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(71) **Applicant** (for AE, AG, AU, BB, BH, BW, BZ, CA, CY, EG, GB, GD, GH, GM, IE, IL, KE, KN, LC, LK, LS, MT, MW, MY, NA, NG, NZ, OM, PG, QA, RW, SC, SD, SG, SL, SZ, TT, TZ, UG, VC, ZA, ZM, ZW only): **UNILEVER PLC** [GB/GB]; Unilever House, 100 Victoria Embankment, London, Greater London EC4Y 0DY (GB).

(71) **Applicant** (for all designated States except AE, AG, AU, BB, BH, BW, BZ, CA, CY, EG, GB, GD, GH, GM, IE, IL, IN, KE, KN, LC, LK, LS, MT, MW, MY, NA, NG, NZ, OM, PG, QA, RW, SC, SD, SG, SL, SZ, TT, TZ, UG, US, VC, ZA, ZM, ZW): **UNILEVER N.V.** [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL).

(71) **Applicant** (for IN only): **HINDUSTAN UNILEVER LIMITED** [IN/IN]; Unilever House, B.D. Sawant Marg Chakala Andheri (East), Mumbai, Maharashtra 400 099 (IN).

(72) Inventors; and

(75) **Inventors/Applicants** (for US only): **BERRY, Mark, John** [GB/GB]; Unilever R&D Colworth, Sharnbrook,

Bedford, Bedfordshire MK44 1LQ (GB). **FOWLER, Mark, Ian** [GB/GB]; Unilever R&D Colworth, Sharnbrook, Bedford, Bedfordshire MK44 1LQ (GB). **HEATH, Alan, David** [GB/GB]; Unilever R&D Colworth, Sharnbrook, Bedford, Bedfordshire MK44 1LQ (GB).

(74) **Agent**: **WURFBAIN, Gilles Lodewijk**; Unilever Patent Group, Olivier van Noortlaan 120, 3133 AT Vlaardingen (NL).

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(54) Title: AN EDIBLE COMPOSITION

(57) **Abstract**: Foods or meals high in available carbohydrate such as sucrose or starch increase postprandial blood glucose concentrations. Repeated high post-prandial plasma glucose "spikes" are associated with an increased risk of developing type II diabetes. Unregulated glycemic excursions are undesirable, and any reduction or "blunting" of the post-prandial glucose concentration in blood is potentially beneficial. This invention relates to an edible composition for delay of intestinal glucose uptake through synergistic inhibition of both active sodium glucose co-transporter 1 (SGLT1) and passive glucose transporter 2 (GLUT2) leading to flattening or blunting of the post-prandial glucose peak. Thus in a first aspect of the invention, an edible composition is provided, the composition comprising at least 5% dry weight at least one flavonoid aglycone and at least 5% dry weight at least one flavonoid glucoside, wherein the flavonoid glucoside is at least 20 %, preferably at least 40 %, most preferably at least 60 % more resistant to hydrolysis by lactase phloridzin hydrolase than quercetin-4-glucoside, and wherein the flavonoid aglycone is a GLUT 2 inhibitor and the flavonoid glucoside is a SGLT 1 inhibitor.



AN EDIBLE COMPOSITION

Foods or meals high in available carbohydrate such as sucrose or starch increase postprandial blood glucose concentrations. According to Node et al. (Cardiovascular diabetology, 8, 23 (2009)), repeated high post-prandial plasma glucose “spikes” are associated with an increased risk of developing type II diabetes. Unregulated glycemic excursions are undesirable, and any reduction or “blunting” of the post-prandial glucose concentration in blood is potentially beneficial. This invention relates to an edible composition for delay of intestinal glucose uptake through synergistic inhibition of both active sodium glucose co-transporter 1 (SGLT1) and passive glucose transporter 2 (GLUT2) leading to flattening or blunting of the post-prandial glucose peak.

Summary of the Invention

In a first aspect of the invention, an edible composition is provided, the composition comprising at least 5% dry weight at least one flavonoid aglycone and at least 5% dry weight at least one flavonoid monoglucoside, wherein the flavonoid monoglucoside is at least 20 %, preferably at least 40 %, most preferably at least 60 % more resistant to hydrolysis by lactase phloridzin hydrolase than quercetin-4-glucoside, and wherein the flavonoid aglycone is a GLUT 2 inhibitor and the flavonoid monoglucoside is a SGLT 1 inhibitor.

Lactase phloridzin hydrolyase (LPH), a beta-galactosidase, is an enzyme found in the small intestine involved in the hydrolysis of the disaccharide lactose into its constituent galactose and glucose monomers. In particular, the enzyme hydrolyses the beta-glycosidic bond in D-lactose. Deficiency of this enzyme causes lactose intolerance. LPH also has glucosidase activity. Thus it is essential that the flavonoid glucoside exhibits a degree of resistance to hydrolysis by LPH in the small intestine where glucose absorption, via the glucose transporters, takes place.

By the term “at least 20 % more resistant to hydrolysis by LPH than quercetin-4-glucoside” is meant that the rate of hydrolysis by LPH is at least 20 % lower than that for quercetin-4'-glucoside. Thus the rate would be zero if 100 % more resistant to hydrolysis by LPH than quercetin-4-glucoside.

By the term “flavonoid aglycone” is meant an unglycosylated flavonoid. By the term “flavonoid monoglucoside” is meant a flavonoid attached to a single glucose unit. By the term “GLUT 2 inhibitor” is meant a compound which inhibits the transmembrane carrier protein known as passive glucose transporter 2. By the term “SGLT1 inhibitor” is meant a compound which inhibits the transmembrane carrier protein known as sodium glucose co-transporter 1.

The flavonoid aglycone may be selected from the group consisting of flavone aglycones, flavanol aglycones, flavanone aglycones, isoflavone aglycones and mixtures thereof. Thus by the terms “flavone aglycone”, “flavanol aglycone”, “flavanone aglycone” and “isoflavone aglycones” are meant an unglycosylated flavone, flavanol, flavanone and isoflavone respectively. In particular, the flavonoid aglycone may be selected from the group consisting of apigenin, luteolin, quercetin, kaempferol, myricetin, naringenin, pinocembrin, hesperetin, genistein and mixtures thereof.

The flavonoid monoglucoside may be selected from the group consisting of luteolin-7-glucoside, apigenin-8-C-glucoside, kaempferol-7-O-glucoside, kaempferol-3-O-glucoside, naringenin-7-O-glucoside, daidzein-8-glucoside, cyanidin-3-glucoside, quercetin-3-glucoside, pelargonidin-3-glucoside, malvidin-3-glucoside, delphinidin-3-glucoside and mixtures thereof.

The molar ratio of flavonoid aglycone to flavonoid monoglucoside may be in the range 4:1 to 1:4, preferably 3:1 to 1:3, most preferably 2:1 to 1:2.

The composition may comprise no more than 50 %, preferably no more than 10 %, most preferably no more than 2 % by weight flavonoid aglycone, and separately no more than 50 %, preferably no more than 10 %, most preferably no more than 2 % by weight flavonoid monoglucoside. Thus at a level of no more than 2 % by weight flavonoid aglycone, the composition must comprise water in order for the composition to comprise at least 5% dry weight at least one flavonoid aglycone.

The composition is preferably in the form of a daily dose, the daily dose comprising at least 50 micromoles, preferably at least 100 micromoles, most preferably at least 250 micromoles

of flavonoid aglycone and at least 50 micromoles, preferably at least 100 micromoles, most preferably at least 250 micromoles of flavonoid monoglucoside.

5 The inventive composition may be in the form of a packaged beverage comprising no more than 99.95 % w/w water. It may also be in the form of a dry powder contained in a sachet, the dry powder suitable for addition to a meal.

10 In a second aspect of the invention, a method of reducing post-prandial blood glucose peak amplitude or glycemic response in a non-diabetic person is provided, the method comprising the steps of:

- (a) oral administration of the composition of the first aspect of the invention to the non-diabetic person; and
 - (b) oral administration of saccharide to the non-diabetic person;
- wherein step (a) is simultaneous with, precedes by 0 to 90, preferably 0 to 60 minutes, or
15 follows by 0 to 30 minutes step (b), and wherein the saccharide comprises or is glucose.

In a third aspect of the invention, a method for treating a person in need thereof for type 2 diabetes is provided, the method comprising the steps of:

- (a) oral administration of the composition of the first aspect of the invention to the person
20 in need thereof; and
 - (b) oral administration of saccharide to the person in need thereof;
- wherein step (a) is simultaneous with, precedes by 0 to 90, preferably 0 to 60 minutes, or follows by 0 to 30 minutes step (b), and wherein the saccharide comprises or is glucose.

25 The saccharide may be selected from the group consisting of polysaccharide, oligosaccharide, disaccharide, monosaccharide and mixtures thereof.

