Universidad de Navarra

Faculty of Pharmacy

ANTI-OBESITY AND ANTI-DIABETIC PROPERTIES OF TWO NATURAL EXTRACTS RICH IN FLAVONOIDS (HELICHRYSUM AND GRAPEFRUIT): PHYSIOLOGICAL AND MOLECULAR MECHANISMS

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Memoria presentada por Dña. **Ana Laura Isabel de la Garza Hernández** para aspirar al grado de Doctor por la Universidad de Navarra

Ana Laura Isabel de la Garza Hernández

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Ciencias de la Alimentación y Fisiología y autorizamos su presentación ante el tribunal que lo ha de juzgar.

Pamplona, ___ de **junio** de 2014.

Dr. D. Fermín I. Milagro Yoldi Prof. Dr. D. J. Alfredo Martínez Hernández

A mi familia,

a tí por tu confianza puesta en mí,

por tu breve y larga acogida.

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Index

VII. *Appendix*

1. Natural inhibitors of pancreatic lipase as a key players in obesity treatment

2. Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase

Other publications

Congress contributions

Oral communications

VIII. *Thematic index*

Thematic index

Abbreviations

11β-HSD-1	11-β-hydroxysteroid dehydrogenase type 1 inhibitors
5-HT2C	5-hydroxytryptamine receptor 2C
β3-ARs	β3-adrenoceptors
ACACA	acetyl-coenzyme A carboxylase alpha
ActB	beta actin
AdipoQ	adiponectin
ADRβ3	β-adrenergic receptor 3 gene
AMPK	AMP-activated protein kinase
ANOVA	ANalysis Of VAriance
ARC	arcuate nucleus
ASP	acylation-stimulating protein
ATP	adenosine triphosphate
AUC	area under curve
BAT	brown adipose tissue
BDNF	brain-derived neurotrophic factor gene
BMR	basal metabolism
C/EBPα	CCAAT/ enhancer-binding protein-α
CAD	coronary artery disease
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CNS	central nervous system
COX2	ciclooxigenase-2
CRP	C-Reactive protein

CVD	cardiovascular disease
DNA	deoxyribonucleic acid
DPP4	dipeptidyl-peptidase-4
ECG	epicatechin gallate
EE	energy expenditure
EGCG	epigallocatechin gallate
ELISA	enzyme-linked-immunosorbent assay
ERK1/2	extracellular signal-regulated protein kinases 1 and 2
FASN	fatty acid synthase
FDA	food and drug administration (USA)
FFA	free fatty acids
FNTA	farnesyltransferase
FPG	fasting postprandial glucose
FTO	fat-mass-and obesity-associated
G6Pase	glucose 6-phosphatase
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GCK	glucokinase
GH	growth hormone
GLP1	glucagon-like peptide-1
GLUT2	glucose transporter-2
GLUT4	glucose transporter-4
GLUT5	glucose transporter-5
GWAS	genome-wide association study
HAT	histone acetyltransferase
HDAC	histone deacetylase

HED	human equivalent doses
HFS	high-fat sucrose
HOMA-IR	homeostatic model assessment-estimated insulin resistance
IC_{50}	concentration estimated to give 50% inhibition
IFG	impaired fasting glucose
IGF1	insulin like growth factor-1
IGT	impaired glucose tolerance
IL6	interleukin-6
InsR	insulin receptor
IPGTT	intraperitoneal glucose tolerance test
IR	insulin resistance
IRS-1	insulin receptor substrates-1
KRT	krebs-ringer-tris
LEP	leptin
LPH	lactase phloridzin hydrolase
MAPK	mitogen-activated protein kinase
MC4R	melanocortin-4 receptor
MCP1	monocyte chemo-attractant protein-1
MG	methylglucoside
MIF	macrophage migration inhibitory factor
mRNA	messenger ribonucleic acid
MRSD	minimum recommended starting dose
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NEAT	non-exercise-induced activity
NFkB	nuclear factor-kappaB

NOAEL	non-observed adverse effect level
NPY	neuropeptide Y
OGTT	oral glucose tolerance test
OMTT	oral maltose tolerance test
OSTT	oral starch tolerance test
oxLDL	oxidized Low-density lipoprotein
PAI1	platelet activator inhibitor-1
PEPCK	phosphoenolpyruvate carboxykinase
PIK	phosphatidylinositol-kinase
PL	pancreatic lipase
*p*NPG	*p*-nitrophenyl α-D-glucopyranoside
POMC	proopiomelanocortin
PPARα	peroxisome proliferator-activated receptor-α
PPARγ	peroxisome proliferator-activated receptor-γ
RBP4	retinol binding protein-4
ROS	reactive oxygen species
RQ	respiratory quotient
RT-PCR	reverse transcription and quantitative real-time polymerase chain reaction
SGLT1	sodium-dependent glucose transporter-1
SGLT2	sodium-dependent glucose transporter-2
SNS	sympathetic nervous system
T2DM	type 2 diabetes mellitus
TBARS	thiobarbituric acid reactive substrate
TGFβ	transforming growth factor-β

TLR2	toll-like receptor-2
TLR4	toll-like receptor-4
TMEM18	transmembrane protein 18
TNFα	tumor necrosis factor-α
TRb	thyroid hormone receptor subtype β-agonists
UCP1	uncoupling protein-1
UCP2	uncoupling protein-2
UPLC	ultraperformance liquid chromatography
VEGF	vascular endothelial growth factor
WAT	white adipose tissue
WHO	World Health Organization

I Introduction

1. *Obesity and type 2 diabetes // Obesity and type 2 diabetes: definitions and prevalence. Physiopathology of obesity. Physiopathology of type 2 diabetes.*
2. *Adipose tissue // Physiological role of adipose tissue. Inflammation in obesity-related diabetes. Adipokines and the role in obesity and type 2 diabetes.*
3. *Therapeutic approaches in obesity and diabetes // Physiological role of digestion and absorption of nutrients in the study of pharmacotherapy of obesity and diabetes. Fat digestion. Digestion and absorption of carbohydrates. Pharmacotherapy of obesity. New advances and mechanisms of action. Pharmacotherapy of diabetes. Mechanisms of current therapies.*
4. *From pharmacotherapy to phytotherapy: Bioactive compounds // Definition and classification. Functional food. Pharmacological effects of flavonoids on obesity and diabetes. Food intake management. Natural inhibitors of digestion and absorption of nutrients. Thermogenic effects. Antioxidant and anti-inflammatory properties. Nutrigenomic and epigenetic effects.*
5. *Helichrysum and grapefruit extracts // Helichrysum (Helichrysum italicum). Grapefruit (Citrus x paradisi)*

1. Obesity and type 2 diabetes

1.1 Obesity and type 2 diabetes: definitions and prevalence

Obesity comes from the latin word *obesitas* (stout, fat or plump) and is defined as an abnormal or excessive fat accumulation that presents a risk to health (World Health Organization, 2014). However, the full acceptance of obesity as a clinical phenomenon has been low and recent (Kushner, 2014), despite that Greeks were the first to recognize obesity as a morbidity. Hippocrates wrote about the relationship between illness and diet, and the Egyptians were the first to recognize that, quantity as well as quality of food were important (Haslam, 2007).

Nevertheless, some cultures throughout history have viewed obesity as a symbol of health and prosperity (Haslam, 2007). The history of mankind in society is in parallel with the study of nutrition. Thus, changes to different conditions of life have obliged to acquire dietary habits necessary for survival and health. Furthermore, after the industrial era, the emergence of attractive and rich food for the palate led to an increase in feed, disassociated with health benefits and involved in the development of obesity (Van Horn, 2013).

Obesity is a multifactorial chronic disease characterized by the hypertrophy and hyperplasia of the adipose tissue, as a result of a positive balance between energy intake and energy expenditure (Chatzigeorgiou et al., 2014). The epidemic of obesity is prevalent worldwide and obesity is considered as the pandemic of XXI century (WHO, 2014). The World Health Organization statistics from 2008 evidenced that more than 1.4 billion adults (20 and older) were overweight, of which 500 million were obese.

Recently, in a systematic analysis for the global burden of disease (Ng et al., 2014) reported that the number of overweight and obese individuals increased to 2.1 billion in 2013 (figure 1).

