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**Promotion of anti-diabetic effects
of flavonoid glycosides by
nondigestible saccharides**

(難消化性糖質によるフラボノイド配
糖体の抗糖尿病作用を高める研究)

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Division of Applied Bioscience
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SUMMARY

Management of Type 2 Diabetes Mellitus (T2DM) requires a combination of lifestyle modifications, exogenous insulin and prescription drugs. Many prescription drugs carry undesirable side effects and potential adverse drug interactions; therefore, researchers directed their attention to naturally occurring flavonoids (e.g., Quercetin 3-*O*-glucoside (Q3G)) and Fructooligosaccharide (FOS) for its potential to manage the insulin levels.

Q3G has shown to decrease plasma glucose level but has poor bioavailability due to poor absorption in the small intestine. FOS has shown to reduce the risk of hyperglycemia and dyslipidemia in animal models, but the evidence in human subjects are limited and endure methodologic limitations. Additionally, FOS enhances secretion of GLP-1, which is an enteroendocrine-derived peptide hormone that reduces blood glucose level by stimulating insulin secretion and regulating lipid metabolism (i.e., anti-diabetic effects).

We have previously demonstrated that FOS promotes the bioavailability of Q3G by way of suppressing the degradation in the caecum. The inter-relationship between Q3G, FOS and GLP-1 suggests an important but not yet realized effects on insulin secretion and glucose metabolism, which in turn has implications for reducing the risk of T2DM as well as hyperglycemia and dyslipidemia.

Investigating the anti-diabetic effects of Q3G, FOS and GLP-1 were conducted via *in vivo*, *in situ* and *in vitro* experiments. Our *in vivo* rat experiment investigated both individual and synergistic effects of Q3G and FOS in diets on visceral (i.e., abdominal) fat deposition, HOMA-IR and oral glucose tolerance test (OGTT). HOMA-IR and OGTT were chosen because they are standard diagnostic

index for insulin resistance. *In situ* rat experiment tested for the synergistic effects of Q3G and FOS on GLP-1 secretion in the distal part of ileum. *In vitro* experiment tested for direct effects of Q3G with- or without FOS on GLP-1 using a murine enteroendocrine cell line, GLUTag cells (i.e., L-cell model).

For the *in vivo* experiment, we hypothesized that supplementation of Q3G+FOS in a sucrose based AIN-93G diet would reduce a) visceral mass, b) OGTTs, c) fasting blood glucose, d) fasting insulin concentration, e) plasma total cholesterol, and f) HOMA-IR when compared to the sucrose-based diet (i.e., Suc). We also hypothesized that Q3G+FOS supplementation would increase in the plasma concentration of Q3G. For the *in situ* experiment, we hypothesized that test solution of Q3G+FOS injected directly into distal ileum would increase the plasma GLP-1.

To conduct our *in vivo* experiment, four groups of rats were fed a normal reference dextrin-based diet (D) or 1 of 3 sucrose-based diets (Suc) (0.3% Q3G; 5% FOS; 0.3% Q3G + 5% FOS (Q3G+FOS)) for 48 days. Oral Glucose Tolerance Tests (OGTTs) were done throughout the experiment and adipose tissue and aortic blood samples were obtained post-experiment. The GLP-1 secretion also was investigated via jugular vein after oral administration of Q3G and FOS (*in vivo* experiment) and via portal vein after ileal administration of Q3G and FOS (*in situ* experiment). *In vitro* experiments were done using GLUTag cells (i.e., L-cell model).

Significantly lower blood glucose level for the Q3G+FOS group was observed from *in vivo* experiment. HOMA-IR value was significantly lower in the Q3G+FOS group than in S group. The plasma quercetin derivatives increased for

FOS diet group on day 48. Plasma total cholesterol levels for the Q3G+FOS group was suppressed compared to the S group. GLP-1 secretion was enhanced in Q3G+FOS group than other groups in *in vivo* experiment. Ileal injection of Q3G with FOS in our *in situ* experiment yielded significantly higher increase and prolonged plasma GLP-1 concentrations, which was much higher than those after oral administration (*in vivo*). GLUTag cells used in our *in vitro* experiment confirmed that Q3G directly stimulated GLP-1 secretion while FOS enhanced the effects of Q3G.

Findings of our study suggests that synergistic effects of Q3G with FOS has the potential for prevention as well as management of T2DM by mediating the GLP-1 secretion and prolongation the high plasma concentration of the antidiabetic hormone with direct stimulation to L-cells.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Akt	Serine/threonine protein kinase
AUC	Area under the curve
BW	Body weight
CFA	Caffeic acid
CF	Caffeine, anhydrous
ChAH	Chlorogenic acid hemihydrate
D	Dextrin
DFA III	Difructose anhydride III
FI	Food intake
FOS	Fructooligosaccharide
G-Hesp	Glucosyl hesperidin
GLP-1	Glucagon-like peptide 1
Gal-M	Galactosyl myricitrin
Glu-M	Glucosyl myricitrin
α -GR	α -Glucosyl rutin
Hesp S	Hesperidin S
HOMA-IR	Homeostasis model assessment-insulin resistance
HQ3GM	High concentration of α -glycosyl-isoquercitrin
KCl	Potassium chloride
LC/MS	Liquid chromatography mass spectrometry
LQ3GM	Low concentration of α -glycosyl-isoquercitrin
M-Hesp	Monoglucosyl hesperidin

Milli Q	Ultra purified water by Milli-Q Integral Water Purification System
OGTT	Oral glucose tolerance tests
Q3G	Quercetin-3- <i>O</i> - β -glucoside (isoquercitrin)
Q3GM	α -Glycosyl-isoquercitrin
S	Saline
Suc	Sucrose
SCFAs	Short chain fatty acids
T2DM	Type II diabetes mellitus

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INTRODUCTION

Type II diabetes mellitus (T2DM), one of the fastest growing health problems [1, 2], increases individual's risk for cardiovascular diseases and cholesterolemia [3, 4]. T2DM can have serious health complications, including excess visceral adipose tissue [5] and hyperglycemia [6]. Many patients with T2DM rely on the use of prescriptions (e.g., sulfonylureas, glucosidase inhibitors, rosiglitazone and metformin) that have adverse side effects, such as weight gain, liver failure or increased risk of heart attacks, to manage their blood glucose levels (i.e., normal range is 4.0 to 5.9 and under 7.8 mmol/L for pre- and post-prandial)[7-12]. To minimize these side effects, researchers are seeking management approaches with minimal or no side effects while helping patients manage blood glucose level within a normal range. The search for effective and safer anti-diabetes is continuing to be an important topic.

Phytochemicals have been reported to be physiologically active compounds, including anti-hyperglycaemic agents [13]. They exert minimal or no adverse effects and are relatively low cost in comparison to synthetic drugs [14]. A diet high in fruits and vegetables intake has received much attention from the researchers and healthcare professionals, to be used for weight management strategy as well as its anti-diabetic effects of flavonoid. Flavonoids are a large group of plant secondary metabolites, widely present in food such as fruits, vegetables and plant-based foods [15, 16]. There are several subclasses of flavonoids including flavonols (e.g., quercetin or myricetin found nearly ubiquitous in foods), flavones (e.g. luteolin or apigenin found in herbs and celery), flavanones (e.g. hesperidin found in citrus fruit), flavan-3-ols (e.g. catechin and

epicatechin found in green tea, cocoa, and apples), anthocyanidins (e.g. cyanidin found in colored berries), isoflavones (e.g. daidzein found in soy products), and polymeric forms which characterize these molecules based on their carbon structure and level of oxidation [17, 18]. Past studies have demonstrated that flavonoids (e.g., Quercetin, Rutin, and Hesperidin) exhibit antioxidant and anti-inflammatory functions [19, 20] with not yet realized potential to reduce the risk of preventable medical conditions (i.e., certain types of cancer [21, 22], cardiovascular disease [23] and T2DM [16, 24]).

Quercetin (Appendix B), one of the most abundant flavonoids, is found naturally as quercetin-3-*O*- β -rutinoside or quercetin-3-*O*- β -glucoside (Q3G, Appendix C). Q3G receives much attention for its protective effects against preventable medical conditions such as coronary heart diseases [25, 26]. Additionally, Q3G in AIN-93G diet given to db/db mice has shown to reduce plasma total cholesterol, glucose, insulin levels, and homeostasis assessment for insulin resistance (HOMA-IR) [27-29]. The evidence supporting the benefits of Q3G in human diet, however, is limited. This may be due to low serum Q3G level in the plasma due to inefficient absorption in the small intestine and degradation by the microflora in the large intestine [30, 31].

Rutin (quercetin-3-*O*-rutinoside, Appendix E), a naturally occurring flavonol consisting of aglycone quercetin and a rutinoside moiety in position 3 of the C ring, is found in many plants such as buckwheat, apple, and tea. Rutin has a potential for glycemic control by increasing glucose uptake in both *in vivo* model of insulin resistance and T2DM in STZ-induced rat experiment [32, 33].

Hesperidin (Appendix F) is a flavanone glycoside, consisting of an

aglycone hesperetin or methyl eriodictyol and an attached disaccharide, rutinose [34]. It is present in citrus fruits, predominantly in oranges, lemons, and pomelos. Hesperidin and its derivatives have been found to have anti-hyperglycemic and anti-hypercholesterolaemic effects [35-37]. Hesperidin decreases blood glucose level by changing the activity of glucose metabolizing enzyme and potentiating the antioxidant defense system in streptozotocin-induced (STZ-induced) Type 1 and Type 2 diabetic rats [38, 39]. Only aglycones and some glucosides can be absorbed in the small intestine, whereas polyphenols linked to a rhamnose moiety must reach the colon and be hydrolyzed by rhamnosidases of the microflora before absorption [40].

Non-digestible oligosaccharides are a saccharide polymer containing a small number (typically 3 to 9) of simple sugars (monosaccharides) that can resist hydrolysis by salivary and intestinal digestive enzymes [41]. In recent times, they are considered to play an important role in human diet and health. Fructooligosaccharide (FOS, Appendix I), consisted of short chains of fructose molecules commonly found in many fruits and vegetables (e.g., banana, onion, and apple) [42]; is one of the most prevalent non-digestible oligosaccharides and well-established to exert physiological effects such as reduce the risk of hyperglycemia and dyslipidemia [31]. FOS in diet also has shown to reduce visceral adipose tissue deposition in mice [43]. The anti-diabetic effects of FOS, however, among human participants are inconclusive (i.e., FOS increases or decreases serum blood glucose level) and endure some methodological limitations [44, 45]. Additionally, it has also been reported that FOS promotes the bioavailability of essential minerals and flavonoids in animal experiments by

modification of the caecal- metabolism by microorganisms [46-48].

The development and approval of the incretin-based therapies may be a promising strategy in prevention and treatment of T2DM [57, 58]. Examples of incretin hormone are glucagon-like peptide-1 (GLP-1)(7-36) amide and GLP-1(7-37) [59], which are produced and secreted by the enteroendocrine L-cells of the small and large intestine in a nutrient-dependent manner. Upon release, GLP-1 stimulates the production and secretion of insulin, pancreatic β -cell proliferation, and inhibits glucagon secretion; all of which are important mechanisms for regulating glycemic level [60-62].

There is limited evidence on flavonoid on GLP-1 secretion. Recent evidence, however, suggests that flavonoid (e.g., Q3G) and non-digestible oligosaccharide (e.g., FOS) exert physiological effects on reducing glycemic level in rats and affects GLP-1 secretion and insulin with different mechanisms [63, 64]. FOS shows indirect effects on enhancing the secretion of GLP-1 as well as the expression of proglucagon gene in the proximal colonic mucosa through production of short-chain fatty acids (SCFA) [65]. In addition, Q3G may have direct effects on postprandial glycemic level by inhibiting carbohydrates digestion or absorption [66]. These findings suggest that Q3G and FOS together may have positive synergistic effects to reduce the glycemic level.

The primary objective of this study was to investigate both individual and combined effects of a flavonoid and a non-digestible saccharide on T2DM indices, such as plasma levels of glucose, total cholesterol and Homeostasis model assessment-insulin resistance (HOMA-IR). The secondary objective was to test the effect of a flavonoid and a non-digestible saccharide modulated glucose

homeostasis associations on enhancing secretion of incretin hormones, GLP-1.

CHAPTER 1: A NONDIGESTIBLE SACCHARIDE AUGMENTS BENEFIT OF QUERCETIN-3-*O*- β -GLUCOSIDE ON INSULIN SENSITIVITY AND PLASMA TOTAL CHOLESTEROL WITH PROMOTION OF FLAVONOID ABSORPTION IN SUCROSE-FED RATS.

We used rats to conduct *in vivo* experiments to compare the effects of Q3G, FOS, and Q3G+FOS on various indicators of anti-diabetic effects. The indicators for the *in vivo* experiment included visceral (i.e., abdominal) adipose and gastrocnemius muscle masses and plasma levels of glucose, total cholesterol, quercetin, and Homeostasis model assessment-insulin resistance (HOMA-IR). Oral glucose tolerance test (OGTT) was also conducted as a standard diagnostic test for insulin resistance. The purpose of this study was to investigate both individual and synergistic effects of Q3G and FOS on various indicators mentioned above for T2DM and to elucidate the potential mechanisms of its action.

MATERIALS AND METHODS

Chemicals

Quercetin-3-*O*- β -glucoside (isoquercitrin, Q3G) used for the *in vivo* experiment and α -glucosyl-isoquercitrin (Q3GM), used for in situ experiment was donated in kind by San-Ei Gen F.F.I., Inc. (Osaka, Japan). Fructooligosaccharide (FOS) (Meiologo-P, Meiji Seika Kaisha, Ltd., Tokyo, Japan) is a mixture of 42 % 1-kestose, 46 % nystose, and 9 % 1F- β -fructofuranosyl nystose. Other reagents and chemicals were of the highest grade commercially available.

Animals

Male Wistar/ST rats, weighing about 200 g (i.e., approximately 7 weeks old), were housed in an individual stainless steel cage with wire-mesh bottom. These cages were placed in a light (i.e., lights on 08:00-20:00), humidity (i.e., 40-60%), and temperature (i.e., 22-24°C) controlled room for a total of 48 days. Prior to the start of the study, rats acclimatized to their cages for 6 days with unrestricted access to tap water. Body weight (BW) and food intake (FI) in individual rats were measured daily. The Hokkaido University Animal Committee approved the study and the rats were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

***In vivo* experiment**

Experimental Diets

Rats were randomly divided on the basis of body weight into one of five groups ($n = 35$), which was based on the type of diet offered: Suc (sucrose), Q3G, FOS, Q3G+FOS and D (dextrin). All diets was based on AIN-93G formula by substituting cornstarch with sucrose or dextrin as a source of carbohydrates [67]. Suc, Q3G, FOS and Q3G+FOS diets used sucrose while D diet used dextrin for the source of carbohydrate. We chose to use sucrose for the experiment diets because it induced diabetic responses (i.e., decreased insulin sensitivity, increased plasma concentration of glucose and total cholesterol) [68, 69]. Dextrin does not induce diabetic response, therefore we chose to use D diet as the normal reference group to compare other 4 sucrose based experimental diets.

The recommendation of flavonoid daily intake is unavailable but we calculated the concentration of Q3G using the Dietary Guideline recommendations for the intake of vegetables and fruits for an adult (i.e., 1.5-2 cups of fruit and 2.5-3.5 cups of vegetables per day) [70]. The concentration for the FOS was based on the ADA recommendation for dietary fiber intake of 25 g for average adults consuming 2,000 kcal per day [71]. Therefore, we supplemented 0.3% of Q3G, 5% of FOS, and 0.3% of Q3G + 5% of FOS by replacing the sucrose for the Q3G, FOS and Q3G+FOS diets, respectively. All five diets had 20% protein and 7% fat content (Table I-1). All diets were given to the rats daily for a total of 48 days.

HOMA-IR and OGTT

OGTT tests were conducted on day 14, 28, and 45 using a 2 g glucose/kg BW with 20% glucose solution administered by gavage after 8 h fasting from early morning [72]. Blood samples were collected from the tail vein at 0 (i.e., before glucose administration), 15, 30, 60 and 120 mins post-glucose administration. Also, insulin and total cholesterol was measured from the tail blood (i.e., 0 min) and we used the following formula to calculate the HOMA-IR:

$$\text{HOMA-IR} = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (}\mu\text{U/mL)} / 22.5$$

Blood and Tissue Sample Collection

On day 48 of the study, the rats were first anesthetized using pentobarbital (40 mg/kg BW) and then injected with insulin (10 units/kg BW) into the portal vein 2 min prior to collecting the aortic blood for assessment of phosphorylation

of an insulin signaling molecule, Akt. The aortic blood was collected using a syringe containing sodium heparin (200 IU/ mL blood; Ajinomoto, Tokyo, Japan) as anticoagulation. We obtained aortic blood sample to assess the plasma concentration of quercetin derivatives (e.g., methyl quercetin and quercetin). We also dissected and weighed visceral adipose tissues (e.g., epididymal, mesenteric, and retroperitoneal), caecum with the contents, liver, and gastrocnemius muscle prior to freezing them in liquid nitrogen for analysis at later time.

Analytical Methods

Blood samples were centrifuged (1300 *g* for 15 min at 4°C) to isolate the plasma. The concentration of insulin was analysed with a commercial Rat Insulin ELISA kit (AKRIN-010T, ShibayakiCo., Ltd., Gunma, Japan), while glucose and total cholesterol concentration in plasma were analysed with commercial kits (Glucose CII Test Wako and Cholesterol E Test Wako, Wako Pure Chemicals, Osaka, Japan). The caecal contents were diluted with 4 volumes of deionized water and homogenized with a Teflon homogenizer. The pH of these caecal homogenates was measured with a semiconducting electrode (ISFET pH sensor 0010–15C, Horiba,Ltd., Kyoto, Japan).