30 In a fourth aspect of the invention, a composition according to the first aspect of the invention is provided for use in reducing post-prandial blood glucose peak amplitude or glycemic response in a non-diabetic person

In a fifth aspect of the invention, a composition according to the first aspect of the invention is provided for use in the treatment of type 2 diabetes.

In a sixth aspect of the invention, use of a composition according to the first aspect of the invention is provided for the manufacture of a medicament for reducing post-prandial blood glucose peak amplitude or glycemic response in a non-diabetic person.

5

In a seventh aspect of the invention, use of a composition according to the first aspect of the invention is provided for the manufacture of a medicament for treatment of type 2 diabetes.

Brief Description of the Figures

10 The invention is illustrated with reference to the figures which show in:

Figure 1 a model of the glucose concentration timeline during a meal;

15 Figure 2 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM phloridzin (Pz)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (125 μM phloretin (Pt)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control);

20 Figure 3 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM luteolin-7-glucoside (L7G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (50 μM genistein (G)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control);

25

Figure 4 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM apigenin-8-C-glucoside (A8G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (50 μM genistein (G)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control);

30

- Figure 5 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM quercetin-3-glucoside (Q3G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (100 μM hesperetin (H)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control);
- Figure 6 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM quercetin-3-glucoside (Q3G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (50 μM luteolin (L)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control);
- Figure 7 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM kaempferol-3-glucoside (K3G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (50 μM hesperetin (H)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control);
- Figure 8 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM quercetin-3-glucoside (Q3G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (50 μM naringenin (N)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control);
- Figure 9 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM naringenin-7-glucoside (N7G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (50 μM apigenin (A)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control); and

Figure 10 the total cumulative glucose transport (μM) across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor (300 μM delphinidin-3-glucoside (D3G)) for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor (50 μM genistein (G)) in 25 mM D-glucose for the remaining 45 minutes (NC = vehicle negative control).

Detailed Description of the Invention

Example 1: Identification of SGLT1 and GLUT2 inhibitors

Routine cell culture

Human epithelial colorectal adenocarcinoma (Caco-2) cells were obtained from the American Type Culture Collection (ATCC) and cultured in Growth Medium consisting of Dulbecco's modified Eagle's medium (containing Glutamax-1, 4.5 g/L D-glucose and 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (Hepes) (Invitrogen)), 10 % foetal bovine serum (Sigma), 1 % non-essential amino acids (Invitrogen) and 1 mM sodium pyruvate (Sigma)). The cells were routinely passaged at approximately 80 % confluence using TrypLE™ Express Stable Trypsin-Like Enzyme (Invitrogen) to detach the cells, and seeded at approximately 114 cells per mm^2 in fresh tissue culture flasks. Only cells between the passage numbers 45 and 49 were used for experiments.

Preparation of differentiated Caco-2 cell monolayers

Corning® HTS Transwell® 96 well permeable insert supports (Sigma) were collagen coated with 40 μl of 50 $\mu\text{g}/\text{ml}$ rat tail collagen type I (BD Biosciences) in 0.02 M acetic acid for one hour at room temperature under sterile conditions. The inserts were washed twice in phosphate buffered saline (PBS (Invitrogen)) and the Caco-2 cells seeded into the inserts at 9.6×10^5 cell/ml (75 μl per insert) in Growth Medium and 30 ml of Growth Medium added to the feeder plate below. The cells were left to attach to the collagen matrix and form monolayers over 48 hours at 37°C, 5 % CO_2 . Both inserts and feeder plate were washed in PBS and the cells incubated with BD Entero-STIM™ Enterocyte Differentiation Medium containing MITO+™ Serum Extender solution (both BD Biosciences), 75 μl per insert and 30 ml in the feeder plate, for a further 48 hours at 37°C, 5 % CO_2 .

Glucose Transport Inhibitor Cell Screening Assay

Differentiated cell monolayers were washed gently in Dulbecco's Phosphate Buffered Saline containing CaCl_2 and MgCl_2 (PBS(+)) (Invitrogen)) and the inserts transferred to a new
5 Corning® HTS Transwell®-96 well receiver plate (Sigma). The cells were incubated with fresh PBS(+) (75 μl per insert and 225 μl per well) for 60 minutes at 37 °C, 5 % CO_2 . The PBS(+) was gently aspirated and replaced with 75 μl per insert of either 5 mM D-glucose (Sigma) $\pm$ test active or 25 mM D-glucose $\pm$ test active in triplicate and 225 μl per well of
10 PBS(+) quickly added to each well. The 5 mM glucose wells and the 25 mM glucose wells were incubated at 37 °C, 5 % CO_2 for 15 minutes and 30 minutes, respectively. Details of all the actives tested are found in table 1. The cell inserts were transferred to a new receiver plate, the supernatant gently aspirated from the cells and replaced with 100 μl of 100 μM of Lucifer Yellow (Sigma) solution to confirm the integrity of the monolayers. 225 μl of PBS(+) was added to each well and incubated at 37°C, 5 % CO_2 for 1 hour. The cell inserts
15 were then discarded and the permeability of the membranes to Lucifer Yellow checked by measuring the fluorescence of the samples at 485 nm (excitation) and 530 nm (emission) on a Spectramax Gemini EM fluorescence microplate reader.

Glucose Assay

20 The amount of glucose transported across the cell monolayers was measured using a glucose assay based on Invitrogen's Amplex Red Glucose/Glucose oxidase Assay Kit. Briefly, 50 μl of each test sample was transferred to a black sided/clear bottom 96-well plate (Greiner Bio-One) to which 100 μl of reaction buffer (0.5 μl 10 mM Ampliflu Red, 1 μl 10U/ml Horse Radish peroxidase, 1 μl 100U/ml glucose oxidase and 97.5 μl PBS (all Sigma)) was added.
25 After 10 minutes incubation at room temperature, the fluorescence of the samples were measured at 530 nm (excitation) and 590 nm (emission) on a Spectramax Gemini EM fluorescence microplate reader and the glucose concentration extrapolated from a standard curve.

30 Table 1 shows the percentage of inhibition of each test active against the transport of glucose across a differentiated Caco-2 cell monolayer. At the lower D-glucose concentration of 5 mM, the early transport of glucose across the cell monolayer is predominantly through the apically expressed, high affinity, low capacity SGLT1 glucose transporter. At higher D-glucose concentrations, the SGLT1 transporter becomes saturated and consequently the

majority of glucose transport across the monolayer is driven by the low affinity, high capacity GLUT2 transporter that is targeted to the apical membrane only following an initial SGLT1-dependent transport of glucose. The screening cell model, detailed in the methods above, is designed to take advantage of these differences in the optimal conditions for each transporter to identify both SGLT1 and GLUT2 specific inhibitors. While both SGLT1 and GLUT2 on the apical membrane transport glucose into the enterocyte, GLUT2 is also expressed in the basolateral membrane where it is essential for the transport of glucose out of the cell. Hence, GLUT2 specific inhibitors will not only block the apically targetted transporters at high D-glucose concentrations (25 mM), they will also enter the cell and block the exit of glucose from the enterocyte at low D-glucose concentrations (5 mM). Therefore, to differentiate between inhibition of apical and basolateral transporters, each active was tested at both 5 mM D-glucose for 15 minutes and 25 mM D-glucose for 30 minutes. Actives were classed as SGLT1 inhibitors if they exhibited at least 20 % inhibition of glucose transport at 5 mM D-glucose and a corresponding no more than 20 % inhibition at 25 mM D-glucose. Actives that were able to inhibit glucose transport by at least 20 % in both conditions were classed as GLUT2 specific inhibitors. This approach was qualified through the use of the widely recognised specific inhibitors of both SGLT1 and GLUT2, namely phloridzin and phloretin respectively.

The foregoing glucose transport cell model was described by Kellett et al. (Diabetes, 54, 10, 3056-62 (2005)) and illustrated by figure 1 designed to mimic the localised changes in glucose concentration in the small intestine during the consumption of a carbohydrate rich meal. Before the meal, the concentration of free glucose in the lumen of the intestine is low (<5 mM) and the apically expressed SGLT1 transporter actively transports any available glucose into the enterocyte. GLUT2 transporters are also active on the basolateral membrane of the enterocyte, transporting glucose from the blood into the cell to maintain cellular metabolism if required. During a meal, the local concentration of glucose begins to increase (5-10 mM) and is transported from the intestinal lumen by SGLT1 and subsequently into the systemic circulation via GLUT2. As a consequence of this initial glucose transport across the enterocyte, intracellular stores of GLUT2 are mobilised and targeted to the apical membrane. Shortly after the meal, very high local concentrations of glucose occur (25-100 mM) as the carbohydrate content of the meal is broken down into monosaccharides by alpha-glucosidase enzymes located on the apical enterocyte membrane. At these high levels of glucose, the high affinity, low capacity transporter SGLT1 becomes saturated and the majority of glucose

transport across the enterocyte is due to the low affinity, high capacity GLUT2 transporters now present in the apical membrane.

