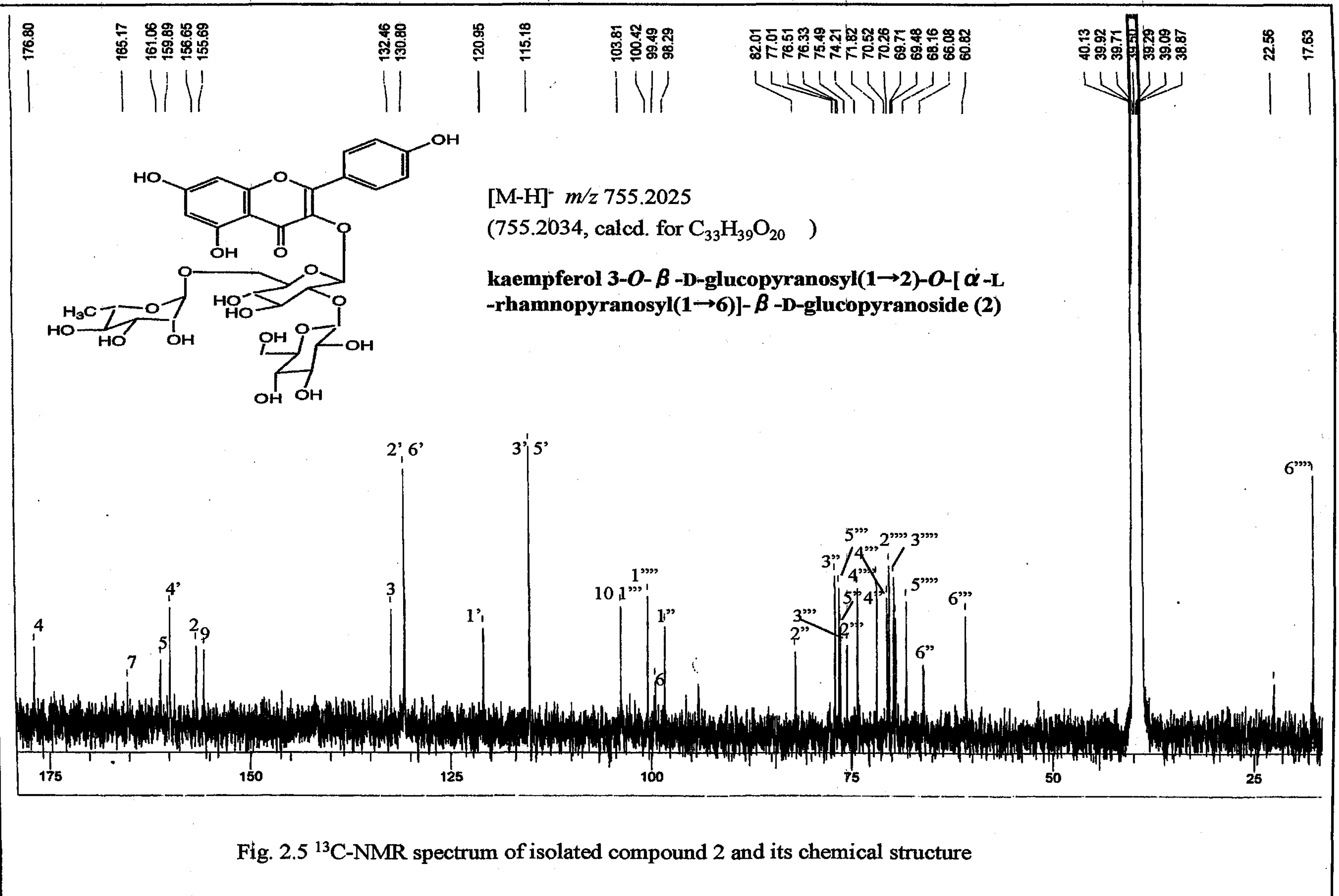


Fig. 2.5 ^{13}C-NMR spectrum of isolated compound 2 and its chemical structure

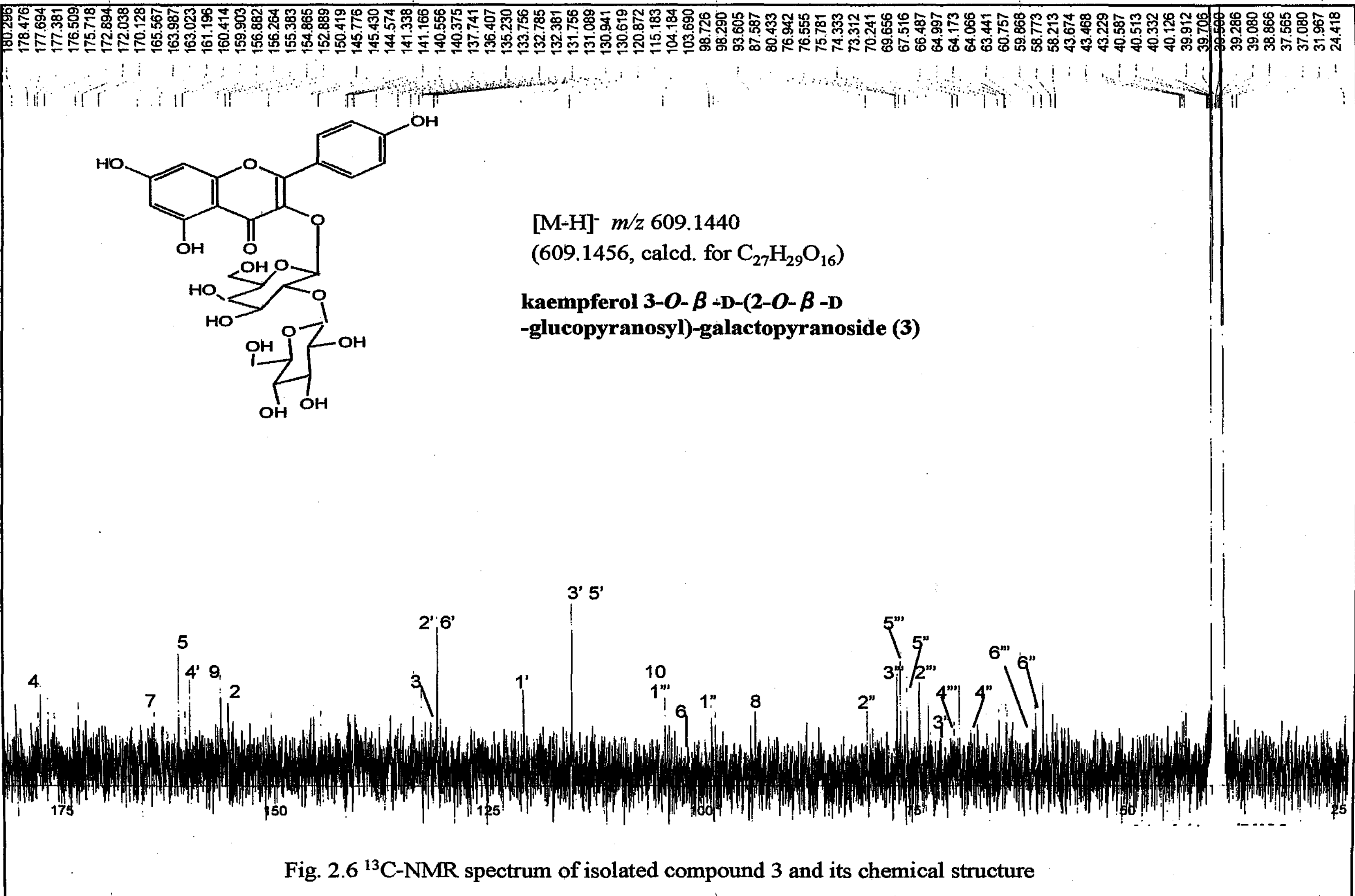


Fig. 2.6 ^{13}C-NMR spectrum of isolated compound 3 and its chemical structure

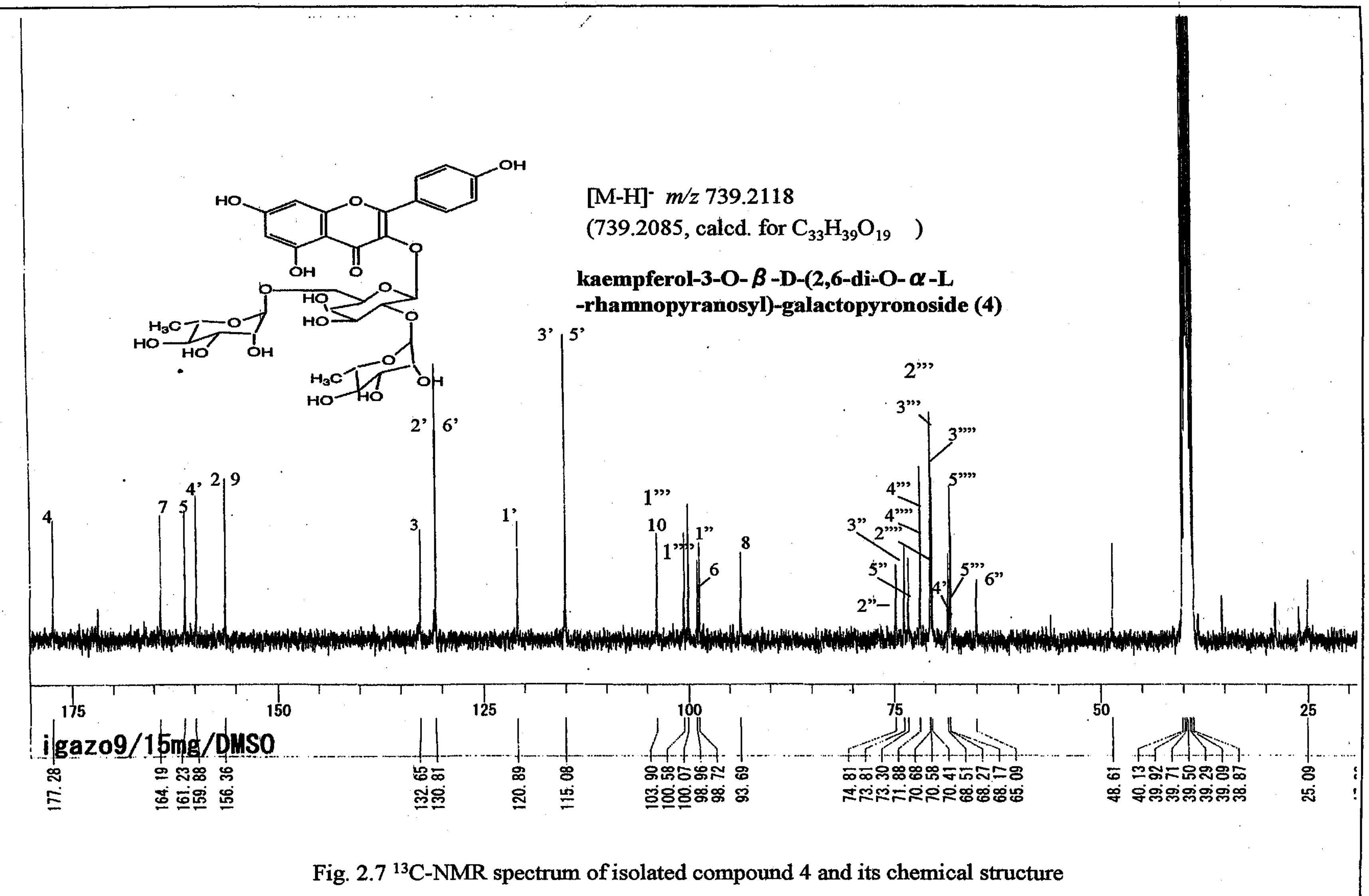


Fig. 2.7 ^{13}C-NMR spectrum of isolated compound 4 and its chemical structure

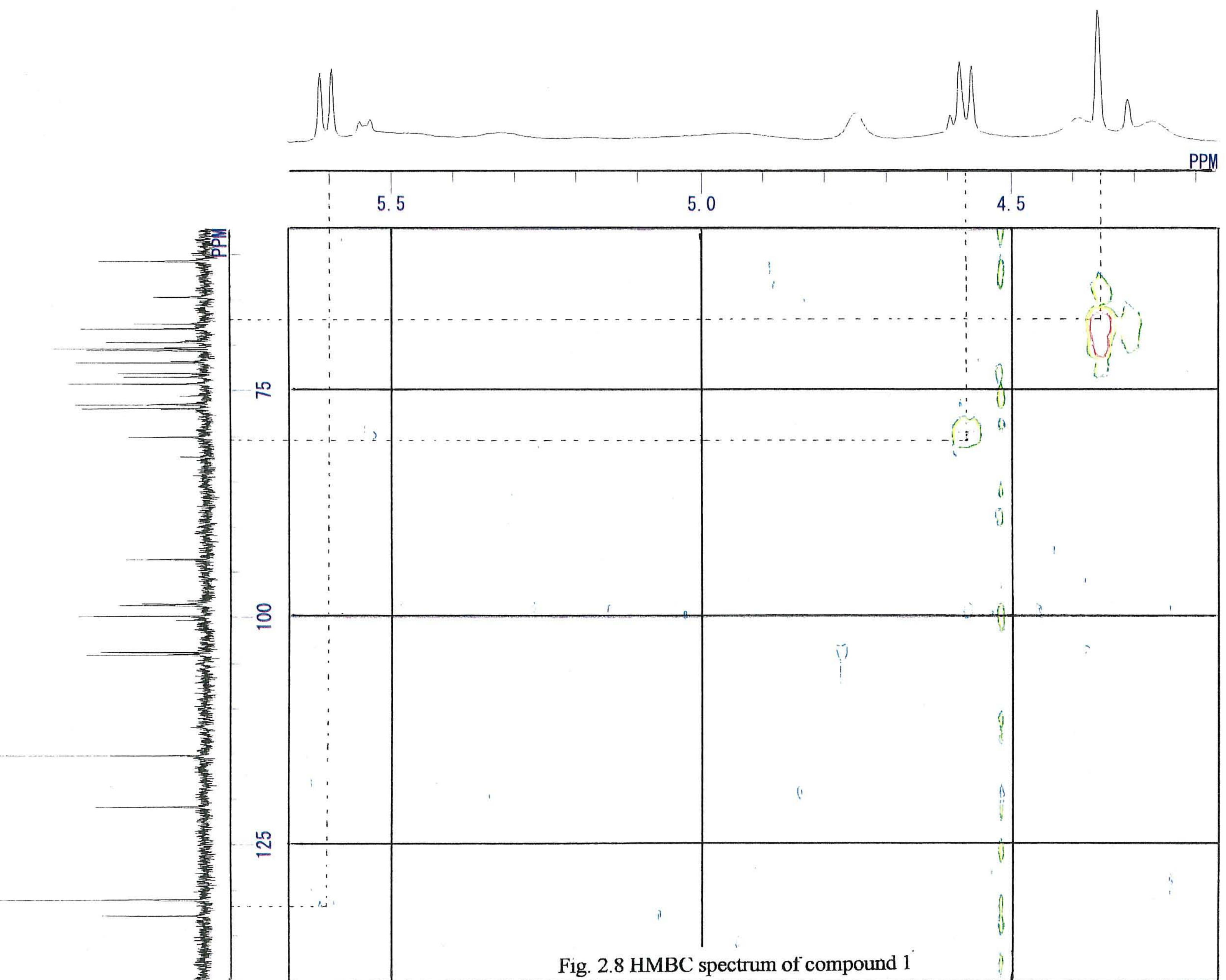


Fig. 2.8 HMBC spectrum of compound 1

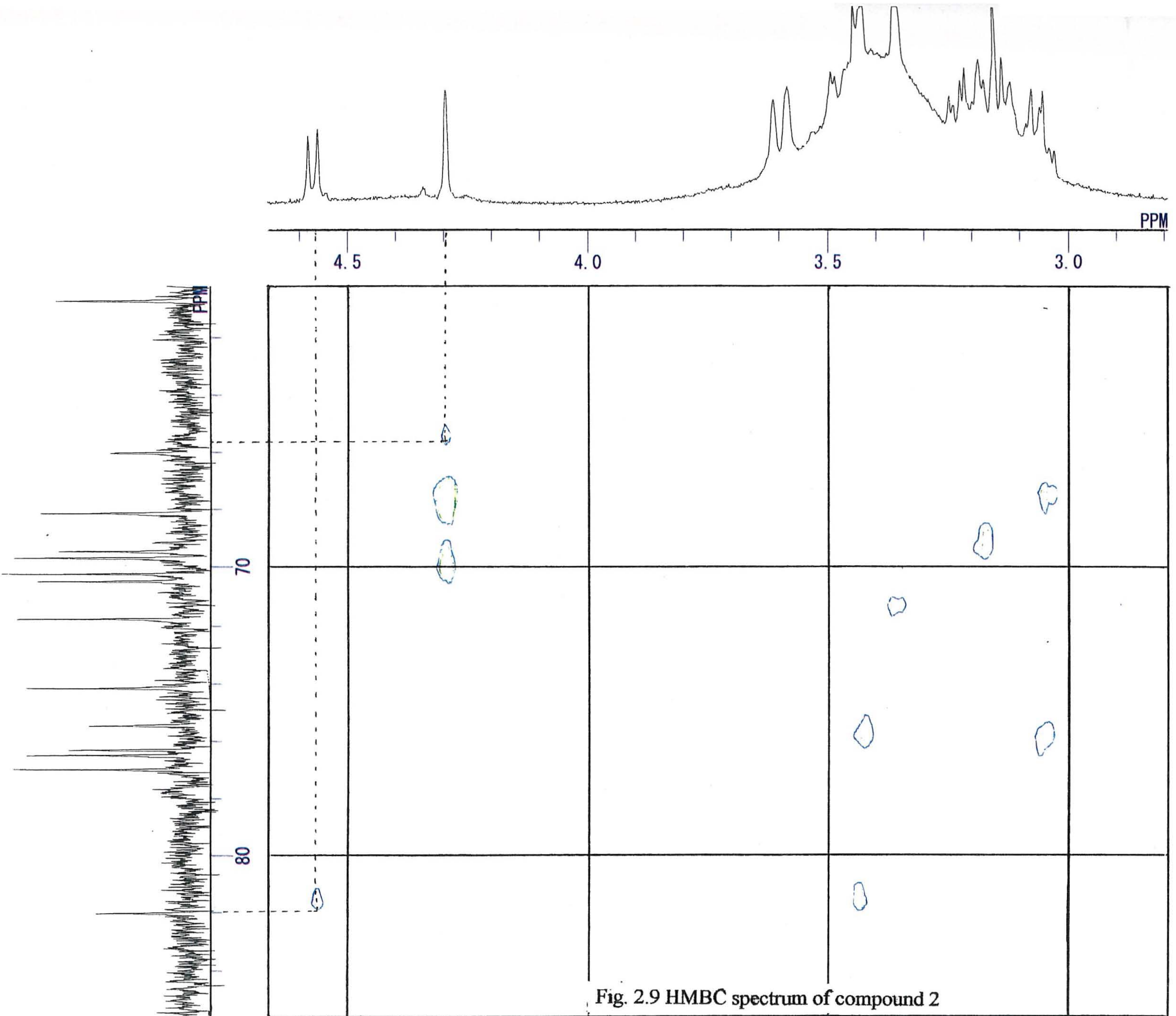


Fig. 2.9 HMBC spectrum of compound 2

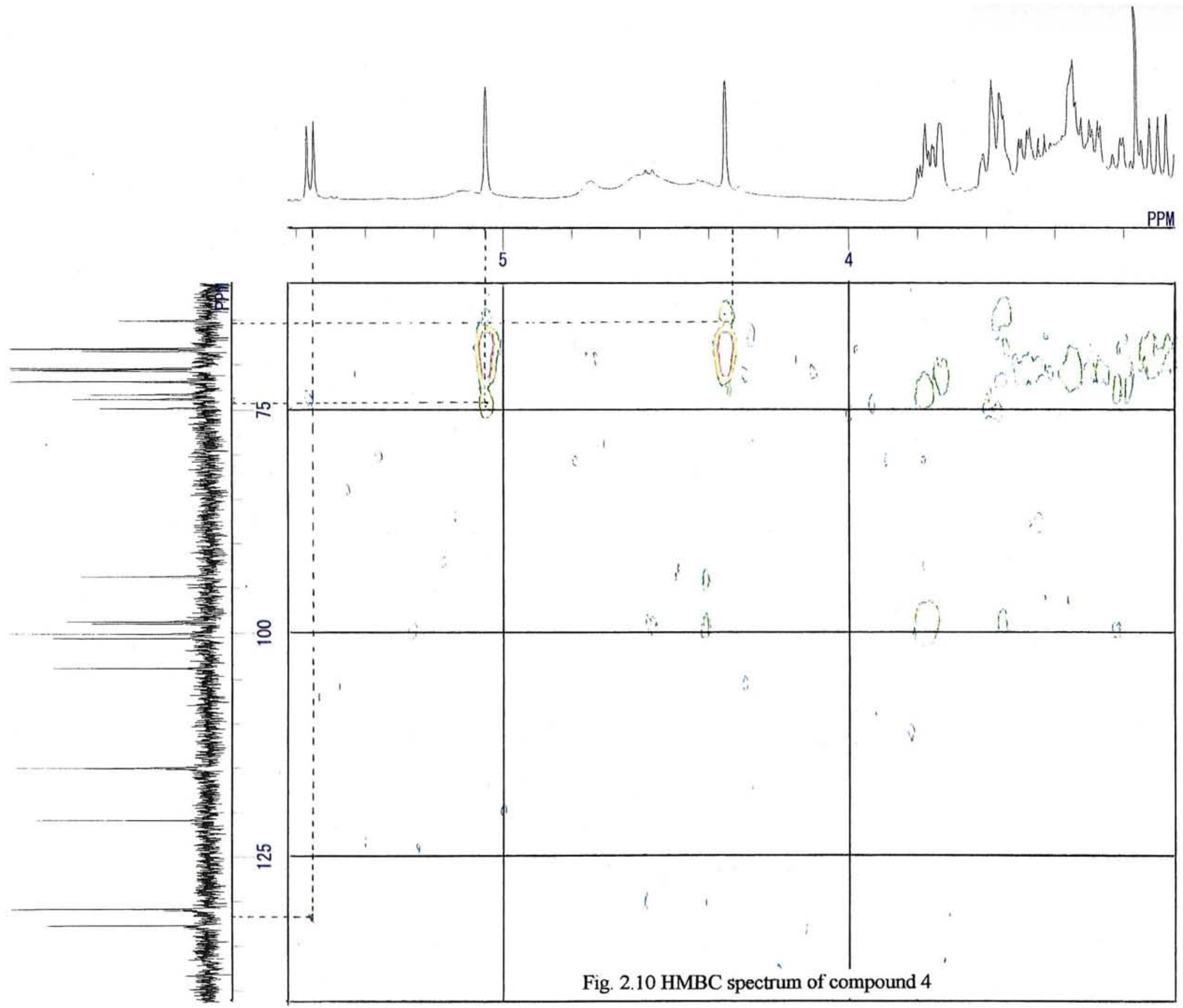


Fig. 2.10 HMBC spectrum of compound 4

Table 2.1. ^{13}C-NMR spectra data for compounds 1-4 isolated from kaempferol glycoside-rich fraction (in DMSO-d6)

Position [a]	Chemical shifts (δppm)			
	Compund 1	Compound 2	Compound 3	Compound 4
Aglycone				
2	155.87	156.65	156.4	156.36
3	132.78	132.46	133.29	132.65
4	177.44	176.8	175.1	177.28
5	161.24	161.06	160.87	161.23
6	98.71	99.49	100.84	98.72
7	164.09	165.17	166.3	164.19
8	93.65	94.08	93.8	93.69
9	156.31	155.69	156.9	156.36
10	104.12	103.81	103.98	103.9
1'	120.88	120.95	121.17	120.89
2'	131.02	130.8	130.55	130.81
3'	115.22	115.18	115.14	115.08
4'	159.96	159.69	159.8	159.88
5'	115.22	115.18	115.14	115.08
6'	131.02	130.8	130.55	130.81
Sugar moiety				
	Galactosyl	Glucosyl	Galactosyl	Galactosyl
1"	98.52	98.28	98.45	98.69
2"	80.13	82	80.23	74.81*
3"	74.29	77.01	73.27	73.81
4"	67.66	70.52	67.43	68.51
5"	73.12	75.49	75.59	73.30*
6"	64.73	66.08	59.78*	65.09
	Glucosyl	Glucosyl	Glucosyl	Rhamnosyl
1"'	103.83	103.81	103.98	100.07
2"'	73.52	74.21	74.23	70.41
3"'	77,70*	76.51	76.98	70.58
4"'	69.9	70.26	69.71	71.88
5"'	76.57*	76.33	76.57	68.17
6"'	60.82	60.82	60,80*	17.92
	Rhamnosyl	Rhamnosyl		Rhamnosyl
1""	99.9	100.42		100.58
2""	70.6	69.71		70.68
3""	70.39	69.48		70.58
4""	71.94	71.82		71.88
5""	68.24	68.16		68.27
6""	17.62	17.63		17.26

[a] See the carbon number in Fig.2.4 for an example. * Assignmets are interchangeable in the same column.

CHAPTER 3

ANTI-DIABETIC EFFECT OF KAEMPFEROL AND KAEMPFEROL GLYCOSIDE-RICH FRACTION FROM JINDAI-SOY LEAVES ON KK-A^y MICE

3.1 Introduction

Type 2 diabetes is the most common type of diabetes and has developed into worldwide (*59*). The number of patients is estimated to increase around 300 million by 2025 in the world. The major pathophysiological background of type 2 diabetes is insulin resistance, obesity, and excessive energy intake (*60*). Diabetes (type 2) has increased in children and adolescents. Thus, effective treatment of type 2 diabetes and the metabolic syndrome as a cause of diabetes would be of great benefit to the individual and society (*61*).

Flavonoids are polyphenolic compounds widely distributed throughout the plant kingdom (*62*). They are important to protect the leaves from UV rays during their growth (*63*). Most flavonoids are natural antioxidants which reduce the risk of cancer, aging and cardiovascular diseases (*64*). Their important roles include free radical scavengers, reducing agents, protectors against lipid peroxidation and quenchers of the reactive oxygen species (*65*).

In the plant kingdom, many compounds are reported to exist in glycoside or in its aglycone form. A previous study has proved that the bioavailability of the aglycone form is higher than the glycoside form on isoflavones (*66*). A study shows that glycosylation attenuated the efficiency in inhibiting the enzyme xanthine oxidase and the aglycone act as an active chelator for metallic ions (*67*). However, to the best of our knowledge, the preventive effect of dietary kaempferol glycoside and its aglycone for diabetes in the type 2 model mice KK-A^y have not yet been studied.

In this study, the effects of kaempferol glycoside-rich fraction, prepared from the leaves of Jindai-soybean, on the markers of diabetes, serum and liver lipid levels were determined in KK-A^y mice, an obese diabetic animal model, comparing with those of kaempferol, an aglycon of kaempferol glycoside.

3.2 Materials and Methods

Materials

Jindai-soybean (*Glycine max.* L. Merr. 'Jindai') leaves were collected in September 2008 at the Mogami area of Yamagata Prefecture, Japan. Leaves were then washed with water and dried in the shady open air. The dried leaves were extracted with 70% methanol (MeOH) at 60-70 °C under reflux for 3 h. The evaporated extract was dissolved with the mixture of $CHCl_3$/MeOH/H_2O (1:1:2). The upper phase of the mixture was applied to Diaion-exchange resin (Diaion, HP-20: Mitsubish Co. Ltd., Japan) column after its concentration. After washing the column with H_2O, phenolic compounds were eluted with aqueous 20%, 50% and 100% MeOH solution, successively. The 20% MeOH fraction was applied to silica gel column and eluted with solvent system *n*-hexane/EtOAc and EtOAc/MeOH systems as developing solvent. The fraction containing kaempferol glycosides were checked by HPLC with diode array detection equipment (Fig. 1(A)), and used in animal experiment as KG. HPLC was performed by using Develosil C30-UG5 column (4.6 i.d. × 250 mm, Nomura Chemical Co., Aichi, Japan) and a solvent system of 5% MeCN in 1% acetic acid (A) and 40% MeCN (B). A linear gradient of 0-100% of solvent B in solvent A over the course of 180 min at a flow rate of 0.8 ml was used.

Animals