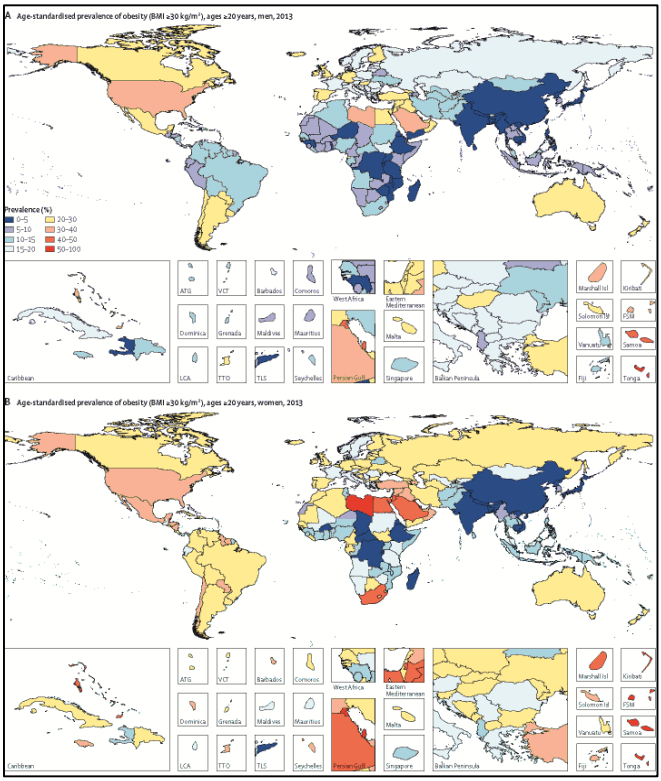

Figure 1. Age-standardised prevalence of obesity (IMC ≥ 30). A) Ages 20+, men, 2013. B) Ages 20+, women 2013. (Ng et al., 2014)

Type 2 diabetes mellitus (T2DM) is a metabolic disorder resulting from impairments in insulin secretion and insulin action in target tissues (Yang et al., 2011). Diabetes is a disease probably known since 3550 years ago, while the Indian physicians Sushruta and Charaka (400-500 AC)

identified and associated, for the first time, T2DM with overweight (Haslam, 2007). T2DM is the main comorbidity linked to obesity and its prevalence has also increased significantly in recent years (Chatzigeorgiou et al., 2014). More than 360 million people have T2DM in the world, and in 2030 it is projected that 552 million people will suffer this disease (table 1) (Varemo et al., 2013).

Table 1. Territory / number of people with diabetes (ages 20-79), 2011 and 2030 (Shaw et al., 2010).

	Country	2011 (millions)		Country	2030 (millions)
1	China	90.0	1	China	129.7
2	India	61.3	2	India	101.2
3	USA	23.7	3	USA	29.6
4	Russian Federation	12.6	4	Brazil	19.6
5	Brazil	12.4	5	Bangladesh	16.8
6	Japan	10.7	6	Mexico	16.4
7	Mexico	10.3	7	Russian Federation	14.1
8	Bangladesh	8.4	8	Egypt	12.4
9	Egypt	7.3	9	Indonesia	11.8
10	Indonesia	7.3	10	Pakistan	11.4

- *Obesity-related diabetes*

Since obesity is strongly linked to T2DM, the term “diabesity” has been coined to include both conditions (Kalra, 2013). Indeed, obesity has become a significant contributing factor to type 2 diabetes. For example, McNaughton, (2013) found that obesity causes or exacerbates many clinical problems including T2DM, cardiovascular disease (CVD), certain forms of cancer and respiratory complications. The idea that a diabetes epidemic is being driven by the earlier and ongoing obesity epidemic has gained considerable ground in the public health (McNaughton, 2013). In this sense, it is necessary to highlight the consideration of obesity in the management of T2DM (Haslam, 2010).

1.2 Physiopathology of obesity

Obesity is the result of a sustained imbalance between energy intake and energy expenditure (figure 2). While in the past the contribution of energy intake has been thought to be the primary factor in the pathogenesis of obesity, energy expenditure is now considered to be equally or even more important (Bensimhon et al., 2006). The energy intake is primarily regulated by the appetite, which in turn is controlled by neuroregulatory factors (hormones and neurotransmitters) (Kalra et al., 1999). Furthermore, the total energy expenditure is constituted by three main components: basal metabolism, thermogenesis and physical activity (Astrup et al., 1997).

Additionally, susceptibility to obesity is complex and involves genetic factors (Razquin et al., 2011). For example, appetite may be regulated by several genetically programmed metabolic pathways, as has been demonstrated with some cases of leptin deficiency (Woods et al., 1998). Thus, gene variants may contribute to produce the energy imbalance that finally leads to obesity (Schuster, 2010).

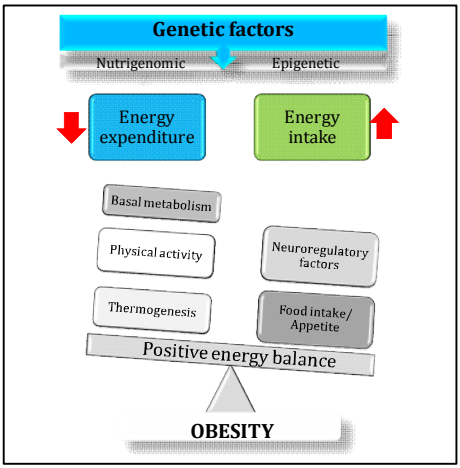

Figure 2. Physiopathology of obesity

In this context, there is evidence that there are multiple plausible causes of obesity, such as sleep hours, infections, maternal age, reproductive fitness and ambient temperature, considered as environmental factors that may contribute to the development of obesity (McAllister et al., 2009).

- *Appetite and neuroregulatory factors*

The brain plays an important role in maintaining energy homeostasis (figure 3). In this sense, signals of appetite and satiety are produced in the hypothalamus (Rios, 2014). The regulation of food intake in the hypothalamus is mainly achieved by altering the expression of neurotransmitters or neuromodulators (Kalra et al., 1999, Hillebrand et al., 2002). The main neurotransmitters that stimulate appetite are norepinephrine, opioid peptides (endorphin) and pancreatic peptides (neuropeptide Y and peptide YY) (Panickar, 2013).

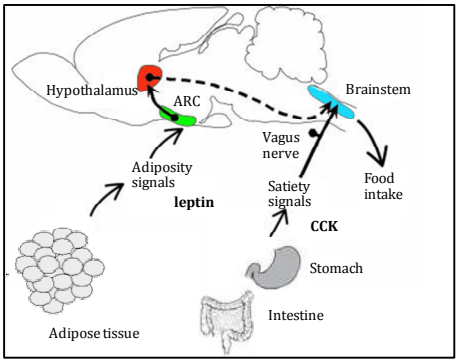

Figure 3. Mechanisms underlying food intake. Modified from (google images). ARC, arcuate nucleus; CCK, cholecystokinin.

The arcuate nucleus (ARC) of the hypothalamus responds to the circulating levels of leptin, ghrelin and insulin. Ghrelin is the only known circulating orexigen hormone. It is mainly released from the stomach, duodenum and ileum, and its expression is increased during fasting but decreased after food intake (Panickar, 2013). In the hypothalamus, ghrelin stimulates the release of neuropeptide Y (NPY) from the NPY neurons

(Solomon et al., 2007). On the other hand, leptin and insulin act by inhibiting orexigenic peptides, including NPY and agouti related protein (AgRP), in the NPY/AgRP neurons (Rios, 2014).

In this sense, the molecules that suppress the appetite are numerous, including also dopamine, serotonin, cholecystokinin (CCK), calcitonin and glucagon. Cholecystokinin and glucagon are examples of gastrointestinal hormones that are released upon food intake and act on the brain to reduce food intake or induce satiety (Panickar, 2013). The cells of the centrolateral nuclei of the ARC are anorexigenic and express proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Rios, 2014).

- *Basal metabolism*

The basal metabolism (BMR) is the minimum energy expenditure required to maintain vital functions. The level of basal metabolism is around 65 and 75% of total energy expenditure (Suarez, 2012). However, an increase or decrease in BMR is not necessarily correlated with the presence of obesity and is most closely associated to the presence of lean mass that consumes more energy (Suarez, 2012). BMR decreases with age but physical exercise can increase BMR (Suarez, 2012).