Table 1 demonstrates that for inhibition of SGLT1, a flavonoid monoglucoside is required as confirmed by luteolin-7-glucoside, apigenin-7-glucoside, apigenin-8-c-glucoside, kaempferol-3-glucoside, kaempferol-7-glucoside, quercetin-3-glucoside, quercetin-4-glucoside, naringenin-7-glucoside, eriodictiol-7-glucoside, daidzein-8-c-glucoside, daidzein-7-glucoside, cyanidin-3-glucoside, malvidin-3-o-glucoside, delphinidin-3-glucoside and pelargonidin-3-glucoside. Indeed, the presence of an additional glucose moiety on the chemical structure destroys this inhibitory action as demonstrated by quercetin-3,4'-diglucoside. The specificity for a glucoside is confirmed by the absence of SGLT1 inhibitory activity by other flavonoid glycosides tested, including cyanidin-3-rutinoside and malvidin-3-O-galactoside. In addition, the lack of SGLT1 inhibitory activity shown by the hydroquinone monoglucoside, arbutin, reinforces the importance of a flavonoid structure in the glucoside molecule. Other non-flavonoid glucosides alleged by Welsch et al. (J. of Nutrition, 119, 11, 1698-704 (1989)) to be glucose transporter inhibitors such as chlorogenic acid, caffeic acid and rosmarinic acid (an ester of caffeic acid) showed no inhibitory activity in this cell model for either SGLT1 or GLUT2 inhibition. Table 1 also shows that all the aglycones tested from each flavonoid class selected, except the anthocyanidins, were confirmed as GLUT2 inhibitors.

Example 2: Synergy between SGLT1 and GLUT2 inhibitors

Preparation of differentiated Caco-2 cell monolayers

Caco-2 cells were cultured and routinely passaged as described in example 1. Caco-2 cells were seeded into BioCoat HTS Fibrillar Collagen Multiwell Inserts (BD Biosciences) at 2.5×10^5 cell/ml (500 μ l per insert) in Growth Medium and 30 ml of Growth Medium added to the feeder plate below. The cells were left to attach to the collagen matrix and form monolayers over 24 hours at 37 °C, 5 % CO₂. Both inserts and feeder plate were washed in PBS and the cells incubated with BD Entero-STIM™ Enterocyte Differentiation Medium containing MITO+™ Serum Extender solution (both BD Biosciences), 500 μ l per insert and 30 ml in feeder plate, for a further 48 hours at 37 °C, 5 % CO₂.

Table 1: Actives tested for SGLT1 and GLUT2 inhibition activity in Caco-2 cells using 5 mM D-glucose for 15 minutes and 25 mM D-glucose for 30 minutes, respectively. The assigned class of transporter inhibited by each active is based on SGLT1 inhibitors having $\geq 20\%$ inhibition of glucose transport at 5 mM D-glucose and $\leq 20\%$ inhibition at 25 mM D-glucose, and GLUT2 inhibitors having $\geq 20\%$ inhibition at both 5 mM and 25 mM D-glucose levels. ND = not detected; Nt = not tested.

Chemical Family:		Test Active ^a	Solvent	% Glucose Transport inhibition		Class ^b	Supplier
				5mM	25mM		
Chalcone		Phloridzin	EtOH	57.57	18.42	SGLT1	Sigma
		Phloretin	EtOH	86.77	76.14	GLUT2	Sigma
Flavonoids:	Flavones	Apigenin	DMSO	56.83	49.28	GLUT2	Sigma
		Luteolin	DMSO	77.42	63.24	GLUT2	Sigma
		Luteolin-7-glucoside	DMSO	44.09	17.03	SGLT1	Extrasynthese
		Apigenin-7-glucoside	DMSO	37.97	12.81	SGLT1	Extrasynthese
		Apigenin-8-C-glucoside	DMSO	33.84	5.76	SGLT1	Extrasynthese
	Flavonols	Quercetin	DMSO	80.78	61.37	GLUT2	Sigma
		Kaempferol	DMSO	25.96	32.44	GLUT2	Sigma
		Myricetin	DMSO	76.80	55.04	GLUT2	Sigma
		Kaempferol 7-O-glucoside	DMSO	43.89	14.42	SGLT1	Extrasynthese
		Kaempferol 3-O-glucoside	DMSO	53.89	19.12	SGLT1	Extrasynthese
		Quercetin-3-glucoside	DMSO	46.40	12.20	SGLT1	PlantChem
		Quercetin-4-glucoside	DMSO	43.20	19.05	SGLT1	PlantChem
		Quercetin-3,4'-diglucoside	DMSO	17.48	Nt	None	PlantChem
	Flavanones	Naringenin	DMSO	68.96	57.05	GLUT2	Sigma
		Pinocembrin	DMSO	47.72	48.07	GLUT2	Sigma
		Hesperetin	DMSO	72.34	74.43	GLUT2	Sigma
		Naringenin-7-O-glucoside	DMSO	29.56	0.06	SGLT1	Extrasynthese
		Eriodictiol-7-O-glucoside	DMSO	38.88	4.98	SGLT1	Extrasynthese
	Isoflavones	Genistein	DMSO	56.53	57.73	GLUT2	Sigma
		Daidzein-8-C-glucoside	DMSO	20.31	12.88	SGLT1	Sigma
		Daidzein-7-glucoside	DMSO	35.03	7.23	SGLT1	Extrasynthese
	Anthocyanidins	Cyanidin	DMSO	7.93	3.21	None	ChromaDex
		Pelargonidin	DMSO	4.07	16.48	None	ChromaDex
		Malvidin	DMSO	14.21	4.99	None	ChromaDex
		Delphinidin	DMSO	0.01	15.86	None	ChromaDex
		Cyanidin-3-glucoside	DMSO	42.48	ND	SGLT1	Extrasynthese
		Cyanidin-3-rutinoside	DMSO	19.42	ND	None	Extrasynthese
		Malvidin-3-O-glucoside	DMSO	22.92	10.91	SGLT1	Extrasynthese
		Delphinidin-3-glucoside	DMSO	41.27	13.58	SGLT1	Extrasynthese
		Pelargonidin-3-glucoside	DMSO	27.30	ND	SGLT1	Extrasynthese
		Malvidin 3-O-Galactoside	DMSO	19.05	10.41	None	Sigma

^a - All actives tested at 150uM and 300uM for GLUT2 inhibition and SGLT1 inhibition assays respectively.

^b - Based on SGLT1 inhibitors having >20% inhibition at 5mM (glucose) and <20% inhibition at 25mM, and GLUT2 having >20% inhibition at both 5mM & 25mM
ND - Not detected, Nt - Not tested

Glucose Transport Cell Model

Differentiated cell monolayers were washed gently in PBS(+) and the inserts transferred to a new standard tissue culture 24-well plate. The cells were incubated with fresh PBS(+) (500 µl per insert and 1 ml per well) for 30 minutes at 37 °C 5 % CO₂. The PBS(+) was gently aspirated and replaced with 250 µl per insert of 5 mM D-glucose ± test active and 1 ml of PBS(+) quickly added to each well below before the cells were replaced in the incubator at 37 °C 5 % CO₂. After 15 minutes, the cell inserts were transferred to a new 24-well plate, and a further 250 µl of 45 mM D-glucose ± test active was added to each insert (resulting in a final concentration of glucose of 25 mM) and again 1 ml of PBS(+) added to the wells. After a further 15 minutes the inserts were again transferred to a new 24-well plate and this time only fresh PBS(+) was added to the wells below. This step was repeated after another 15 minutes. The cell inserts were transferred to a new 24-well plate, the supernatant gently aspirated from the cells and replaced with 500 µl of 100 µM of Lucifer Yellow (Sigma) solution to confirm the integrity of the monolayers. 1 ml of PBS(+) was added to each well and incubated at 37 °C, 5 % CO₂ for 1 hour. The cell inserts were then discarded and the permeability of the membranes to Lucifer Yellow was checked by measuring the fluorescence of the samples at 485 nm (excitation) and 530 nm (emission) on a Spectramax Gemini EM fluorescence microplate reader.

Glucose Assay

After the last incubation, all of the retained PBS(+) from each step (i.e. at 15, 30, 45 and 60 minutes) was assayed for glucose levels as described in example 1, and the total cumulative glucose transport calculated. The localised changes in luminal glucose concentrations described and illustrated in example 1 are mimicked in-vitro through an initial short incubation of differentiated Caco-2 cells with a low level of D-glucose (5 mM for 15 minutes) immediately followed by a sustained incubation with a high level of D-glucose (final concentration of 25 mM for 45 minutes).