Plasma quercetin derivatives

The plasma sample (100 μ L) of the aortic blood was treated enzymatically to release quercetin from glucuronic acid and sulfate conjugates, and subsequently solid-phase extracted. The plasma was first acidified by adding 10 μ L of 0.58 mol/L acetic acid and total flavonoid extraction was treated with 10 μ L of β -

glucuronidase/sulfatase (*Helix pomatia* extract, Sigma G0876, 5,106 U/L of β -glucuronidase and 25,105 U/L of sulfatase) for 30 min at 37°C, but not for extracting the unconjugated flavonoids. In order to extract the quercetin derivatives, prepared mixture was added to 100 μ L of methanol, heated at 100°C for 1 min, and then centrifuged. The extraction procedure was repeated in triplicate.

The combined supernatant was transferred to a C18 cartridge (Oasis HLB, Waters Co. LTD, Milford, MA, USA) and after rinsing it with 1 mL of water, the eluent with methanol was dried and dissolved in 100 μ L of 50% methanol (i.e., sample solution). The supernatant was examined for quercetin and its metabolites using the LC/MS system with an electric spray ionization (ESI) interface (Acquity UPLC, Waters Co. Ltd., Milford, MA) [46]. Concentrations of Q3G, monomethylquercetin (i.e., isorhamnetin and tamarixetin), and quercetin were calculated from the peak area of each mass spectrum with calibration curves of each standard compound. The concentrations of conjugated derivatives in the plasma were estimated as quercetin or monomethylquercetin concentrations after the enzymatic treatment subtracted the values without enzyme treatment.

Western blot for Akt phosphorylation after an insulin injection

Gastrocnemius muscle samples (100 mg) were collected and put in 1 mL lysis buffer (Appendix N). Then, 10 μ L of 100 mmol/L phenylmethylsulfonyl fluoride was added and homogenized for 15 seconds by polytron homogenizer. Homogenates were centrifuged at 8500 *g* at 4°C for 10 min. The supernatants were added and mixed thoroughly with equal volume of 2X Laemmli sample

buffer. The solution was then heated for 3 mins before loading 50 µg of protein per lane on to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide gel) and transferred to a nitrocellulose membrane. Phosphorylated protein kinase B (Akt) (Ser-473) and total Akt were detected after the membranes were incubated with the respective primary antibodies (Rabbit anti-*p*-Akt-ser-473, Cell Signalling Technology, MA, USA, 1:1000 dilution and Rabbit anti-Akt, Rockland, PA, USA), and HRP-conjugated anti-rabbit IgG antibodies (1:20000, Sigma-Aldrich, MO, USA) as a secondary antibody. Protein bands were detected by the enhanced chemiluminescence method (GE Healthcare), and quantified by densitometric analysis of specific bands on the immunoblots using Image J software version 1.44 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Daily body weight gain was calculated by differences between final and initial body weight divided by days of test period. Also, daily food intake was calculated from accumulated food consumption divided by days of test period.

We employed one-way ANOVA with Tukey-Kramer's post-hoc test, with a statistically significant *P*-value of < 0.05. Differences in anti-diabetic indicators (i.e., body weight, food intake, tissue weights, plasma glucose, insulin and Q3G concentration) among 4-sucrose based diet groups of rats and the normal reference D (i.e., dextrin) group were examined. Two-way ANOVA was used to verify the effect of the time (i.e., day 0, 14, 28, and 45) and diet treatment (i.e., Suc, Q3G, FOS, Q3G+FOS, and D), and time-by-diet treatment interaction on

fasting glucose, fasting insulin, HOMA-IR, and total cholesterol. Differences in post-mortem tissue weights (i.e., abdominal fat pads, gastrocnemius muscle, and liver) and quercetin derivative in plasma were examined among 5 diet groups.

RESULTS

Change in body and tissue weight, food intake, and caecal pH

Average initial BWs were similar in four sucrose-fed groups (236.4 , $SE = 1.3$, $n = 28$) as well as between Suc and D diet groups. Weight gain was observed for all diet groups, but less weight gain was observed for the Q3G+FOS diet (2.5 ± 0.2 , $P < 0.05$) compared to the Suc (3.2 ± 0.2) and D diet groups and (3.5 ± 0.2 , Table I-2). The weight gain was lowest for the Q3G+FOS group compared to other diet groups. These changes in BWs were observed without significant differences in food and energy intakes between Suc and Q3G+FOS diet groups.

Weights of epididymal and mesenteric fat pads were lower for the Q3G+FOS diet group but the mesenteric and retroperitoneal pad weights were lower for the FOS diet group compared to those in Suc diet group (Table I-3). Gastrocnemius muscle weights were lower in Suc group compared to all other diet groups. Liver weights in four sucrose-base diet groups were higher than that in D diet group (Table I-3).

The caecal pH values were significantly lower in the FOS-fed groups with higher weights in both caecal tissue and contents compared to Suc group ($P < 0.05$) (Table I-4). Weight of the caecal contents in FOS group (2.02 ± 0.08), but not Q3G group, was higher than Suc (0.46 ± 0.04) and D (0.71 ± 0.06) groups, and the weight of Q3G+FOS group (2.70 ± 0.16) was further increased compared to that of only FOS group (Table I-4).

Oral glucose tolerance test (OGTT)

In the oral glucose tolerance test (OGTT) on day 14 (Fig. I-1 A), the level

of plasma glucose reached its peak at 30 min after administration with the greatest value (14.75 ± 0.43 mmol/L) in the Suc group without significant difference between treatments. The reduction from the peak to 60 min tended to be greater in the Q3G+FOS group than in the Q3G or FOS group despite similar peak values among the 3 other diet groups and the normal reference, D group. At 60 min, the value of Q3G+FOS group was significantly lower (8.44 ± 0.27 mmol/L, $P < 0.005$) than those of Suc (11.00 ± 0.44) and D groups (11.34 ± 0.65 mmol/L). Two-way ANOVA for time and diet treatments showed significant effect on plasma glucose at 60 min in OGTTs ($P < 0.0001$ and 0.0052 , respectively), but no interactions between time and diet treatments on day 14.

On day 28 and 45, changes in plasma glucose level at 60 min among the groups were similar to the day 14. The glucose levels for the Q3G+FOS at 60 min in OGTTs was significantly lower ($P < 0.05$) than Suc group on day 28, and than Suc and D groups on day 45 (Fig. I-1 B). The AUC (0-120 min) of plasma glucose for the Suc diet group was highest among other diet groups on day 14 and 45. Also, the glucose AUC (0-120 min) of Suc diet group was significantly higher than Q3G+FOS ($P < 0.005$), but no differences when compared to the Q3G or FOS group (Fig. I-1 C). Two-way ANOVA for time and diet treatments showed significant effect on AUC of plasma glucose ($P < 0.0001$), but no interactions between time and diet treatments.

Insulin sensitivity

The Q3G+FOS diet group had significantly lower fasting plasma glucose level (5.82 ± 0.17 mmol/L, $P < 0.005$) than the Suc group (7.26 ± 0.07 mmol/L)

on day 14 (Fig. I-2 A). We also observed significant effects of time (i.e., day 0, 14, 28, and 45) ($P = 0.0022$) and diet treatment ($P < 0.0001$). Insulin concentrations at fasting state showed no significant difference on day 0 and 14. The fasting insulin level in the Q3G+FOS was statistically lower ($P < 0.05$) than the Suc diet group from day 28 to 45 (Fig. I-2 B). Two-way ANOVA showed significant interactions between time and diet treatments on fasting insulin ($P = 0.0036$).

An insulin resistance index, homeostatic model assessment of insulin resistance (HOMA-IR), was calculated from fasting levels of plasma glucose and insulin. The HOMA-IR index for the Q3G+FOS diet group was significantly lower ($P < 0.05$) than Suc diet group on day 14, 28 and 45 (Fig. I-2 C). Two-way ANOVA for time ($P < 0.0001$), diet treatments ($P < 0.0001$), and interactions ($P = 0.0051$) showed significant on HOMA-IR.

Plasma total cholesterol level

Plasma total cholesterol level on days 14, 28, and 45 was significantly lower ($P < 0.05$) in the Q3G+FOS diet group than the Suc diet group (Fig. I-3). The total cholesterol level in the Suc diet group was highest among all groups throughout experimental period but we observed similar level of total cholesterol for both Q3G+FOS and the normal reference D group throughout the experiment. Two-way ANOVA for time and diet treatments showed significant effects on total cholesterol ($P < 0.0001$), but no interactions between time and diet treatments.

Effects on plasma quercetin derivative levels

Quercetin and methylquercetin was detected in conjugated forms, while

Q3G was not detected in the blood plasma among rats given experimental diets containing Q3G (i.e., Q3G and Q3G+FOS diet groups) for 48 days (Fig. I-4). None of the quercetin derivatives was detected in the blood among rats given a Suc or D diet. The concentration of methylquercetin conjugates was much higher than that of quercetin conjugates in both Q3G and Q3G+FOS groups. The sum of methylquercetin and quercetin values in the 5% FOS supplemented groups were 8-fold higher than the Q3G diet groups ($P < 0.05$).

Changes in Akt phosphorylation

Akt phosphorylation level under insulin stimulation was measured to evaluate insulin-signaling activity in gastrocnemius muscle, which is the most important tissue to determine whole body insulin sensitivity. Akt phosphorylation level in sole Q3G group was highest among all groups, and higher with significant difference (2.15 ± 0.27 , $P < 0.05$) compared to the Suc (1.13 ± 0.25) and D (i.e., normal reference) groups (0.92 ± 0.12), but not in the FOS or Q3G+FOS groups (Fig. I-5).

DISCUSSION

We conducted *in vivo* experiment to test whether Q3G, FOS, or Q3G+FOS, have anti-diabetic effects on rats. In the *in vivo* experiment, we found that sucrose-based AIN-93G diet supplemented with Q3G+FOS minimized overall body weight and abdominal fat pads weight compared to rats given Suc, Q3G, or FOS diet. Moreover, we observed significant improvements in anti-diabetic indicators for rats given Q3G+FOS diet but not for rats given a Q3G or FOS diet. Q3G+FOS diet group showed reduction of plasma total cholesterol with increase in plasma quercetin concentration and cecal weight with lower luminal pH as well as improvements in glucose tolerance (i.e., OGTT), insulin sensitivity.

An increase in plasma quercetin concentration level that we observed for rats given Q3G+FOS diet was consistent with our previous published study, which may be associated with greater effect on metabolic syndrome-related indicators. Our finding suggests that cecal bacterial suppressed quercetin aglycone degradation which increased the bioavailability of quercetin in the plasma [46]. The increase in the bioavailability of quercetin may also reduce the total cholesterol concentration in the plasma by suppressing the synthesis or catabolism of cholesterol. Past studies have reported that quercetin is responsible for regulating lipid metabolism at the transcription level (i.e., *Fnta*, *Pon1*, and *Ppara* expression) [73, 74]. Quercetin may have a role in reducing the expression of *Ppara* gene, which affects fat accumulation in the liver and induces the *Cd36* gene (i.e., the *Ppara* target genes) [29]. The expression of these genes may have a role in the reduction of cholesterol level.

Likewise, dietary effects and the periodic changing pattern in HOMA-IR is

very similar to those of total cholesterol, which suggests that similar mechanism may be used to regulate insulin sensitivity and cholesterol levels. Only the Q3G+FOS diet group showed significant improvements in HOMA-IR values on day 14, 28, and 45. Q3G+FOS improved glucose tolerance and insulin resistance index impaired by high sucrose. These improvements, however, were not clear in the Q3G or FOS groups compared to the Q3G+FOS group. The tendency of improvement in HOMA-IR for Q3G group was observed on day 45. A prolonged exposure of low level of quercetin in blood may adaptively improve insulin sensitivity on day 45.

Quercetin (10 mg/kg of body weight/day) reportedly increased plasma concentration of adiponectin but reduced TNF- α secretion and the expression of the pro-inflammatory inducible nitric oxide synthase (iNOS) in visceral adipose fat pads [75]. Our finding suggests that Q3G in diet increased the phosphorylation of Akt, which is a main downstream target molecule of PI3-kinase [76, 77]. PI3-kinase is an essential molecule for insulin-stimulated glucose transporter 4 translocation in skeletal muscle [78, 79]. The activation of insulin signaling pathway with Akt phosphorylation due to quercetin intake may contribute to improvement in insulin sensitivity in the Q3G diet group.

The time-dependent improvement in HOMA-IR value in our previous study suggests that FOS feeding takes time to affect insulin sensitivity [80]. Our result also showed a tendency to improve HOMA-IR in FOS diet group only on day 45. FOS has shown to promote proliferation of bifidobacteria and lactobacilli associated with caecal hypertrophy and lowered the pH due to higher level short chain fatty acid (SCFA) production in the caecum [81]. Moreover, SCFA (i.e.,

butyrate) supplementation at 5% (w/w) in a high-fat diet has shown to reduce the development of dietary obesity and insulin resistance [82]. FOS intake also reduced fat absorption [43] and induced the reduction of the abdominal adipose tissue with increasing SCFA production. Taken together, FOS promoting the absorption of quercetin glycosides and reducing abdominal fat by the cecal fermentation may improve the insulin sensitivity in Q3G+FOS diet group.

In conclusion, FOS enhances the beneficial effects of Q3G on insulin sensitivity and reduces plasma total cholesterol levels. Also, we observed that FOS in the diet reduced the abdominal fat deposition. The anti-diabetic effects of Q3G+FOS in rat may be applicable to human subjects; however, additional investigation in human study may need and warrant. Our findings suggest that Q3G and FOS may serve as a non-invasive approach to managing or reducing the risk of T2DM.

Table I-1 Composition of normal reference⁵ and experimental diets

Diet ingredients	Experimental Diets				Normal Reference Diet
	(g/kg)				(g/kg)
	Suc ¹	Q3G ²	FOS ³	Q3G+FOS ⁴	D ⁵
Dextrin⁶	-	-	-	-	629.5
Sucrose⁷	629.5	626.5	579.5	576.5	-
Casein⁸	200	200	200	200	200
L-cystine	3	3	3	3	3
Soybean oil	70	70	70	70	70
Crystallized cellulose⁹	50	50	50	50	50
Mineral mixture¹⁰	35	35	35	35	35
Vitamin mixture¹⁰	10	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
Q3G¹¹	-	3	-	3	-
FOS¹²	-	-	50	50	-
Energy (kJ/kg)	16,553	16,517	16,135	16,099	16,276

1. Suc is a modified AIN-93G diet with sucrose as source of carbohydrate.
2. Q3G is Suc with 0.3% Q3G.
3. FOS is Suc with 5% FOS.
4. Q3G+FOS is Suc with 0.3% Q3G and 5% FOS.
5. D is a modified AIN-93G diet with dextrin as source of carbohydrate as a normal reference group.
6. Dextrin (TK-16; Matsutani Chemical Industry).
7. Sucrose
8. Casein (ALACID; New Zealand Daily Board).
9. Crystallized cellulose (Avicel PH102, Asahi Chemical Industry).

10. Mineral and vitamin mixtures were prepared according to the AIN-93G formulation (Appendix K and L).
11. Quercetin-3-O- β -glucoside (San-Ei Gen F.F.I., Inc.; Osaka, Japan)
12. Fructooligosaccharide (Meiji Seika Kaisha, Ltd.)

Table I-2 Changes in body weights, average food intake, and average energy intake of rats fed a sucrose-based experiment diet (Suc) supplemented either with or without 0.3% Q3G (Q3G), 5% FOS (FOS), or 0.3% Q3G + 5% FOS (Q3G+FOS), and a dextrin (D) as normal reference group for 48 days.

	Initial Body Weight (g)	Final Body Weight (g)	Body Weight Gain (g/d)	Food Intake (g/d)	Energy Intake (KJ/d)
Suc	236.3 ± 2.8	394.9 ± 8.4 ^{ab}	3.2 ± 0.2 ^a	19.1 ± 0.5	315.9 ± 7.6
Q3G	236.4 ± 2.2	379.7 ± 9.2 ^{ab}	2.9 ± 0.2 ^{ab}	18.7 ± 0.4	308.8 ± 6.1
FOS	236.6 ± 3.0	378.2 ± 8.4 ^{ab}	2.9 ± 0.2 ^{ab}	17.8 ± 0.4	286.9 ± 6.2
Q3G+FOS	236.4 ± 3.0	358.6 ± 11.8 ^b	2.5 ± 0.2 ^b	18.9 ± 0.6	303.6 ± 9.1
D	236.9 ± 2.7	406.5 ± 8.3 ^a	3.5 ± 0.2 ^a	18.7 ± 0.4	309.9 ± 7.0

Mean ± SE (n = 6-7) with unlike superscript letters were significantly different ($p < 0.05$) for 4 sucrose-based diet groups and a normal reference group (D) performed by Tukey-Kramer's post hoc tests.

Table I-3 Abdominal fat pads (Epididymal, Mesentric, and Retroperitoneal), gastrocnemius muscle, and relative liver weights of rats fed a sucrose-based experiment diet (Suc) supplemented either with or without 0.3% Q3G (Q3G), 5% FOS (FOS), or 0.3% Q3G + 5% FOS (Q3G+FOS), and a dextrin (D) as normal reference for 48 days.