6 weeks old KK-A^y (TaJcl) male mice (average weight, 29 g) and 6 weeks old C57BL/6J male mice (average weight, 19 g), were purchased from Clea Ltd. (Tokyo, Japan) and housed individually under a 12:12 h light-dark cycle at 22 ± 2 °C and 40-60% humidity. After acclimatizing for 5 d, the KK-A^y mice were divided into 3 groups of 6 each: basal diet group (CON group); basal diet plus kaempferol glycoside-rich fraction (KG group)

and basal diet plus kaempferol (K group). The C57BL/6J mice group was fed with the basal diet (6 mice). In the K and KG groups, K and KG were added at 0.095% and 0.15% levels in the respective diets. The composition of each experimental diet is shown in Table 3.1. The diets and water were given for 30 d *ad libitum*. The body weight was measured every other day and the diet was measured every day. At the end of the feeding period, the mice were anesthetized with Nembutal (Dainippon Pharmaceutical Co. Osaka, Japan) after 10 h of fasting, and the blood was collected by cardiac puncture, followed by detaching the liver. Liver lipids were extracted by the method of Floch (*68*). The serum was prepared by centrifuging the blood at 3000 × *g* for 10 min. Kaempferol was purchased from Extrasynthese, (Genay, France).

The mice were cared for according to the institutional guidelines of Yamagata University.

Measurement of fasting blood glucose levels and glucose tolerance test

The blood samples were collected from the tail vain after 10 h fasting every week, and the levels of fasting blood glucose were measured, using a commercial glucose analyzer Medesafe GR-102 (Termo Co., Tokyo, Japan, 20-600 mg/dl measuring range). An oral glucose tolerance test (OGTT) was performed on day 26 after 10 h of fasting. The mice were administered with glucose solution (1g/kg of body weight). Blood samples were collected from the tail vein at 0, 30, 60 and 120 min, after glucose loading, and its glucose levels were measured with the Medesafe GR-102.

Measurements of the insulin and adiponectin levels

The serum levels of insulin, adiponectin, high molecular weight (HMW) adiponectin, and

leptin were measured with commercial ELISA kits (each, Mouse insulin (S-Type) Kit, Shibayagi Co., Gunma, Japan; Mouse adiponectin kit, Otsuka Co., Tokyo, Japan; Mouse high molecular weight adiponectin kit, Shibayagi Co., Gunma, Japan; Mouse leptin Kit, Morinaga Co., Tokyo, Japan).

Hemoglobin A_{1c} (HbA_{1c}) levels were measured with a Micromat II (Bio-Rad Laboratories, California, USA) at 10:00-11:00 am after 10 h of fasting at the end of the 4th week of the feeding period.

Lipid analyses

The serum and liver total-cholesterol (T-Chol), HDL-cholesterol (HDL-Chol), triglyceride (TG), and total non-esterified fatty acids (NEFA) levels were measured with commercial kits (Cholesterol E test, HDL-cholesterol E test, Triglyceride E test, and NEFA C test kits, respectively; Wako pure Chemical Industries, Osaka, Japan).

Measurements of liver enzyme activities

Samples to measure the liver enzyme activities were prepared by homogenizing the liver in a 3 mM Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose and 1mM EDTA. The supernatant of the homogenate obtained by centrifuging at 500 × *g* for 10 min at 4 °C was re-centrifuged at 9,000 × *g* for 10 min at 4 °C, and further centrifuged at 105,000 × *g* for 60 min.

The fatty acid synthase (FAS) activity was determined in terms of malonyl-CoA-and acetyl-CoA-dependent oxidation of NADPH according to the method of Kumer *et al.* and Carey *et al.* (*69, 70*). The reaction mixture was composed of a 0.1M phosphoric buffer (pH 7.0) containing 0.2 mM EDTA, 0.3 mM NADPH, 0.05 mM acetyl-CoA, 0.2 mM

malonyl-CoA. The rate of decrease on the absorbance at 340 nm was measured.

The reaction mixture to measure the carnitine palmitoyl transferase (CPT) activity, was composed of a 58 mM Tris-HCl buffer (pH 8.0) containing 0.25 mM DTNB, 0.04 mM palmitoyl-CoA, 1.25 mM EDTA and 1.25 mM L-carnitine. The CPT activity was determined from the rate of change in absorbance at 412 nm (*71*).

Fecal lipid analyses

Feces were collected on day 26-27 for 48 h. Fecal total lipids was extracted by the method of Folch *et al.* (*68*). The lipids in the extracts were measured with commercial kits as described above. Total bile acid (T- BA) levels were measured by the Total bile acid test kit (Wako pure Chemical Industries, Osaka, Japan).

Statistical analysis

Each value is given as the mean ± SEM. The homogeneity of the variance between treatments was verified by the Bartlett's test. Data were statistically analyzed by a one-way analysis of variance (ANOVA). A *Post–hoc* analysis of significance was made by using the Fisher's PLSD test, where the differences were considered significant at $p < 0.05$.

3.3 Results

Properties of kaempferol glycoside-rich fraction

HPLC chromatograms of 70% MeOH extract of the leaves of unripe Jindai-soybean, and KG prepared from it are shown in Fig. 3.1 (A) and 3.1 (B). A comparison of the two HPLC chromatograms shows that compounds 1, 2, 3, and 4, which are the major

components in the KG, are also contained as major components in the 70% MeOH extracts of the leaves, indicating that the major components in the leaves are recovered in the KG which is used in the animal experiments. The amounts of compounds 1, 2, 3 and 4 in the dry leaves were 6.8, 6.7, 10.1, and 5.7 mg/g, and in the KG were 244, 241, 99, and 49 mg/g dry weight, respectively.