Figure 2 plots the total cumulative glucose transport across differentiated Caco-2 monolayers in the presence or absence of a SGLT1 inhibitor for the first 15 minutes in 5 mM D-glucose and subsequently in the presence or absence of a GLUT2 inhibitor in 25 mM D-glucose for the remaining 45 minutes. To validate this cell system, phloridzin (Pz) and phloretin (Pt) were used as the widely accepted specific inhibitors of SGLT1 and GLUT2 respectively. The SGLT1 specific inhibitor, phloridzin (Pz), shows a reduction in total Cumulative Glucose

Transport (tCGT) when added from the beginning of the cell system (Pz NC – horizontal striped bar), demonstrating the inhibition of SGLT1 which is the dominant active transporter at low glucose levels. However Pz exhibits no inhibition on tCGT when added later with the high concentration of D-glucose (NC Pz – dotted bar) since the SGLT1 transporters are now saturated and glucose transport is now dependent on the high capacity GLUT2 transporters. The GLUT2 specific inhibitor, phloretin (Pt), demonstrates a significant ($p \leq 0.05$) reduction in tCGT when added with the high concentration of D-glucose as expected (NC Pt – diagonal striped bars). However a combination of 300 uM Pz at low glucose concentrations, followed by 125 uM Pt at the high glucose concentration appears to significantly ($p \leq 0.01$) and synergistically inhibit the tCGT. This synergy exploits the requirement of an initial transport of glucose into the enterocyte by SGLT1 before the high-capacity GLUT2 can be targeted to the apical membrane. Used in combination, both SGLT1 and GLUT inhibitors can synergistically inhibit the localised uptake of glucose from the intestinal lumen and hence reduce the high ‘spikes’ of postprandial blood glucose associated with the onset of type 2 diabetes.

Hence any flavonoid monoglucoside identified in example 1 as specific SGLT1 inhibitors, can be combined with any flavonoid aglycone identified in example 1 as specific GLUT2 inhibitors to demonstrate the synergistic inhibition of glucose transport across a differentiated Caco-2 cell monolayer as detailed above for the combination of Pz and Pt. This is confirmed by combining the flavone monoglucosides luteolin-7-glucoside (L7G) and apigenin-8-C-glucoside (A8G) with the isoflavone aglycone genistein (Figures 3 and 4 respectively). Similarly, the flavonol monoglucosides quercetin-3-glucoside (Q3G) and kaempferol-3-glucoside (K3G) can be combined with the flavone aglycone luteolin and the flavanone aglycones hesperetin and naringenin to demonstrate the synergy as shown in Figures 5, 6, 7 and 8. Furthermore, the flavanone monoglucoside naringenin-7-glucoside (N7G) demonstrates synergistic glucose transport inhibition when combined with the flavone aglycone apigenin (Figure 9). Finally, Figure 10 shows the synergistic inhibition of glucose transport demonstrated by the anthocyanin delphinidin-3-glucoside (D3G) and the isoflavone aglycone genistein.

Example 3: Resistance of flavonoid glucosides to human lactase phloridzin hydrolase (hLPH) degradation

Preparation of human Lactase Phloridzin Hydrolase (LPH) extract

Human epithelial colorectal adenocarcinoma (Caco-2) cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified eagle medium (DMEM) (with GlutaMAX™ I, 4500 mg/L D-glucose, 25 mM HEPES, Invitrogen) +10 % foetal calf serum (FCS) (Sigma), MEM non essential amino acids (Fisher Scientific UK Ltd,) and 1.0 mM sodium pyruvate (SIGMA) at confluence for 21 days prior to extraction with a Novagen ProteoExtract™ trans-membrane Protein Extraction kit using "Reagent A" (a mild extraction reagent for recovery of fragile protein complexes) and protease inhibitor cocktail (included in the kit). 200 µl of extract were generated from each T175cm² flask. Extracts were stored at -80 °C in 50 µl aliquots. Prior to use extracts were semi-purified, removing low molecular weight interfering components by passing through a small G-25 Sephadex 50-100 µl spin-column (Roche or ThermoFisher Scientific).

hLPH Enzyme Assay

10 µl of semi-purified LPH extract was added to 90 µl of glucoside (1.0 mM final concentration) in 0.1 M maleate (Sigma) buffer, pH 6.0 and incubated for 60 minutes at 37 °C. The reaction was terminated by addition of 200 µl 2M Tris (Sigma), pH 8.0. Concurrent blanks were run for each glucoside (1.0 mM) at 37 °C for 60 minutes adding Tris 2 M and then LPH extract only at the end. The 200 µl resultant reaction mix was passed through a small C-18 column (Sep-Pak® Light C18 cartridge 55-105 µm, Waters Ltd.) prepared with (HPLC grade methanol (VWR) and maleate buffer) x5 times to remove potential interfering hydrophobic LPH extract, substrate (glucoside) and reaction product (aglycone) components. This allowed a full recovery of glucose which was measured using the Amplex® Red Glucose/Glucose Oxidase Assay. Briefly, 100 µl of reaction reagent (0.5 % 10 mM Ampliflu Red; 1.0 % 10 U/ml horseradish peroxidase and 1.0 % 100 U/ml glucose oxidase in phosphate buffered saline (PBS), all reagents from SIGMA) was added to 50 µl of sample and incubated at room temperature on an orbital shaker for 20 minutes. Fluorescence was read on a SpectraMax Gemini EM SN plate reader (Molecular Devices) (excitation. 530 nm and emission.at 590 nm).

Table 2: Glucoside hydrolysis to LPH and resistance relative to Q4G. Glucosides (1.0 mM) were mixed with 10 % LPH extract (Caco2 cell-line) for 1 hour at 37 °C. The extent of LPH hydrolysis was assessed by measuring glucose reaction product using the Amplex® Red Glucose/Glucose Oxidase Assay. The table lists those glucosides showing greater resistance to LPH hydrolysis than Q4G.

<u>Substrates</u> <u>(1.0mM)</u>	<u>μM substrate</u> <u>hydrolysed / hr</u>	<u>Resistance to</u> <u>hydrolysis-</u> <u>% Q4G</u>	<u>Supplier</u>
Q3G Quercetin-3-glucoside	0.178	94.26	SIGMA
K3G Kaempferol-3-glucoside	0.224	92.78	Extrasynthese
PLZ Phloridzin	0.507	83.64	SIGMA
D8G Daidzein-8-glucoside	0.552	82.19	SIGMA
K7G Kaempferol-7-glucoside	0.618	80.05	Extrasynthese
A8G Apigenin-8-glucoside	0.685	77.90	SIGMA
L7G Luteolin-7-glucoside	0.814	73.72	Extrasynthese
P3G Pelargonidin-3-glucoside	1.272	58.94	Polyphenols
N7G Naringenin-7-glucoside	1.564	49.52	Extrasynthese
Q4G Quercetin-4-glucoside	3.098	-	SIGMA

Table 2 demonstrates the variation in the degradation rates of different flavonoid glucosides by hLPH. Whilst Q4G demonstrates the highest degree of degradation by hLPH, Q3G appears to be approximately 95 % more resistant to hLPH in this assay than Q4G. Similarly, the other eight glucosides all exhibit increased resistance to hLPH relative to Q4G, ranging from 49.5 % for N7G to 92.8 % for K3G. Consequently, these glucosides would be less liable to hydrolysis by hLPH in the intestine during a meal and thus potentially prolonging its activity as a SGLT1 inhibitor. Therefore selecting flavonoid glucosides with at least a 20 % increased resistance to hLPH than Q4G would be beneficial in prolonging any glucoside-specific activity in vivo.

Example 4: Bottled water

A dry powder comprising 200 mg quercetin-3-glucoside per gram and 125 mg luteolin per gram and also comprising flavours is added to water at a level of 2 grams of dry powder per litre of water. The formulation is dispensed into 250mls bottles and sealed. Each bottle is labelled “1-a-day” or words to that effect. Each bottle therefore delivers a daily dose of 100mgs quercetin-3-glucoside and 62.5mgs of luteolin.

Example 5: Sachets for adding to a meal

A dry powder comprising 100 mg of luteolin-7-glucoside per gram and 60 mg of genistein per gram and also comprising flavours is dispensed into sachets at the level of 1 gram per sachet and sealed. Each sachet is labelled “1-a-day” or words to that effect.