	Abdominal Fat Pads			Gastrocnemius muscle	Liver
	Epididymal	Mesentric	Retroperitoneal		
	(g/100g BW)				
Suc	1.88 ± 0.13 ^a	1.26 ± 0.08 ^a	2.31 ± 0.29 ^a	0.54 ± 0.04 ^b	3.64 ± 0.13 ^a
Q3G	1.82 ± 0.17 ^a	1.16 ± 0.10 ^{ab}	1.99 ± 0.15 ^{ab}	0.61 ± 0.01 ^a	3.54 ± 0.08 ^a
FOS	1.41 ± 0.10 ^{ab}	0.89 ± 0.05 ^b	1.40 ± 0.12 ^b	0.60 ± 0.01 ^a	3.60 ± 0.13 ^a
Q3G+FOS	1.30 ± 0.08 ^b	0.89 ± 0.07 ^b	1.44 ± 0.24 ^{ab}	0.58 ± 0.02 ^a	3.40 ± 0.06 ^a
D	1.71 ± 0.08 ^{ab}	1.22 ± 0.09 ^{ab}	2.31 ± 0.27 ^{ab}	0.59 ± 0.02 ^a	2.95 ± 0.07 ^b

Mean ± SE (n = 6-7) with unlike superscript letters were significantly different ($p < 0.05$) for 4 sucrose-based diet groups and a normal reference group (D) performed by Tukey-Kramer's post hoc tests.

Table I-4 Caecal with content weight and pH of the caecal of rats fed with experimental diets supplemented either with or without 0.3% Q3G, 5% FOS, or 0.3% Q3G + 5% FOS, and a dextrin (D) as normal reference for 48 days.

	Caecal + content	Caecal tissue	Caecal content	pH
	(g/ 100 g Body Weight)			
Suc	0.71 ± 0.06^c	0.25 ± 0.03^b	0.46 ± 0.04^c	7.97 ± 0.04^{ab}
Q3G	0.90 ± 0.06^c	0.26 ± 0.02^b	0.64 ± 0.07^c	7.84 ± 0.03^b
FOS	2.51 ± 0.08^b	0.49 ± 0.04^a	2.02 ± 0.08^b	6.76 ± 0.08^c
Q3G+FOS	3.32 ± 0.18^a	0.61 ± 0.05^a	2.70 ± 0.16^a	6.83 ± 0.13^c
D	0.93 ± 0.05^c	0.22 ± 0.03^b	0.71 ± 0.06^c	8.22 ± 0.05^a

^{a,b,c} Mean \pm SE within a row with unlike superscript letters were significantly different ($p < 0.05$) for 4 sucrose-based diet groups and a normal reference group (D) performed by Tukey-Kramer's post hoc tests.

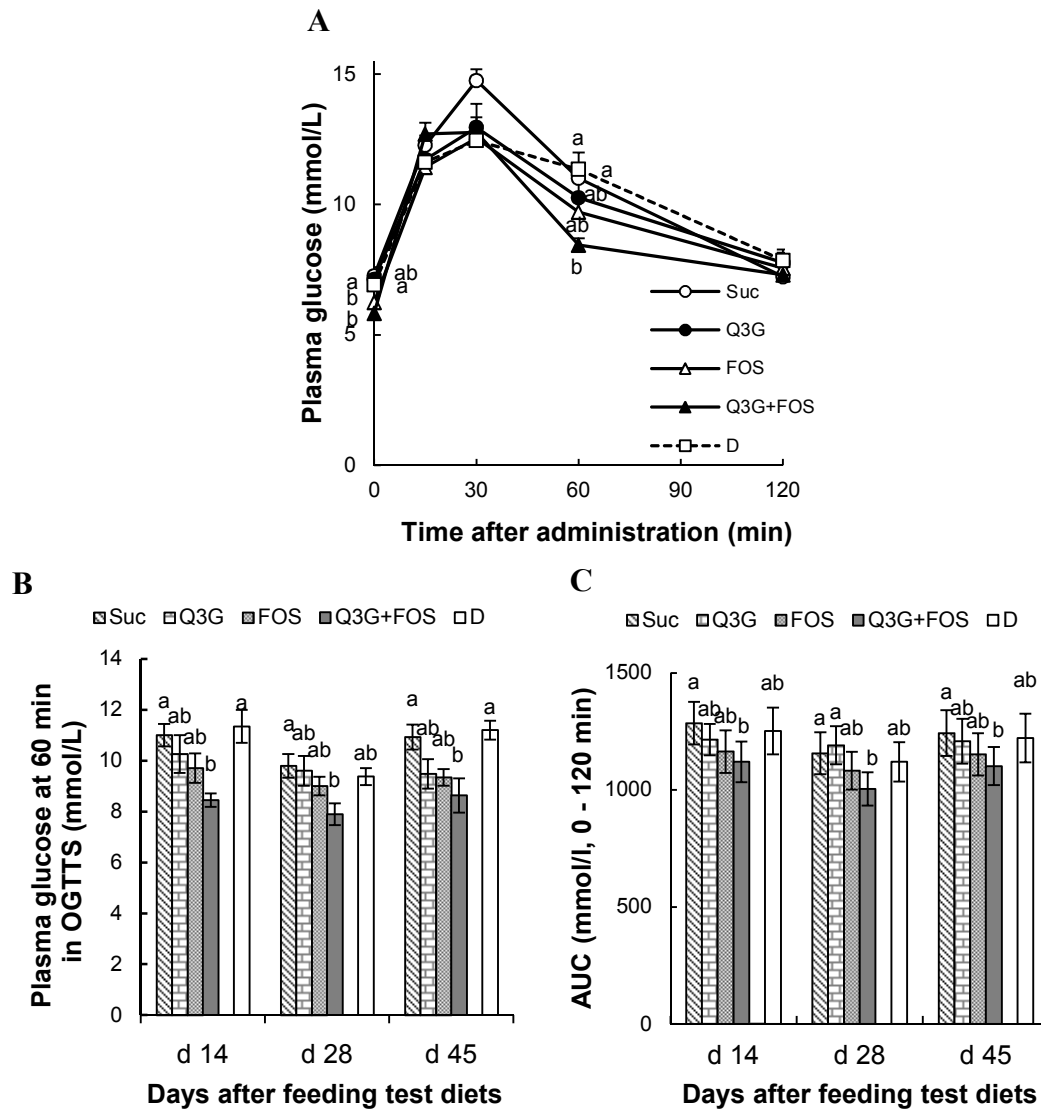


Figure I-1 Plasma glucose concentration in rats fed with test diets in response to OGTT with an oral glucose load (2 g/kg BW) on day 14 (**A**), plasma glucose concentration at 60 min (**B**) and AUC of glucose concentrations in OGTT from 0-120 min performed on day 14, 28 and 45 (**C**).

Where Suc is sucrose diet, Q3G is 0.3% Q3G in sucrose based diet, FOS is 5% FOS in sucrose based diet, Q3G + FOS is 0.3% Q3G + 5% FOS in sucrose based diet, and D is dextrin diet. 2-way ANOVA was used to reveal significance of time (0.0052 and < 0.0001 for **B** and **C** respectively), treatment (< 0.0001 for **B** and **C**) and interaction of time \times treatment (0.9166 and 0.8618 for **B** and **C** respectively). Values are the means \pm SE depicted by vertical bar ($n = 6-7$). ^{a,b} Mean values within a row with unlike alphabetical letters denote significant differences ($P < 0.05$) among all groups.

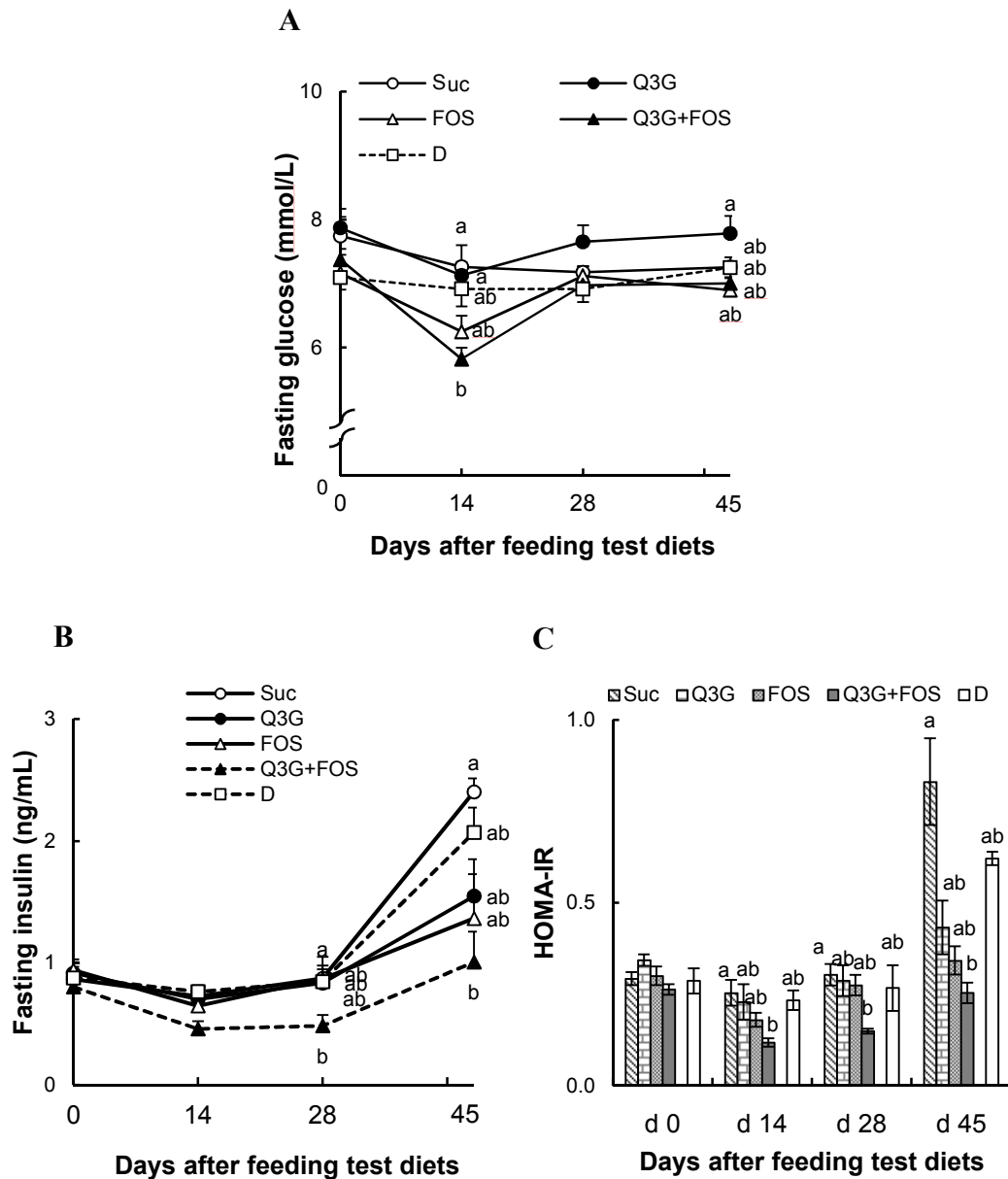


Figure I-2 Fasting glucose concentration (**A**), fasting insulin concentration (**B**), and HOMA-IR (**C**) of rats fed with experimental diets on day 0, 14, 28, and 45.

$$\text{HOMA-IR} = \text{fasting glucose (mM)} \times \text{fasting insulin } (\mu\text{U/mL}) / 22.5.$$

Where Suc is sucrose diet, Q3G is 0.3% Q3G in sucrose based diet, FOS is 5% FOS in sucrose based diet, Q3G + FOS is 0.3% Q3G + 5% FOS in sucrose based diet, and D is dextrin diet. 2-way ANOVA was used to reveal significance of time (0.0022, < 0.0001, and < 0.0001 for **A**, **B** and **C** respectively), treatment (< 0.0001 for **A**, **B** and **C**) and interaction of time \times treatment (0.0036, 0.0036, and 0.0051 for **A**, **B** and **C** respectively). Values are the means \pm SE depicted by vertical bar ($n = 6-7$). ^{a,b} Mean values within a row with unlike alphabetical letters denote significant differences ($P < 0.05$) among all groups.

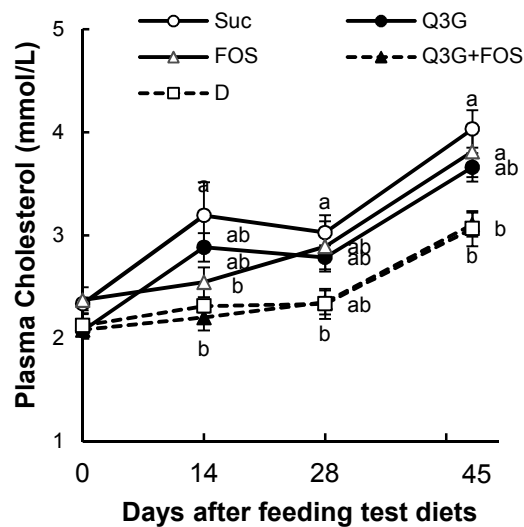


Figure I-3 Plasma total cholesterol concentration of rats fed with experimental diets on day 0, 14, 28, and 45.

Where Suc is sucrose diet, Q3G is 0.3% Q3G in sucrose based diet, FOS is 5% FOS in sucrose based diet, Q3G + FOS is 0.3% Q3G + 5% FOS in sucrose based diet, and D is dextrin diet. 2-way ANOVA was used to reveal significance of time (< 0.0001), treatment (< 0.0001) and interaction of time \times treatment (0.4412). Values are the means \pm SE depicted by vertical bar ($n = 6-7$). ^{a,b} Mean values within a row with unlike alphabetical letters denote significant differences ($P < 0.05$) among all groups.

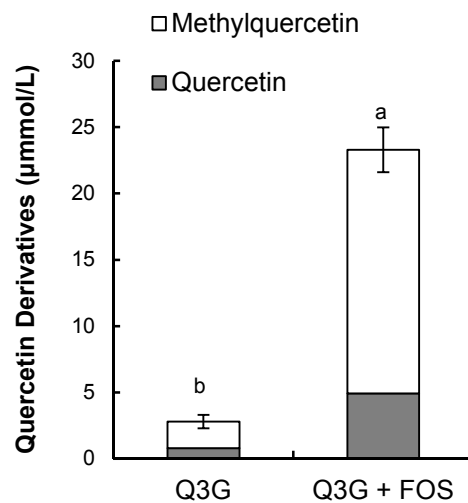


Figure I-4 Plasma concentration of quercetin derivatives in the aortic blood of rats fed Q3G-containing experimental diets with or without FOS on day 48. Non-conjugated forms of methylquercetin and quercetin were not detected and quercetin derivatives were not detected in plasma of those without Q3G.

Where Q3G is 0.3% Q3G in sucrose based diet and Q3G + FOS is 0.3% Q3G + 5% FOS in sucrose based diet. Values are the means \pm SE depicted by vertical bar ($n = 6-7$). ^{a,b} Mean values of total quercetin derivatives with unlike alphabetical letters denote significant differences ($P < 0.05$) by Student's *t*-test.

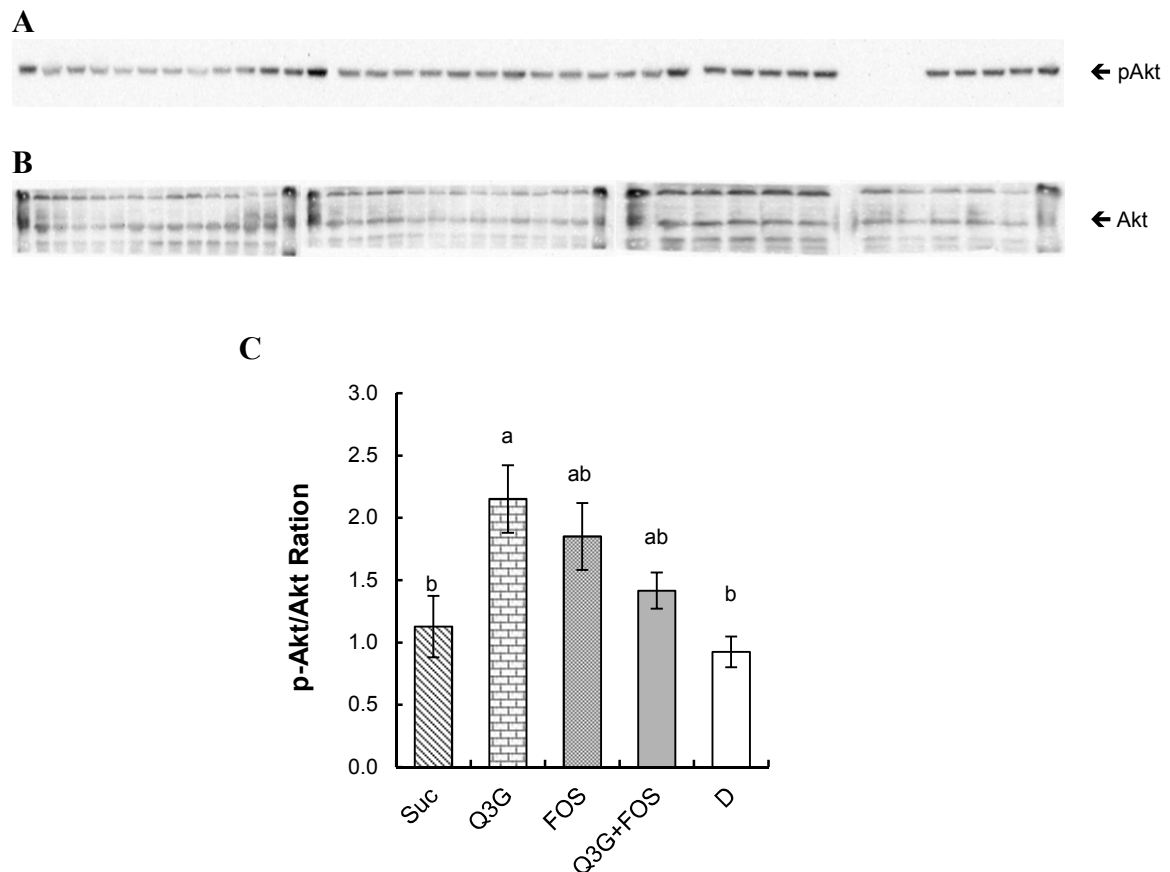


Figure I-5 Western Blot analysis of the phosphorylated (A) and non-phosphorylated forms of Akt expression (B). Relative ratio of the phosphorylated and non-phosphorylated forms of Akt (C) after exogenous insulin stimulation in gastrocnemius muscle of rats-fed experimental diets for 48 days. The rats under a pentobarbital anaesthesia (40 mg/kg BW) were injected insulin (10 units/kg BW) into portal vein

Where Suc is sucrose diet, Q3G is 0.3% Q3G in sucrose based diet, FOS is 5% FOS in sucrose based diet, Q3G + FOS is 0.3% Q3G + 5% FOS in sucrose based diet, and D is dextrin diet. Values are the means \pm SE depicted by vertical bar ($n = 6-7$). ^{a,b} Mean values within a row with unlike alphabetical letters denote significant differences ($P < 0.05$) among all groups.