- *Thermogenesis*

In addition of basal metabolism, there are certain factors that can also induce energy expenditure. This is the case of thermogenesis, which is defined as the heat production occurs primarily in BAT and skeletal muscle in response to the diet. However, thermogenesis not only depends on the supply of nutrients, but also the specific regulation of its use through nervous, endocrine and enzyme processes and genetic factors (Dulloo and

Montani, 2010). In this sense, activation of β3-adrenoceptors (β3-ARs) has been reported to induce anti-obesity effects mediated by a stimulation of brown adipose tissue thermogenesis leading to increased energy expenditure (Ricquier, 2002, Arch, 2008). On the other hand, the implication of the AMP-activated protein kinase (AMPK) signaling pathway in the stimulation of fatty acid oxidation plays an important role because the activation of AMPK facilitates the entry of fatty acyl-CoA into the mitochondria leading to increased fat oxidation (Dulloo, 2011).

Likewise, adipose tissue plays a central role in the regulation of energy storage and expenditure (Sammons and Price, 2014). Two types of adipose tissue are known, white adipose tissue (WAT) and brown adipose tissue (BAT), which have separate developmental origins. However, beige adipocytes have been recently identified , which are more similar to brown adipose tissue as they have increased mitochondrial biogenesis and the expression of the uncoupling protein 1 (UCP1) (Keipert and Jastroch, 2014). UCP1 is activated by fatty acids and inhibited by nucleotides. Fatty acids can undergo β-oxidation, serving as the fuel for thermogenesis, and finally activate UCP1 (Keipert and Jastroch, 2014). In summary, beige adipocytes respond to stimulation by cAMP or β-adrenergic receptor agonists by increasing UCP1 expression and respiration rate (Sammons and Price, 2014).

- *Physical activity*

Physical activity is any movement of muscle mass that results in energy expenditure (Hills and Byrne, 2004). Thereby, according to Levine, (2004), activity thermogenesis can be divided into exercise and non exercise-induced activity (NEAT). NEAT concerns activities which are

physical but fall outside of exercise, such as pacing, fiddling, typing, talking, standing, and other occupational activities performed at work or school. As it ranges from 15-50 % of total calories, expended NEAT might explain the majority of the difference in daily energy expenditure between individuals (Levine et al., 2005).

Moreover, exercise is defined as vigorous activity, scheduled and repetitive in order to maintain or improve physical health (Hills and Byrne, 2004). Exercise can improve metabolic fitness and optimize the body composition maximizing the proportion of fat mass loss. However, the capacity to increase total energy expenditure is variable, depending on various factors (the level of physical activity and the exercise dose) as well as the individual circumstances (Hills and Byrne, 2004). Considerable data show that moderate, daily exercise can greatly improve insulin sensitivity, and even a single exercise session can increase insulin-stimulated glucose uptake in previously sedentary adults (Ross, 2003).

- *Genetic factors: nutrigenomics and epigenetics*

Although environmental influences are an interfering factor in the development of obesity, the occurrence of a genetic background may also be part of the causes of obesity (Marti et al., 2010). Genetics is the science that studies the process of trait inheritance from parents to offspring, including the function of genes (Hunter, 2005).

Between 40 and 70% of the variability in body weight has been attributed to genetic inheritance (Walley et al., 2006). Thus, more than 600 chromosomal regions have been described affecting the regulation of body weight (Rankinen et al., 2006). In this sense, genetics plays a role in modulating individual susceptibility to obesity. The study of monogenic

obesity has been providing evidence of the role of appetite / satiety regulation in obesity susceptibility (El-Sayed Moustafa and Froguel, 2013). On the other hand, in genome-wide association studies (GWAS), a cluster of genes has been associated with obesity including fat mass and obesity associated gene (FTO), melanocortin 4 receptor (MC4R), brain-derived neurotrophic factor gene (BDNF), transmembrane protein 18 (TMEM18), β-adrenergic receptor 3 gene (ADRB3), or peroxisome proliferator-activated receptor γ gene (PPARγ) (El-Sayed Moustafa and Froguel, 2013).

In latest years, it has been developed the study of how gene sequence may affect the response to the diet altering the susceptibility to develop obesity (nutrigenetics) (Razquin et al., 2011). Moreover, it has been shown that nutrient intake can also influence the gene expression (nutrigenomics) (Bouchard and Ordovas, 2012). In this sense, nutrigenomics is the science that provides a possible justification at the molecular level of how nutrients and other food components interact with the genes (Marti et al., 2005). Among the different mechanisms that may be involved in the interindividual differences occurring in obesity, epigenetics has recently emerged (Milagro et al., 2013). Epigenetics is the study of heritable changes in gene expression which occur in the absence of a change in the deoxyribonucleic acid (DNA) sequence (*epi* = "above" or "adittion" gene) (Mansego et al., 2013). Epigenetic mechanisms include DNA methylation, histone modifications and several types of regulatory RNAs, such as microRNAs (Milagro et al., 2011). Most studies have focused on the study of the methylation of CpG islands (genomic regions with a high frecuency of CG dinucleotides present in a large majority of promoters) in the genomic DNA (Campion et al., 2009, Lomba et al., 2010, Milagro et al., 2012). Campion et al., (2009) have proposed the existence of epiobesigenes,

such as PPARGC1A, PPARγ, SOCS1/SOCS3, TNFα and CAV1, with tight epigenetic regulation.

1.3 Physiopathology of type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by chronic hyperglycemia as a result of impairments in insulin secretion from pancreatic β-cells and insulin action in target tissues (Yang et al., 2011). Insulin is a hormone secreted by the pancreatic β-cells, whose main function is the regulation of carbohydrate and fat metabolism in the body. Thus, insulin resistance (IR) is defined as the inability of cells to respond adequately to normal levels of insulin, and occurs primarily in muscle, liver and fat tissue (figure 4).

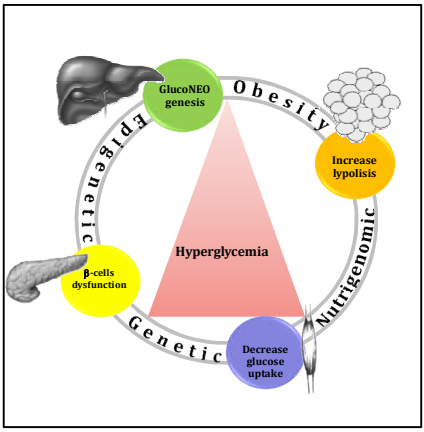

Figure 4. Physiopathology of T2DM

- *Glucose homeostasis and insulin function*

The origin of the word "hyperglycemia" is Greek (*hyper-*, excessive; *-glyc-*, sweet; and *–emia*, of the blood). A subject with a consistent glycemia range between 100 mg/dL (5.6 mmol/L) and 125 mg/dL (6.9 mmol/L), in fasting conditions, is considered hyperglycemic, while with basal levels equal or higher than 126 mg/dL (7 mmol/L) are generally considered

diabetic (American Diabetes Association, 2014). The maintenance of normal glucose levels is mainly balanced by the liver (endogenous glucose production), intestine (glucose absorption) and other peripheral tissues (glucose uptake). Thus, an increase in plasma glucose levels after its absorption in the intestine, stimulates the pancreas to secrete insulin (American Diabetes Association, 2014).

Chronic hyperglycemia induces pancreatic insulin secretion, which in turn leads to an increase in blood insulin levels (hyperinsulinemia), thereby binding to insulin receptor in each tissue and stimulating glucose uptake by peripheral tissues (muscle and fat tissue) (Garber, 2000). Insulin secretion can be divided into two phases. First-phase insulin secretion is triggered by the rise in [Ca(2+)] that occurs synchronously in all β-cells of every islet in response to a sudden increase in the glucose concentration. During the second phase, synchronous [Ca(2+)] oscillations in all β-cells of an individual islet induce pulsatile insulin secretion (Henquin, 2009). In T2DM, the early-phase of the insulin secretion is delayed and blunted (Polonsky et al., 1988). The second phase of insulin secretion occurs in the period of hyperglycemia and is longer that the early-phase produced after β-cell stimulation (Polonsky et al., 1996). Thus, in patients with higher concentrations of fasting blood glucose levels, total insulin release decreased progressively to subnormal levels (Dowse et al., 1996).

Insulin suppresses hepatic glucose production. Thus, an important cause of the hyperglycemia characteristic of T2DM appears to be a failure in this process (Garber, 2000). In the liver, glucose is metabolised by glucokinase (GCK) and, depending on the requirements of the body, it can be stored as glycogen, or oxidized to generate ATP (glycolysis) (de la Iglesia et

al., 2000). Abnormal glucose sensing in T2DM results in fat accumulation and excessive glucose production in liver. In this regard, it ends up becoming a vicious circle, because the accumulation of triglycerides in the liver lead to increased hepatic glucose output resulting in hyperglycemia (Oosterveer and Schoonjans, 2014).