Claims

1. An edible composition comprising at least 5 %, preferably at least 10 % dry weight at
5 least one flavonoid aglycone and at least 5 %, preferably at least 10 % dry weight at
least one flavonoid monoglucoside, wherein the flavonoid monoglucoside is at least
20 %, preferably at least 40 %, most preferably at least 60 % more resistant to
hydrolysis by lactase phloridzin hydrolase than quercetin-4-glucoside, and wherein
the flavonoid aglycone is a GLUT 2 inhibitor and the flavonoid monoglucoside is a
10 SGLT 1 inhibitor.
2. A composition according to claim 1 wherein the flavonoid aglycone is selected from
the group consisting of flavone aglycones, flavanol aglycones, flavanone aglycones,
isoflavone aglycones and mixtures thereof.
- 15 3. A composition according to claim 2 wherein the flavonoid aglycone is selected from
the group consisting of apigenin, luteolin, quercetin, kaempferol, myricetin,
naringenin, pinocembrin, hesperetin, genistein and mixtures thereof.
- 20 4. A composition according to any one of the preceding claims wherein the flavonoid
monoglucoside is selected from the group consisting of luteolin-7-glucoside,
apigenin-8-C-glucoside, kaempferol-7-O-glucoside, kaempferol-3-O-glucoside,
naringenin-7-O-glucoside, daidzein-8-glucoside, cyanidin-3-glucoside, quercetin-3-
glucoside, pelargonidin-3-glucoside, malvidin-3-glucoside, delphinidin-3-glucoside
25 and mixtures thereof.
5. A composition according to any one of the preceding claims wherein the molar ratio
of flavonoid aglycone to flavonoid monoglucoside is in the range 4:1 to 1:4,
preferably 3:1 to 1:3, most preferably 2:1 to 1:2.
- 30 6. A composition according to any one of the preceding claims wherein the composition
comprises no more than 50 %, preferably no more than 10 %, most preferably no
more than 2 % by weight flavonoid aglycone.

7. A composition according to any one of the preceding claims wherein the composition comprises no more than 50 %, preferably no more than 10 %, most preferably no more than 2 % by weight flavonoid monoglucoside.
- 5 8. A composition according to any one of the preceding claims in the form of a daily dose, the daily dose comprising at least 50 micromoles, preferably at least 100 micromoles, most preferably at least 250 micromoles of flavonoid aglycone and at least 50 micromoles, preferably at least 100 micromoles, most preferably at least 250 micromoles of flavonoid monoglucoside.
- 10 9. A method of reducing post-prandial blood glucose peak amplitude or glycemic response in a non-diabetic person comprising the steps of:
- (a) oral administration of the compositions of any one of the preceding claims to the non-diabetic person; and
- 15 (b) oral administration of saccharide to the non-diabetic person;
- wherein step (a) is simultaneous with, precedes by 0 to 90, preferably 0 to 60 minutes, or follows by 0 to 30 minutes step (b), and
- wherein the saccharide comprises or is glucose.
- 20 10. A method for treating a person in need thereof for type 2 diabetes, the method comprising the steps of:
- (a) oral administration of the compositions of any one of claim 1 to 8 to the person in need thereof; and
- (b) oral administration of saccharide to the person in need thereof;
- 25 wherein step (a) is simultaneous with, precedes by 0 to 90, preferably 0 to 60 minutes, or follows by 0 to 30 minutes step (b), and
- wherein the saccharide comprises or is glucose.
- 30 11. A method according to claim 9 or claim 10 wherein the saccharide may be selected from the group consisting of polysaccharide, oligosaccharide, disaccharide, monosaccharide and mixtures thereof.

12. Composition according to any one of claims 1 to 8 for use in reducing post-prandial blood glucose peak amplitude or glycemic response in a non-diabetic person.
- 5 13. Composition according to any one of claims 1 to 8 for use in the treatment of type 2 diabetes.
14. Use of compositions according to any one of claims 1 to 8 for the manufacture of a medicament for reducing post-prandial blood glucose peak amplitude or glycemic response in a non-diabetic person.
- 10 15. Use of compositions according to any one of claims 1 to 8 for the manufacture of a medicament for treatment of type 2 diabetes.