CHAPTER 2: A NONDIGESTIBLE SACCHARIDE INCREASE THE PROMOTIVE EFFECT OF FLAVONOIDS ON GLUCAGON-LIKE PEPTIDE 1 (GLP-1) SECRETION

The aim of the study was to conduct *in vivo*, *in situ* and *in vitro* studies, which builds on our previously published work [83], that investigates the effects of flavonoid with- or without non-digestible saccharide on GLP-1 secretion.

MATERIALS AND METHODS

Chemicals

Alpha-glycosyl-isoquercitrin (Q3GM), Galactosyl myricitrin (Gal-M), and Glucosyl myricitrin (Glu-M) provided in kind, by San-Ei Gen F.F.I., Inc. (Osaka, Japan), was a mixture of 1-7-D-glucose adducts for isoquercetrin (i.e., quercetin-3-*O*-glucoside). Fructooligosaccharide (FOS) (Meiologo-P, Meiji Seika Kaisha, Ltd., Tokyo, Japan) was a mixture of 42% 1-kestose, 46% nystose, and 9% 1F- β -fructofuranosyl nystose. di-D-fructose anhydride III ((DFA III; di-d-fructofuranosyl 1,2'/2,3' dianhydride, Appendix J), a disaccharide comprising two fructose residues with two glycoside linkages, was provided by Fancel Co. (Yokohama, Japan). α -Glucosyl rutin (α -GR) was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Hesperidin S (HespS Appendix H), and Monoglucosyl hesperidin (M-Hesp, Appendix G) were provided in kind by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Caffeic acid (CFA, 031-06792) was purchased from Sigma-Aldrich (MO, USA). Other flavonoids (i.e. Caffeine, Anhydrous (CF), Chlorogenic acid hemihydrate (3-

Caffeoylquinic acid hemihydrate, ChAH 033-14241)) and reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless specified otherwise.

Animals

Male Wistar/ST rats, weighing about 220-260 g (7-8 wk old), were purchased from Japan SLC (Hamamatsu, Japan). The rats were caged individually and were fed with an AIN-93G based diet (Table II-1) and unrestricted access to water [84]. Prior to start of experiments (both *in vivo* and *in situ*), rats were acclimatized for 3–7 d in a temperature-controlled room (i.e., $23 \pm 2^{\circ}\text{C}$) that was maintained with a 12-h light-dark cycle (i.e., 8:00 – 20:00, light period). The protocols of our experiments were approved by the Hokkaido University Animal Committee and all animals were maintained in accordance to the Hokkaido University guidelines for the care and use of laboratory animals.

In vivo (experiment with conscious rats): The purpose of this experiment was to examine the effects of oral administration of flavonoid with- or without FOS increases the GLP-1 secretion.

Jugular vein cannulation procedure: To obtain multiple blood samples from experimental rats, rats were first anesthetized using sodium pentobarbital (50 mg/kg body weight, Somnopentyl injection; Kyoritsu Seiyaku Co., Tokyo, Japan) before inserting the silicone catheter [Silascon no. 00; internal diameter (ID), 0.5 mm; outer diameter (OD), 1.0 mm; Dow Corning Co., Kanagawa, Japan] into the

right jugular vein and immobilized with a suture on the sternocleidomastoid muscle. This catheter was led subcutaneously behind the neck to collect blood samples without physical restraints. During the post-operative recovery period of 3-4 days, the catheter was flushed daily with sterilized saline containing heparin (50 IU/mL; Ajinomoto, Tokyo, Japan) to maintain the patency. Moreover, rats were trained daily by an orogastric administration with distilled water using a feeding tube (Safeed Feeding Tube Fr. 5, 40 cm; Terumo, Tokyo, Japan) during post-surgery recovery period (3-7days).

Experiment 1: Effect of oral administration flavonoids with- or without fructooligosaccharides (FOS) on GLP-1 secretion in conscious rats.

After recovery from jugular canulation, the rats were fasted for 24-hours before being randomly assigned into one of four groups: Saline as a control (S), FOS (100 mM of fructooligosaccharide), Q3GM (20 mM of α -glucosyl-isoquercitrin) and Q3GM+FOS (a mixture of 20 mM of α -glucosyl-isoquercitrin and 100 mM fructooligosaccharide). Blood samples (300 μ L) were drawn through a jugular catheter before (i.e., 0 min) and at 15, 30, 60, 90 and 120 min after oral gavage of 2 mL of test solutions (i.e., S, FOS, Q3GM, and Q3GM+FOS). The jugular catheter was flushed with saline containing heparin upon each blood sample.

In situ (experiment with anesthetized rats): The purpose of *in situ* experiment was to examine the effect of flavonoid administration into the ileal lumen with- or without FOS on GLP-1 secretion.

Surgical procedure for *in situ* experiment: Rats acclimatized for 3-7 d with a standard AIN-93G diet, were anesthetized with ketamine (80 mg/kg body wt ip; Ketalar, Daiichi Sankyo, Tokyo, Japan) containing xylazine (12 mg/kg ip; MP Biomedicals, Irvine, CA) after 24 hr fast. The body temperature was maintained with a heating pad during experiment. To obtain blood samples, a small midline incision was made to insert a 6-7 mm polyethylene catheter (SP 10; ID 0.28 mm, OD 0.61 mm; Natsume Seisakusyo, Tokyo, Japan) connected to a silicone tube (Silascon no. 00, ID 0.5 mm, OD 1.0 mm; Dow Corning Co.) into the hepatoportal vein.

Experiment 2: Effect of the flavonoid with or without fructooligosaccharides on GLP-1 secretion into the ligated ileal loops in anesthetized rats.

The catheter was inserted into hepatoportal vein as described above procedure. The ligated loop (15 cm) was prepared in the upper ileum (45 cm distal from the ligament of Treitz) by ligating it with a silk suture after flushing the lumen by 5 mL of saline.

Before injecting the test solutions, a 250 μ L of basal blood sample was drawn from the portal catheter. Test solutions were: 1) 100 mM of FOS, 2) low concentration of Q3GM (10 mM, LQ3G), 3) high concentration of Q3GM (20 mM, HQ3G), 4) 10 mM of Q3GM and 100 mM FOS (LQ3G+FOS), and 5) 20 mM of Q3GM and 100 mM FOS (HQ3G+FOS). Two milliliter of saline (i.e., control) or test solutions (i.e., FOS, LQ3G, LQ3G+FOS, HQ3G, or HQ3G+FOS) was directly injected at the proximal end of the ligated ileum segment. A saline

containing heparin (50 IU/mL; Ajinomoto, Tokyo, Japan) was used to maintain the patency of the catheter used for drawing blood samples and between each blood sampling. Blood samples were drawn prior to the administration of test solutions (i.e., 0 min) and subsequently at 15, 30, 60, and 120 min after the administration.

Experiment 3: Effect of the flavonoid with or without fructooligosaccharides on GLP-1 secretion into non-ligated ileal loops in anesthetized rats.

Three milliliter of flavonoid luminal fluid sample (20 mM flavonoid: α -GR and G-Hesp) with- or without 100 mM fructooligosaccharides (FOS) was directly administered into the ileum (45 cm distal to the ligament of Treitz) since the highest numbers of L-cells, GLP-1 production site, can be found in the ileum [85]. Portal blood was collected through the portal catheter at 0 (i.e., before administration), 15, 30, 45, 60, 90, and 120 min after the administration.

Plasma total GLP-1. Blood samples were collected using a syringe containing EDTA (final concentration 1 mg/mL), aprotinin (final concentration at 500 kIU/mL), and DDP-4 inhibitor (i.e., a dipeptidyl peptidase IV inhibitor, final concentration 50 μ mol/L, Millipore Co, Billerica, Massachusetts). The blood sample was transferred to a 1.5 mL tube and went through centrifugation at 2,500 \times g for 15 min at 4°C to separate plasma. Plasma sample was frozen at -80°C until analyses at later time. The GLP-1 concentration was measured with a commercial enzyme immunoassay kit (GLP-1 Total ELISA kit) (Millipore Co, Billerica, Massachusetts).

In vitro (experiment using a cultured enteroendocrine cell, GLUTag cell): This experiment is to examine the direct activities for releasing GLP-1 by flavonoid glucoside with and without FOS.

Experiment 4: Examination of GLP-1 secretion by dose dependence of various flavonoid solutions.

We prepared test solutions by diluting 50 mM of stock flavonoid solutions (i.e., Q3GM, α -GR, G-Hesp, Glu-M, Gal-M, ChAH, CF, and CFA) in deionized water (1 mL) and later diluted with HEPES contained 115 mM NaCl buffer to prepare 0.1, 1.0 and 10 mM solution respectively. The pH of flavonoid solutions was adjusted to 7.0-7.2 ranges with 0.01 M NaOH.

Experiment 5: Examination of GLP-1 secretion by flavonoid with or without fructooligosaccharide or difructose anhydride III.

We prepared test solutions by diluting 20 mM of stock flavonoid solutions (i.e., Q3GM, α -GR, G-Hesp, Hesp S) with 2 mL of HEPES containing 115 mM NaCl buffer. Twenty mM flavonoid stocks were diluted with 40 mM fructooligosaccharide (FOS) or difructose anhydride III (DFA III) in HEPES to make the mixture of 10 mM flavonoid solutions with 20 mM FOS or DFA III (i.e., Q3GM+, α -GR+, G-Hesp+, CF+, CFA+, ChAH+, and Hesp S+). The pH of flavonoid solutions was adjusted to 7.0-7.2 ranges with 0.01 mM NaOH.

GLP-1 secretion via GLUTag cell

GLUTag cells (a gift from Dr. D. J. Drucker, University of Toronto, Toronto, Canada) were grown in Dulbecco's modified Eagle's medium (Invitrogen, cat. no. 12100-038) supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, and 500 µg/mL streptomycin in a humidified 5% CO₂ atmosphere in 48-well culture plates at a density of 1.25×10^5 cells/well at 37°C. Cells were routinely subcultured by trypsinization upon reaching 80–90% confluency. Cells were rinsed twice with HEPES buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM D-glucose and 0.1% BSA, pH 7.4) to remove the culture media and exposed to test agents, as described above in Experiment 6 and 7, for 60 min at 37°C. Supernatants were collected from the wells, centrifuged at 800 *g* for 5 min at 4°C to remove remaining cells, and stored at –50°C until the GLP-1 concentration was measured with a commercial enzyme immunoassay kit (EIA kit) (Yanaihara Institute Inc., Shizuoka, Japan).

Measurement of cytotoxicity in GLUTag cells

Cytotoxic effects on GLUTag cells were determined by measuring the release of lactate dehydrogenase (LDH) into the supernatant of GLUTag cells exposed to test agents, as described above in GLP-1 secretion via GLUTag cell section. The measurement of LDH was performed using a cytotoxicity detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cytotoxicity was calculated as the relative release (%) of LDH after exposure to the test agents compared to the total LDH (100%) released upon treatment with lysis reagent.

Statistical analysis.

All values represent the means \pm SEM. Area under the curve ($AUC_{0-120 \text{ min}}$) was calculated using the trapezoidal rule for knowing the average concentration over a time interval. Two-way ANOVA followed by Tukey-Kramer post-hoc test was used to analyze for differences in GLP-1 level among the group and Dunnett was used to test the differences from the basal blood sample. $AUC_{0-120 \text{ min}}$ of GLP-1 excursion and GLP-1 secretion via GLUtag cell line were analyzed by one-way ANOVA, followed by Tukey-Kramer post-hoc test. The results were statistically significant with a p -value less than 0.05.

RESULT

Examination of oral administration of Q3GM with and without FOS on GLP-1 secretion in conscious rats (*in vivo*, experiment 1).

Baseline concentration of total GLP-1 was 22.0-25.6 pM. Administering S and FOS did not result in any significant change in GLP-1 level (Fig. II-1 A). The administration of S, however, suppressed the GLP-1 level, which showed a consistence in both the changes (i.e., Δ GLP-1) and its AUC (0-120 min) (Fig II- 1 B and D). GLP-1 level of Q3GM+FOS group immediately increased and peaked (35.0 ± 4.3 pM) at 15 min, which was significantly higher than that of control (S) group. After 90 min post-administration, Q3GM+FOS exhibited clear enhancement of GLP-1 secretion compared with Q3GM. AUC (0-120 min) of GLP-1 and Δ GLP-1 in Q3GM+FOS group were also much higher than S and FOS groups and had higher tendency than those of Q3GM group. Two-way ANOVA values revealed significant differences for time ($p < 0.0001$ and < 0.05), treatment ($p < 0.05$ and < 0.0001), and interactions between time and treatment ($p < 0.05$) for GLP-1 and Δ GLP-1 respectively (Fig II-1 A and B). Two-way ANOVA values for AUC revealed significant differences for Q3GM ($p < 0.0001$ and < 0.05), FOS ($p < 0.001$ and < 0.0001), and interactions between Q3GM and FOS ($p = 0.0578$ and < 0.05) for GLP-1 and Δ GLP-1 respectively (Fig II-1 C and D).

Examination of the flavonoid with or without FOS on GLP-1 secretion into the ligated ileal loops in anesthetized rats (*in situ*, experiment 2).

Dose dependence of α -glucosyl-isoquercitrin (i.e., Q3GM) with or without FOS was studied in the rat ileum, where GLP-1 producing cells are abundant.

Basal GLP-1 levels varied in range of 89.0–104.3 pM without significant differences among groups. Both low concentration (LQ3GM, 10 mM of Q3GM) and high concentration (HQ3GM, 20 mM of Q3GM) themselves, slightly but not significantly, potentiated absolute and Δ GLP-1 levels (Fig. II-2 and 3). Δ GLP-1 levels after luminal injection of LQ3GM+FOS (i.e., 10 mM of Q3GM and 100 mM of FOS) were significantly higher at 15 and 30 min against S and FOS, and values of absolute GLP-1 showed similar manners (Fig. II-2 A and B). HQ3GM injection significantly increased absolute GLP-1 and Δ GLP-1 at 15 min and 30 min, against S and FOS (Fig. 3 A and B). HQ3GM+FOS (i.e., 20 mM of Q3GM and 100 mM of FOS) significantly enhanced only in the Δ GLP-1 secretion at 15 min points. Furthermore, 2-way ANOVA revealed significant difference in low dose of Q3GM (i.e., LQ3GM) for time ($p < 0.0001$ and < 0.05), treatment ($p = 0.01$ and < 0.0001), and interactions between time and treatment ($p < 0.05$) for GLP-1 and Δ GLP-1, respectively in Fig. 2 A and B. Both time ($p < 0.05$) and treatment ($p < 0.0001$) affected GLP-1 and Δ GLP-1 whereas their interaction didn't affect absolute GLP-1 of HQ3GM but affected Δ GLP-1 ($p < 0.05$) in Fig. II-3 A and B.

Both single administration of LQ3GM and HQ3GM resulted an increase in AUC of total GLP-1, which 2-way ANOVA revealed significant difference in both doses of Q3GM (i.e., LQ3GM and HQ3GM) for Q3GM ($p < 0.0001$), FOS ($p < 0.05$), and interactions between Q3GM and FOS ($p < 0.05$) in Fig. 2 C and 6C. Δ AUC of LQ3GM+FOS was significantly higher against S, FOS, and individual groups (Fig. II-2 D), whereas Δ AUC of HQ3GM+FOS was significantly higher against S and FOS (Fig. II-3 D). 2-way ANOVA results were

Q3GM ($p < 0.05$), FOS ($p < 0.0001$), and interactions between Q3GM and FOS ($p < 0.05$) for Δ AUC of total GLP-1 of LQ3GM and HG3GM in Fig. II-2 D and 3 D.

Examination of the flavonoid with or without FOS on GLP-1 secretion into non-ligated ileal loops in anesthetized rats (*in situ*, experiment 3).

We examined the effect of flavonoid with or without non-digestible saccharide on GLP-1 secretion in the rat non-ligated intestine. Flavonoid (i.e., α -GR, and Hesp S) solutions with or without FOS were administered into the ileum of anesthetized rats. No significant difference could be observed in total GLP-1 secretion in α -GR (Fig. II-4 A). α -GR+FOS potentially increased Δ GLP-1 secretion at 30 and 90 min after administration (Fig. II-4 B). Hesp S (Fig. II-5 A and B), however, did not cause any significant change and those Hesp S+FOS tended to reduce Δ GLP-1 secretion in the rat intestine.