Glucose transporter-4 (GLUT4) is an insulin sensitive glucose transporter that plays an important role in glucose uptake in peripheral tissues (Huang and Czech, 2007). In addition, the binding of insulin to its receptor also triggers multiple signaling pathways, such as tyrosine phosphorylation of insulin receptor substrates (IRS) (White, 2003) and mitogen-activated protein kinase (MAPK) pathway, that may be involved in the regulation of glucose uptake (Bazuine et al., 2005).

- *Insulin resistance*

Long term hyperinsulinemia induces peripheral insulin resistance by impairing insulin-signaling pathways (Mlinar et al., 2007). Insulin resistance (IR) is a physiological condition in which insulin concentration reflects an impaired biological response (Sesti, 2006). Insulin resistance and insulin deficiency lead to a dysregulation in glucose metabolism to finally maintain optimal blood glucose levels, and are manifested by an impaired suppression of hepatic glucose production (Sesti, 2006). Several procedures have been developed to quantify the sensitivity to insulin action. One is the HOMA, which was first developed in 1985 by Matthews et al., (1985). HOMA is a model that describes the balance between glucose and insulin action with a mathematically derived nonlinear equations (Eslam et al., 2011).

HOMA-IR measures the resistance or sensitivity to insulin in a basal state of fasting (Eslam et al., 2011):

HOMA-IR = [glucose (mmol/L) * insulin (μU/L)]/22.5

Likewise, β-cell function (insulin secretion) is also calculated using the HOMA index (Eslam et al., 2011):

HOMA1 - %B = [20 * insulin (μU/L)] / [glucose (mmol/L) – 3.5]

HOMA β-cell is another calculated index to evaluate insulin activity (Eslam et al., 2011):

HOMAβ-cell = [20 * insulin (μU/L)] / glucose (mmol/L) – 3

However, a recent study highlighted that the leptin / adiponectin ratio (L / A) is associated with IR. In obesity, both hormones fluctuate in the opposite direction depending on the amount of visceral fat (Oda et al., 2008). Therefore, Oda et al., (2008) demonstrated that L / A may be more powerful than HOMA-IR for evaluating insulin resistance in diabetic patients.

Likewise, insulin modulates free fatty acid levels (FFA) (Bajaj and Defronzo, 2003). Thus, in hyperinsulinemia, increased concentrations of FFA, accompanied by decreased adiponectin levels and increased leptin levels contribute to the state of insulin resistance (Oda et al., 2008). The accumulation of triglycerides in liver is also associated with insulin resistance leading to fasting hyperglycemia (Oosterveer and Schoonjans, 2014).

The physiopathology of insulin resistance involves mechanisms regulated by genetic and environmental factors (Mlinar et al., 2007). There are few cases of T2DM that result from genetically determined abnormalities of insulin action (American Diabetes Association, 2014). On the other hand, obesity state is usually accompanied by an impairment in insulin action that is linked to the development of insulin resistance (Schuster, 2010). In this context, another mechanism for obesity-induced insulin resistance is related to the overproduction of proinflammatory cytokines and hormones that directly or indirectly induce insulin resistance (Kahn et al., 2006), such as CRP (Xi et al., 2011), TNFα (Hotamisligil, 1999) and IL6 (Senn et al., 2002). Likewise, insulin resistance promotes inflammation because insulin has anti-inflammatory effects at the cellular and molecular levels (Dandona et al., 2004b). Moreover, a circle between inflammation and oxidative stress occurs in obesity.

Oxidative stress is defined as an imbalance between the production and accumulation of reactive species and the body's ability to handle them by endogenous and exogenous antioxidants (Valko et al., 2007). The reactive species, including free radicals, reactive oxygen and nitrogen species, are produced as a result of normal physiological processes and have important roles in cell signaling, gene transcription and immune response (Valko et al., 2007). Production or accumulation of reactive species could have deleterious effects by participating in redox reaction, inducing DNA damage (Bondia-Pons et al., 2012). This may alter the biological properties of enzymes and receptors, affect cellular function, and cause cell death (Dalle and Claude, 2006). Ogihara et al., (2004) suggested that NFkB activation is involved in this process. On the other hand, oxidative stress can activate multiple serine kinase cascades, which increases the serine phosphorylation

of insulin receptor subtrates (IRS) proteins and impairs insulin signaling and phosphatidylinositol-kinase (PIK), which is followed by reduced protein kinase-B phosphorylation and GLUT4 (Bloch-Damti and Bashan, 2005).

In this sense, oxidative stress may be one of the mechanistic links between obesity and its comorbidities, including T2DM (Crujeiras et al., 2013, Savini et al., 2013), altering normal insulin action by the insulin-signaling pathway (Hulsmans et al., 2011).

- *Complications of T2DM*

Obesity-related insulin resistance is associated with progression of endothelial dysfunction (Belin de Chantemele and Stepp, 2012), which in turn plays a key role in the pathogenesis of diabetic complications (Zhang et al., 2012). Likewise, obesity is often associated with a chronic low-grade inflammatory state in adipose tissue and peripheral tissues that increases insulin resistance (Baker et al., 2011). The combination of insulin resistance and β-cell dysfunction leads to the development of T2DM (Schuster, 2010).

On the other hand, T2DM must be considered as an independent cardiovascular risk factor (Schlienger, 2013). It is usually linked to micro-vascular (retinopathy, nephropathy and neuropathy) and macro-vascular complications (coronary artery disease (CAD), cerebrovascular disease, peripheral vascular disease and myocardial ischemia). Also, some cancers may be considered as an emerging complication of type 2 diabetes, as well as cognitive decline, sleep apnea syndrome, mood disorders and bone metabolism impairments (Schlienger, 2013).

Impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), are two terms that indicate high blood glucose levels but not as high as

those of T2DM (American Diabetes Association, 2014). However, both conditions are at high risk of developing T2DM (table 2), although, not everyone with IGT goes on to develop T2DM.

The high blood glucose, undiagnosed and without showing typical symptoms of type 2 diabetes, can - silently - damage the body and diabetes complications may be developed. This data supports the importance to prevent the progression to diabetes and identify people with risk factors (International Diabetes Federation, 2013).

Table 2. Categories and complications in the development of T2DM (American Diabetes Association, 2014).

Categories	Diagnosis	Complications
Impaired fasting glucose (IFG)	FPG levels of 100 mg/dL to 125 mg/dL	Hyperglycemia; risk factor for diabetes, CVD. Obesity; dyslipidemia; triglyceridemia and hypertension.
Impaired glucose tolerance (IGT)	2-h OGTT values of 140 mg/dL to 199 mg/dL	Hyperglycemia; risk factor for diabetes, CVD. Obesity; dyslipidemia; triglyceridemia and hypertension.
T2DM	FPG levels equal or higher than 126 mg/dL	Hyperglycemia; oxidative stress; inflammation. Obesity; dyslipidemia; triglyceridemia; insulin resistance. Hypertension Inflammation, atherosclerosis; CVD

IFG, impaired fasting glucose; IGT, impaired glucose tolerance; T2DM, type 2 diabetes mellitus; FPG, fasting postprandial glucose; OGTT, oral glucose tolerance test; CVD, cardiovascular disease.

2. Adipose tissue

2.1 Physiological role of white adipose tissue

White adipose tissue (WAT) serves as the major storage site for fuel, mainly in the form of triglycerides, when energy expenditure exceeds energy intake (Schuster, 2010). Similarly, WAT controls energy metabolism through endocrine, paracrine and autocrine signals that allow the adipocyte to regulate the metabolism of other fat cells located in brain, liver, muscle or pancreas (Vazquez-Vela et al., 2008). Adipose tissue is constituted mainly by adipocytes, but other cell types are also present, such as fibroblasts, macrophages, stromal cells, monocytes and preadipocytes (Divoux and Clement, 2011). Adipocytes secrete multiple metabolically important proteins known as "adipokines" (Antuna-Puente et al., 2008), which include hormones, cytokines and other proteins with specific biological functions (Vazquez-Vela et al., 2008). The size of the adipose tissue is a function of both adipocyte number and size (Schuster, 2010). Adipocyte number is controlled by apoptosis/necrosis and through development and differentiation of progenitor cells, preadipocytes (Ali et al., 2013). In this sense, adipogenesis is the adipocyte formation (fat tissue) from precursor cells and peroxisome proliferator-activated receptor-γ (PPARγ) plays a central role in this process (Tamori et al., 2002). On the other hand, tumor necrosis factor-alpha (TNFα) is responsible for adipocyte apoptosis (Schuster, 2010).