Food intake, body weight gain and organ weights

There were no significant differences in the food intake and body weight gain among the 3 groups except the C57BL group. The liver, kidney and white adipose tissue weights also did not differ to a significant level among the 3 groups except the C57BL group (Table 3. 2).

Fasting blood glucose levels, and glucose levels in oral glucose tolerance test (OGTT)

The fasting blood glucose levels in the K and KG groups tended to be lower than that of the CON group, but no significant difference was found (Fig. 3.3). Blood glucose levels on the OGTT carried out on the 26th day did not differ among the CON, K and KG groups (Fig. 3.4 (A)). The area under the curve (*72*) on the OGTT tended to be lower in the K and KG groups than in the CON group when compared with those of the CON group ($0.05 < p < 0.1$) (Fig. 3.4 (B)).

Serum lipid insulin, HbA_{1c}, and cytokine levels

As shown in Table 3.3, there were no statistically significant differences in the serum T-Chol, HDL-Chol, TG, LDL-Chol and NEFA levels, and atherogenic index among K, KG and CON groups.

The leptin, adiponectin and HMW-adiponectin levels also did not differ among the K, KG and CON groups, although the HMW adiponectin level tended to increase in the K and KG groups ($0.05 < p < 0.1$). The serum insulin level measured on day 26 during the feeding period did not differ among the K, KG and CON groups (Table 3.3). The HbA_{1c} levels measured on day 26 during the feeding periods were significantly lower in the K and GK groups, compared with that of the CON group (Fig 3.3 (B)).

Liver lipid levels, and liver FAS and CPT activities

The liver lipid levels were shown in Table 3.3. The liver TG, T-Chol, PL and NEFA levels were significantly lower in the K and KG groups than in the CON group.

The activities of the liver FAS and CPT enzymes were also shown in Table 3.3. The FAS activity was significantly lower in the K and KG groups than in the CON group, but the CPT activities did not differ among those 3 groups.

There was no difference in the fecal excretion of the total lipid, T-Chol, TG and T-BA (Table 3.4).

3.4 Discussion

The fasting blood glucose levels of the K and KG groups showed a lower tendency than in the CON group during the feeding period, although a statistically significant difference was not observed (Fig. 3.3). In the OGTT, blood glucose levels in the K and KG groups tended to be suppressed, compared with the CON group. The AUC in the K and KG groups, calculated from the blood glucose levels in the OGTT, showed lower tendencies ($0.05 < p < 0.1$) in the K and KG groups than in the CON group (Fig. 3.4). These results may suggest that both K and KG have an ameliorating effect for glucose tolerance and

diabetes, as flavonoids have been reported their activity to improve glucose tolerance (*73, 74*), but, anti-diabetic effect of kaempferol and its triglucosides–rich faction which prepared in this experiments, and isolated kaempferol triglycoseds have not yet been examined in KK-A^y mice.

The lower level of HbA_{1c} in the K and KG groups in comparison with the CON group, measured after 4 weeks at the end of the feeding period may indicate that feeding the K and KG for longer periods might be effective for mitigating diabetes (Fig. 3.3). The HMW-adiponectin levels, which are known to show a higher level in KK-A^y mice with diabetes improved (*72*), tended to be increased by K and KG feeding (Table 3.3). These results may suggest that the anti-diabetic effects of K and KG are weak, although experiments with a higher dose level of K and KG might be necessary. No significant difference between the K and KG groups in the ability to improve the diabetic markers, indicate that the effect of KG may depend on its aglycone, kaempferol.

Although the body weight gain, food intake and water intake during the feeding periods did not differ among the CON, K and KG groups, the white adipose tissue including pararenal, epididymal and mesenteric adipose tissues of K and KG groups tended to be a little lower than those of the CON group (Table 3.2), suggesting that K and KG have activities to inhibit lipid accumulation or to promote lipid β-oxidation. The reports, in which an anti-obese effect of kaempferol glycoside(kaempferol 3-*O*-β-D-glucopyranoside) that differs from KG identified in this experiment in its chemical structure, caused little decreases in visceral fat weight (*75*), and a decrease in serum and liver TG levels, may suggest that K and KG may contribute to the improvement of diabetes through the suppression of TG accumulation, because mesenteric fat weight tended to decrease and the liver TG level decreased significantly when compared with those of the CON group (Table

3.3). Lowering of the FAS activity and no significant change in CPT activity by feeding K and KG may indicate the inhibition of FAS by K and KG, but not the activation of CPT mainly involved with lowering the liver TG levels (Table 3.3). A significant lowering of the liver total lipid and TG levels by feeding K and KG were similar to those by feeding with kaempferol which were reported previously (*75*), indicating that the effect of KG might be mainly demonstrated by the aglycone of KG, kaempferol. No differences in the fecal excretion of lipids and bile acids (Table 3.4) may indicate that the improvement of the liver lipids levels by K and KG did not ascribe to their inhibitory activity on the intestinal absorption of dietary lipids.

The results of this study demonstrate that dietary kaempferol, and KG which were mainly composed of compounds 1, 2, 3, and 4, and differ from kaempferol 3-*O*-β-D-glucoside in their chemical structures are available to improve HbA_{1c} levels and glucose tolerance and liver lipid levels. As it is considered that there are many foodstuffs containing kaempferol with two or three sugar moieties *via* its 3-position, analyses of the physiological functions of KG may be helpful to understand the properties of foodstuffs containing kaempferol glycoside with three sugar moieties. No significant differences in OGTT, HbA_{1c}, serum and liver lipid levels between mice fed with K and KG, suggested that the effects of KG was mainly due to those of an aglycone moiety of kaempferol glycoside in KG, kaempferol. The lowering effects of K and KG on liver lipids and the tendency to improve diabetes by those compounds were considered partly due to the suppression of FAS activity by K and KG, however, it is necessary to investigate the other effects of KG on lipid metabolism. Although the identification of kaempferol 3-*O*-β-D-glucopyranosyl (1→2) -*O*-[α-L-rhamnopyranosyl (1→6)]-β-D-galactopyranoside, kaempferol 3 - *O* - β - D - glucopyranosyl (1→2) - *O* - [α - L - rhamnopyranosyl

(1→6)]- β- D- glucopyranoside, kaempferol- 3- *O*- β- D- (2- *O*- β- D- glucopyranosyl) galactopyranoside and kaempferol- 3- *O*- β- D- (2,6- di- *O*- α- L- rhamnopyranosyl) galactopyronoside from KG may suggest that some or all of them are concerned with the activity of KG. Further studies should be done to investigate the principle and exact action mechanism. In addition, the former 3 compounds have not yet been reported on the leaves of unripe soybeans.

3.5 Summary

Anti-diabetic effects of kaempferol glycoside-rich fraction containing kaempferol 3-*O*-β-D-glucopyranosyl (1→2)-*O*- [α-L-rhamnopyranosyl(1→6)] -β-D-galactopyranoside, kaempferol 3 -*O* -β -D -glucopyranosyl (1→2)- *O* -[α-L-rhamnopyranosyl (1→6)] -β -D-glucopyranoside, kaempferol 3-*O*-β-D- (2-*O*-β-D-glucopyranosyl) galactopyranoside and kaempferol 3-*O*-β-D-(2,6-di-*O*-α-L-rhamnopyranosyl) galactopyronoside as major flavonoids, prepared from leaves of unripe Jindai-soybean, and kaempferol, aglycone of kaempferol glycoside, were determined in genetically type 2 diabetic KK-A^y mice. Hemoglobin A_{1c} level was decreased and tended to be decreased by feeding KG and kaempferol (K), respectively. The AUC (area under the curve) in the oral glucose tolerance test (OGTT) tended to be decreased by feeding K and KG. The liver triglyceride level and fatty acid synthase activity decreased in both of the mice fed with KG and K, compared to those in the control mice. From these results, it was suggested that KG and K are useful to improve diabetes and the KG contained in Jindai-soybean leaves may be available as components of functional food.

Table 3.1 Composition of the diet (%)

Dietary group	C57BL	KK-A^y		
		CON	K	KG
Casein	20	20	20	20
α-Cornstarch: Sucrose=2:1	62.95	62.95	62.8	62.855
Cellulose powder	5	5	5	5
Corn oil	7	7	7	7
Mineral mixture [1]	3.5	3.5	3.5	3.5
Vitamin mixture [2]	1	1	1	1
L-Cystine	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25
Kaempferol (K)			0.095	
Kaempferol glycoside -rich fraction (KG)				0.15

[1] AIN-93G-MX and [2] AIN-93-VX were purchased from Clea Japan (Tokyo, Japan)

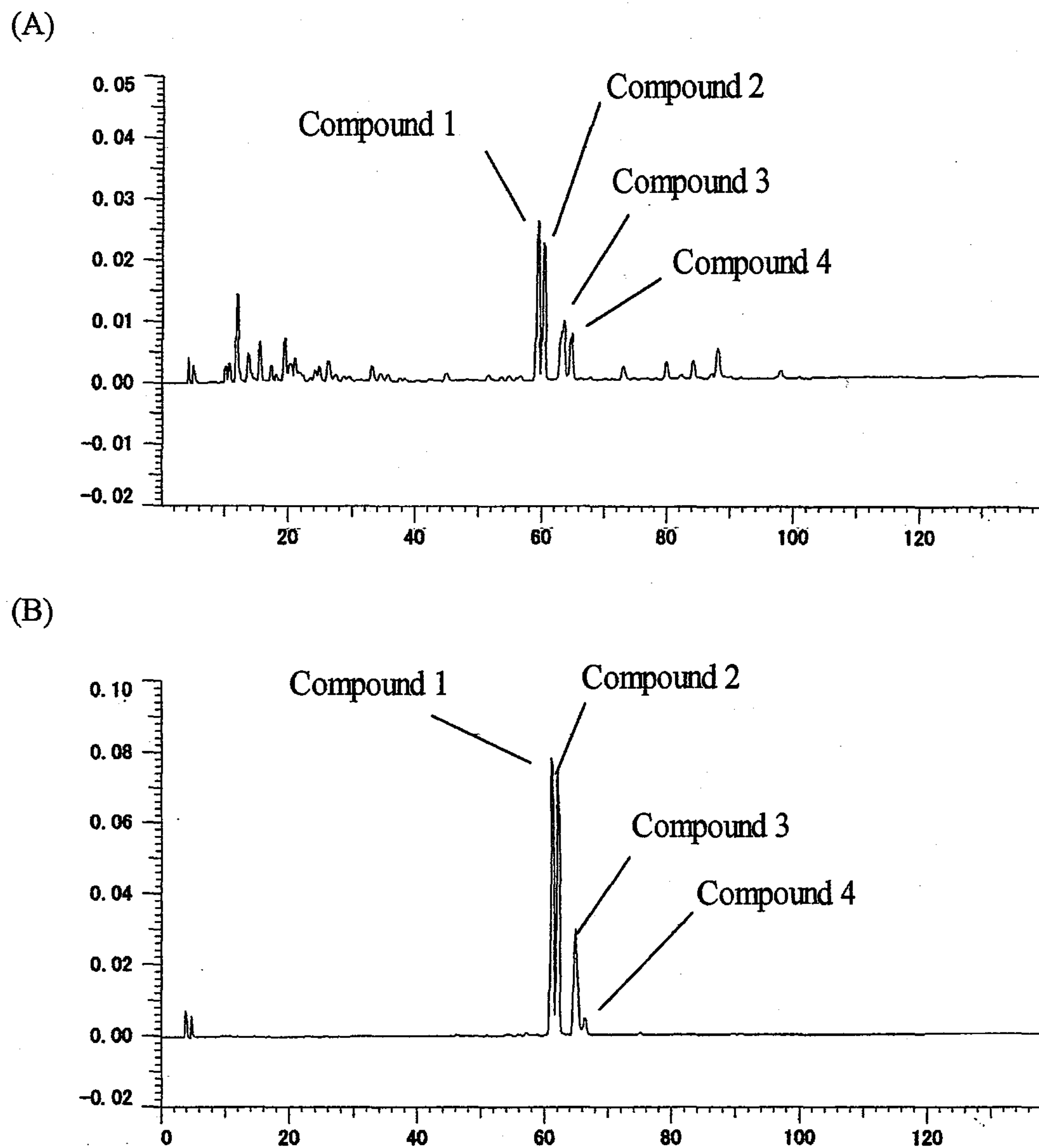


Fig. 3.1. HPLC chromatograms of 70% MeOH-extract of the leaves of unripe soybean (Jindai-mame) (A) and its kaempferol glycoside-rich fraction (KG) (B)

HPLC conditions: column, Develosil C-30-UG-5 (4.6 i.d. × 250 mm, Nomura Chemical Co., Aichi, Japan); development, a linear gradient of 0-100% of solvent B (40% CH_3CN) in solvent A (5% CH_3CN in 1% acetic acid) over the course of 180 min at a flow rate of 0.8 ml/min; detection at 330 nm.

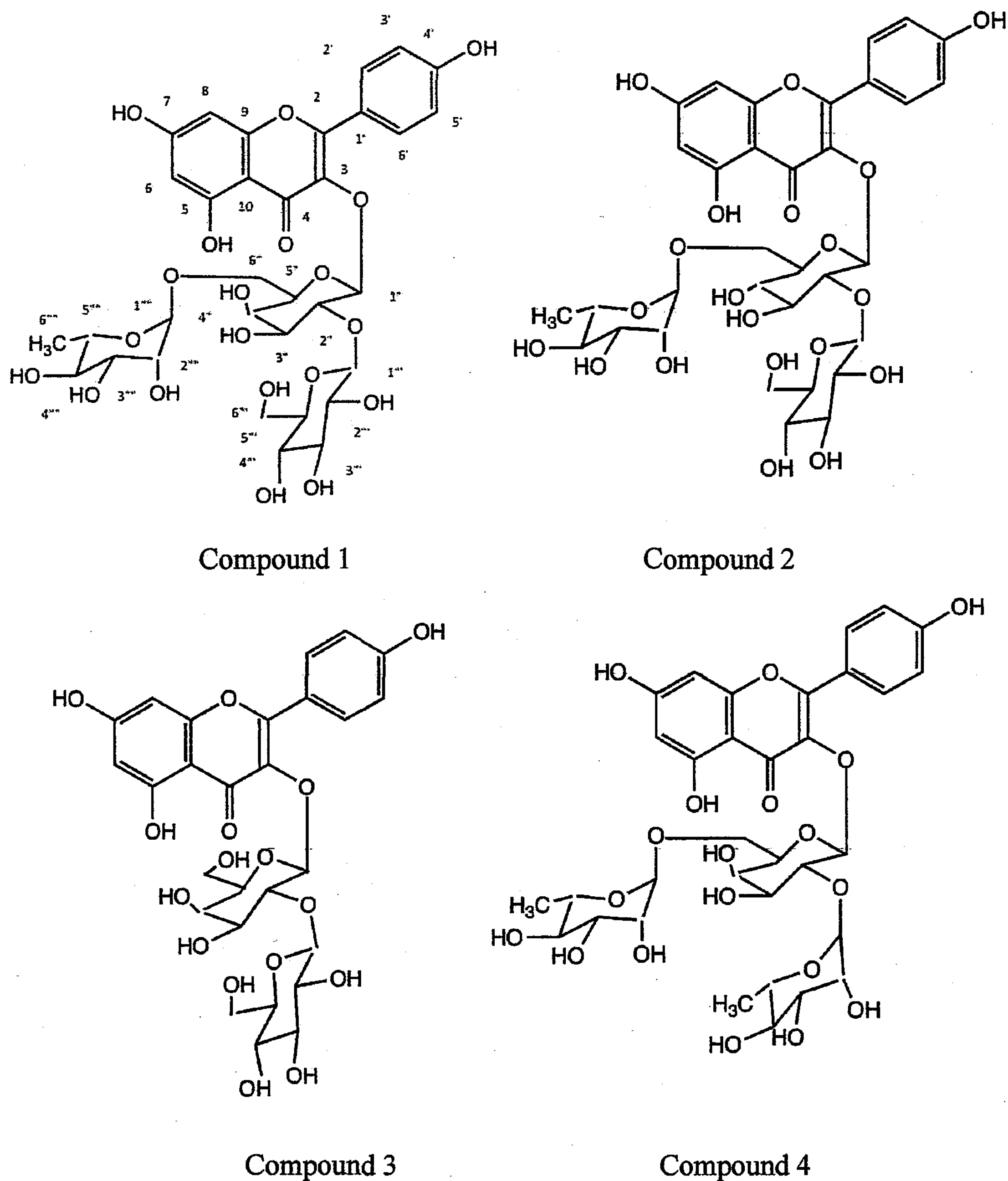


Fig. 3.2. Chemical structures of the compounds 1, 2, 3 and 4

Compound 1, kaempferol 3-*O*-β-D-glucopyranosyl(1→2)-*O*-[α-L-rhamnopyranosyl (1→6)]-β-D-galactopyranoside