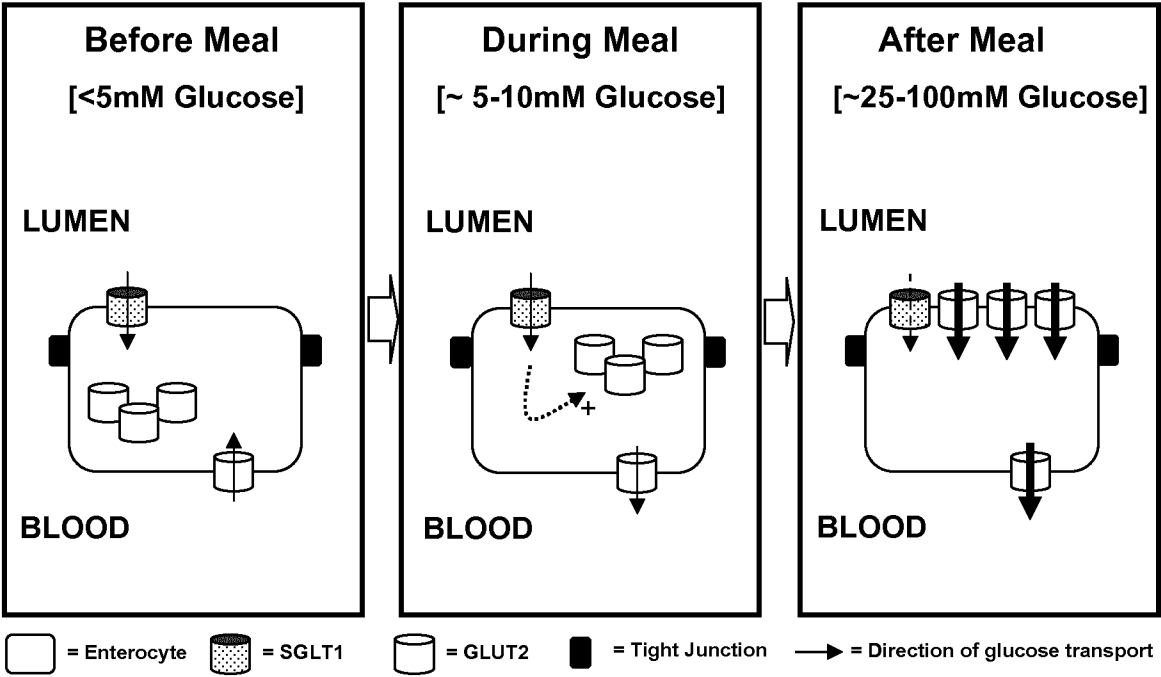


Figure 1

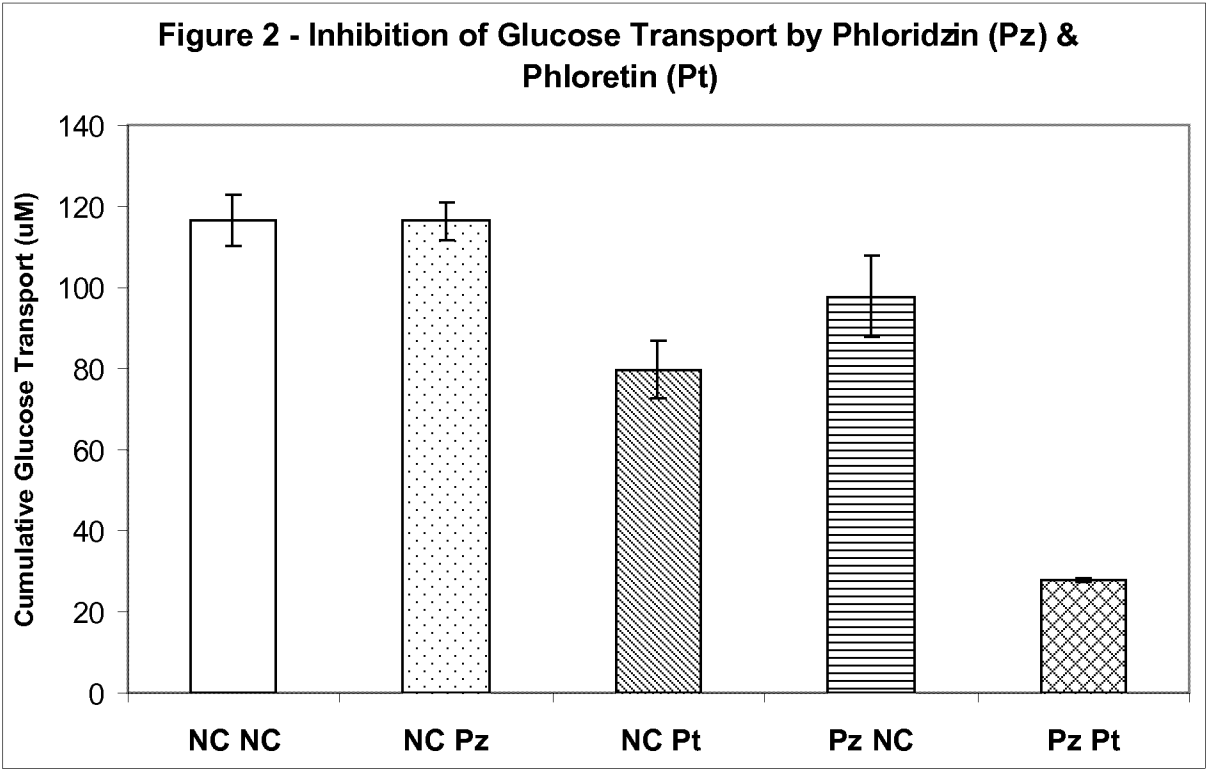


Figure 2

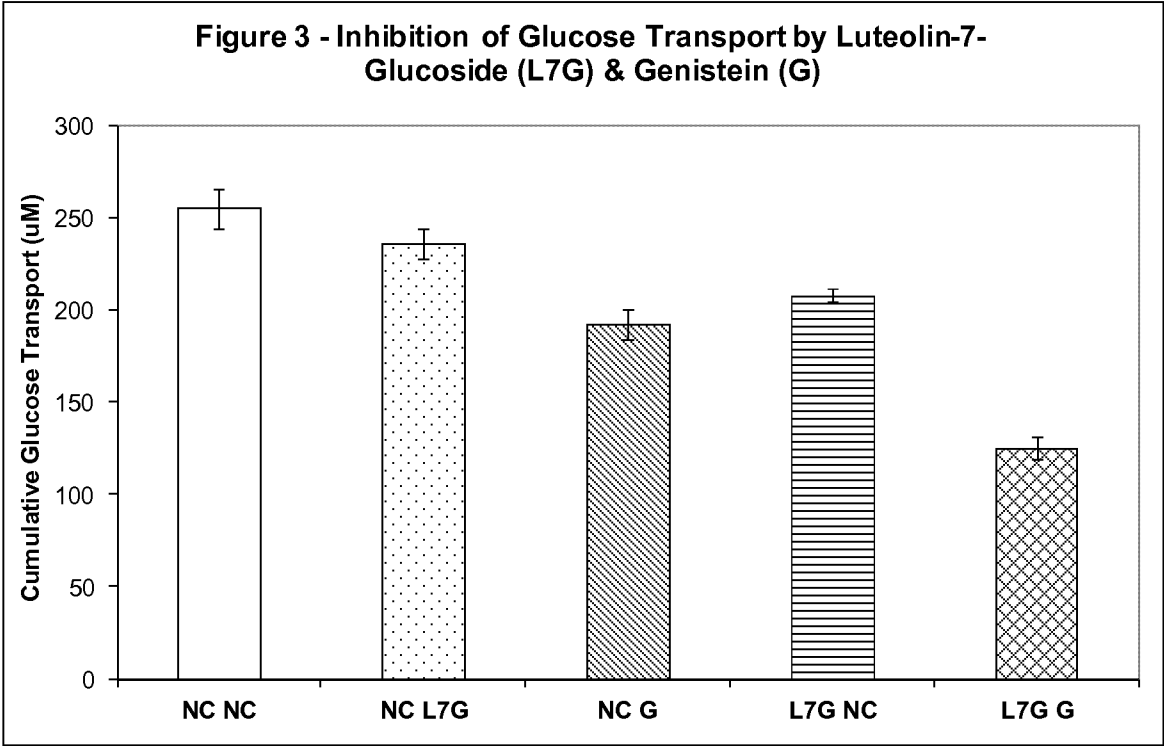


Figure 3

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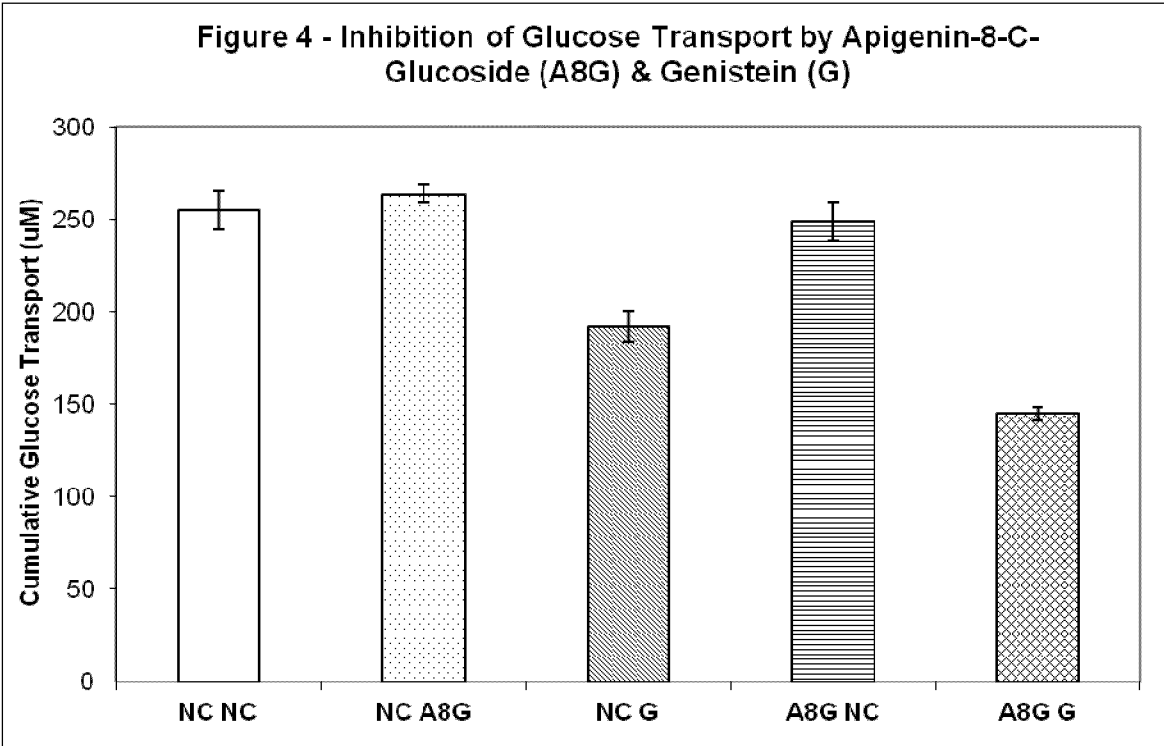