Two metabolic pathways are key elements in the regulation of triglyceride storage and mobilization. Lipogenesis is the synthesis of esterified fatty acids, which form triglycerides from different energy sources

(Vazquez-Vela et al., 2008). On the other hand, lipolysis is the catabolic process leading to the breakdown of triglycerides stored in fat cells and release of free fatty acids and glycerol (Holm, 2003). Insulin is an anabolic hormone that stimulates lipogenesis but suppresses lipolysis through the activation of its downstream kinase, Akt, resulting in the inhibition of protein kinase A (PKA) (Choi et al., 2010).

TNFα induces triglyceride mobilization by both the stimulation of hepatic *de novo* fatty acid synthesis and an increase in lipolysis. Other cytokines including IL1, IL6, and α-interferon increase hepatic de novo fatty acid synthesis, whereas cytokines such as IL1 and α, β, and γ-interferon also increase lipolysis. Thus, a variety of cytokines acting at different receptors can affect multiple processes related to lipid homeostasis, and the overproduction of cytokines in diabetic condition could lead to inappropriate metabolic effects, including hyperlipidemia, that in the long run could have adverse consequences, such as accelerated atherosclerosis (Feingold and Grunfeld, 1992).

2.1.1 *Inflammation in obesity-related diabetes*

Recently, genome-wide association studies (GWAS) have focused to identify obesity susceptibility genes (El-Sayed Moustafa and Froguel, 2013). In this sense, studies of obese adipose tissue have shown the overexpression of a number of inflammatory genes associated with obesity, including TNFα (Emilsson et al., 2008).

Obesity is usually accompanied by low-grade chronic inflammation, while T2DM is considered another inflammatory state condition (Gregor and Hotamisligil, 2011). Energy imbalance results in adipocyte hypertrophy,

initiating a state of cellular stress and activation of proinflammatory pathways, including NFkB (Bondia-Pons et al., 2012). This situation leads to upregulate the production of proinflammatory adipokines that also co-activate the recruitment of macrophages (Dandona et al., 2004a) (figure 5). On the other hand, obesity is a major risk factor for T2DM. In this sense several studies confirmed that, during obesity, the presence of inflammatory conditions predicts the development of insulin resistance (Gregor and Hotamisligil, 2011). Furthermore, proinflammatory adipokines compromise insulin signaling blocking insulin/insulin receptor (InsR) action by inducing suppresors of cytokine signaling, which inactivated insulin receptor substrates-1 (IRS1) via proinflammatory pathway (NFkB), as described elsewhere (Ding and Lund, 2011).

There is also evidence that glucose intake induces oxidative stress at cellular level and that chronic macronutrient intake leads to proinflammatory state (Tilg and Moschen, 2008). Likewise, in T2DM, the presence of hyperglycemia further exacerbates the proinflammatory state (Dandona et al., 2004a). On the other hand, insulin resistance promotes inflammation by itself, due to the inability of insulin to enter into the cells to exert its anti-inflammatory effects (Dandona et al., 2004b).

In this context, obesity and T2DM are proinflammatory states in which inflammatory mechanisms may greatly contribute to insulin resistance (Bondia-Pons et al., 2012).

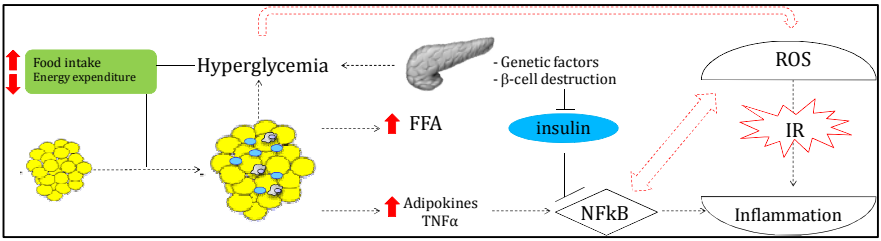

Figure 5. The induction of reactive oxygen species (ROS) and inflammation (NFkB) by increase food intake, hyperglycemia, free fatty acids (FFA), obesity and proinflammatory changes leads to insulin resistance (IR). TNFα, tumor necrosis factor-α; NFkB, nuclear factor-kappaB. Based on (Dandona et al., 2004a).

2.1.2 *Adipokines and the role in obesity and diabetes*

Since latest 1980s, some studies demonstrated that adipose tissue can secrete some factors that can help the maintenance of energy homeostasis (Cook et al., 1987). However, it was not until the middle of nineties that the adipokines were identified and the role of adipose tissue as an endocrine organ was accepted (Scherer et al., 1995). These adipokines interact with peripheral tissues (brain, muscle, liver and pancreas) and participate in different metabolic pathways, such as carbohydrate metabolism, lipid metabolism, inflammation, among others (Ran et al., 2006) (figure 6).

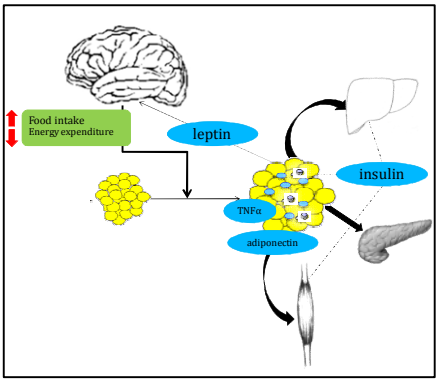

Figure 6. Role of some adipokines from WAT with other peripheral tissues (brain, muscle, liver and pancreas). Tumor necrosis factor-α (TNFα).

In this sense, the dysregulation of adipokine secretion/action that occurs in obese adipose tissue plays an important role in the development of T2DM, inflammatory diseases and other metabolic disorders (Antuna-Puente et al., 2008).

The major adipokines identified (table 3) are adipocyte-derived and have endocrine and non-endocrine functions, while others are produced by the infiltrated macrophages and interact in a paracrine fashion to control adipocyte metabolism (Harwood, 2012, Zulet et al., 2007).

Table 3. Some adipokines and their metabolic effects (Harwood, 2012, Zulet et al., 2007).

Secreted proteins	Metabolic effects	Secreted by
Adiponectin	Increases insulin sensitivity; anti-inflammatory action	Adipocytes
Adipsin	Stimulates triglyceride storage in adipocytes; activates alternate complement pathway	Adipocytes
ASP	Stimulates triglyceride synthesis in white adipose tissue; antilipolytic	Adipocytes
IGF1	Stimulates proliferation and differentiation of adipocytes	Adipocytes
IL6	Proinflammatory; lipolytic; reduces insulin sensitivity	Macrophages
Leptin	Satiety signal; inhibits lipogenesis; stimulates lipolysis; improves insulin sensitivity	Adipocytes
MCP1	Recruits monocytes to sites of injuri and inflammation	Macrophages
MIF	Immunoregulator with paracrine actions in white adipose tissue	Endothelial cells
Omentin	Believed to enhance the actions of insulin	Adipocytes
PAI1	Inhibits plasminogen activation; blocks fibrinolysis	Adipocytes
RBP4	Increases insulin resistance	Adipocytes
Resistin	Increases insulin resistance; promotes endothelial dysfunction	Adipocytes
TGFβ	Regulates preadipocyte proliferation and differentiation and also adipocytes apoptosis	Macrophages
TNFα	Lipolytic; increases energy expenditure; induces insulin resistance	Macrophages
VEGF	Stimulates angiogenesis (vascular proliferation) in WAT	Endothelial cells
Visfatin	Insulin mimetic produced primarily by visceral AT	Adipocytes

ASP, acylation-stimulating protein; IGF1, insulin like growth factor-1; IL6, interleukin-6; MCP1, monocyte chemo-attractant protein-1; MIF, macrophage migration inhibitory factor; PAI1, platelet activator inhibitor-1; RBP4, retinol binding protein-4; TGFβ, transforming growth factor-β; TNFα, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; WAT, white adipose tissue.