Compound 2, kaempferol 3-*O*-β-D-glucopyranosyl(1→2)-*O*-[α-L-rhamnopyranosyl (1→6)] -β-D-glucopyranoside

Compound 3, kaempferol 3-*O*-β-D-(2-*O*-β-D-glucopyranosyl)-galactopyranoside

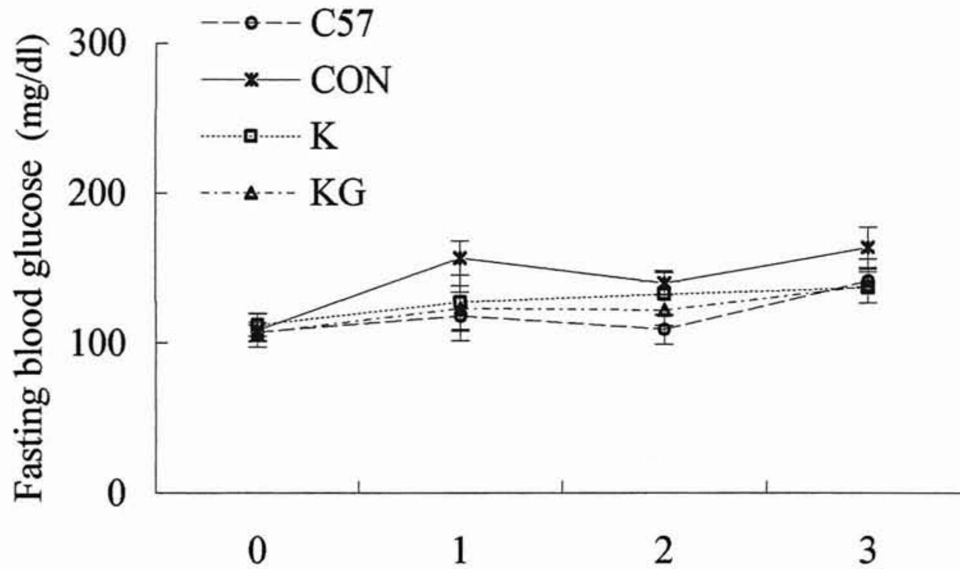
Compound 4, kaempferol 3-*O*-β-D-(2,6-di-*O*-α-L-rhamnopyranosyl)-galactopyronoside

Table 3.2. Effects of dietary kaempferol and kaempferol glycoside-rich fraction on the liver, kidney and white adipose tissue weight

Dietary group	C57BL	$KK\text{-}A^y$		
		CON	K	KG
Total food intake (g)	133±1	$187±5^{a}$	$176±5^{a}$	$176±5^{a}$
Body weight gain (g)	4.43±0.37	$7.65±0.37^{a}$	$7.35±1.01^{a}$	$8.43±0.85^{a}$
Feed efficiency[1]	0.0332±0.0023	$0.0410±0.0020^{a}$	$0.0422±0.0063^{a}$	$0.0476±0.0043^{a}$
Water intake (g)	95.8±5.3	$523±31^{a}$	$501±45^{a}$	$551±55^{a}$
Liver weight (% of body weight)	3.65±0.07	$3.95±0.08^{a}$	$3.85±0.06^{a}$	$3.88±0.08^{a}$
Kidney weight (% of body weight)	0.590±0.048	$0.687±0.016^{b}$	$0.698±0.019^{ab}$	$0.725±0.032^{a}$
Pararenal adipose tissue (% of body weight)	0.204±0.037	$0.375±0.055^{a}$	$0.345±0.036^{a}$	$0.274±0.052^{a}$
Epididymal adipose tissue (% of body weigh	0.928±0.062	$2.03±0.06^{a}$	$1.97±0.08^{a}$	$1.96±0.14^{a}$
Mescenteric adipose tissue (% of body weigl	0.640±0.056	$2.04±0.13^{a}$	$1.95±0.28^{a}$	$1.86±0.26^{a}$

C57BL and $KK\text{-}A^y$ mice (6 weeks old) were given the diets for 29 d. Each value is the mean ± SEM, n = 5-6 for each group. Values without a common letter differ significantly ($p < 0.05$) when compared CON, K, and KG groups. [1] body weight gain (g)/food intake (g)

(A)



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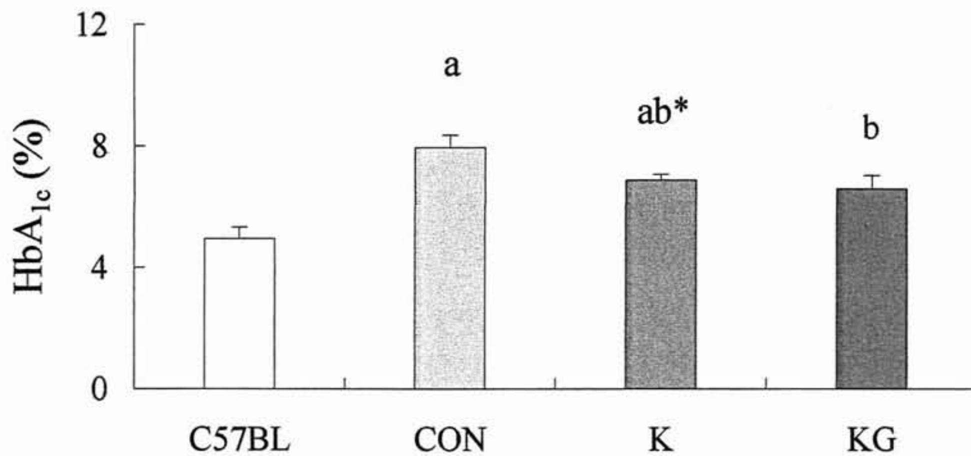
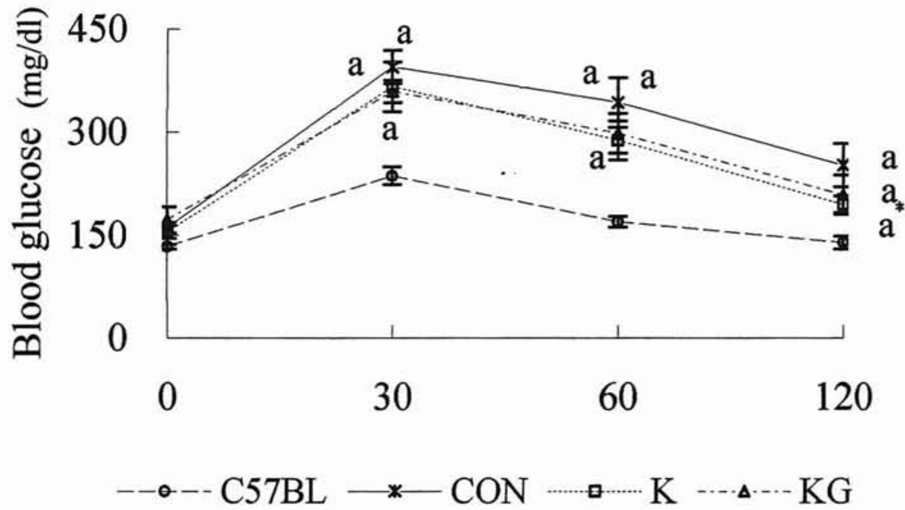


Fig. 3.3. Effects of dietary K and KG on the fasting blood glucose and HbA_{1c} levels (Fasting blood glucose was measured after 10 h of fasting at the end of every week. Each value is mean ± SEM (n = 5-6 for each group). HbA_{1c} was measured at the end of the feeding period. Values without a common letter differ significantly ($p < 0.05$) when compared CON, K, and KG groups.

(A)



(B)

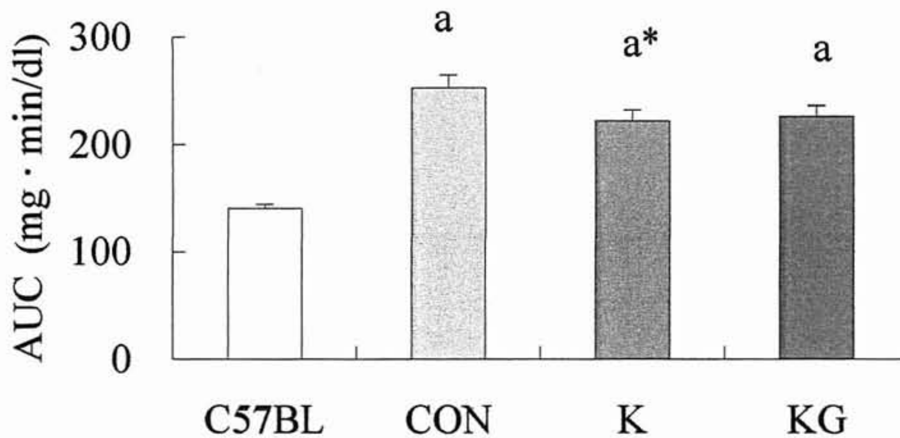


Fig. 3.4. Effect of dietary kaempferol and kaempferol glycoside-rich fraction on the blood glucose levels (A) and AUC (B) in the oral glucose tolerance test

(A) Glucose loading was carried out on day 26 of the feeding period;

(B) AUC: Area under the curve of changes in the blood glucose.

Each value is mean ± SEM (n = 5- 6 for each group). Values without a common letter differ significantly ($p < 0.05$) when compared CON, K, and KG groups. * ($0.05 < p < 0.1$) when compared with the CON group.

Table 3.3. Effects of dietary kaempferol and kaempferol glycoside-rich fraction on the serum and liver lipid levels, the serum adipocytokine and leptin levels, and on the liver enzyme activities

Dietary group	C57BL	KK-A^y		
		CON	K	KG
Serum				
T-Chol (mg/dl)	132±4	148±5^{a}	152±3^{a}	144±8^{a}
HDL-C (mg/dl)	97.8±3.4	95.2±8.3^{a}	93.6±2.9^{a}	90.7±8.8^{a}
Atherogenic index	0.346±0.022	0.548±0.086^{a}	0.619±0.051^{a}	0.520±0.073^{a}
TG (mg/dl)	60.5±5.6	135±20^{a}	124±7^{a}	123±6^{a}
NEFA (mEq/l)	2.80±0.16	4.02±0.24^{a}	4.54±0.36^{a}	4.56±0.42^{a}
Insulin (ng/ml)	0.499±0.108	4.86±0.96^{a}	4.76±1.10^{a}	4.73±0.69^{a}
Adiponectin (μg/ml)	27.0±1.5	8.97±0.57^{a}	9.92±0.96^{a}	8.98±0.30^{a}
HMW Adiponectin (μg/ml)	2.63±0.15	2.43±0.15^{a}	2.78±0.16^{a*}	2.83±0.09^{a*}
HMW Adipo/Adiponectin(%)	9.56±0.85	27.0±2.4^{a}	33.2±5.0^{a}	31.1±1.7
Leptin (ng/ml)	3.50±0.46	32.5±2.2	36.1±3.4	34.1±2.2
Liver				
Total lipid (mg/g of liver)	52.2±3.5	62.6±3.8^{a}	51.0±3.8^{b}	47.1±2.5^{b}
T-Chol (mg/g of liver)	1.12±0.16	7.41±0.55^{a}	3.28±0.26^{b}	3.43±0.51^{b}
TG (mg/g of liver)	34.7±3.5	30.7±2.5^{a}	22.3±3.5^{ab}	24.0±2.2^{b}
NEFA (mEq/g of liver)	0.201±0.018	0.292±0.026^{a}	0.224±0.047^{b}	0.157±0.051^{b}
PL (mg/g of liver)	11.9±0.9	13.8±0.3^{a}	11.4±0.5^{b}	11.2±0.8^{b}
Liver enzyme				
FAS(μmol/min/mg of protein)	0.432±0.095	0.966±0.129^{a}	0.365±0.119^{b}	0.545±0.041^{b}
CPT(μmol/min/mg of protein)	1.57±0.10	1.24±0.03^{a}	1.50±0.13^{a}	1.45±0.08^{a}

KK-A^y and C57BL mice (6 weeks old) were each given the diets for 29 d. Each value is the mean ± SEM. n = 5-6. Values without a common letter differ significantly ($p < 0.05$) when compared CON, K, and KG groups. $^{*}0.05 < p < 0.1$ when compared with the CON group. [1] (T-Chol−HDL-Chol)/HDL-Chol).

Table 3.4. Effects of dietary kaempferol and kaempferol glycoside-rich fraction on the fecal excretion of lipids

Dietary group	C57BL	KK-A^y		
		CON	K	KG
T-Chol (mg/2 days of feces)	4.87±0.45	8.33±0.48[a]	7.63±0.43[a]	7.53±0.45[a]
TG (mg/2 days of feces)	2.06±0.36	5.15±0.71[a]	4.53±0.56[a]	5.42±0.65[a]
T-BA (mg/2 days of feces)	0.480±0.022	1.15±0.09[a]	1.26±0.12[a]	1.15±0.14[a]

Faces were collected for 2 days from day 27 to day 28. Each value is the mean ± SEM, n = 5-6. Values without a common letter differ significantly ($p < 0.05$) when compared CON, K, and KG groups.

CHAPTER 4

ANTI-OBESE AND ANTI-DIABETIC EFFECTS OF KAEMPFEROL GLYCOSIDES FRACTION FROM JINDAI-SOY LEAVES ON C57BL MICE

4.1 Introduction

Obesity usually results from an energy imbalance due to excessive energy storage and insufficient energy expenditure, and develops a number of risk factors for cardiovascular diseases, arteriosclerosis osteoarthritis, and certain cancers. The clustering of several cardiovascular risk factors for abdominal obesity, dyslipidemia, glucose intolerance, and hypertension in the same individual is called the metabolic syndrome (*76, 77*). The prevalence of obesity has been increasing worldwide, with 136 million obese individuals in 2006 and an estimated 162 million by 2015 (*78*). Obesity has increased at an alarming rate in recent years and it is now a worldwide health problem.

Recently, more attention has been focused on flavonoids that might be beneficial in reducing the risk of obesity (*79*). Flavonoids are polyphenolic compounds widely distributed throughout the plant kingdom (*62*). They are important to protect the leaves against UV rays during their growth (*63*). Flavonoids are natural antioxidants which reduce the risk of cancer, the effects of aging and the risk of cardiovascular diseases (*64*). Their important roles include free radical scavengers, reducing agents, protectors against lipid peroxidation and quenchers of reactive oxygen species (*65, 80*).

In this study, anti-obese and anti-diabetic effects of kaempferol glycoside-rich fraction, prepared from leaves of unripe Jindai-soybean were determined, on markers of obesity, body weight, adipose tissue, serum and liver lipid levels were determined. Furthermore, the anti-diabetic effect were determined by measuring the relevant markers of diabetes as glucose tolerance, hemoglobin A_{1c}, insulin, fasting glucose and adipocytokine levels, and further enzyme activities concerned with glucose and lipid metabolism.

4.2 Materials and Methods

Materials

Preparation of kaempferol glycoside-rich fraction (KG) Jindai-Soybean (Glycine max. L. Merr. 'Jindai') was shown in chapter 3.

Animals

Male C57BL/6J mice, average weighing 19 g (6 weeks old) were purchased from Clea (Tokyo, Japan) and housed individually under a 12:12 h light-dark cycle at 22 ± 2 °C and 40-60% humidity.

After acclimatizing for 5 d, the C57BL/6J mice were randomly divided into 3 groups of 7 each, and fed on either the basal diet control group (CON) or high fat diet (HF) or high fat diet containing kaempferol glycosides fraction (HFKG) at 0.15%. The composition of each experimental diet is shown in Table 4.1. The diets and water were given for 92 d *ad libitum*. The body weight was measured every other day and the diet was measured daily. At the end of the feeding period, the mice were anesthetized with Nembutal (Dainippon Pharmaceutical Co. Osaka, Japan) after 10 h of fasting, and the blood was collected by cardiac puncture, followed by detaching the liver. The detached live was stored at -80 °C until needed for analysis. The left kidney, left pararenal adipose tissues, left epididymal adipose tissue and mesenteric adipose tissue were also detached and stored at -80 °C until needed for analysis.

The mice were cared according to the institutional guidelines of Yamagata University.

Measurements of fasting and fed blood glucose levels and blood glucose tolerance test

Fasting blood glucose levels were measured with a Medesafe GR-102, 20-600 mg/dl

measuring range (Termo Co., Tokyo, Japan) on the 1st, 42nd and the 92nd day after 10 h of fasting. An oral glucose tolerance test (OGTT) was performed on day 42 after 10 h of fasting.

Serum was prepared by centrifuging the collected blood at 1,000 × g for 15 min. The liver was detached and stored at -80 °C until needed for analysis. The left kidney, left pararenal adipose tissues, left epididymal adipose tissue and mesenteric adipose tissue were also detached and stored at -80 °C after being weighed.