GLP-1 secretion by various dose of flavonoids and with or without FOS / DFA III in GLUTag cell (*in vitro*, experiment 4 and 5).

Direct effect of various flavonoids (i.e., Q3GM, α -GR, G-Hesp, G-M, Gal-M, ChAH, C, and CA) on the enteroendocrine cells was subsequently examined by using GLP-1 producing enteroendocrine cell line, GLUTag cells. GLUTag cells were exposed to various dose flavonoid and depolarization stimuli (70 mM KCl) as a positive control. Application of KCl showed inconsistency results (Fig. II-6 A-C), where no stimulation in the first set of various flavonoid solution (Fig. II-6 A) whilst, highly stimulated in the second set (Fig. II-6 B). GLP-1 secretion from various flavonoid solutions with dose dependence via GLUTag cells (Fig. II-

6 C) found that 10 mM of α -G-rutin solely stimulates GLP-1 secretion whereas glucosyl hesperidin shows a tendency of GLP-1 stimulation. 10 mM dose of flavonoid solutions (Q3GM, α -GR, and G-Hesp) were then chosen to observe with or without the presence of 20 mM FOS for the GLP-1 secretion in GLUTag cells (Fig. II-7 A). 10 mM dose of flavonoid solutions (Q3GM, α -GR, G-Hesp and Hesp S) were also observed with or without the presence of 20 mM DFA III for the GLP-1 secretion in GLUTag cells (Fig. II-7 B). GLP-1 secretion of those with FOS found to be greater than those without FOS in all flavonoid solutions (Fig. II-7 A). In figure 7 B, there was no different of GLP-1 secretion between flavonoid with- and without DFA III. The treatment of GLUTag cells with up to 10 mM of Q3G produced no cytotoxicity (cell viability was greater than 98%) when the release of lactate dehydrogenase was measured using a cytotoxicity detection kit (Table II-2).

DISCUSSION

The development and progression of type 2 diabetes first involves postprandial hyperglycemia and eventually increase in fasting hepatic glucose production leading to the elevated fasting glucose levels [86]. Quercetin has shown to improve postprandial hyperglycemia in STZ diabetic model rats [87]. GLP-1 is one of incretin hormone released from the enteroendocrine L cells in response to the presence of luminal nutrients, such as glucose, proteins and fats [88, 89]. GLP-1 production site or L-cells lie scattered along the length of the intestinal epithelium and increases the density along the length of the gastrointestinal tract, with the highest numbers being found in the ileum [85, 90].

We found that oral administration of Q3GM transiently stimulated GLP-1 secretion and co-administration with FOS enhanced Q3GM-induced GLP-1 secretion, especially in the later phase of the GLP-1 response (Fig.II-1). In several animal models, the intestinal fermentation induced by non-digestible saccharides like FOS is correlated with GLP-1 production [65, 91-93]. Additionally, previous studies showed that FOS increased plasma GLP-1 and L-cell proliferation [65], and free fatty acid receptor-2 (FFAR-2), which is activated by short-chain fatty acids (SCFAs) is expressed in L-cells in the lower intestine [94]. Therefore, fermentation of FOS might be involved in prolonged effect of FOS on Q3GM-induced GLP-1 secretion *in vivo*. We, however, did not observe any significant increases in GLP-1 secretion by only FOS in rat studies. Possibly, products of SCFA were not sufficient for stimulation of GLP-1 secretion in this study.

We examined direct infusion of flavonoid with or without FOS into the ligated ileum to determine direct effect on ileal L-cells and involvement of the

cecal fermentation of FOS for GLP-1 secretion after oral gavage. Q3GM+FOS administered into the ligated ileum loop induced increment of plasma GLP-1 in anesthetized rats (Fig. II-2 and 3), which indicate that Q3GM and FOS directly stimulate GLP-1 secretion in the ileum, not indirect stimulation through the upper small intestine. In the *in situ* intestinal loop experiments, to prevent SCFA production by fermentation with intestinal microorganism in the ileum, the intestinal loop was sufficiently washed out with saline. The results using the ileum loop showed that FOS significantly enhanced increase in GLP-1 release induced by Q3GM, consistently with those after oral gavage (i.e., Exp. 1). These results indicate that augmentation of GLP-1 secretion by FOS may not depend on intestinal fermentation.

We also found that Q3GM itself dose-dependently induces transient GLP-1 secretion. Even though a high level of Q3GM (i.e., 20 mM HQ3GM) induced the higher GLP-1 secretion till 30 min after administration (Fig. II-3), the lower level of Q3GM (i.e., 10 mM, LQ3GM) with the presence of FOS (i.e., LQ3GM+FOS, Fig. II-2) had very similar secretion of GLP-1 to HQ3GM with FOS, which indicates the FOS is more effective in LQ3GM than HQ3GM. The presence of Q3GM and FOS in the ileum possibly recognize as a representative signal for the presence of carbohydrates. FOS itself, however, did not stimulate the GLP-1 secretion by both oral gavage and ileal injection. These results suggest that FOS and Q3GM have synergistic effects on GLP-1 secretion. Some humoral or nervous factors may partake in the synergistic effects of FOS and Q3GM in the rat intestine. The mechanism and how the combination of Q3GM and FOS affect GLP-1 should be clarified in future.

We also examined another flavonoids (i.e., α -G rutin and hesperidin S), which also have been found to anti-hyperglycemic effects [33, 95]. Our results of *in situ* (i.e., Experiment 3) of α -GR and Hesp S with and without FOS together (Fig. II-4 and 5) FOS induced GLP-1 secretion in non-ligated anesthetized rats, which is consistent with previous studies [63, 91]. Results also showed the tendency of GLP-1 secretion by α -GR and Hesp S itself, compared to FOS. When combination of FOS, FOS enhanced the GLP-1 stimulation of α -GR, similar to the results of Q3GM+FOS in *in vivo* experiment (Fig. II-1). Hesp S+FOS, however, did not enhance GLP-1 secretion. Hesp S+FOS rather showed the suppression of GLP-1 secretion compared to individual Hesp S and FOS. The hypoglycemic effect of Hesp S may not involve incretin hormone, GLP-1; the mechanism by which hesperidin decreases plasma glucose remains to be verified

We also observed direct effects of various flavonoids on GLP-1 producing enteroendocrine cell line; GLUTag cells (Fig. II-6). The secretion of GLP-1 from enteroendocrine L cells is stimulated by the luminal nutrients and neuroendocrine factors [96]. Q3GM, α -GR, and G-Hesp are water-soluble flavonoids which are known to have protective effects to many diseases, including the management of type 2 diabetes [31, 97, 98]. One possible mechanism is stimulation of GLP-1 secretion. In this study, we found that a flavonoid glycoside, α -GR, potentially stimulate GLP-1 secretion in GLUTag cell. For further understanding about the combination of nondigestible saccharide and flavonoid on the mechanisms of GLP-1 secretion, direct effect of flavonoid solutions with and without nondigestible saccharide (FOS and DFA III) on GLUTag cells was examined (Fig. II-7). We found that presence of FOS additionally increased GLP-1 secretion in

the GLUTag cell line, not with DFA III. There was tendency of GLP-1 stimulation by Q3GM+FOS (Fig. II-7 A). The cytotoxicity of 10 mM of Q3G produced to GLUTag cell showed no cytotoxicity (cell viability was greater than 98%) according to measuring the release of lactate dehydrogenase (LDH) using a cytotoxicity detection kit (Table.II-2). Experiment conducted with GLUTag cells suggested that FOS alone also stimulated the GLP-1 secretion. Contrariwise, FOS did not stimulate the GLP-1 secretion as mentioned above in rat studies. This difference is possibly associated with a higher expression of proglucagon gene in GLUTag cells than in rat intestine [21]. Factors that contributed to the differences between rats and GLUTag cell experiments are not well known and warrant further examination.

In conclusion, our results provide evidences that but additional research is needed to understand the molecular mechanisms of the combined effects of Q3G+FOS. Future studies should investigate the response of Q3G+FOS in diet among T2DM patients to aid in preventing or reducing the use of pharmaceuticals to manage their glycemic levels.

Table II.1 Composition of basal diet

Ingredients	g/ kg diet
Sucrose ¹	629.5
Casein ²	200
L-cystine	3
Soybean oil	70
Crystallized cellulose ³	50
Mineral mixture ⁴	35
Vitamin mixture ⁴	10
Choline bitartrate	2.5

¹Sucrose as the carbohydrate source.

²Casein (ALACID; New Zealand Daily Board).

³Crystallized cellulose (Avicel PH102, Asahi Chemical Industry).

⁴Mineral and vitamin mixtures were prepared according to the AIN-93G formulation (Appendix K and L).

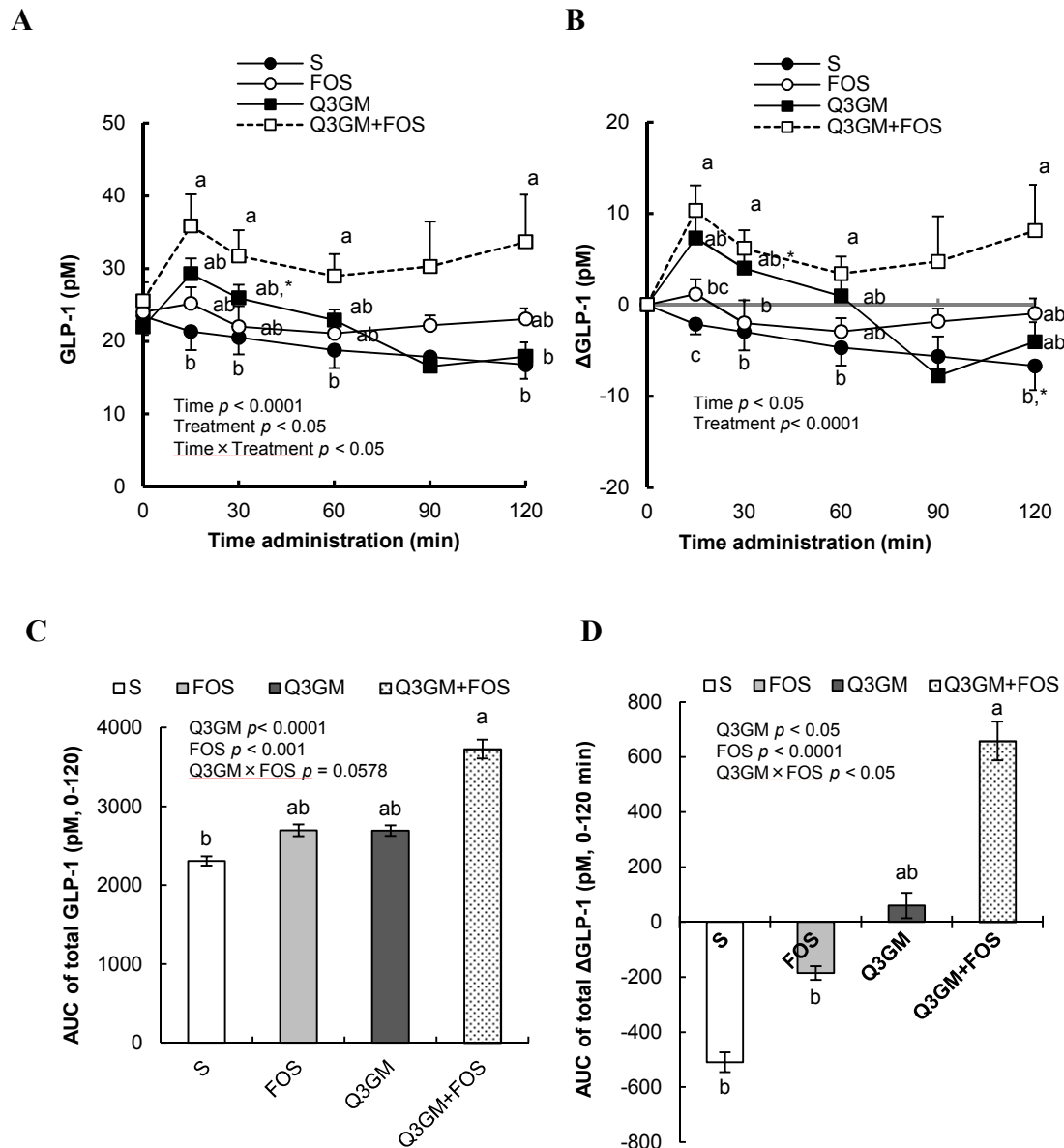


Figure II-1 Plasma total GLP-1 levels (**A**) and total Δ GLP-1 (**B**), AUC_{0-120 min} of total GLP-1 (**C**) and AUC_{0-120 min} of total Δ GLP-1 (**D**) in conscious rats after oral gavage of saline as a control, FOS (100 mM solution), and Q3GM (20 mM solution) without or with 100 mM FOS (Q3GM+FOS)

Values are displayed as the means \pm SEM ($n = 8$). Two-way ANOVA results (p -values; time and treatment for (**A**) and (**B**), and FOS and Q3GM for (**C** and **D**) are shown in graphs. Asterisk (*) signs indicate significant differences from the basal value (0 min) in each group (Dunnett's test; $p < 0.05$). Plots at the same time point not sharing the same letter differ significantly between treatments (Tukey–Kramer's significant difference test; $p < 0.05$).

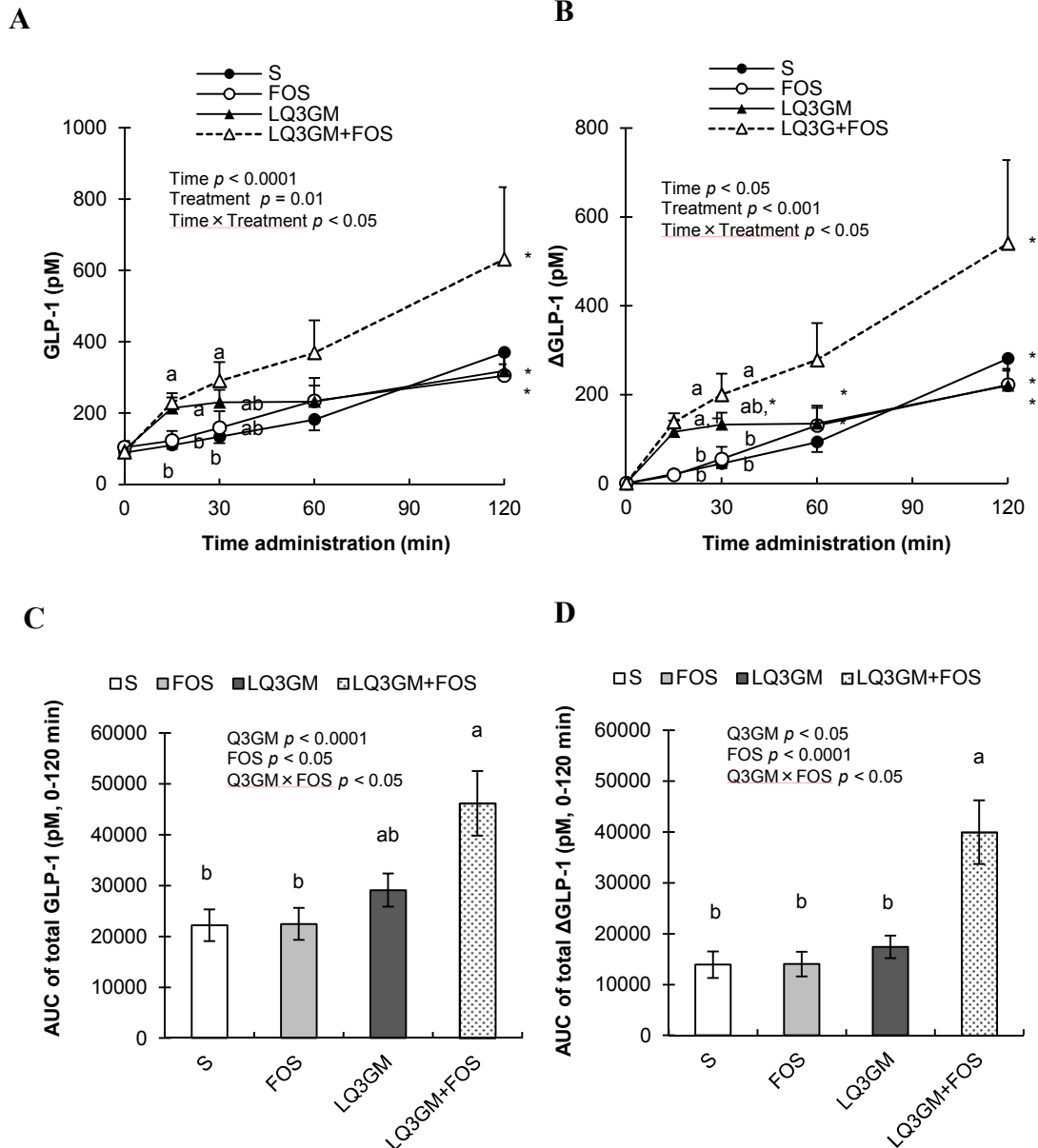


Figure II-2 Plasma total GLP-1 (**A**), total Δ GLP-1 (**B**), AUC_{0-120 min} of total GLP-1 (**C**) and AUC_{0-120 min} of total Δ GLP-1 (**D**) in anesthetized rats after the ileal administration of saline as a control, FOS, and low dose (10 mM) of Q3GM (LQ3GM) without or with 100 mM FOS (LQ3GM+FOS).