Leptin (from Greek "*leptos*" meaning "thin"), a protein whose gene is located at chromosome 7, was first identified in 1994 (Zhang et al., 1994). Leptin is produced and expressed mainly by differentiated adipocytes (Proenca et al., 2014), although it is also produced by stomach, salivary glands, placenta and kidney (Barrenetxe et al., 2002). Leptin production is also stimulated by insulin and inflammatory cytokines, among other hormones (Fonseca-Alaniz et al., 2007). Its principal role is the regulation of energy homeostasis (Mlinar et al., 2007), but also increases hepatic lipid oxidation and lipolysis in muscle and adipocytes (Long and Zierath, 2006). The plasma levels of leptin in normal weight are positively correlated with total fat mass. However, obesity is associated with high circulating levels of leptin, which is accompanied by leptin resistance in the peripheral tissues (Tilg and Moschen, 2008). The development of leptin resistance is characterized by reduced rates of leptin-stimulated AMPK signaling in muscle and hypothalamus under obese conditions (Martin et al., 2006). In this sense, the leptin insufficiency in the brain due to leptin resistance and hyperleptinemia, results in the inhibition of the signal to reduce insulin secretion (pancreas) and the glucose uptake by peripheral tissues, inducing hyperinsulinemia and hyperglycemia that leads to develop T2DM (Kalra, 2009) (figure 7).

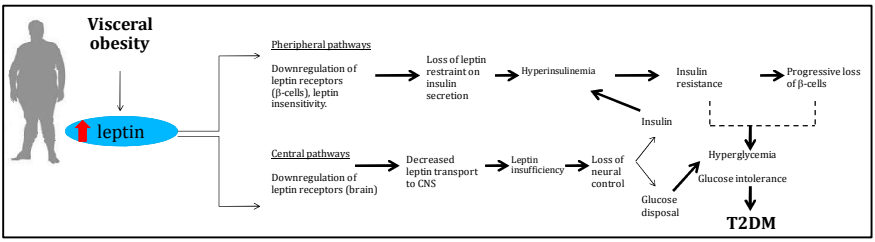

Figure 7. Peripheral and central mechanisms mediating the obesity-induced T2DM. CNS, central nervous system; T2DM, type 2 diabetes mellitus. Modified from (Kalra, 2009).

Adiponectin, a protein whose gene is located at chromosome 3, was first identified in 1995 (Scherer et al., 1995). It is secreted only by adipocytes and has been shown to increase insulin sensitivity with effects on fatty acid oxidation in muscle, glucose uptake and utilization in WAT and muscle, and hepatic glucose production in liver (Ruan and Lodish, 2003). In addition to these positive properties on glucose metabolism, adiponectin modulates the activity of nuclear factor-kappaB (NFkB) and inhibits the actions of tumor necrosis factor-α (TNFα) (Ruan and Lodish, 2003). It also reduces the secretion of cytokines from the macrophages (Fonseca-Alaniz et al., 2007). Indeed, adiponectin has been shown to improve insulin sensitivity and inflammation in animal models of genetic and diet-induced obesity (Proenca et al., 2014).

TNFα is a proinflammatory cytokine synthesized as a transmembrane protein and mainly secreted by the macrophages of the adipose tissue (Kriegler et al., 1988). TNFα induces lipolysis and inhibits lipogenesis, increases leptin secretion and decreases adiponectin secretion on adipose tissue (Proenca et al., 2014). These effects contribute to decrease glucose uptake by peripheral tissues due to impairing the ability of insulin to suppress hepatic glucose production, decreasing glucose transporter-4 (GLUT4) expression in muscle and adipose tissue, and also inhibiting insulin secretion from pancreatic β-cells (Mlinar et al., 2007, Hotamisligil, 2006). In this sense, TNFα is usually elevated in obese adipose tissue and is considered an important mediator of insulin resistance, being the first adipose tissue molecule that connected obesity, inflammation and T2DM (Hotamisligil et al., 1993). Other mechanisms for the induction of insulin resistance include the enhancement of serum FFA and the activation of serine protein kinases (Proenca et al., 2014).

3. Therapeutic approaches in obesity and diabetes

The therapeutic approach to obesity includes dietary, behavioral, pharmacological and in some cases surgical strategies (Halford, 2006). The main treatment for obesity consists in changes in lifestyle habits (including the diet and physical activity) to produce energy deficit. Unfortunately, this clinical approach is not usually long-term lasting and sometimes drugs or surgery appear necessary in obesity treatment (Chatzigeorgiou et al., 2014). The history of drug treatment in obesity is complex because some of them have shown severe toxicity or side effects with health damage (Halford, 2006). Currently, there are few options for obesity pharmacotherapy. The only available approved drug for weight management in Europe is Orlistat (Xenical®) (Yumuk et al., 2014).

Furthermore, individuals who have diabetes should receive individual nutrition treatment. In this sense, low-calorie diets and exercise are also a treatment for overweight and obese T2DM patients (Hayes and Kriska, 2008). In overweight and obese insulin resistant people, modest weight loss has been shown to reduce insulin resistance. Thereby, physical activity is an important component in the weight maintenance and weight loss in these patients (American Diabetes Association, 2014). Furthermore, the use of drugs combined with healthy lifestyle is the best treatment for T2DM and the hyperglycemic control. According with the clinical guidelines (Spain, 2014), the oral hypoglycemic agents marketed are classified as follows: α-glucosidase inhibitors (acarbose and miglitol); thiazolidinediones (pioglitazone); glucagon-like peptide 1 (GLP1) receptor agonists (exenatide, liraglutide and lixisenatide); dipeptidyl-peptidase-4 inhibitors (sitagliptin,

vildagliptin, saxagliptin and linagliptin); sulphonylureas (glibenclamide), meglitinidines (repaglinide and nateglinide); biguanides (metformin) and sodium-dependent glucose transporter-2 (SGLT2) inhibitors (dapagliflozin) (Israili, 2011). Frequently, these drugs can be combined to make the treatment more efficient due to synergistic effects (Israili, 2011).

Probably, the drugs with less secondary effects are those targeting macronutrient digestion and uptake. Thus, Orlistat acts by inhibiting pancreatic lipase activity and finally reduces the triglyceride digestion and absorption by 30% (Yumuk et al., 2014). The most common adverse effects are a range of gastrointestinal sympomts, including steathorrhea, bloating, fecal urgency and fecal incontinence (Filippatos et al., 2008, de la Garza et al., 2011). Likewise, α-glucosidase inhibitors can delay the absorption of carbohydrates and thus, reduce postprandial hyperglycemia (Thule, 2012). Therefore, similar mechanisms of action were found in other anti-obesity and anti-diabetic drugs whose main mechanism of action is the inhibition of the digestion and absorption of nutrients.

3.1 Physiological role of digestion and absorption of nutrients in anti-obesity and anti-diabetic drugs

3.1.1 *Fat digestion*

The fat content in the human diet represents for about 40% of total energy, in a major form as triglycerides (90-95%) and the rest as phospholipids, cholesterol, fatty acids and fat-soluble vitamins (Mu and Hoy, 2004). Additionally, 5-10 g of fat are of endogenous origin (phospholipids, cholesterol and membrane lipids). In normal conditions, fat is absorbed

with 95% efficiency, the remainder being excreted in the feces (Mu and Hoy, 2004).

Fat digestion starts in the mouth, then through the stomach (by an acid stable gastric lipase), where lipids of all sorts tend to form large fatty globules. As fat globules enter the duodenum, they are coated with bile salts of hepatic origin. As soon as fatty acids reach the duodenum and jejunum, CCK is released into the portal circulation and stimulates the pancreas to release triglyceride lipase and co-lipase. Both enzymes hydrolyze triglycerides leading to the formation of monoglycerides and fatty acids (Lowe, 1994). The interaction of fatty acids and monoglycerides with bile salts form mixed micelles also necessary to absorb fat-soluble vitamins (D, E and K) (Carey et al., 1983). On the other hand, after digestion with pancreatic lipase, fatty acids may form vesicles to begin the process of absorption (de la Garza et al., 2011).

Free fatty acids (FFA) are absorbed by passive diffusion, facilitated diffusion and active transport through the enterocyte. FFA and monoglycerides are synthesized into new triglyceride molecules and phospholipids, which are transported to the different organs via lipoproteins, especially chylomicrons (Armand, 2007) (figure 8).