Measurements of the insulin and adiponectin levels

The serum level of insulin, adiponectin, high molecular weight (HMW) adiponectin, and leptin were measured with commercial ELISA kits (each, Mouse insulin (S-Type) Kit, Shibayagi Co., Gunma, Japan; Mouse adiponectin kit, Otsuka Co., Tokyo, Japan; Mouse high molecular weight adiponectin kit, Shibayagi Co., Gunma, Japan; Mouse leptin Kit, Morinaga Co., Tokyo, Japan).

Hemoglobin A_{1c} (HbA_{1c}) levels were measured with a Micromat II (Bio-Rad Laboratories, California, USA) at 10:00-11:00 am after 10 h of fasting at the end of the 4th week of the feeding period.

Lipid analysis

Total-cholesterol and HDL-cholesterol (T-Chol and HDL-Chol), triglyceride (TG), phospholipid (PL), and total non-esterified fatty acids (NEFA) in the serum and liver, and feces T-Chol, TG and total bile acid (T-BA) levels were measured with commercial kits (cholesterol E test, HDL-cholesterol E test, triglyceride E test, phospholipid B test, NEFA C test and total bile acid test; Wako pure Chemical Industries, Osaka, Japan). Liver and

feces lipids were measured by using the isopropyl alcohol-soluble fraction of the lipids, which was prepared by removing the solvent in the lipid fraction obtained by the method of Folch *et a*l (71, 84).

Measurements of liver enzyme activities

A sample to measure the liver enzyme activities was prepared by homogenizing the liver in a 3 mM Tris-HCl buffer (PH 7.2) containing 0.25 M sucrose and 1mM EDTA. The supernatant of the homogenate obtained by centrifuging at 500 × *g* for 10 min at 4 °C was re-centrifuged at 9,000 × *g* for 10 min at 4 °C, and further centrifuged at 105,000 × *g* for 60 min.

The FAS (fatty acid synthesis) activity was determined in terms of malonyl-CoA-and acetyl-CoA-dependent oxidation of NADPH following the methodology of Kumer et *al.* and Carey et *al.* (*69, 82*). The reaction mixture was composed of a 0.1M phosphoric buffer (PH 7.0) containing 0.2mM EDTA, 0.3mM NADPH, 0.05mM acetyl-CoA, 0.2mM malonyl-CoA. The rate of decrease on absorbance at 340 nm was measured.

Fecal lipid analyses

Feces were collected on day 86-88 for 72 h. Fecal total lipids were extracted by the method of Folch *et al.* (*68*). The lipids in the extracts were measured with commercial kits as described above.

Statistical analysis

Each value is given as the mean ± SEM. The homogeneity of variance between treatments was verified by Bartlett's test. Data were statistically analyzed by a one-way analysis of

variance (ANOVA). A *Post-hoc* analysis of significance was made by using Fisher's PLSD test, with significant difference at $p < 0.05$.

4.3 Results

Body weight, body weight gain and feed efficiency of the HFKG group were significantly lower than those of the HF group, but there were no significant differences between CON and HFKG groups. Food intake tended to be decreased in the HFKG group than in the HF group (Fig. 4.1) (Table 4.2).

Although the liver and kidney weights did not differ among the three groups, the pararenal adipose tissue, epididymal adipose tissue, mesenteric adipose tissue and visceral adipose tissue weights of the HFKG group were significantly lower than those of the HF group (Table 4.2).

Fasting blood glucose levels, and oral glucose tolerance test

The fasting blood glucose levels on day 1 did not differ among the CON, HF and HFKG groups, but those on day 42 tended to be decreased in the HFKG group compared with the HF group, and those on day 92 showed significantly lower levels in the HFKG group than in those in the CON group (Fig. 4.2).

Blood glucose levels on the OGTT carried out on day 42 were not different among the CON, HF and HFKG groups (Fig. 4.3 (A)). The area under the curve (AUC) on the OGTT showed lower tendency in the HFKG group than in the CON and HF groups, but no significant differences were found (Fig. 4.3 (B)).

Serum HbA_{1c} levels

Serum HbA_{1c} levels measured on day 42 and 92 were significantly lower in the HFKG and CON groups than in the HF group (Fig. 4.4).

Serum lipid levels and cytokine

As shown in Table 4.3, the serum TG, HDL-Chol, LDL-Chol and NEFA levels did not differ among the CON, HF and HFKG groups. The serum adiponectin and HMW adiponectin levels also did not differ among the three groups. Leptin and Insulin levels of the HFKG group were significantly lower than those of the HF group. The TNF-α level tended to be lower in the HFKG group than in the HF group.

Liver lipid levels, liver FAS and CPT activities

The liver lipids levels were not different among the CON, HF and HFKG groups as shown in Table 4.3. The activities of the liver FAS and CPT enzymes are shown in Table 4.4. Although the FAS activities did not differ significantly among the CON, HF and HFKG groups, the CPT activities in the HFKG group showed a significantly higher level than those in the HF group.

There were no differences on the fecal excretion of total lipid, T-Chol, TG and total bile acids between the HFKG and HF groups (Table 4.5).

4.4 Discussion

Obesity is defined as an accumulation of excess adipose tissue, and decreasing of body weight is recommended as first-line treatment to reduce it (*83*). In the present study, the body weight significantly decreased from week 4 to the end of the feeding period (Fig.

4.1). The visceral adipose tissue was significantly decreased in the HFKG group when compared the HF group (Fig. 4.5). These results suggest that the KG (kaempferol glycoside-rich fraction) can suppress the increase in weight gain or fat deposition induced by high fat diet.

Obesity is highly associated with insulin resistance and it is the biggest risk factor for non-insulin-dependent diabetes mellitus (*84*). In the present study, the serum and liver lipid levels, and the liver FAS activity did not differ between the HF and HFKG groups, in spite of that adipose tissues (viseral adipose tissue) weights declined to a decrease in the HFKG group in the comparison with HF group, suggesting that adipose tissue weight does not always reflect the serum and liver lipid levels and FAS activity. The higher activity of CPT, which catalyzes the transfer of fatty acid from CoA to carnitine, allowing the initial transport of fatty acids into mitochondria for β-oxidation, showed higher level in the HFKG group than in the HF group (Table 4.4). This result may indicate that lower level of the viseral adipose tissue weight in the HFKG group (Fig.4.5) might be brought by enhancement of ß-oxidation by KG rather than inhibition of FAS activity. The serum insulin level decreased significantly in the HFKG group in the HF group (Table 4.3). As it has been reported that feeding of a high fat diet could increase the serum insulin level, inducing insulin resistance on C57BL/6J mice (*85*), the lower insulin level in the HFKG group may indicate that KG is available to improve insulin resistance. Lowering of the fasting blood glucose level on day 92, and of HbA_{1c} level on day 42 and 92 in the HFKG group may also indicate availability of KG for improvement of diabetes induced by the HF diet (Fig. 4.2 and Fig. 4.4).

Adipocytokines with numerous functions such as regulations of metabolism, inflammatory process, and body weight (*86*) are released from adipose tissue. Leptin as

adipocytokine plays key roles in regulating the food intake and energy expenditure, and it is known that the serum leptin level is altered depending on the adipose tissue weight (*87*). Similar to the previously study (*88*), leptin level in the HF group was significantly increased by about 6-folds when compared with that of the CON group, suggesting that HF diet induced leptin resistance. The lower serum leptin level in the HFKG group in the comparison with the HF group seems to suggest that the leptin resistance has been improved by KG consumption. The lower tendency in the food intake in the HFKG group may also support availability of the KG in mitigation of leptin resistance. As it is reported that the serum insulin level is affected (determined) by the serum leptin level (*85*), decrease in the serum insulin level in the HFKG group might have been caused by an improvement of the leptin resistance by KG.

It is known that TNF-α level increases in rodent with obesity and inflammation, resulting in enhancement of insulin resistance. Therefore, lower tendency of the TNF-α level in the HFKG group in this experiment, compared with HF group (Table 4.3), also suggest that the insulin resistance was involved with the obesity, and an increase in the adipose tissue weight might be improved by the dietary KG. However, as the blood glucose levels in OGTT carried out on day 42 did not differ between the HF and HFKG groups (Fig. 4.3), complications of diabetes may occur during the latter stage of the feeding period. Changes in the adiponectin and HMW adiponectin levels, in which the latter is pointed out to be more sensitive than the adiponectin for the insulin resistance (*89*), were little than those in the insulin and leptin levels, showing that the effect of the KG to improve diabetes may be not so strong to suppress a decrease in adiponectin level.

No difference in the amounts of excreted lipids (Total-Chol, TG and Total bile acid) between the HF and HFKG groups (Table 4.5) may indicate that KG added to the diet

almost dose not affect intestinal absorption of lipids..

4.5 Summary

The anti-obese and anti-diabetic activities of kaempferol glycoside-rich fraction (KG) prepared from unripe Jindai-soybean (Edamame, *Glycine max.* L. Merrill. 'Jindai') leaves were investigated in C57BL/6J mice fed high fat diet for 92 days to develop their use as raw materials for functional food.

The visceral adipose tissue weight was lower in the KG group than in the CON group. Fasting blood glucose and HbA_{1c} levels were also lower in KG group, suggesting that KG is available to mitigate both obesity and diabetes. The serum leptin and insulin levels increased by feeding with high fat diet returned to the level of the CON group by adding the KG to the diet, suggesting that both the leptin and insulin resistance might had been induced by high fat diet could be improved by the dietary KG, and that the anti-obese and anti-diabetic activities of KG might be concerned closely with its regulation of leptin and insulin levels.

Table 4.1. Ingredient composition of the diet (%)

Constituent	CON	HF	HFKG
Casein	20	20	19.85
α-Cornstarch	53	8	8
Sucrose	10	25	25
Cellulose powder	5	5	5
Corn oil	7	7	7
Tallow	0	30	30
Mineral mixture[1]	3.5	3.5	3.5
Vitamin mixture[2]	1	1	1
L-Cystine	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2
Kaemperol glycosides-rich fraction			0.15

[1] AIN-93G-MX and [2] AIN-93-VX were purchased from Clea Japan (Tokyo, Japan)

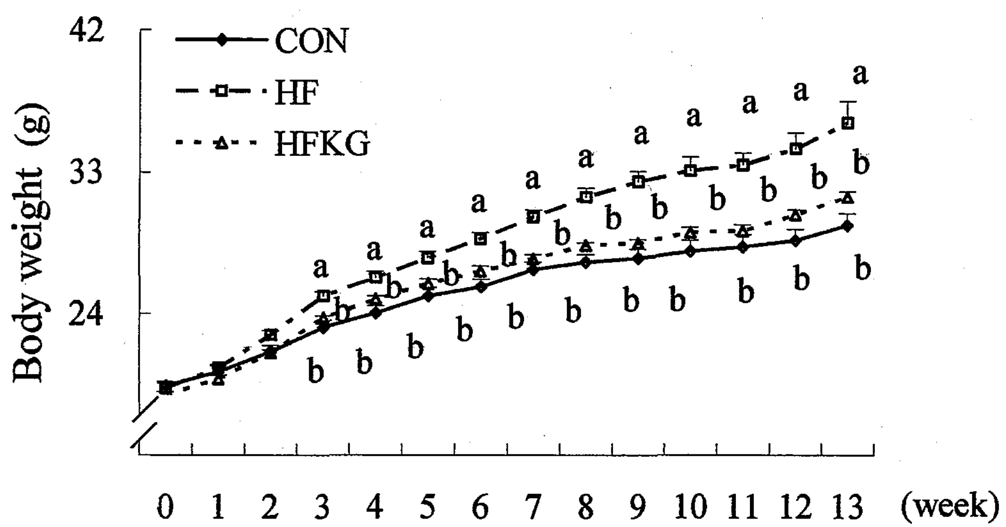


Fig. 4.1. Effects of dietary kaempferol glycosides-rich fraction on body weight
Each value is mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

Table 4.2. Effect of dietary kaempferol glycosides-rich fraction on the total food intake, body weight gain and feed efficiency

Dietary group	CON	HF	HFKG
Total food intake (g)	455±10^{a}	424±20^{ab}	388±13^{b}
Body weight gain (g)	10.1±0.4^{b}	16.8±1.3^{a}	12.6±0.5^{b}
Feed efficiency (%)[1]	2.23±0.09^{b}	4.00±0.30^{a}	3.25±0.12^{b}
Liver (% of body weight)	3.54±0.05	3.38±0.06	3.31±0.11
Kidney (% of body weight)	0.652±0.023	0.581±0.026	0.646±0.020

[1] (body weight gain (g)/food intake (g)). Each value is the mean ± SEM. n = 6 for each group. Values without a common letter differ significantly ($p < 0.05$).

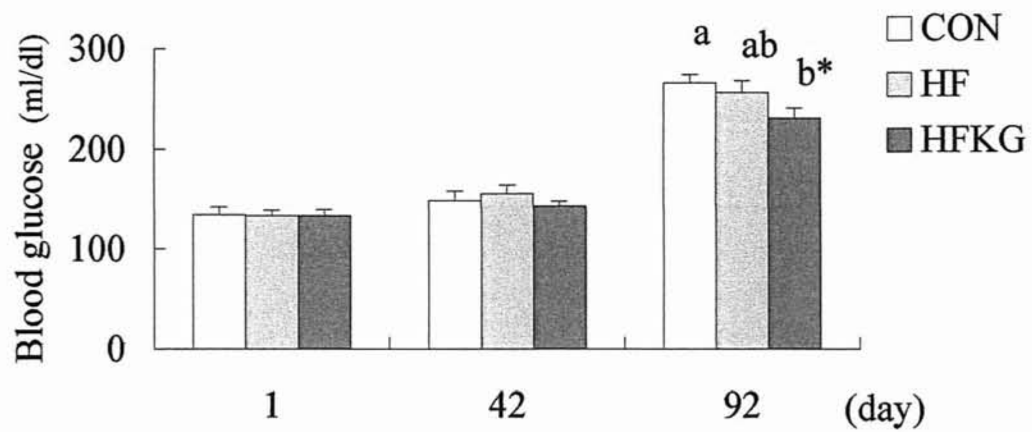
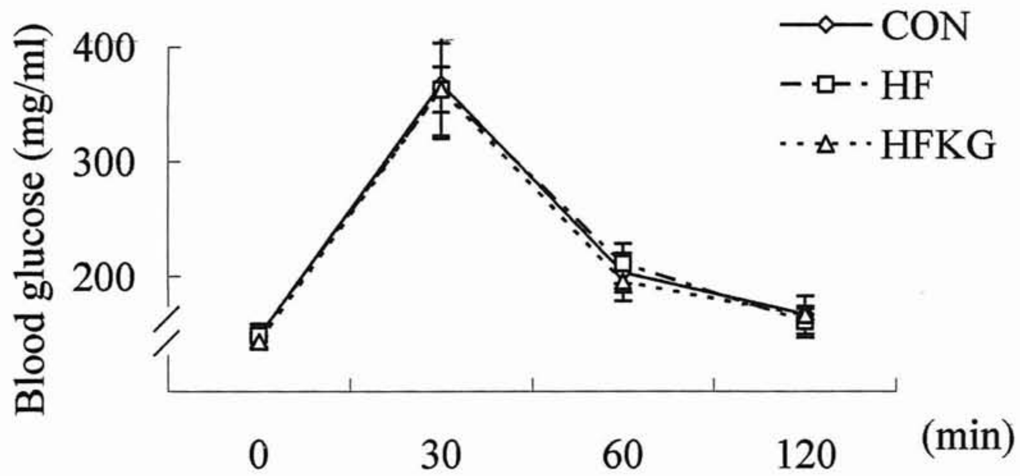


Fig. 4.2. Effect of dietary kaempferol glycosides-rich fraction on the fasting blood glucose levels

Each value is mean ± SEM. n = 7 for each group. Values without a common letter differ significantly ($p < 0.05$) *Compare with HF group ($0.05 < p < 0.1$)

(A)



(B)

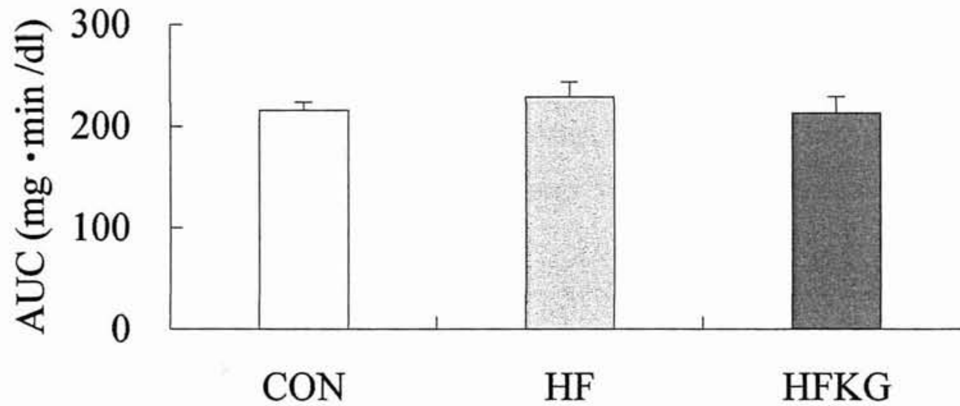


Fig. 4.3. Effect of dietary kaempferol glycosides-rich fraction on the blood glucose levels and AUC during the glucose tolerance test