Values are displayed as the means \pm SEM ($n = 8$). Two-way ANOVA results (p -values; time and treatment for (**A**) and (**B**), and FOS and Q3GM for (**C** and **D**) are shown in graphs. Asterisk (*) signs indicate significant differences from the basal value (0 min) in each group (Dunnett's test; $p < 0.05$). Plots at the same time point not sharing the same letter differ significantly between treatments (Tukey–Kramer's significant difference test; $p < 0.05$).

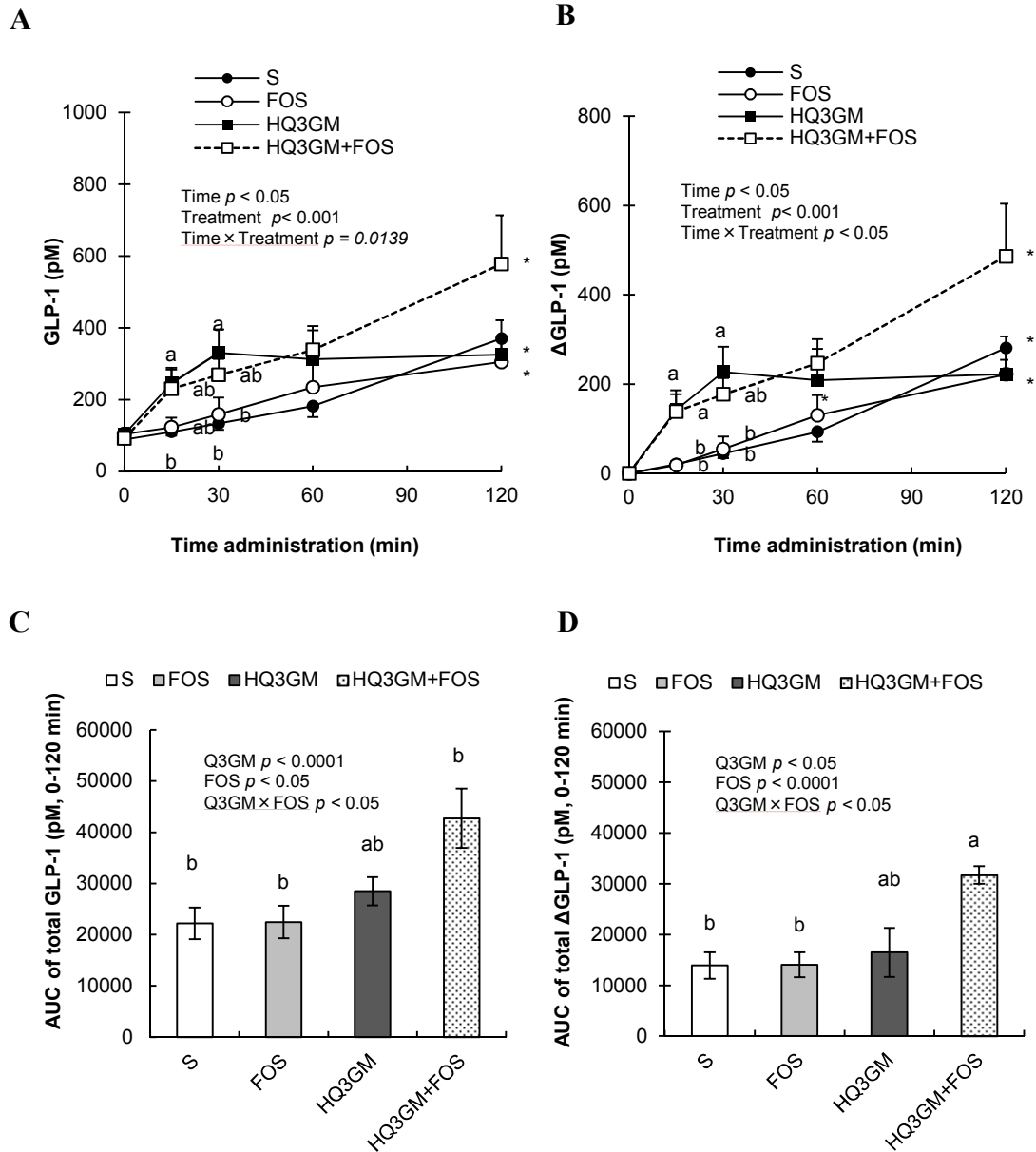


Figure II-3 Plasma total GLP-1 (**A**), total Δ GLP-1 (**B**) and AUC_{0-120 min} of total GLP-1 (**C**) and total Δ GLP-1 (**D**) in anesthetized rats after the ileal administration of saline as a control, FOS, and high dose (20 mM) of Q3GM solution (HQ3GM) without or with 100 mM FOS (HQ3GM+FOS).

Values are displayed as the means \pm SEM ($n = 8$). Two-way ANOVA results (p -values; time and treatment for (**A**) and (**B**), and FOS and Q3GM for (**C** and **D**) are shown in graphs. Asterisk (*) signs indicate significant differences from the basal value (0 min) in each group (Dunnett's test; $p < 0.05$). Plots at the same time point not sharing the same letter differ significantly between treatments (Tukey–Kramer's significant difference test; $p < 0.05$).

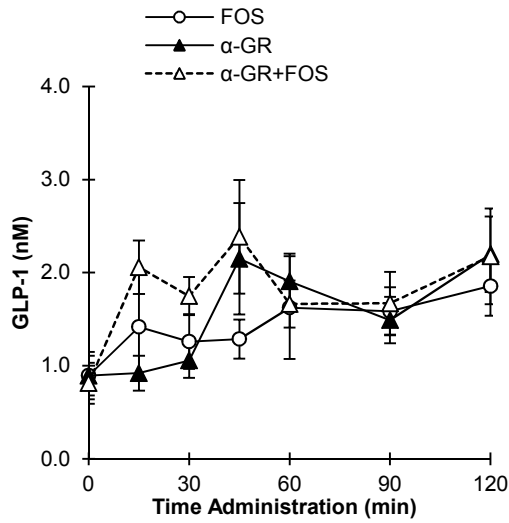
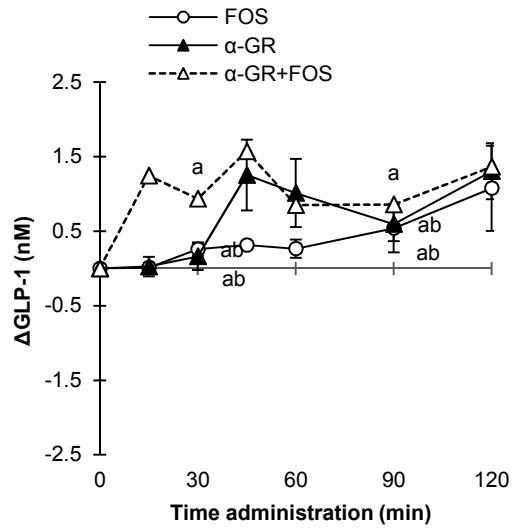
A**B**

Figure II-4 GLP-1 (A) and Δ GLP-1 (B) secretions of α -G rutin with or without FOS administration into non-ligated ileum of anesthetized rats

Values are displayed as the means \pm SEM ($n = 8$). Plots at the same time point not sharing the same letter differ significantly between treatments (Tukey–Kramer’s significant difference test; $p < 0.05$).

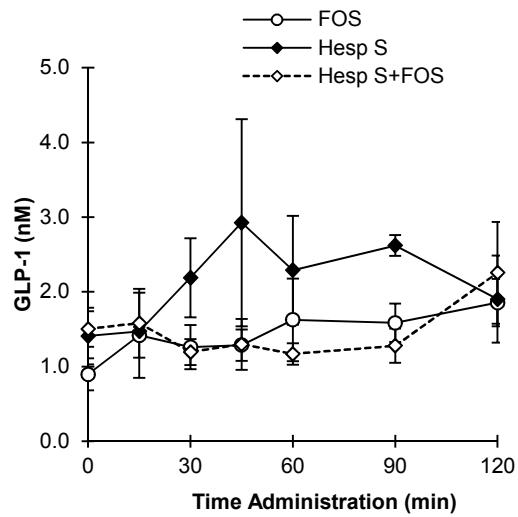
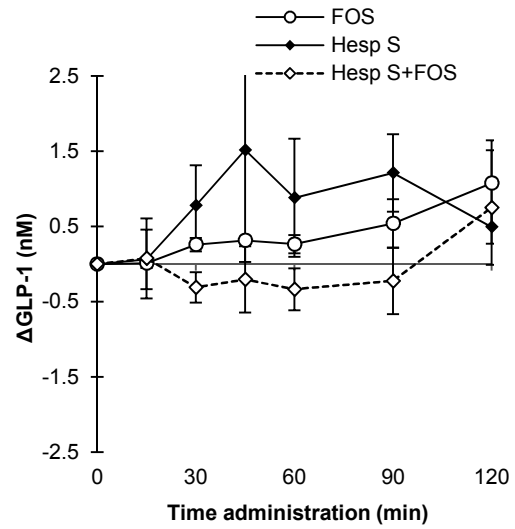
A**B**

Figure II-5 GLP-1 (A) and Δ GLP-1 (B) secretions of Hesperidin S with or without FOS administration into non-ligated ileum of anesthetized rats.

Values are displayed as the means \pm SEM ($n = 8$). Plots at the same time point not sharing the same letter differ significantly between treatments (Tukey–Kramer’s significant difference test; $p < 0.05$).

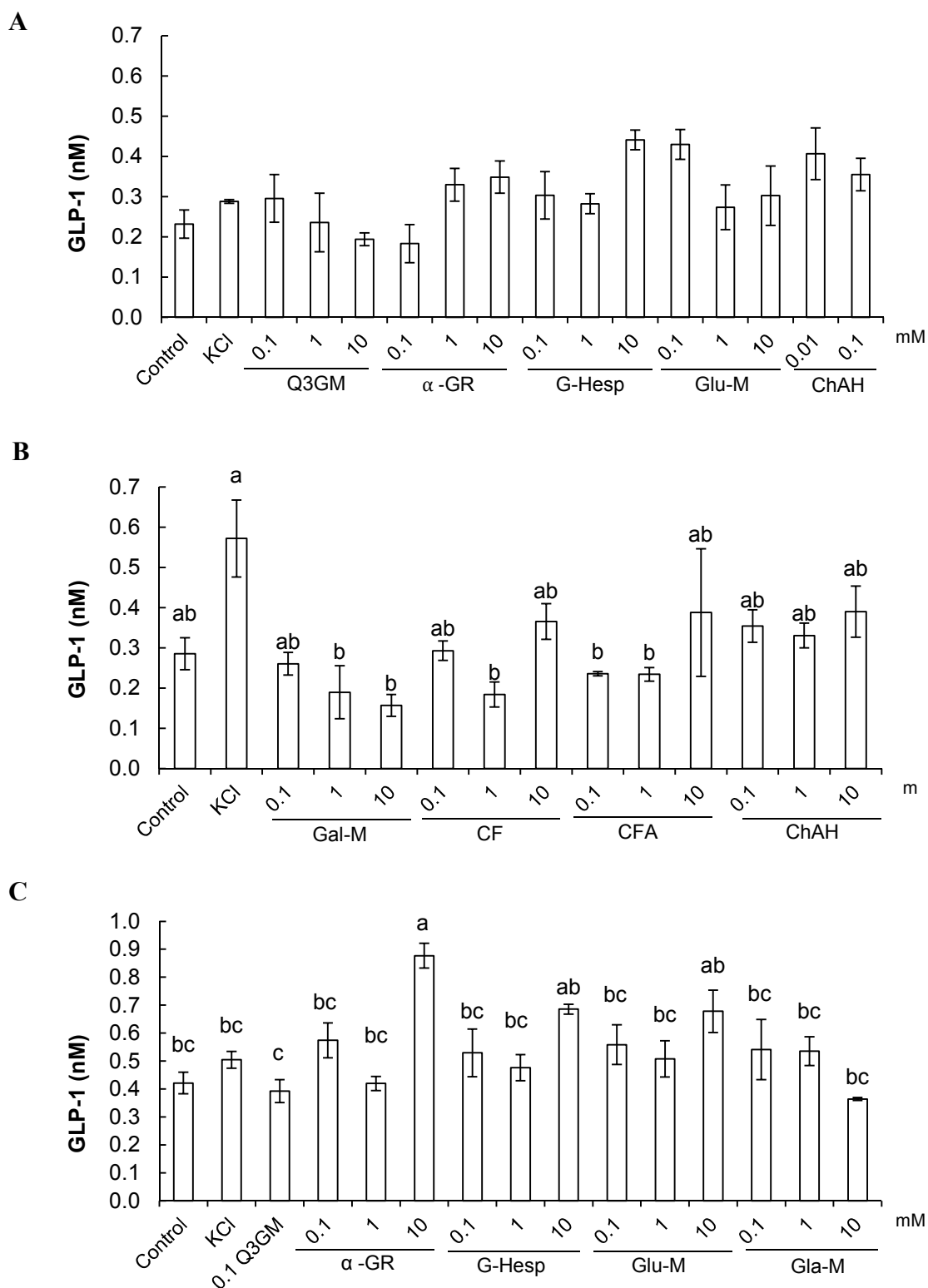


Figure II-6 GLP-1 secretion on dose dependence of flavonoid solutions in enteroendocrine cell line, GLUTag cell

Where Q3GM is α -Glycosyl-isoquercetin, α -GR is α -Glucosyl rutin, G-Hesp is Glucosyl

hesperidin, Glu-M is Glucosyl myricitrin, ChAH is Chlorogenic acid hemihydrate, Gal-M is Galactosyl myricitrin, CF is Caffeine, anhydrous, CFA is Caffeine acid. Values are displayed as the means \pm SEM ($n = 4$). Different letters indicate significantly between treatments (Tukey–Kramer’s significant difference test; $p < 0.05$).

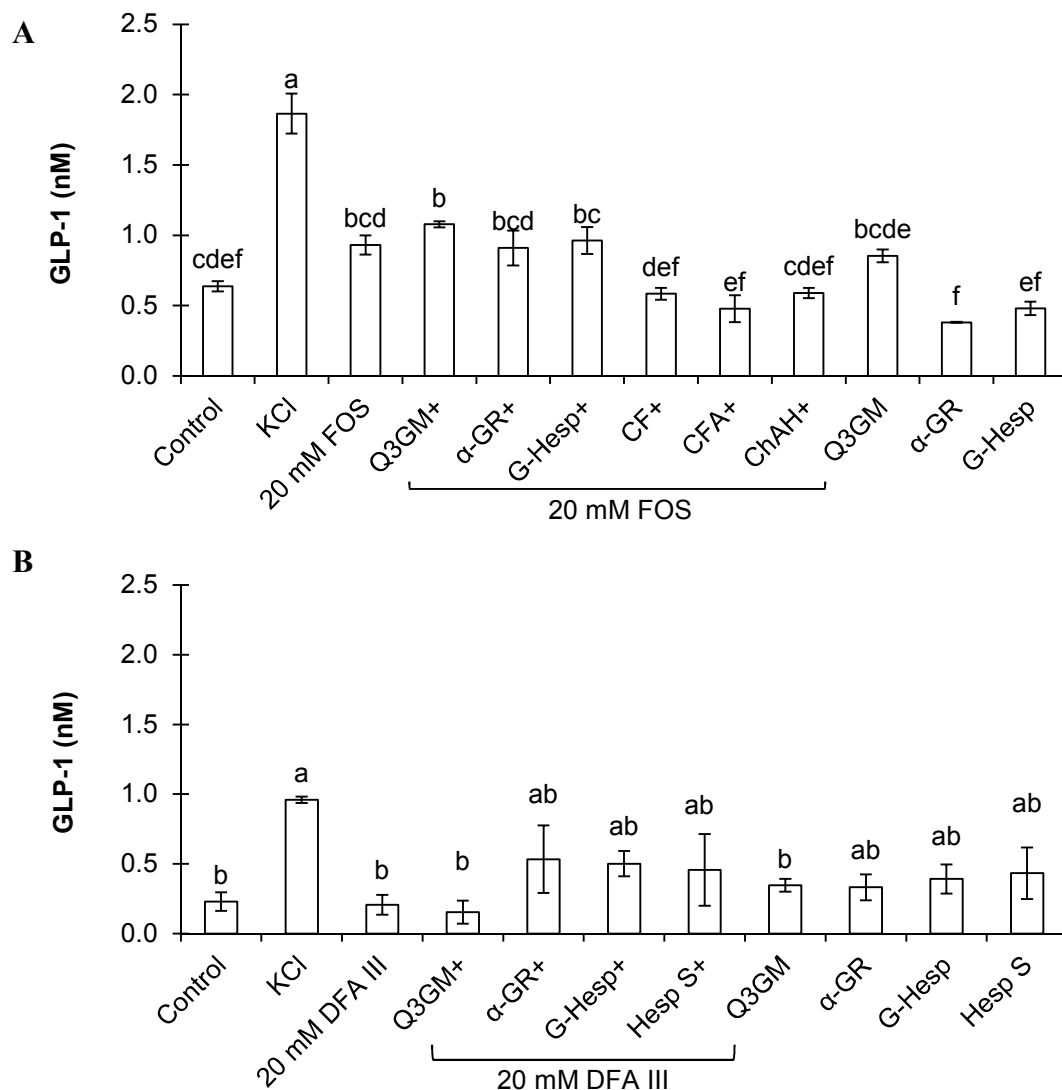


Figure II-7 GLP-1 secretion from various flavonoid solutions (10 mM) with or without fructooligosaccharides (20 mM FOS, **A**) and difructose anhydride III (20 mM DFA III, **B**) in enteroendocrine cell line, GLUTag cell

Where Q3GM is α -Glycosyl-isoquercetin, α -GR is α -Glucosyl rutin, G-Hesp is Glucosyl hesperidin, Hesp S is Hesperidin S, CF is Caffeine, anhydrous, CFA is Caffeine acid, ChAH is Chorogenic acid hemihydrate. Values are displayed as the means \pm SEM ($n = 4$). Different letters indicate significantly between treatments (Tukey–Kramer’s significant difference test; $p < 0.05$).