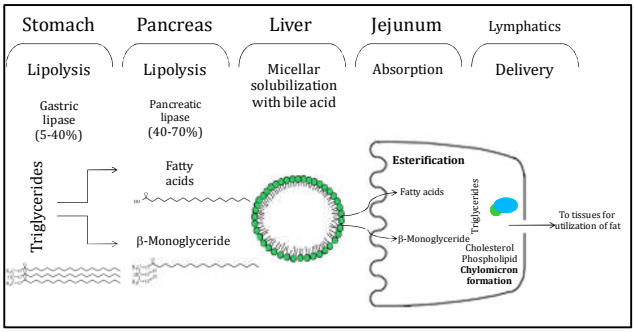

Figure 8. Fat digestion. Modified from (google images).

3.1.2 *Digestion and absorption of carbohydrates*

Carbohydrates constitute much of the normal human diet and usually account more than 50% of the total energy from food (Leturque et al., 2012). Starch is the most abundant carbohydrate in the diet. It consists of two polysaccharides, amylase (α-1,4-linked glucose polymer) and amylopectin (α-1,4-linked glucose chains with α-1,6-linked branch chains) (Sacks et al., 2009). In mammals, dietary carbohydrates are hydrolyzed by different enzymes, principally α-amylase and α-glucosidase. Salivary and pancreatic α-amylases catalyze the endo-hydrolysis of α-1,4-glucosidic linkages in both amylase and amylopectin, and the products that are formed following the action of α-amylases are digested by other enzymes (Etxeberria et al., 2012). Thus, after hydrolysis of starch, the products are malto-oligosaccharides. Further digestion continues with the α-glucosidase enzyme, which is located in the brush-border surface membrane of intestinal cells. This enzyme catalyzes the final step of carbohydrate digestion. After digestion, glucose is the main product from dietary starch and disaccharides (figure 9).

Likewise, galactose and fructose are also end products of enzymatic hydrolysis of carbohydrates. These molecules are absorbed into the small intestine via specific transporters (Zhang and Hamaker, 2009). In the intestine, glucose is absorbed mainly by two transporters, depending on the luminal glucose concentration. At low concentration, glucose is transported against a concentration gradient of active transport mechanism sodium-dependent glucose transporter-1 (SGLT1). Glucose is then transported across the basolateral surface via the glucose carrier GLUT2 (figure 9). At higher concentrations, glucose is transported mainly by the low-affinity

facilitated transporter, glucose transporter-2 (GLUT 2). These GLUT2 carriers in the apical membrane then permit glucose to diffuse into the cells down its concentration gradient (Kellett and Brot-Laroche, 2005). These are the main transporters for glucose, but fructose is absorbed from the intestinal lumen by sodium-independent facilitated diffusion. It is taken up into the enterocytes mainly via a specific carrier called glucose transporter-5 (GLUT5) (Brown, 2000). As the plasma concentration of fructose is usually very low, the gradient for passive fructose absorption is almost always favorable (Levin, 1994).

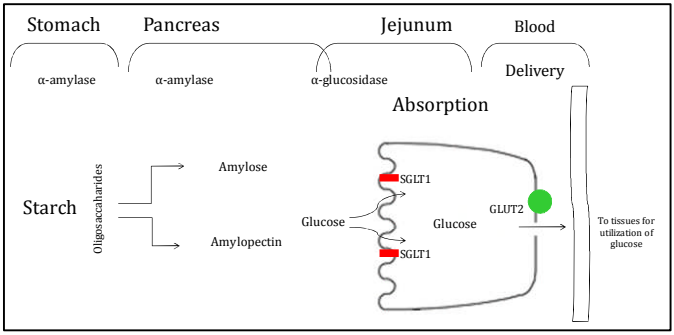

Figure 9. Starch digestion and absorption. Based on (google images). SGLT1, sodium dependent glucose transporter-1; GLUT2, glucose transporter-2.

3.2 Pharmacotherapy of obesity. New advances and mechanisms of action.

According to current Food and Drug Administration (FDA) guidelines, anti-obesity drugs are indicated for those who are obese (BMI of 30 kg/m^2) or overweight (BMI of 25 kg/m^2) with at least one weight-related comorbid condition (Patham et al., 2014).

In this regard, in addition to anti-obesity drugs focused on reduction of fat absorption, there are other categories of drugs acting by suppressing appetite, by increasing energy expenditure or by redistributing adipose tissue (Chatzigeorgiou et al., 2014). Potential drug mediators of this effort are described in table 4.

Sibutramine is a central inhibitor of monoamine uptake. Likewise, rimonabant is an antagonist of CB1 cannabinoid receptors that has been marketed as a treatment in obesity patients (Hofbauer et al., 2007). However, the Spanish Agency for Medicines and Health Products suspended marketing of sibutramine in Spain on January 2010 due to higher risk than the expected benefit. Rimonabant also was suspended in Spain since October 2008 for causing serious psychiatric effects (Chatzigeorgiou et al., 2014).

Table 4. Anti-obesity drugs involved in increasing energy expenditure or adipose tissue redistribution. Based on (Chatzigeorgiou et al., 2014).

Drug category	Drug/compound	Clinical trials	Reference
Thyroid hormone receptor subtype β-agonists	Eprotirome (KB-141)	Terminated at phase III	(Sharma et al., 2014)
Growth hormone analogues	AOD9604	Insufficient efficacy in clinical trials	(Khan et al., 2012)
11β-HSD1 inhibitors	BVT-3498	Terminated at phase III	(Wang, 2006)
ADRβ3	L-796568	Not effective in clinical trial	(Larsen et al., 2002)
Diazoxide	Diazoxide choline	Completed several phase I and II clinical studies	(Alemzadeh et al., 2008)
Sirtuin 1 activators	SRT2104	In phase II	(Baksi et al., 2014)
Angiogenesis inhibitors	TNP-470	In phase II	(Kim et al., 2007)
Inhibitors of methionine aminopetidase 2	Belonarib	In phase II	(Hughes et al., 2013)

In 2012, the FDA approved two new anti-obesity drugs: phentermine (an appetite-suppressant amphetamine) and topiramate (an antiepileptic-controlled release drug). Moreover, submission for marketing approval is expected for other appetite suppressants, including lorcaserin, lisdex-amfetamine, liraglutide and a combination of naltrexone and bupropion (Chatzigeorgiou et al., 2014). However, the European organizations still seem skeptical due to these medications' unwanted effects (Chatzigeorgiou et al., 2014).

- *Topiramate*

Topiramate is an anticonvulsant approved in the USA for the treatment of epilepsy since 1996. This drug is rapidly absorbed after ingestion. The mechanism by which it exerts anti-obesity effect is still unknown. However, some pathways, such as decreased lipogenesis, modification of food taste via inhibition of carbonic anhydrase isoenzymes, and increased energy expenditure are related with the positive effects (Rueda-Clausen et al., 2013).

- *Phentermine*

Similarly to topiramate, this drug is rapidly absorbed after oral administration. Phentermine is an amphetamine analogue that acts as an appetite suppressant. This effect is attributed to its sympathomimetic action, which is related to catecholamine release in the hypothalamus (Kim et al., 2006). However, this action can cause high blood pressure, tachycardia, insomnia and psychological dependence. Thereby it is only recommended for short-term use (Rueda-Clausen et al., 2013).

- *Naltrexone*

Naltrexone is an opioid receptor antagonist that has been used to treat alcohol addiction. Although its administration alone has no effect on body weight, recent studies showed that the co-administration with bupropion (a noradrenaline and dopamine reuptake inhibitor) reduces β-endorphin levels, thereby suppressing the negative-feedback regulating from elevated POMC levels (Apovian et al., 2013).

- *Lorcaserin*

Lorcaserin is a selective agonist of 5-hydroxytryptamine receptor 2C (5-HT2C) that is thought to decrease food intake through the POMC system of neurons. It is rapidly absorbed, reaching its peak circulating concentration 2h after ingestion. Nowadays, lorcaserin has undergone two pivotal randomized, placebo-controlled, double-blind trials to assess efficacy and safety (Kushner, 2014). In mouse models of diabetes, lorcaserin also seems to improve glucose tolerance and hepatic insulin sensitivity (Morton et al., 2006). FDA approval of lorcaserin was based on results of two phase II studies and three phase III randomized controlled trials (Rueda-Clausen et al., 2013). Regarding side effects, initial preclinical studies of lorcaserin suggest that at high doses this molecule has potential oncogenic effects. While phase III studies did not detect any neoplasm, the observational period was too short to make any definitive conclusion (Rueda-Clausen et al., 2013).