A: loading was carried out on day 42 of the feeding period; B: AUC (area under the curve). Each value is mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

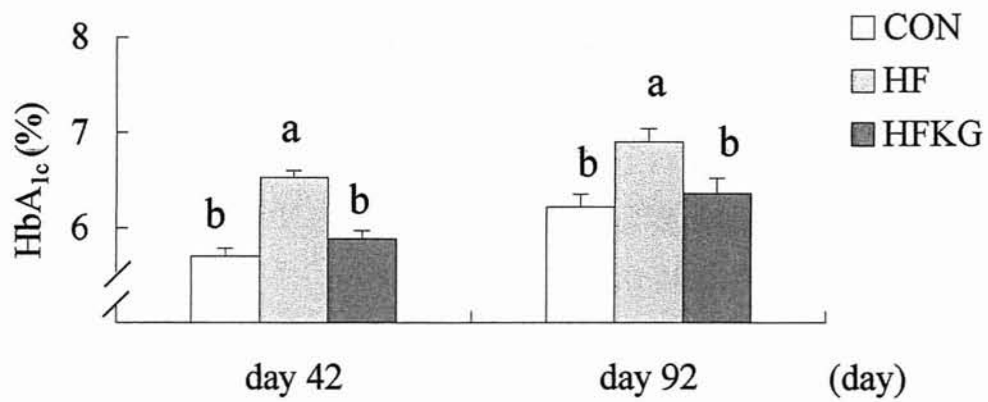


Fig. 4.4. Effect of dietary kaempferol glycosides-rich fraction on the HbA_{1c} levels
Each value is mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

Table 4.3. Effect of dietary kaempferol glycosides-rich fraction on the serum and liver lipids

Dietary group	CON	HF	HFKG
Serum			
TC (mg/dL)	106±3	116±7	102±3*
TG (mg/dL)	50.8±3.5^{a}	38.1±3.5^{b}	45.6±1.1^{ab}
NEFA (mEq/dL)	0.686±0.035	0.719±0.033	0.651±0.039
HDL (mg/dL)	91.0±9.0	68.9±8.0	79.1±14.2
LDL (mg/dL)	43.1±7.5	49.7±6.6	37.0±8.2
Adiponectin (ug/mL)	1.96±0.07	1.77±0.07	1.86±0.05
HMW Adiponectin (ug/mL)	0.308±0.102	0.173±0.001	0.215±0.049
Leptin (ng/mL)	13.4±4.6^{b}	81.0±26.8^{a}	25.1±8.5^{b}
Insulin (pg/ml)	148±68^{b}	344±72^{a}	77.0±14.9^{b}
TNF-α (pg/ml)	181±7	252±49	169±10*
Liver			
Total lipid (mg/g of liver)	85.6±3.2	77.1±3.6	84.1±3.3
TC (mg/g of liver)	2.71±0.24	2.48±0.20	2.72±0.18
TG (mg/g of liver)	42.3±2.9	35.5±2.9	35.6±2.8
PL (mg/g of liver)	16.5±0.6^{a}	13.8±1.0^{b}	15.5±0.6^{ab}
NEFA (mEq/mg of liver)	8.40±0.90^{ab}	7.52±0.98^{b}	11.8±1.7^{a}

[1]((T-Chol-HDL-Chol)/HDL-Chol). Each value is the mean ± SEM. n = 3-7 for each group. Values without a common letter differ significantly ($p < 0.05$). *Compare with HF group ($0.05 < p < 0.1$)

Table 4.4. Effect of dietary kaempferol glycosides-rich fraction on the liver FAS, and CPT activities

Dietary group	CON	HF	HFKG
CPT (umol/ min/mg protein)	0.708 ± 0.039^{ab}	0.674 ± 0.062^{a}	0.778 ± 0.037^{b}
FAS (umol/min/mg protein)	1.93 ± 0.20^{a}	1.00 ± 0.13^{b}	1.17 ± 0.06^{b}

Each value is the mean ± SEM. n = 6-7 for each group.

Values without a common letter differ significantly ($p < 0.05$).

Table 4.5. Effect of dietary kaempferol glycosides-rich fraction on the fecal excretion of lipids

Dietary group	CON	HF	HFKG
Total lipid (mg/3 days of feces)	50.5 ± 2.1^{b}	65.2 ± 4.8^{a}	73.6 ± 3.3^{a}
T-Chol (mg/3 days of feces)	5.92±0.31	5.11±0.59	5.97±0.7
TG (mg/3 days of feces)	1.30 ± 0.19^{b}	3.98 ± 0.63^{a}	3.38 ± 0.63^{a}
Total bile acid (mg/3 days of feces)	0.893 ± 0.070^{b}	1.75 ± 0.21^{a}	1.56 ± 0.19^{a}

Each value is the mean ± SEM. n = 6-7 for each group.

Values without a common letter differ significantly ($p < 0.05$).

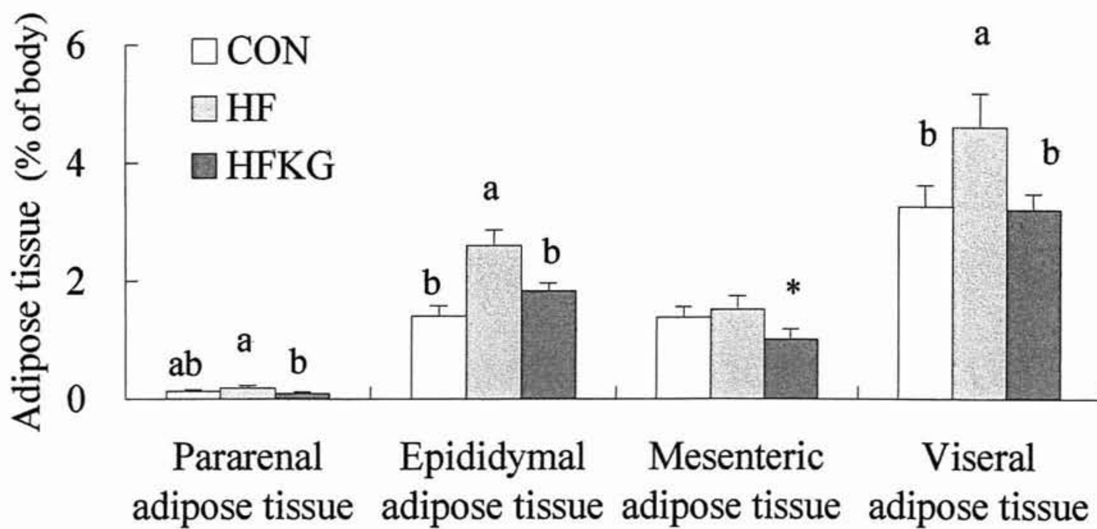


Fig. 4.5. Effects of dietary kaempferol glycosides-rich fraction on adipose tissue

Each value is mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$). *Compare with HF group ($0.01 < p < 0.05$)

CHAPTER 5

ANTI-OBESE AND ANTI-DIABETIC EFFECTS OF ISOFLAVONE MIXTURE (DAIDZIN+GLYCITIN) FROM JINDAI-SOY LEAVES ON C57BL MICE

5.1 Introduction

In recent years, the prevalence of obesity and its related diseases has been increasing worldwide. Obesity is a disorder of energy balance and is associated with hyper insulinemia, insulin resistance, and abnormalities in lipid metabolism, and it is one of the most important risk factors in the development of type 2 diabetes, atherosclerosis, and certain cancers (*74*). Obesity is defined as having excess adipose tissue that can produce adverse health effects. The mechanisms that control the number and size of adipocytes are not fully understood, despite major efforts. Adipose tissue is also considered a major endocrine organ that secretes a number of cytokines and hormones. Therefore, the modulation of adipose tissue mass is critical for anti-obesity efforts in controlling energy balance and endocrine function (*90*).

A lower frequency of obesity and related diseases in Asian countries were found, so attention has been turned toward to the diet. The reason for this is considered to be the high consumption of soy and soy-based products. Soybeans (*Glycine max* L. Merril) have long been an important protein source, complementing grain proteins in Asian countries. Asians typically consume 9 to 30 g soybeans per day. In addition to protein, soybeans also contain various nutrients and functional components (*58*). The health benefits associated with soy consumption have been linked to the content of isoflavones, the main class of the phytoestrogens (*74*). Soybean isoflavones are structurally similar to estrogen, bind to estrogen receptors, and exhibit weak estrogenic activity. It has been reported that isoflavones play an important role in the prevention of hormone-dependent diseases, including osteoporosis, cardiovascular diseases, cancer, and postmenopausal syndrome (*91*).

There are aglycone and its glycoside form in isoflavones. Some studies have shown no

differences in bioavailability between glycosides forms and their aglycones (*92*). On the contrary, other studies have proven that the aglycone form is superior than its glycoside form in preventing some diseases (*61*). Many studies on the physiological functions of isoflavones have been done by using aglycones like genistein and daidzein (*93, 94*), despite that isoflavones could be found as glycoside in many foodstuffs. In the present study, we investigated the anti-obese and anti-diabetic effects of an isoflavone mixture composed of daidzin and glycitin, which are glycosides and found in the leaves of unripe-soybeans (Edamame, *Glycine max*. L. Merrill. 'Jindai') as well as soybeans, but did not investigate precisely their anti-diabetic effects in relation to their effects on an oxidative status in C57BL/6J mice fed with a high fat diet. Addtionally, although the effects of several isoflavones on anti-oxidative enzyme activities in diabetic mice and the suppressive effects of daidzin and glycitin for production of reactive oxygen species *in vitro* were investigated, the anti-diabetic effects of daidzin and glycitin were not investigated precisely in relation to their suppressive effects on oxidative stress *in vivo*, taking such measurements as the serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) and liver glutathione levels (*95, 96*).

5.2 Materials and Methods

Animals and diet

Male C57BL/6J mice with an average weight of 19 g (6 weeks old) were purchased from CLEA Japan Inc (Tokyo, Japan) and housed individually under a 12:12 h light-dark cycle at 22 ± 2 °C and 40-60% humidity. The diet and water were given *ad libitum*, and the body weight was measured every two days and the food intake was measured daily.

After acclimatizing for 5 d, the C57BL/6J mice were randomly divided into 3 groups of

7 each, which fed on either the basal diet (control (CON) group) or high fat diet (HF group), or on the high fat diet containing the isoflavone mixture (daidzin and glycitin) at 0.06% (HFISO group). The composition of each experimental diet is shown in Table 5.1. The isoflavone mixture provided by Fuji Oil Co. Ltd (Osaka, Japan) was composed of daidzin and glycitin with a ratio of about 3:2. The diets and water were given for 92 d *ad libitum*. The body weight was measured every two day and the food intake was measured daily. At the end of the feeding period, the mice were anesthetized with Nembutal (Dainippon Pharmaceutical Co. Osaka, Japan) after 10 h of fasting, and the blood was collected by a cardiac puncture, followed by detaching the liver and stored at –80 °C until needed for analysis. The left pararenal adipose tissues, left epididymal adipose tissue and mesenteric adipose tissue were also detached and stored at –80 °C after weighing. The serum was prepared by centrifuging the blood at 3000 × *g* for 10 min. Liver lipids were extracted by the method of Floch *et al.* (*68*)

The mice were cared for according to the institutional guidelines of Yamagata University.

Fasting blood glucose levels and hemoglobin A_{1c} (HbA_{1c}) levels

Fasting blood glucose levels were measured with a Medesafe GR-102, 20-600 mg/dL measuring range (Termo Co., Tokyo, Japan) 10 h after fasting on day 1, 42 and 92. HbA_{1c} levels were measured with a Micromat II (Bio-Rad Laboratories, California, USA) at 10:00-11:00 am after 10 h of fasting on day 42 and 92 of the feeding period.

Lipid analysis

Total- and HDL-cholesterol (T-Chol and HDL-Chol), triglyceride (TG), phospholipid (PL),

and total non-esterified fatty acids (NEFA) in the serum and liver, and feces T-Chol, TG and total bile acid levels were measured with commercial kits (cholesterol E test, HDL-cholesterol E test, triglyceride E test, phospholipid B test, NEFA C test and total bile acid test; Wako pure Chemical Industries, Osaka, Japan). Liver and feces lipids were measured by using the isopropyl alcohol-soluble fraction of the lipids, which was prepared by removing the solvent in the lipid fraction obtained by the method of Folch *et al* (*68*).

Measurements of the insulin and the other cytokines

The serum levels of insulin, adiponectin, high molecular weight (HMW) adiponectin, and leptin were measured with commercial ELISA kits (each, Mouse insulin (S-Type) kit, Shibayagi Co., Gunma, Japan; Mouse adiponectin kit, Otsuka Co., Tokyo, Japan; Mouse high molecular weight adiponectin kit, Shibayagi Co., Gunma, Japan; Mouse leptin kit, Morinaga Co., Tokyo, Japan).

Measurements of glutathione and 8-OHdG

For the measurement of liver glutathione, 50 mg of liver was homogenized with 500 μl of MeOH containing each of (20 μM of) methionine sulfone, MES and D-Camphol-10-sulfonic acid, followed by the addition of 500 μl of chloroform and 200 μl of Milli-Q water and by mixing. Subsequently the mixture was centrifuged at 4.600 × *g* at 4 °C for 15min. The upper phase was ultra-filtered with an ultra centrifugal filter (Millipore 5-KDa cutoff filter; Millipore Co., Japan) at 9,000 × *g* for 3.5 h at 4 °C. The obtained filtrate was concentrated to dryness by centrifugation at 35 °C for 3 h, followed by solublization with Milli-Q water containing 3-aminopyrrolidine and trimesate (Benzene-1,3,5-tricarboxylic acid triethyl ester) (each, 200 μM), and analyzed by

CE-TOFMS (capillary eletrophoresis-time of flight-mass spectrometry) (*97*).

The serum 8-OHdG was measured by using an ELISA kit, 8-OGdG Check (Japan Institute for the Control of Aging, NIKKEN SEIL Co. Ltd., Fukuroi–shi, Shizuoka Prefecture, Japan)

Measurements of liver enzyme activities

A sample to measure the liver enzyme activities was prepared by homogenizing the liver in a 3 mM Tris-HCl buffer (PH 7.2) containing 0.25 M sucrose and 1mM EDTA. The supernatant of the homogenate obtained by centrifuging at 500 × *g* for 10 min at 4 °C was re-centrifuged at 9,000 × *g* for 10 min at 4 °C, and further centrifuged at 105,000 × *g* for 60 min.

Fatty acid synthesis (FAS) activity was determined in terms of malonyl-CoA- and acetyl-CoA-dependent oxidation of NADPH following the methodology of Kumer *et al.* and Carey *et al.* (*69, 82*). The reaction mixture was composed of a 0.1M phosphate buffer (PH 7.0) containing 0.2mM EDTA, 0.3mM NADPH, 0.05mM acetyl-CoA, 0.2mM malonyl-CoA, and the sample solution. The rate of decrease in the absorbance at 340nm was measured.

Fecal lipid analyses

Feces were collected on day 86-88 for 72 h. Fecal lipid totals were extracted by the method of Folch *et al.* (*68*). The lipids in the extracts were measured with commercial kits as described above. Total bile acid (T- BA) levels were measured by the Total bile acid test kit (Wako pure Chemical Industries, Osaka, Japan).

Statistical analysis

Each value was given as the mean ± SEM. The homogeneity of variance between treatments was verified by Bartlett's test. Data were statistically analyzed by a one-way analysis of variance (ANOVA). A *Post–hoc* analysis of significance was made by using Fisher's PLSD test, where differences were considered significant at $p < 0.05$.

5.3 Results

Food intake, body weight gain and organ weights

Body weight after 4 weeks in the HFISO group was significantly lower than in the HF group, and feed efficiency tended to be lower in the HFISO group compared with the HF group, suggesting that the isoflavone mixture is applicable to suppress an increase in body weight due to the feeding of a high fat diet (Fig.5.2, Table 5.2).

Liver and kidney weights did not differ among the CON, HF and HFISO groups. Pararenal, epididymal and mesenteric adipose tissues weights also did not differ between the HF and HFISO groups, but the visceral adipose tissue weight in the HFISO group tended to be lower than that of the HF group (Fig. 5.3).

Fasting blood glucose and HbA_{1c} levels

The fasting blood glucose levels on day 1 did not differ among CON, HF and HFISO groups, but those on day 42, tended to be lower in the HFISO group. The fasting blood glucose level on day 92 was significantly lower in the HFISO group when compared with the CON group (Fig. 5.4).

Although the serum HbA_{1c} level of the HFISO group measured on day 42 showed a significant lower level than that of the HF group, measured on day 92, it did not differ

between the HF and HFISO groups (Fig.5.6).

OGTT was carried out on day 42 were not differences among the CON, HF and HFKG groups (Fig. 5.5 (A)). The area under the curve (AUC) on OGTT was also no differences among the three groups (Fig. 5.5 (B)).