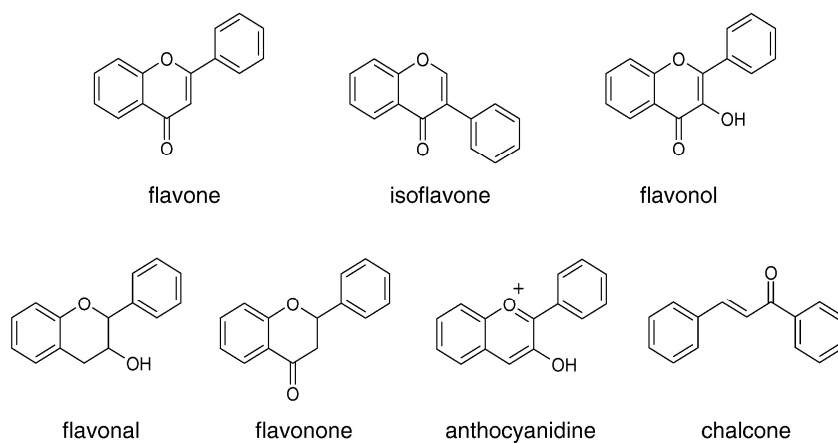
Table II-2 Cytotoxicity of Q3GM in GLUTag cells.

Cytotoxicity (%)= $\frac{(\text{Sample Absorbance})-(\text{Sample Blank Absorbance Average})}{(\text{Total LDH Absorbance})-(\text{Blank Absorbance Average})} \times 100$

	HEPES	KCl	Q3GM	Total
1	5.9	4.5	0.2	125.8
2	5.5	3.3	0.3	108.5
3	4.7	3.3	0.5	113.0
4	3.1	2.3		52.7
Average	4.8	3.4	0.3	100.0

APPENDIX A

Structures of the major classes of flavonoids.



Reference: Boots AW, Haenen GR, Bast A.

Health effects of quercetin: From antioxidant to nutraceutical.

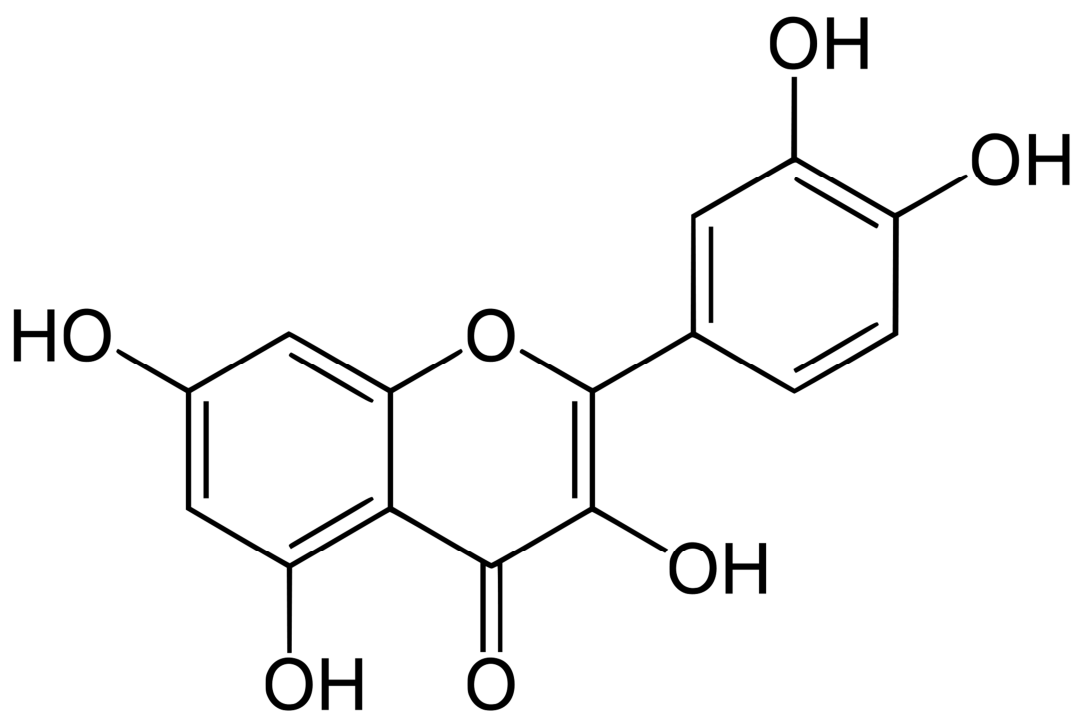
European Journal of Pharmacology, Volume 585, Issues 2–3, 2008, 325 – 337.

<http://dx.doi.org/10.1016/j.ejphar.2008.03.008>

APPENDIX B

Flavonoid: Quercetin aglycone

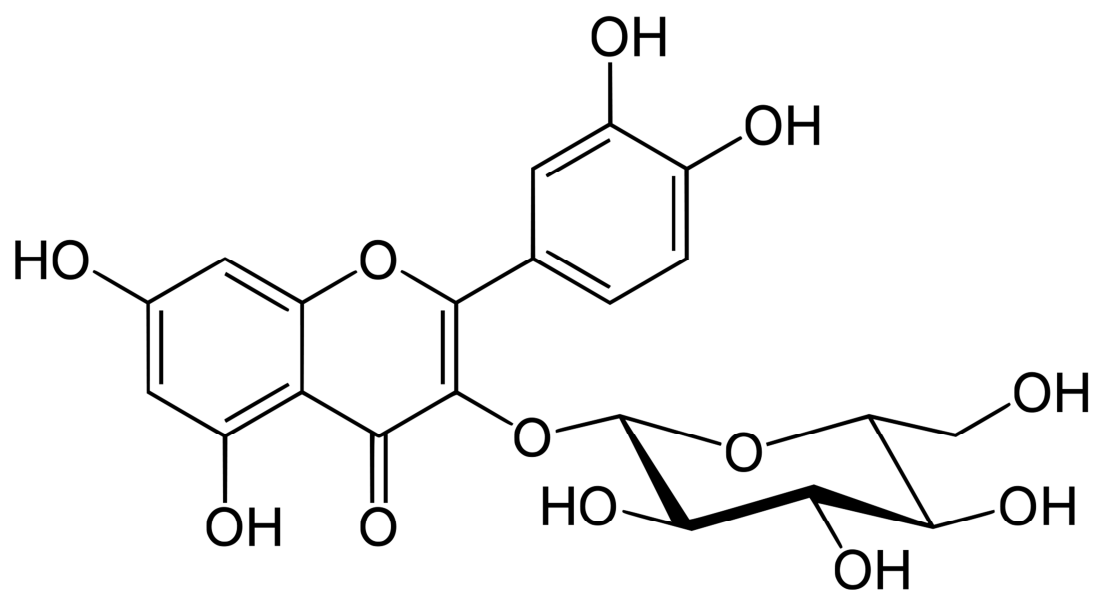
IUPAC name: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one



APPENDIX C

Flavonoid: Isoquercetin (Q3G)

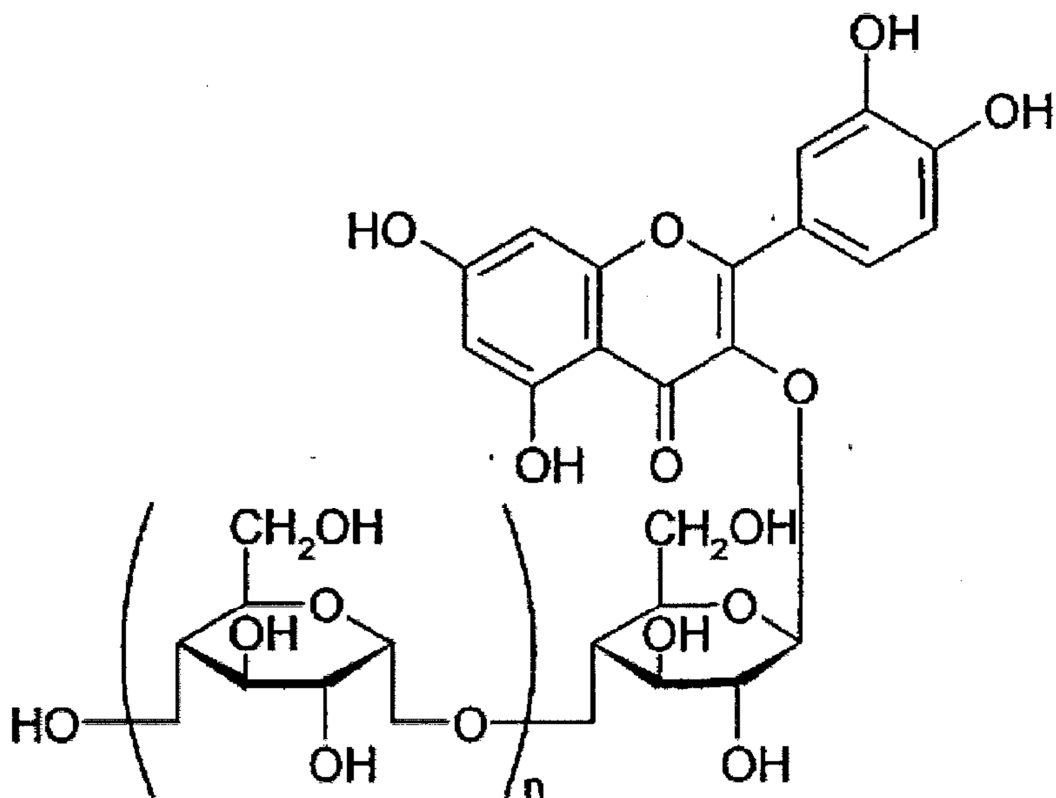
IUPAC name: 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one



APPENDIX D

Flavonoid: α -Glycosyl-isoquercitrin (Q3GM)

Other names: Enzymatically modified isoquercitrin or EMIQ. It is also sold under the trade name SANMELM.

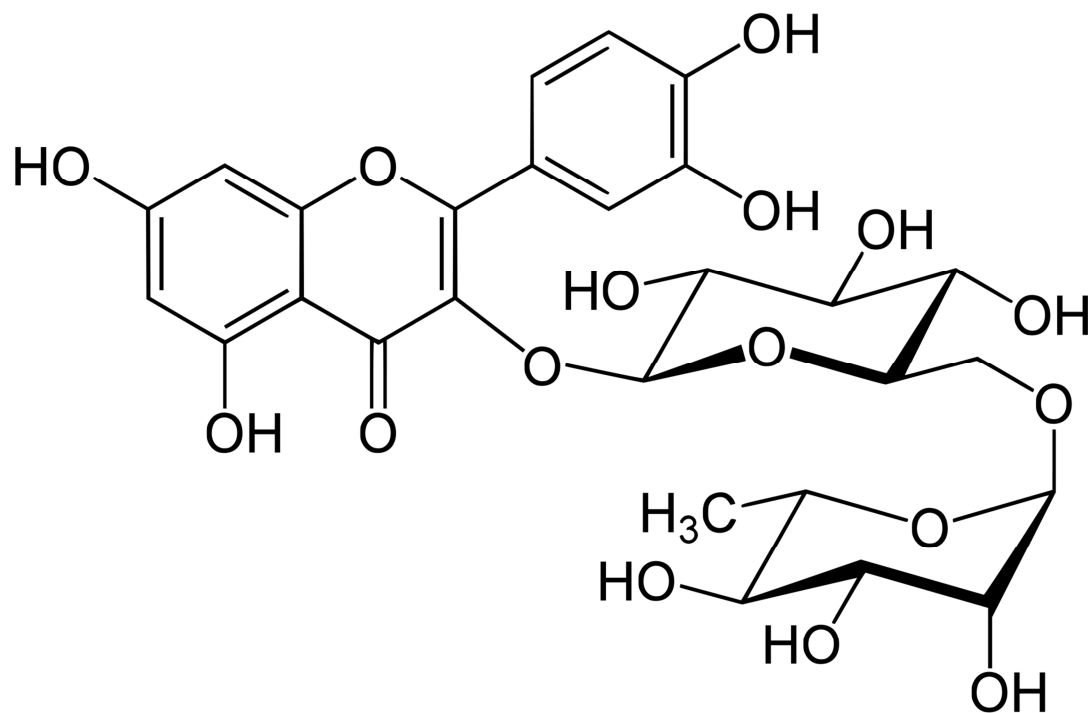


The number of glucose units may vary from 1 to 11 (n= 0-10)

APPENDIX E

Flavonoid: Rutin

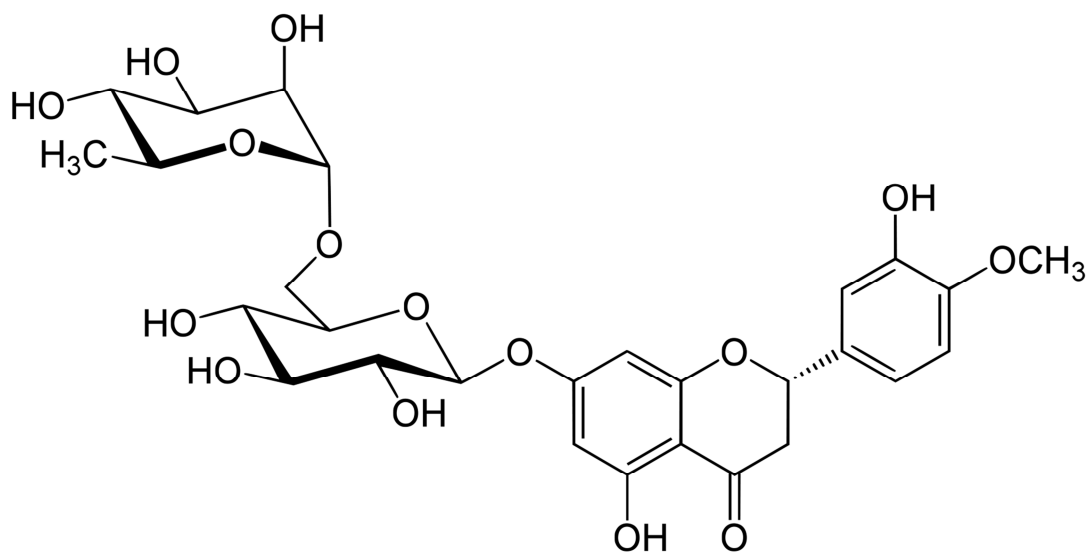
IUPAC name: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranosyloxy]-4H-chromen-4-one



APPENDIX F

Flavonoid: Hesperidin

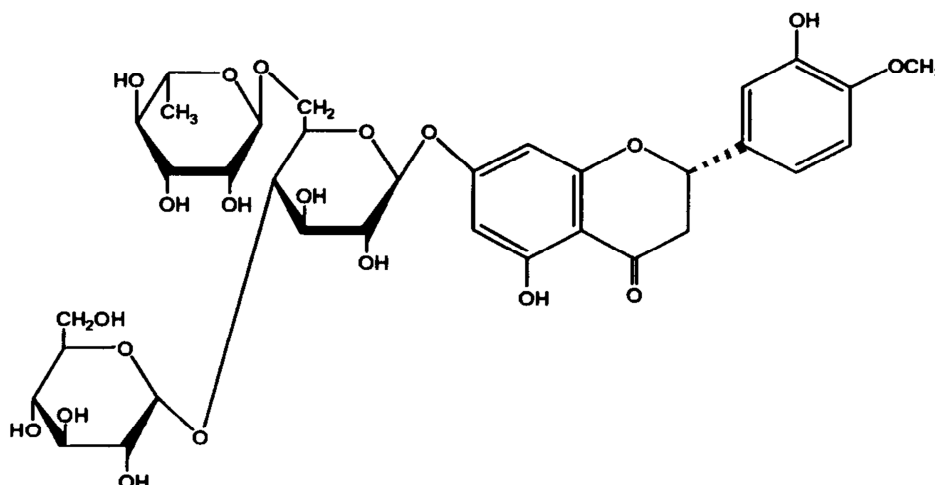
IUPAC name: Hesperetin 7-rutinoside



APPENDIX G

Flavonoid: Monoglucosyl Hesperidin

It is hesperidin derivative wherein equimolar or more D-glucose residues are bound to hesperidin via the d-bond, is formed by a saccharide-trans-ferring enzyme in a liquid containing hesperidin and α -glucosyl saccharide