Figure 4

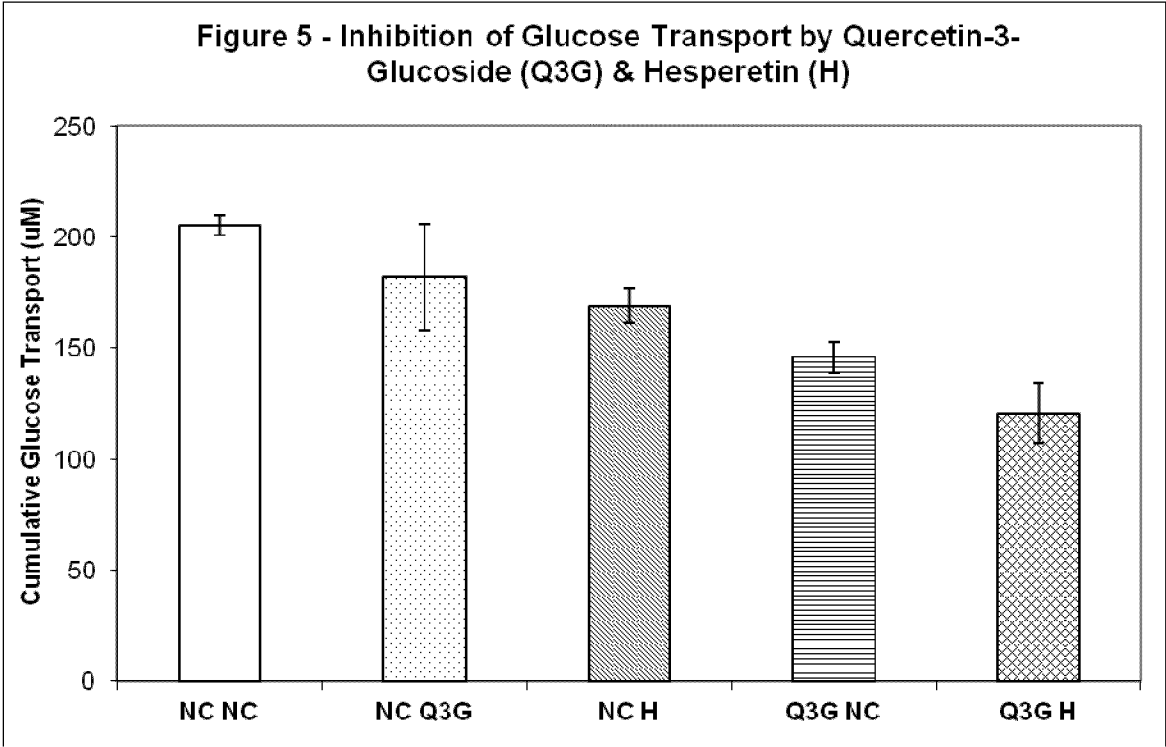


Figure 5

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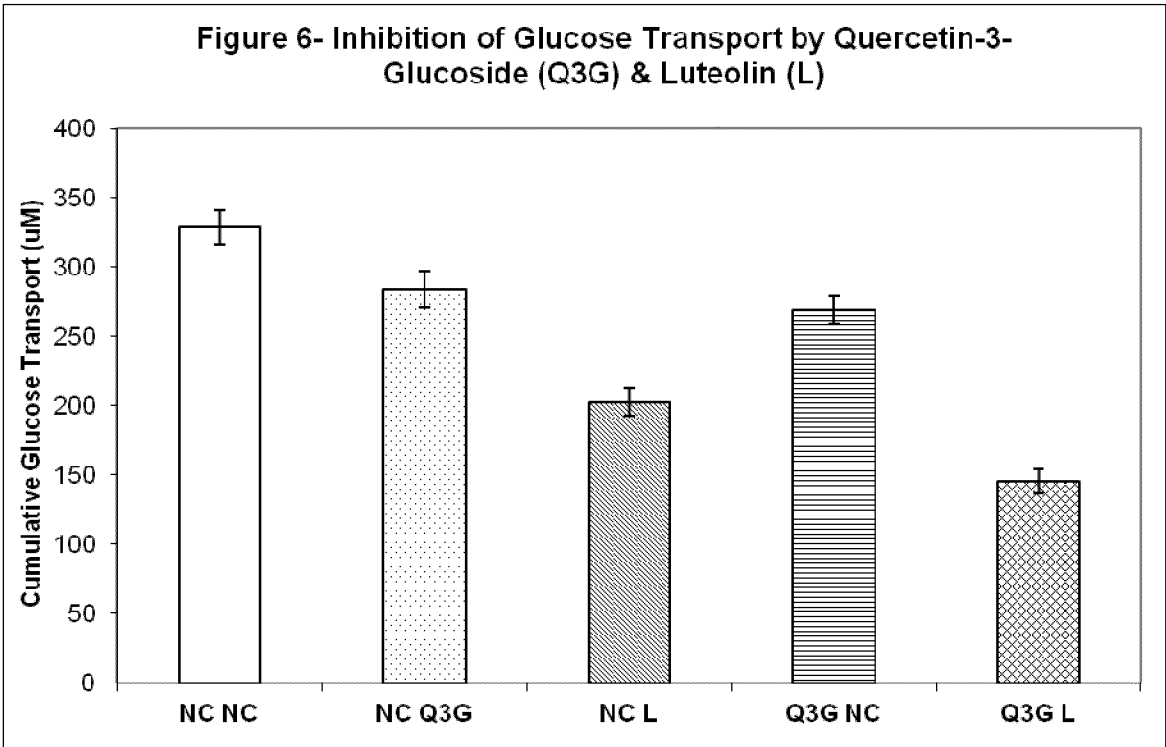


Figure 6

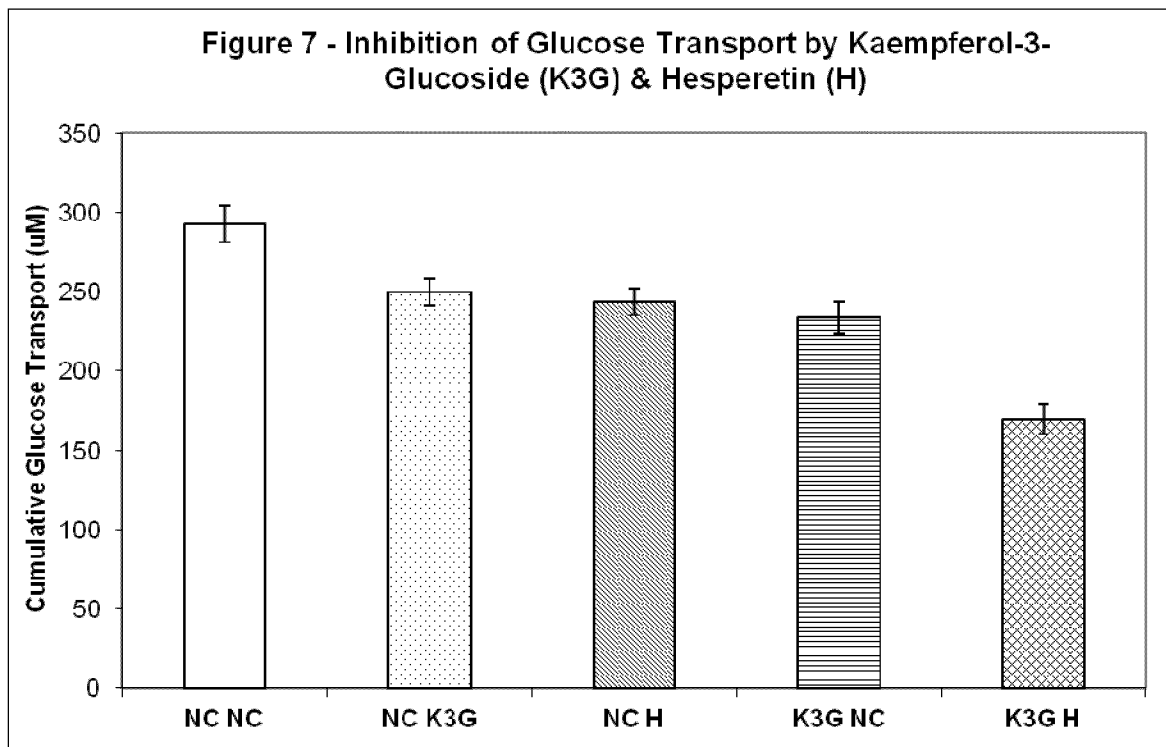


Figure 7

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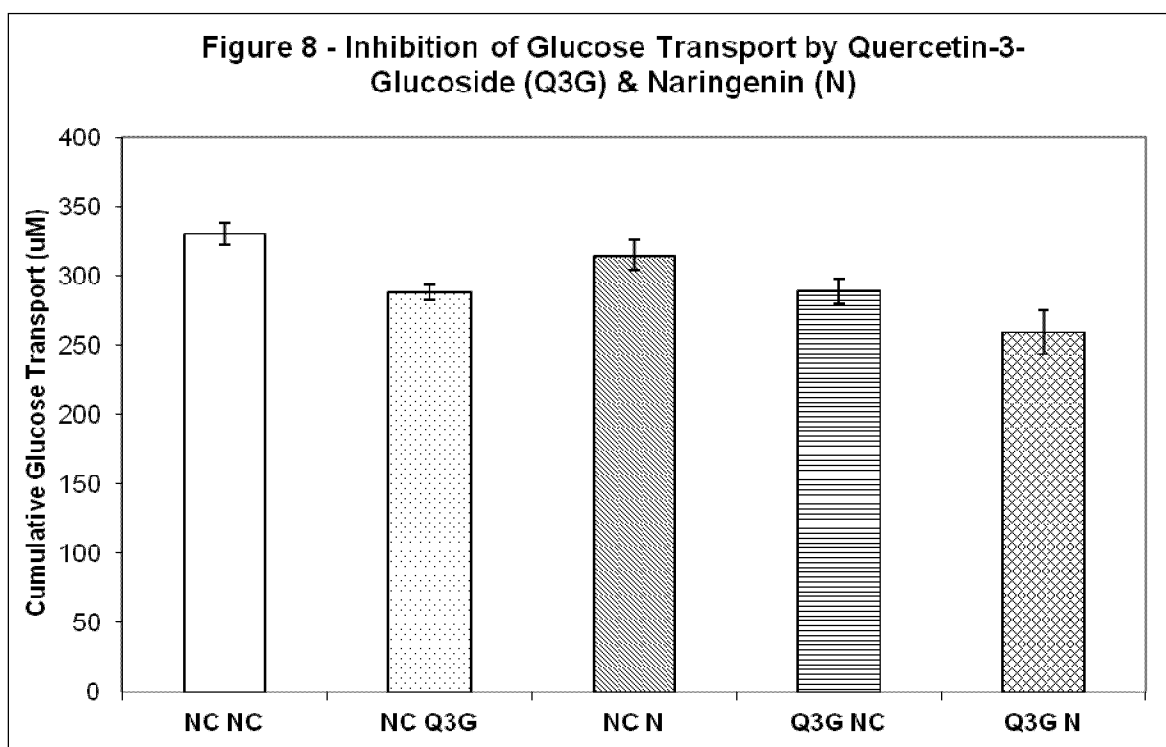