Serum lipid and cytokine levels

As shown in Table 2, there were no statistical differences in the serum TC, TG and NEFA levels among the CON, HF and HFISO groups. The HDL-Chol level was significantly higher in the HFISO group, compared with the HF group. The Atherogenic index was significantly lower in the HFISO group than in the HF group. Leptin level which was significantly higher in the HF group in comparison with the CON group, showed a lower tendency in the HFISO group than in the HF group ($0.05 < p < 0.1$). The adiponectin levels, HMW adiponectin levels, and insulin levels did not differ among the three groups (Table 2).

Liver lipid levels, and liver FAS, and CPT activities

The liver lipids levels are shown in Table 2. The liver TC, TG, and PL levels, except NEFA level, did not differ between the HFISO and HF groups. The activities of the liver FAS and CPT enzymes are also shown in Table 2. Although CPT activity of the HFISO group did not differ from that of the HF group, the FAS activity tended to be higher in the HFISO group ($0.05 < p < 0.1$). There was no difference in the fecal excretion of T-Chol, TG and total bile acids between the HFISO and HF groups (Table 2).

Serum 8-OHdG and liver glutathione levels

As shown in the Fig.4 (A), the level of reduced form glutathione (GSH) of the HF group was lower than that of the CON group, but its level in the HFISO group almost turned to the level of the CON group. In contrast to the GSH level, the oxidized form glutathione (GSSG) level of the HFISO group, showed a tendency to be lower than that of the HF group.

As shown in the Fig.4 (B), the serum 8-OHdG level of the HFISO group was significantly lower than that of the HF group, in which the 8-OHdG level was higher than that of the CON group.

5.4 Discussion

Obesity is considered to be the cause of metabolic syndromes because it causes the accumulation of visceral lipids, insulin resistance, increased serum low-density lipoprotein (LDL) cholesterol level, and decreased serum high-density lipoprotein (HDL) cholesterol levels, which are all closely concerned with complications of metabolic syndrome (*74*). Several authors reported benefits of isoflavone consumption on improvement of the serum triglyceride (*93, 98*) and on the prevention of obesity and diabetes (*99, 100*). However, negative effects of isoflavones have also been reported (*101*), suggesting a necessity to determine the effects of isoflavones more precisely. Almost all of the experiments concerned with these reports were carried out using genistein or its related compounds, which are dominant in soybeans, and using daidzein and genistein as aglycones, but determination of the physiological functions of daidzin and glycitin, which are isoflavone glycosides and found in leaves of unripe soybeans as well as soybeans, have not yet been fully determined in C57BL/6J mice which are susceptible to diet-induced obesity, type 2

diabetes, and atherosclerosis.

Body weight and feed efficiency were lower in the HFISO group than in the HF group after week 4 of the feeding period (Fig.5.2). This phenomenon resembled that in the previous report, in which it was reported that daidzein causes depression in food intake as well as body weight in mice accompanied leptin resistance (*102, 103*), suggesting that daidzin might decreased food intake and body weight gain. However, the suppressive effects of daidzin, a glucoside of daidzein, on food intake and body weight gain have not yet been examined. Higher serum leptin levels in the HF group in the comparison with the CON group may have indicated that being fed with the high fat diet is prone to cause leptin resistance (*85*). Return of the leptin level in the HFISO group to the level of the CON group may suggest that isoflavone mixture is able to improve leptin resistance, although it can not be excluded that the suppression effect of the isoflavone mixture for food intake caused a lower level of the leptin. A lower visceral adipose tissue weights in the HFISO group, compared to those in the HF group, may partly be due to a decrease in the food intake, and partly due to regulation of lipid metabolism by the isoflavone mixture. However, as the CPT activity did not show any significant difference between the HF and HFISO groups, and FAS activity showed a rather higher value in the HFISO group (Table 2), the effects of isoflavone mixture on visceral adipose tissue weights may be necessary to investigate in the future.

It is known that a high fat diet increases adipose tissue, which is a crucial factor in the development of obesity (*65*). Visceral adipose tissue weight in the HFISO group showed a tendency to be decreased (Table 5.3), showing a possibility that the isoflavone mixture had activity to inhibit the accumulation of adipose tissue. However, in contrast to visceral adipose tissue, serum TG and NEFA levels and liver lipid levels, except NEFA, did not

differ by a significant level between the HF and HFISO groups (Table 5.3). Although there is a report in which the serum triglyceride was increased by subcutaneous administration of genistein and daidzein in rats (*101*), the reason why the serum and liver triglyceride levels did not differ between the HF and HFISO groups in this experiment may be necessary to investigate in future. An increase in the liver NEFA level in the HFISO group may indicate that the isoflavone mixture has a weak activity to promote lipid synthesis as reported (*101*). Suppression in the decrease in the HDL-Chol level by feeding the isoflavone mixture (HFISO group), resulting in a decrease in the atherogenic index, also may suggest that isoflavone might be concerned with regulation of lipid metabolism as well as suppression of the progress of diabetes. No difference in the fecal excretion of lipids between the HF and HFISO groups (Table 5.5) indicates that the effects of isoflavone mixture on absorption of lipids from the intestinal tract may be small, but may affect lipid metabolism because the serum HDL-Chol level was higher in the HFISO group than in the HF group.

Lower HbA_{1c} and fasting glucose levels, and a lower tendency in the insulin level in the HFISO group in comparison with the HF group, in which those levels in the HF group was higher than those in the CON group (Fig.5.4, Fig.5.6) may indicate that the isoflavone mixture was able to suppress progression of diabetes induced by feeding a high fat diet. It was also considered that the function of the isoflavone mixture to improve leptin resistance, not a function to decrease food intake, might have improved diabetes markers, resulting in mitigation in the diabetes.

It has been reported that HbA_{1c} and serum leptin levels are increased in type 2 diabetic mice and that dietary genistein and daidzein has an activity to normalize its increase, and further, to normalize plasma and hepatic lipid levels, suggesting that these isoflavones, in

the form of aglycone, are effective to improve diabetes and lipid levels (*93*). However, those effects of daidzin and glycitin, which are glycosides of daidzein and glycitein, respectively, have not yet been examined precisely in C57BL/6J mice fed with a high fat diet. Lower serum 8-OHdG and higher GSH levels in the HFISO group in the comparison with the HF group may also suggest that generation of reactive oxygen species (ROS), which are concerned with production of 8-OHdG, is suppressed by feeding of the isoflavone mixture, and that ROS is efficiently scavenged by antioxidant enzymes necessary GSH in its action.

In this present report, it was demonstrated that the mixture of daidzin and glycitin, found in leaves of unripe soybeans as well as soybeans, is able to improve obesity, but its effects may be weak, and to improve diabetes, leptin resistance, and higher atherogenic index induced by a high fat diet, and further, to improve oxidative stress shown as an increase of 8-OHdG and decrease in GSH level. However, it may be necessary to investigate which isoflavone is more effective.

5.5 Summary

The anti-obese and anti-diabetic effects of a mixture of diadzin and Glycitin (isoflavone mixture) were examined in C57BL/6J mice fed with a high fat diet. The body weight gain, feeding efficiency and visceral adipose tissue weight of mice fed with the high fat diet with the isoflavone mixture for 92 days tended to be lower than those of mice fed with the high fat diet. The serum leptin and TNF-α levels showed significantly lower values and a tendency to be lowered in the isoflavone mixture-fed mice, respectively, than those of mice fed with the high fat diet. The liver glutathione (GSH) and serum 8-OHdG levels showed a higher and a lower level in the isoflavone mixture-fed mice. Furthermore, HbA_{1c}

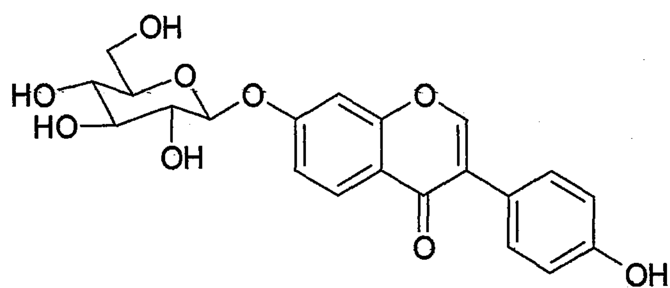
and fasting blood glucose levels showed significantly lower levels in the isoflavone mixture-fed mice than those of mice fed with the high fat diet. These results suggest that dietary daidzin and/or glycitin may be effective to improve diabetes through the improvement of leptin-resistance and oxidative stress caused by consumption of a high-fat diet for a long time, and that isoflavone mixture may be more effective to improve diabetes rather than obesity.

Table 5.1 Ingredient composition of the diet (%)

Constituent	CON	HF	HFISO
Casein	20	20	19.94
α-Cornstarch	53	8	8
Sucrose	10	25	25
Cellulose powder	5	5	5
Corn oil	7	7	7
Tallow	0	30	30
Mineral mixture[1]	3.5	3.5	3.5
Vitamin mixture[2]	1	1	1
L-Cystine	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2
Isoflavone mixture			0.06

[1] AIN-93G-MX and [2] AIN-93-VX were purchased from Clea Japan (Tokyo, Japan)

(A)



(B)

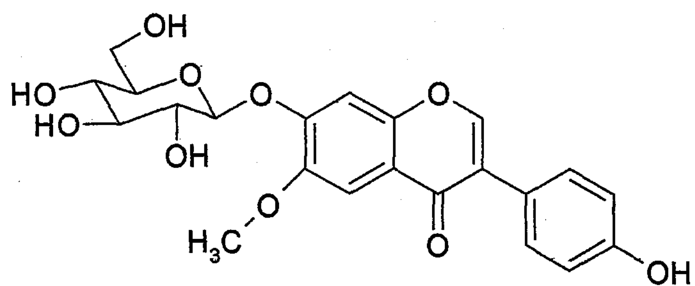


Fig. 5.1. Chemical structures of the isoflavones

A: Daidzin B: Glycitin

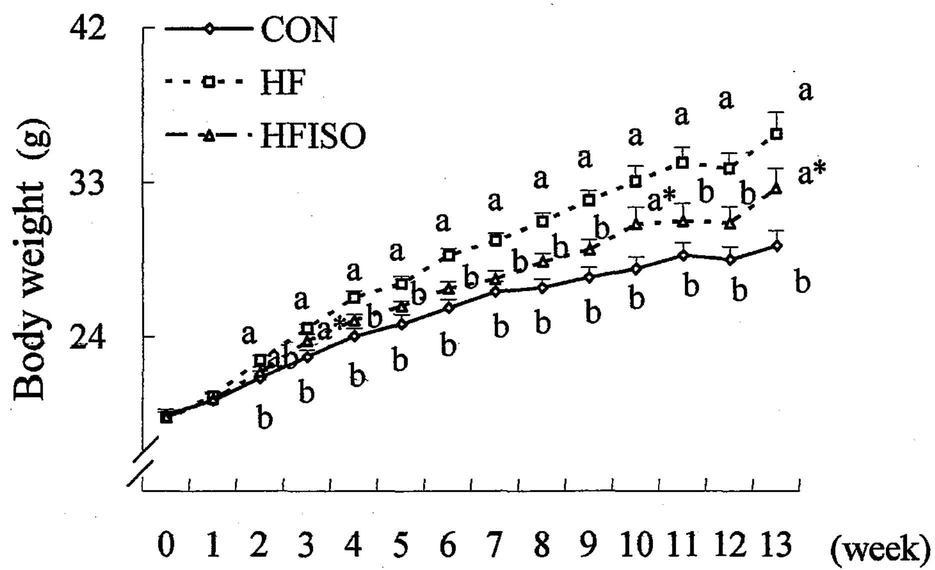


Fig. 5.2. Effects of dietary isoflavones on body weight

Each value is mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

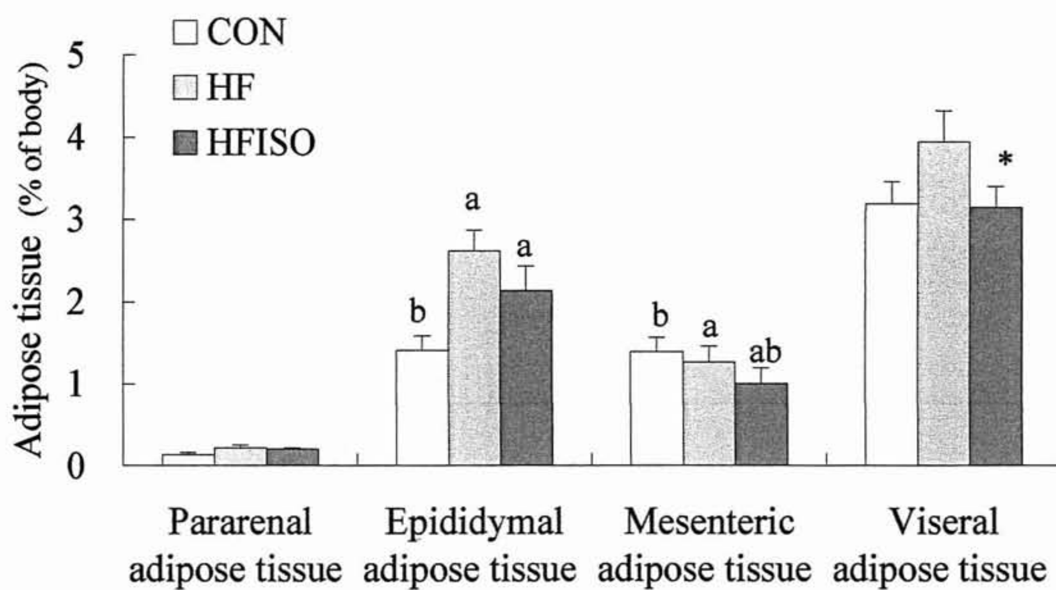


Fig. 5.3. Effects of dietary isoflavones on adipose tissue

Each value is mean ± SEM. n = 5-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

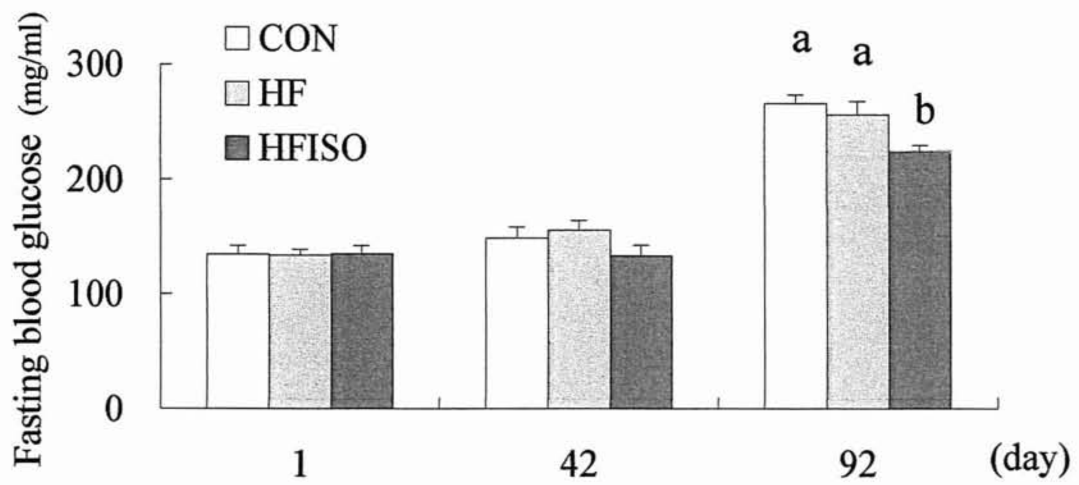


Fig. 5.4. Effects of dietary isoflavones on the fasting blood glucose levels

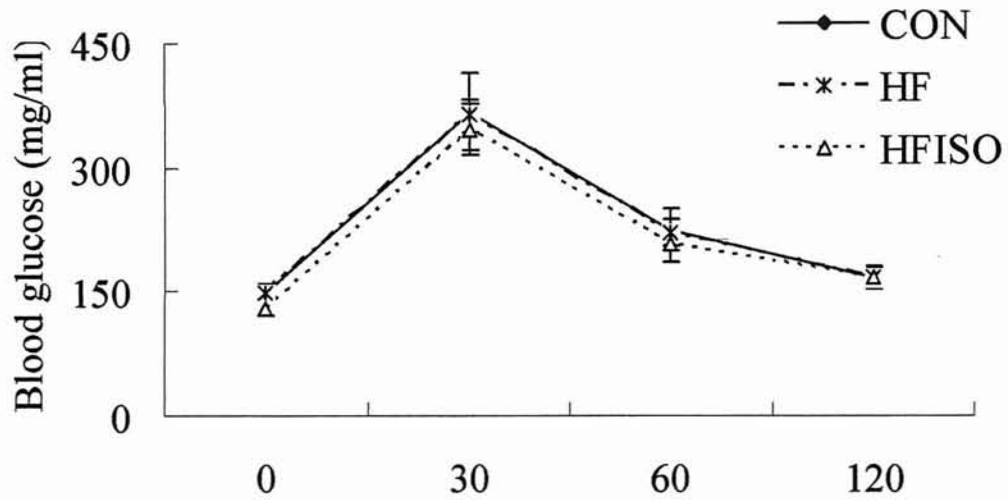
Each value is mean ± SEM. n = 7 for each group. Values without a common letter differ significantly ($p < 0.05$)