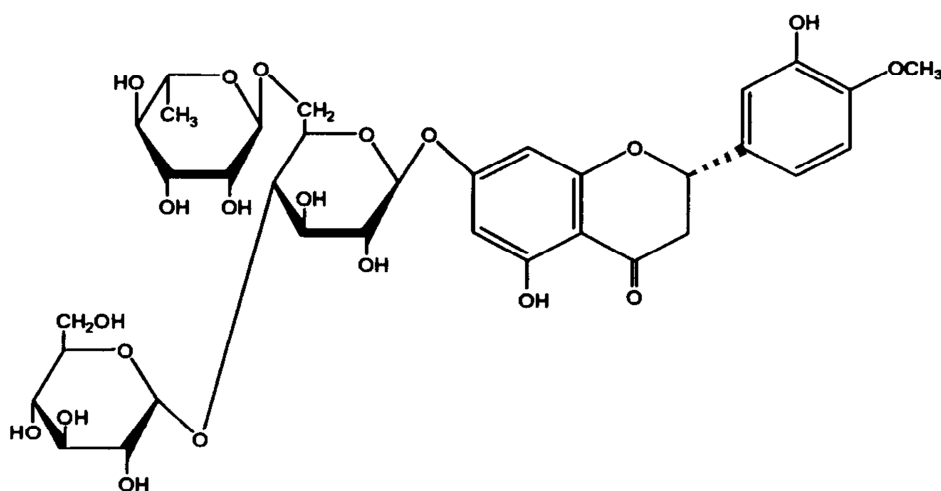
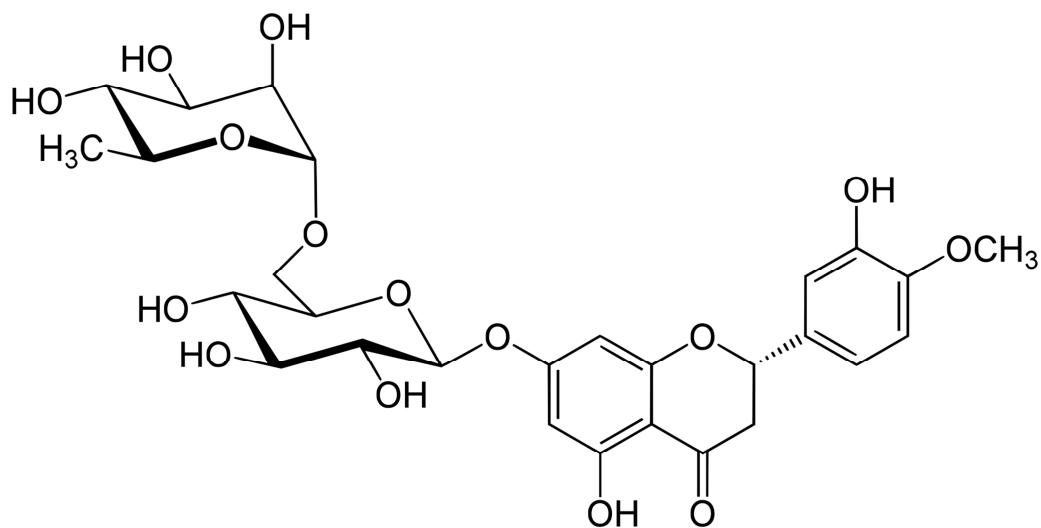


Code No.	:	HG131
Formula	:	$C_{34}H_{44}O_{20}$
Molecular Weight	:	772.70
Purity	:	Not less than 98.0 %
Loss on Drying	:	Not more than 6.0 %
Appearance	:	Light yellow to yellowish brown powder
Availability	:	250 mg

APPENDIX H

Flavonoid: Hesperidin S (Hesp S)

It is a mixture of Hesperidin (25%) and Monoglucosyl Hesperidin (75%)

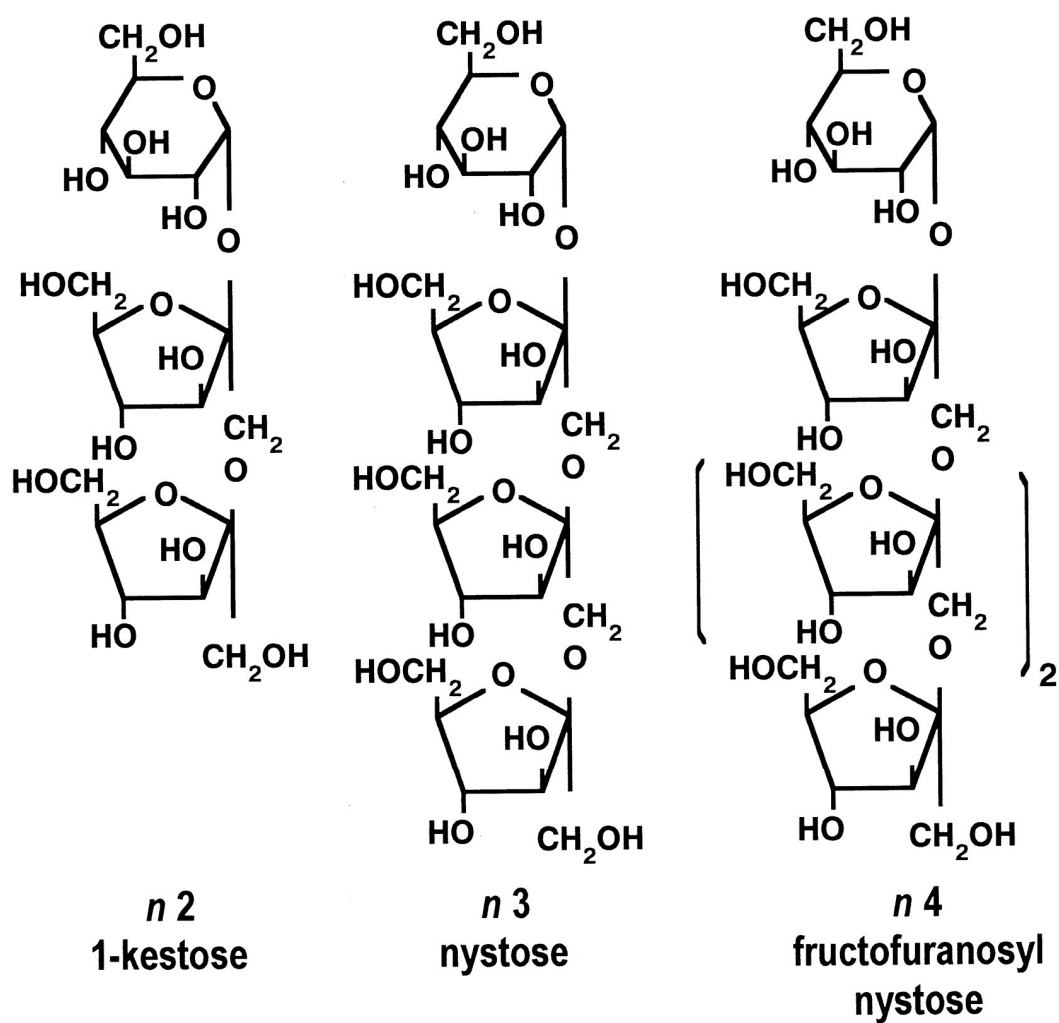


APPENDIX I

Non-digestible saccharide: Fructooligosaccharides (FOS)

FOS are a mixture of 34% 1-kestose, 53% nystose and 10% 1F- β -

fructofuranosylnystose (Meiologo-P, Meiji Seika Kaisha, Tokyo, Japan).

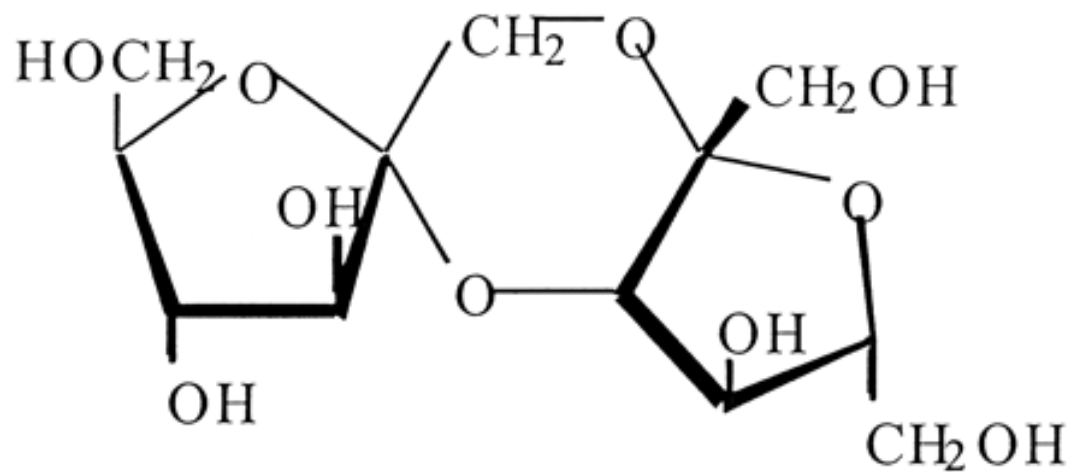


n = 1- 10 of fructosyl (fructose residue) molecules

APPENDIX J

Non-digestible saccharide: Difructose anhydride III (DFA III)

Other name: di-D-fructofuranose 1,2':2,3' dianhydride



APPENDIX K Composition of AIN-93G Vitamin mixture

Ingredients		g/1 kg Diet
Nicotinic acid	Niacin	3.000
Ca Pantothenic acid	パントテン酸	1.600
Pyridoxine hydrochloride	V.B ₆	0.700
Thiamin hydrochloride	V.B ₁	0.600
Riboflavin	V.B ₂	0.600
Folic acid	葉酸	0.200
D-Biotin	V.H	0.020
Cyanocobalamin ¹	V.B ₁₂	2.500
All-rac- α -tocopherylacetate	V.E (ユベラ顆粒)	75.000
All-trans-retinyl acetate ²	V.A (Retinyl acetate)	0.842
Cholecalciferol	V.D ₃	0.250
Phylloquinone	V.K ₁ (カチーフ N)	7.500
Sucrose		907.188

¹ Cyanocobalamin 10 mg と Sucrose 9.99 g を混合したもの

² Sigma 475000 IU

APPENDIX L Composition of AIN-93G Mineral mixture

Ingredients		g/1 kg
CaCO ₃	炭酸カルシウム	357
KH ₂ PO ₄	リン酸二水素カリウム	196
KOOCCH ₂ C(OH)(COOK)CH ₂ C	クエン酸三カリウム	70.78
NaCl	塩化ナトリウム	74
K ₂ SO ₄	硫酸カリウム	46.6
MgO	酸化マグネシウム	24
FeC ₆ H ₅ O ₇ · nH ₂ O	クエン酸鉄(□)n水和物	6.06
ZnCO ₃	炭酸亜鉛	1.65
MnCO ₃ · H ₂ O	炭酸マンガン	0.63
CuCO ₃ · Cu(OH) ₂ · H ₂ O	塩基性炭酸銅(□)	0.3
KIO ₃	ヨウ素酸カリウム	0.01
Na ₂ SeO ₄	セレン酸ナトリウム	0.0102
(NH ₄)Mo ₇ O ₂₄ · 4H ₂ O	七モリブデン酸六アンモニウム	0.0079
Na ₂ SiO ₃ · 9H ₂ O	メタケイ酸ナトリウム	1.45
CrK(SO ₄) ₂ · 12H ₂ O	硫酸カリウムクロム(□) · 12水	0.275
LiCl	塩化リチウム	0.0174
H ₃ BO ₃	ホウ酸	0.0815
NaF	フッ化ナトリウム	0.0635
NiCO ₃ · 2Ni(OH) ₂ · 4H ₂ O	炭酸ニッケル	0.0318
NH ₄ VO ₃	バナジン酸アンモニウム	0.0066
Sucrose	スクロース	221.02

APPENDIX M Protease inhibitor cocktail preparation

1. Protease inhibitor cocktail (100×)

	MW	1×	100×	For 10 mL Milli Q
Aprotonin from bovine lung	6500	5 µg/ mL	500 µg/ mL	5 mg
Leupeptin hemisulfate	493.6	3 µg/ mL	300 µg/ mL	3 mg
Benzamidine hydrochloride (monohydrate)	174	5 mM	500 mM	0.873 g



Aliquot the cocktail 0.5 mL each and store at -40 °C

Note: Add 10 µL of the cocktail into 1 mL lysis buffer to prepare cell lysates

2. 100 mM PMSF in methanol (MW: 174.2, 100×)

0.741 g PMSF

↓ ← 10 mL methanol



Dissolve by vortex

Store at room temperature in dark condition

Note: Add 10 µL of 100 mM PMSF into 1 mL lysis buffer to prepare cell lysates

APPENDIX N Lysis buffer composition

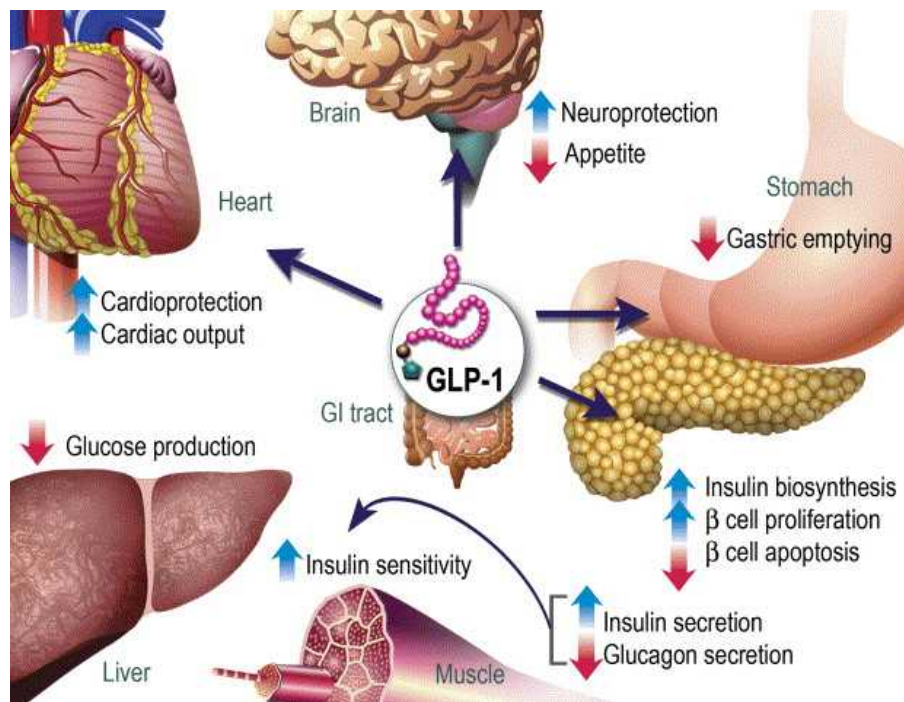
HEPES ^{*1}	10 mM	1× Lysis buffer stock solution
EGTA	5 mM	
EDTA	1 mM	
MgCl ₂	10 mM	
KH ₂ PO ₄	10 mM	
NaCl	150 mM	
NaF	100 mM	
Triton X-100	1 %	
Benzamidin	5 mM	100× Protease inhibitor cocktail
Leupeptin	3 µg/ mL	
Aprotonin	5 µg/ mL	
Na orthovanadate (Va)	2 mM	200 mM stock
B-glycerophosphate	50 mM	
PMSF ^{*2}	2 mM	100 mM stock

^{*1} N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4

^{*2} Phenyl methyl sulfonyl fluorid

APPENDIX O GLP-1 action

GLP-1 acts directly on the endocrine pancreas, heart, stomach, and brain, whereas actions on liver and muscle are indirect.



Reference: Daniel J. Drucker

The biology of incretin hormones

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PUBLICATIONS

Published Peer Reviewed Research Articles (from most recent)

1. **Panchita Phuwamongkolwiat, Tohru Hira, Hiroshi Hara.**
Fructooligosaccharides augment the effect of a flavonoid, α -glucosyl-isoquercetin, on glucagon-like peptide 1 (GLP-1) secretion in rat intestine and enteroendocrine cells. Molecular Nutrition & Food Research. Volume 58, Issue 7, July 2014, pages 1581–1584
2. **Panchita Phuwamongkolwiat, Takuya Suzuki, Tohru Hira, Hiroshi Hara.**
Fructooligosaccharides augment benefits of quercetin-3-O- β -glucoside on insulin sensitivity and plasma cholesterol with promotion of flavonoid absorption in sucrose-fed rats. European Journal of Nutrition. Volume 53, Issue 2, March 2014, Pages 457-468

PROFESSIONAL CONFERENCES

- 2014 The XXVIIth International Conference on Polyphenols (ICP 2014) and 8th Tannin Conference” in Nagoya, Japan. (2013/9/2-6)
- 2013 IUNS 20th International Congress of Nutrition" organized by, at Granada Congress Center, Granada, Spain (2013/9/15-20)
- 2013 Poster presentation in “Experimental Biology (EB) 2013” in Boston, USA.
- 2012 Poster presentation in “Experimental Biology (EB) 2012” in San Diego, USA.
- 2011 Oral presentation in “The International Conference on Food Factors (ICoFF) 2011” in Taipei, Taiwan.
- 2011 Poster presentation in “The Asian Congress of Nutrition (ACN) 2011” in Singapore

AWARDS

- 2014 A Junior Grant in “The XXVIIth International Conference on Polyphenols (ICP 2014) and 8th Tannin Conference” in Nagoya, Japan.
- 2013 Hokkaido University Grant for Attending International Conference for “IUNS 20th International Congress of Nutrition" organized by, at Granada Congress Center, Granada, Spain (2013/9/15-20)
- 2013 Hokkaido University Grant for Attending International Conference for “Experimental Biology 2013” organized by American Society

- for Nutrition (ASN), at Boston Convention and Exposition Center,
Boston USA (2012/04/20-24)
- 2012 Hokkaido University Grant for Attending International Conference
for “Experimental Biology 2012” organized by American Society
for Nutrition (ASN), at San Diego Convention Center, San Diego
USA (2012/04/21-25)
- 2011 Travel Award grant in “The International Conference on Food
Factors (ICoFF) 2011” in Taipei, Taiwan (2011/11/21-23)