Figure 8

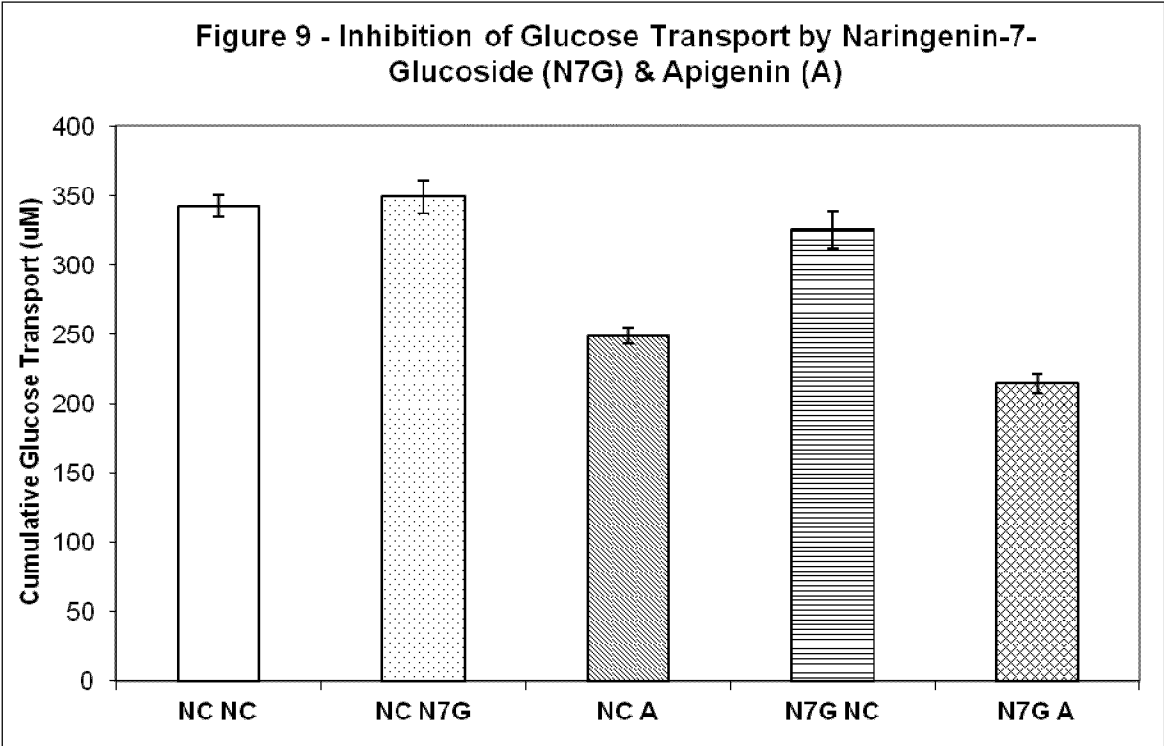


Figure 9

5

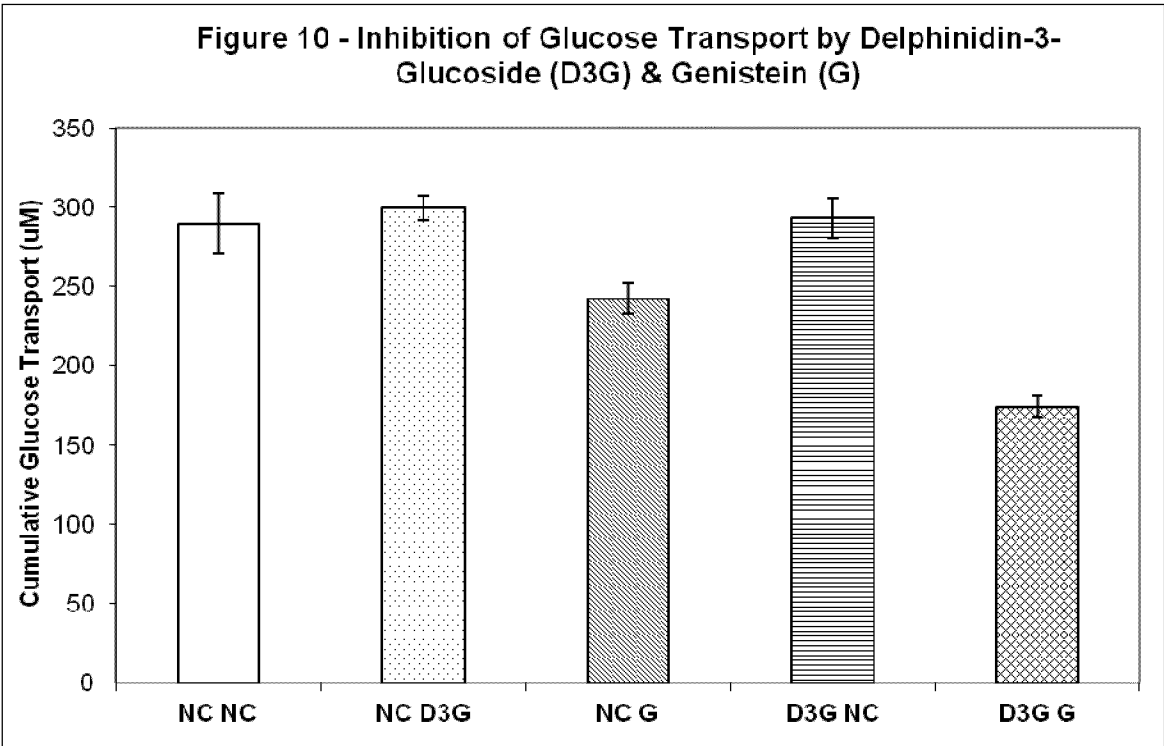


Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/059956

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-15(partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059956

A. CLASSIFICATION OF SUBJECT MATTER

INV. A23L1/30 A61K31/352 A61K31/7048
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, FSTA, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/25795 A1 (MERCK PATENT GMBH [DE]; BUCHHOLZ HERWIG [DE]; MEDUSKI JERZY [US]) 11 May 2000 (2000-05-11) page 3, line 20 - line 36 page 4, line 19 - page 5, line 16; claims 1-12	1-11
X	FR 2 935 096 A1 (COOPERATIVE AGRICOLE D ELLE ET [FR] COOPERATIVE AGRICOLE D ELLE ET VIR) 26 February 2010 (2010-02-26) page 4, line 17 - line 27; claims 1-11; examples 11-13 page 16, line 28 - page 17, line 6; example 7	1-3,5-15
	----- -/-	



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

31 August 2012

Date of mailing of the international search report

14/09/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Fischer, J

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059956

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/135643 A1 (NUTRACEUTIC ET BUSINESS CONSUL [FR]; DALLAS CONSTANTIN [FR]) 13 November 2008 (2008-11-13) page 5, line 7 - line 34 page 15, line 21 - page 19, line 29 page 21, line 3 - page 30; claims 1,6-22; table 1 -----	1-11
X	EP 1 576 893 A1 (FUJI OIL CO LTD [JP]) 21 September 2005 (2005-09-21) paragraph [0015] - paragraph [0018] paragraph [0034] - paragraph [0036]; examples 1-4; table 3 -----	1-3,5-7, 9,10
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059956

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 2005/070441 A1 (LICHTWER PHARMA GMBH [DE]; EICH JUERGEN [DE]; GOERKE THOMAS [DE]; HAFF) 4 August 2005 (2005-08-04) the whole document -----	1-4,9-15
X,P	JP 2011 140457 A (UNIV KOBE) 21 July 2011 (2011-07-21) the whole document & DATABASE WPI 2011 Thomson Scientific, London, GB; AN 2011-J34984 & JP 2011 140457 A (UNIV KOBE) 21 July 2011 (2011-07-21) -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/059956

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Information on patent family members

International application No

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-15(partially)

Present claim 1 relates to a product defined (inter alia) by reference to the following unusual parameters: P1: more resistant to hydrolysis by lactose phlorizin hydrolysate than quercetin- 4'-glucoside or phlorizin. P2: the flavonoid aglycone is a GLUT 2 inhibitor and the flavonoid glucoside is a SGLT 1 inhibitor. The use of this unusual parameters in the present context is considered to lead to a lack of clarity, because the claim does not clearly identify the products encompassed by it as the parameter cannot be clearly and reliably determined by indications in the description or by objective procedures which are usual in the art. This makes it impossible to compare the claim with the prior art. As a result, the application does not comply with the requirement of clarity under Article 84 EPC. The lack of clarity is such that a meaningful search of the whole claimed subject-matter of claims 1-17 could not be carried out (Rule 63 EPC). The extent of the search was consequently limited to the examples clearly defined in, and supported and disclosed by, the description and to the broad concept of a composition comprising compounds having the desired properties or effects. (see claims 3-4). Furthermore claim 10 is not supported by the description as required by Article 84 EPC, as its scope is broader than justified by the description and drawings and has been limited to the broad concept of the claimed composition.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.