Table 5.2. Effects of dietary isoflavones on the total food intake, body weight gain and feed efficiency

Dietary group	CON	HF	HFISO
Total food intake (g)	$455±10^{a}$	$423±20^{ab}$	$382±13^{b*}$
Body weight gain (g)	$10.1±0.43^{b}$	$16.8±1.3^{a}$	$15.0±1.22^{a}$
Feed efficiency (%)[1]	$2.23±0.09^{b}$	$4.00±0.30^{a}$	$3.91±0.26^{a*}$
Liver (% of body weight)	$3.54±0.05^{ab}$	$3.38±0.06^{b}$	3.61±0.09
Kidney (% of body weight)	0.652±0.023	0.581±0.026	0.637±0.029

[1](body weight gain (g)/ food intake (g)). Each value is the mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$). *Compare with HF group ($0.05 < p < 0.1$)

(A)



(B)

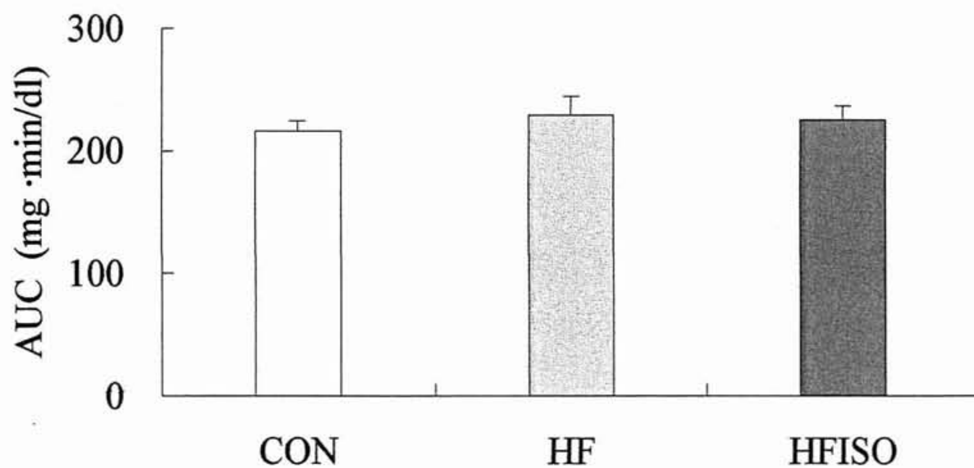


Fig. 5.5. Effects of dietary isoflavones on the blood glucose levels and AUC during the glucose tolerance Test

A: loading was carried out on days 42 of the feeding period B: AUC (area under the curve). Each value is mean ± SEM. n = 5-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

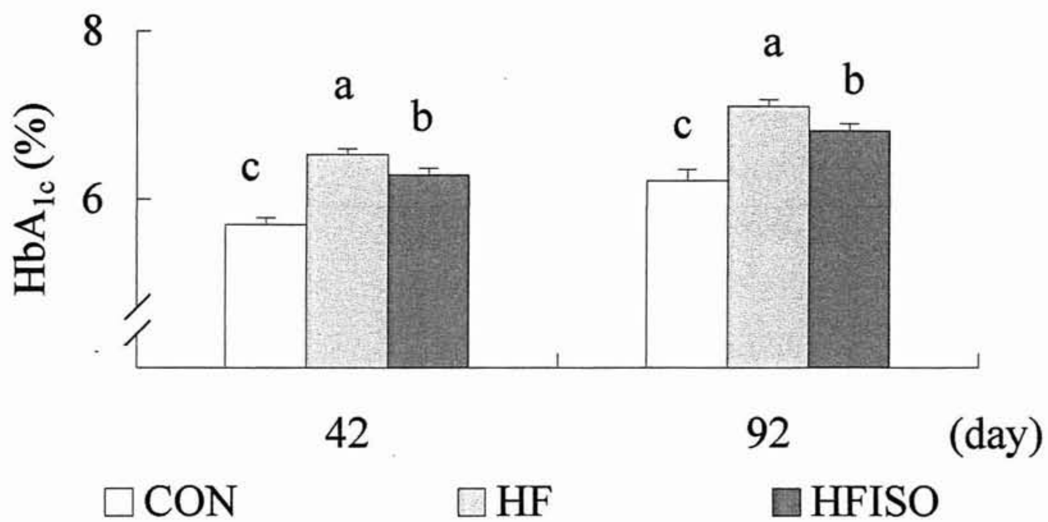


Fig. 5.6. Effects of dietary isoflavones on the HbA_{1c} levels

Each value is mean ± SEM. n = 5-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

Table 5.3. Effects of dietary isoflavones on the serum lipids and adipocytokine levels

Dietary group	CON	HF	HFISO
TC (mg/dL)	106±3	112±7	117±7
TG (mg/dL)	50.8±3.5[a]	38.1±3.5[b]	46.0±5.2[ab]
NEFA (mEq/dL)	0.686±0.035	0.719±0.033	0.717±0.033
HDL (mg/dL)	91.0±9.0[ab]	68.9±8.0[b]	98.9±8.8[a]
LDL (mg/dL)	43.1±7.5	49.7±6.6	23.3±7.9*
Atherogenic index	0.301±0.192[b]	0.899±0.232[a]	0.212±0.085[b]
Adiponectin (ug/mL)	2.02±0.08	1.91±0.10	1.91±0.06
HMW Adiponectin (ug/mL)	0.370±0.069	0.428±0.175	0.557±0.093
Leptin (ng/mL)	13.3±4.6[b]	81.08±26.8[a]	30.1±10.9[ab]*
Insulin (pg/mL)	148±68	344±72	290±57
TNF-α (pg/mL)	181±7	252±75	174±16*

[1]((T-Chol-HDL-Chol)/HDL-Chol) Each value is the mean ± SEM. n = 4-7 for each group. Values without a common letter differ significantly ($p < 0.05$). *Compare with HF group ($0.05 < p < 0.1$)

Table 5.4. Effects of dietary isoflavones on the liver lipid levels and CPT, FAS activities

Dietary group	CON	HF	HFISO
Total lipid (mg/g of liver)	85.6±3.2	77.1±3.6	84.1±3.3
TC (mg/g of liver)	2.71±0.24	2.48±0.20	2.72±0.18
TG (mg/g of liver)	42.3±2.9	35.5±2.9	35.6±2.8
PL (mg/g of liver)	16.5±0.6^{a}	13.8±1.0^{b}	15.5±0.6^{b}
NEFA(mEq/mg of liver)	8.40±0.90^{b}	7.52±0.98^{b}	11.8±1.7^{a}
CPT (umol/ min/mg protein)	0.708±0.039	0.674±0.062	0.644±0.063
FAS (umol/min/mg protein)	1.93±0.20^{a}	1.00±0.13^{b}	1.22±0.14^{b}

Each value is the mean ± SEM. n=6-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

Table 5.5. Effects of dietary isoflavones on the fecal excretion of lipids

Dietary group	CON	HF	HFISO
Total lipid (mg/3 days of feces)	50.5±2.1^{b}	65.2±4.8^{a}	73.6±3.3^{a}
T-Chol (mg/3 days of feces)	5.92±0.31	5.11±0.59	5.97±0.70
TG (mg/3 days of feces)	1.30±0.19^{b}	3.98±0.63^{a}	3.38±0.63^{a}
Total bile acid (mg/3 days of feces)	0.89±0.07^{b}	1.75±0.21^{a}	1.56±0.18^{a}

Each value is the mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$).

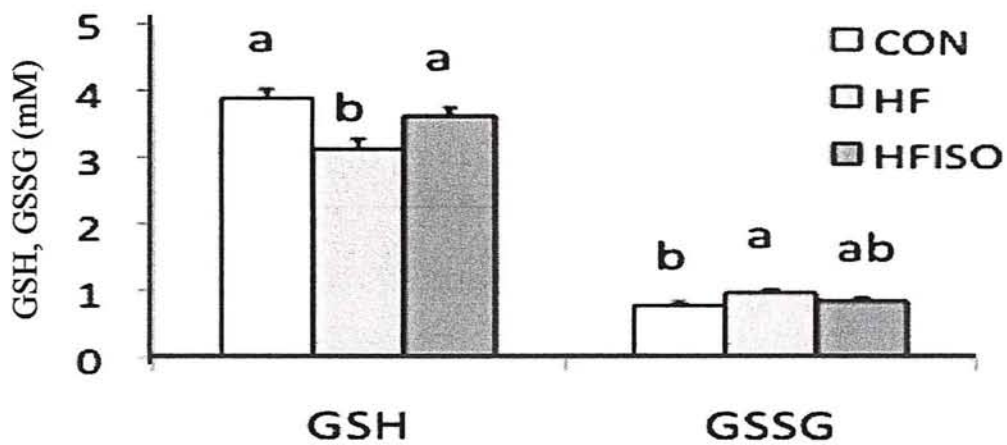


Fig. 5.7. Effects of the dietary isoflavone mixture on the liver glutathione levels in C57BL mice fed with the high-fat diet

GSH, glutathione reduced form; GSSG, glutathione oxidized form. Each value is the mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$)

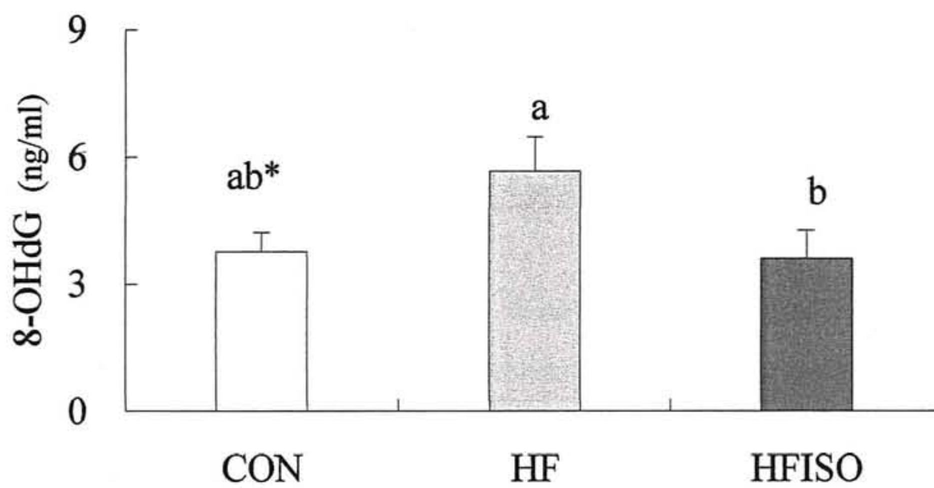


Fig.5.8. Effects of the dietary isoflavone mixture on the serum 8-hydroxy 2'-deoxyguanosine levels in C57BL mice fed with the high-fat diet

Each value is the mean ± SEM. n = 6-7 for each group. Values without a common letter differ significantly ($p < 0.05$). *Compare with HF group ($0.05 < p < 0.1$)

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Summary

Soybeans (*Glycine max* (L.) Merrill) can provide important protein sources and are an important part of diets worldwide. The physiological functions of soybeans have been studied, but no interest has been paid to the utilization of soybeans leaves, especially for leaves of unripe Jindai-soybean (Edamame, *Glycine max* (L.) Merrill. 'Jindai'). Jindai-soybeans are a local variety, cultivated in Shinjo, in Yamagata Prefecture, Japan, and the unripe soybeans are consumed as Edamame in Japan. However, Leaves of Jindai-soybean at the unripe stage are discarded without being used. Therefore, the use of the leaves and their extracts and components as raw food materials and food ingredients is very important from the standpoint of the efficient use of bio-resources and for prevention of lifestyle diseases such as obesity and diabetes. In this study, the isolation and identification of major flavonoids in unripe soybean leaves, and the examination of leave extracts, components were carried out. The purpose was to determine the activities that prevent diabetes and obesity and their action mechanism.

First, the polyphenolic fraction of Jindai-mame leaves was extracted with 70% methanol (MeOH) at 60-70 °C under reflux, and was applied to the Diaion-exchange resin column. The eluted fraction from the Diaion column was then further fractionated through various chromatographic techniques, such as Silica gel partition chromatography, gel filtration and high performance liquid chromatography, to isolate each compound. Isolated polyphenols were characterized by ^{1}H-, ^{13}C-, ^{1}H-^{1}H COSY-, ^{1}H-^{13}C COSY-, DEPT-, HMQC-, and HMBC-NMR, LC-Mass and HPLC. Several flavonoids, isoflavones and coumestan were identified. Major flavonoids in the kaempferol glycoside-rich fraction

were identified as kaempferol 3 -*O*- β -D-glucopyranosyl(1→2) -*O*-[α-L-rhamnopyranosyl (1→6)] -β -D -galactopyranoside, kaempferol 3-*O* -β -D-glucopyranosyl(1→2)-*O*-[α-L -rhamnopyranosyl (1→6)] -β -D -glucopyranoside, Kaempferol 3-*O*-β-D-glucopyranosyl (1→2)-β -D- galactopyranoside, and kaempferol-3-*O*-(2,6-di-*O*-α-L-rhamnopyranosyl) -β-D-galactopyronoside. The former 3 compounds had not yet been reported on the leaves of unripe soybeans.

Second, the anti-diabetes effects of the kaempferol glycoside-rich fraction from Jindai-mame leaves on KK-A^y mice were studied. As a result, there were no significant differences in body weight gain or blood glucose or serum lipid levels among the Kaempferol (K)-, KG- and basal diet-fed mice (each, K, KG and CON groups). The serum leptin, adiponectin, HMW-adiponectin and insulin levels also did not differ among the K, KG and CON groups. The HbA_{1c} levels measured on day 26 during the feeding periods were significantly lower in the K and KG groups, compared with that of the CON group. The liver TG, T-Chol, PL and NEFA levels were significantly lower in the K and KG groups than in the CON group. The liver FAS activity was significantly lower in the K and KG groups than in the CON group, but the CPT activities did not differ among those 3 groups. Comparing the effects of the K and KG groups, no significant differences in OGTT, HbA_{1c} level, serum and liver lipid levels between mice fed with kaempferol as aglycon (K) and fed with kaempferol glycoside-rich fraction , suggests that the effects of KG was mainly due to an aglycon moiety of kaempferol glycoside in KG, kaempferol. It is considered that the activities of K and KG lower liver lipids and have a tendency to improve diabetes by these compounds was partly due to the suppression of FAS activity by K and KG.

Thirdly, the anti-obesity and anti-diabetic activities of kaempferol glycoside-rich fraction were investigated in C57BL/6J mice fed with a high fat diet for 92 days. The visceral adipose tissue weight was lower in the KG group than in the CON group. Fasting blood glucose and HbA_{1c} levels were also lower in the KG group, suggesting that KG is available to mitigate both obesity and diabetes. The serum leptin and insulin levels increased by feeding with a high fat diet in K and KG groups, and returned to the level of the CON group by adding K and KG to the diets. This suggests that both the leptin and insulin resistance that might be induced by the high fat diet could be improved by the dietary KG, and that the anti-obese and anti-diabetic activities of KG might be related to its regulation of leptin and insulin levels.

Fourth, the anti-obese and anti-diabetic effects of a mixture of daidzin and glycitin (isoflavone mixture) were examined in C57BL/6J mice fed with a high fat diet. The body weight gain, feeding efficiency and visceral adipose tissue weight of mice fed with the high fat diet with the isoflavone mixture for 92 days tended to be lower than those of mice fed with the high fat diet. The serum leptin levels showed significantly lower values and TNF-α tended to be low in the isoflavone mixture-fed mice than the mice fed with the high fat diet. The liver reduced-form glutathione (GSH) and serum 8-OHdG levels showed a higher and a lower level in the isoflavone mixture-fed mice, respectively. Furthermore, HbA_{1c} and fasting blood glucose levels showed significantly lower levels in the isoflavone mixture-fed mice than those of the mice fed with the high fat diet. These results suggest that dietary daidzin and/or glycitin may improve diabetes through the improvement of leptin-resistance and oxidative stress caused by consumption of a high-fat diet for a long

time, and that an isoflavone mixture may be more effective to improve diabetes rather than obesity.

From these results, it is indicated that leaves of unripe Jindai-soybean (Edamame, *Glycine max.* L. Merrill. 'Jindai') contain 4 deferent types of kaempferol glycosides as major flavonoids, as well as isoflavones such as daidzin and glycitin. These flavonoids have activities that mitigate obesity and diabetes in obese mice that induced with being fed with a high fat diet and in type 2 diabetes mice. These results showed the possibility that the utilized Edamame leaves and its components can be used as functional food that prevent diseases.

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