- *Glucagon-like peptide-1 (GLP1) receptor agonists*

GLP1 is a 31-amino acid polypeptide originated from the proglucagon gene, involved in regulation of food intake and energy

homeostasis. GLP1 receptor agonists and dipeptidyl peptidase-4 (DPP4) inhibitors are widely used in the treatment of T2DM due to their action on insulin secretion in pancreatic β-cells and also on gluconeogenesis from the liver (Chatzigeorgiou et al., 2014). However, GLP1 can be used as an anti-obesity drug, delaying gastric emptying (satiety) and reducing postprandial hyperglycemia and hyperlipidemia (Chatzigeorgiou et al., 2014).

3.3 Pharmacotherapy of diabetes. Mechanisms of current therapies.

Pharmacologic therapy for patients with type 2 diabetes must necessary take into account that, most of the time, diabetes status may also be accompanied with obesity, dyslipidemia, hypertension, insulin resistance, and/or hyperinsulinemia, which further complicates diabetes status (Kaput et al., 2007). Table 5 summarizes the various types of anti-diabetic drugs with their respective mechanisms of action.

Table 5. Drug classes for the treatment of T2DM based on (Tahrani et al., 2011).

Treatment	Target tissue	Indications	Mechanism of action
Insulin secretagogues	Pancreas	T2DM	Insulin secretion
GLP1 agonists	Pancreas	T2DM	Insulin secretion with high glucose levels
Amylin analogs	Pancreas	Obesity, T2DM	Glucagon secretion
DPP4 inhibitors	Pancreas	T2DM	Insulin secretion with high glucose levels
Biguanides	Liver	Obesity, insulin resistance	Glucose production
Bile acid sequestrants	Liver	T2DM	Incretin secretion
PPARγ agonists	Adipose, muscle	Obesity, insulin resistance	Insulin sensitivity
SGLT2 inhibitors	Kidney	T2DM	Reduces renal glucose reabsorption
Dopamine agonists	Brain	T2DM	Reduction of central adrenergic tone
α-glucosidase inhibitors	Intestine	T2DM	Inhibit the absorbtion of carbohydrates

Metformin, an oral **biguanide**, is recommended as the first line drug and is also used in overweight or obese subjects as its use is usually accompanied by weight loss. However, the use of metformin is contraindicated in patients with high creatinine levels (kidney disease) (Thule, 2012). The **insulin secretagogues** (sulphonylureas and meglitinidines) act by stimulating insulin release from pancreatic β-cells. However, they may cause weight gain and increase the risk of hypoglycemia (Thule, 2012). Thiazolidinediones or glitazones (**PPARγ agonist**) act mainly by increasing glucose uptake in peripheral tissues without stimulating insulin secretion. However, their use is associated with decreased bone density. Rosiglitazone has been associated in a meta-analysis to an increase in the incidence of coronary events and recently hs been suspended in Europe. Pioglitazone is actually comerciable but with particular attention in patients with risk of heart failure (Consoli, 2013). Finally, a newly approved agonist of **glucagon-like-peptide 1** (GLP1), exenatide, acts in the pancreas to stimulate insulin production, but only when circulating glucose levels are high (Kaput et al., 2007). Some of the last anti-diabetic drugs developed are the following:

- *Incretin enhancers (dipeptidyl-peptidase-4 inhibitors)*

Similar to GLP1 agonists, DPP4 inhibitors raise serum incretin levels and may normalize glucose levels after insulin secretion (Thule, 2012).

- *Amylin analog (pramlintide)*

Although amylin is not an incretin hormone, it is included among the incretins because its mechanism of action is similar to GLP1 agonists. Amylin is secreted from pancreatic β-cells and the principal effects are enhanced satiety, diminished glucagon secretion and delayed gastric

emptying. Regarding this, pharmaceutical industry developed pramlintide, a synthetic peptide similar to amylin (Thule, 2012). In this context, in one study with patients with T2DM, pramlintide administration reduced the postprandial glucagon area under the curve in T2DM patients as compared with placebo administration (Schmitz et al., 2004).

Approximately 50% of T2DM patients take medications, 16.4% are treated with insulin, about 11% take drugs with insulin, and the remainders take no medications (20%) (American Diabetes Association, 2014).

In recent years, it has been growing interest on natural products as potential candidates for the use as a coadjuvant treatment for non communicable diseases such as obesity and diabetes. In this sense, countless studies *in vitro* have shown that bioactive compounds present in natural products, such as flavonoids, have biological and pharmacological properties (Pan et al., 2010, Panickar, 2013, Etxeberria et al., 2012, de la Garza et al., 2011). However, claims for a beneficial effect of a food/constituent, need well-designed human studies for the extrapolation of the results obtained in *in vitro* and *in vivo* animal models to consider the natural products as a certain targeted drug product for the treatment of obesity and diabetes.

4. From pharmacotherapy to phytotherapy: Bioactive compounds

4.1 Definition and classification

In recent years, "bioactive compounds" have been defined as non-nutritive constituents in food plants, which are associated with effects on human health and other beneficial effects (Kitts, 1994). Bioactive compounds are common molecules found as a mixture in plants and may influence structure of plant, colour and flavor, or participate in the plants' defense systems (Feeney, 2004). Plant bioactives can be divided into several groups, including polyphenols, terpenoids and phytosterols. The most relevant family of phytochemicals with regard to health benefits are polyphenols, which occurs in all plants, including vegetables, fruits and grains. Flavonoids are the most abundant phenolic compounds. They are present in a wide range of plant-derived foods, mainly in the skin of fruits and in the epidermis of leaves, but also in the root, stem, flowers and plant seeds (Zamora-Ros et al., 2010). The primary structure of flavonoids comprise two benzene rings linked through a heterocyclic pyran or pyrone (with a double bond) ring in the middle. Based on the variation in the type of heterocycle involved, more than 5,000 different flavonoids have been identified and categorized into six subclasses: flavonols, flavones, flavanones, flavan-3-ols or flavanols, anthocyanins and isoflavones (figure 10).

In addition to this diversity, they can be hydroxylated, methoxylated, glycosylated or acetylated (Beecher, 2003).

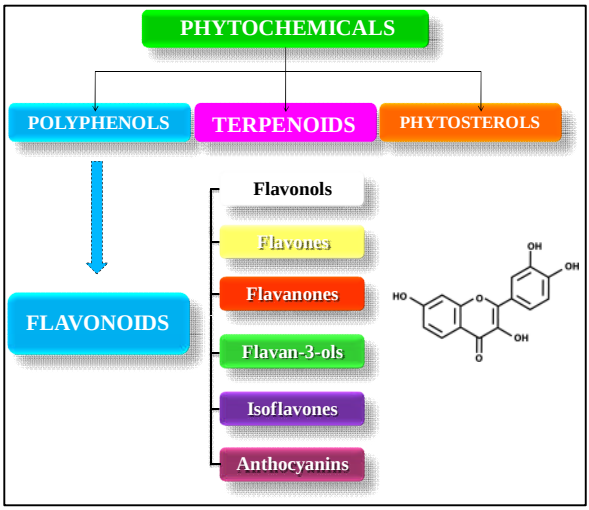

Figure 10. Flavonoids classification

The USDA databases are the most complete, updated, and used databases in the estimation of flavonoid intake (Chun et al., 2007). The mean of the total flavonoid intake was 313.26 mg/day, of which 70.4% correspond to flavan-3-ols, followed by flavanones (16.9%), flavonols (5.9%), anthocyanidins (5.8%) and flavones (1.1%) (table 6) (Zamora-Ros et al., 2010).

Table 6. Estimated flavonoid intake (mg/day) in the European Prospective Investigation into Cancer (EPIC)-Spanish cohort (Zamora-Ros et al., 2010).

Flavonoid compound (mg/day)	Men (n=15,446)	Women (n=25,237)	Total (n=40,683)
Isoflavones	0.11 ± 0.44	0.04 ± 0.16	0.08 ± 0.33
Anthocyanidin	26.34 ± 26.29	11.38 ± 10.93	18.88 ± 21.5
Flavan-3-ols	273.01 ± 228.25	169.99 ± 179.57	221.64 ± 215.87
Flavanones	50.44 ± 48.59	50.66 ± 45.67	50.55 ± 47.15
Flavonols	22.44 ± 12.22	14.93 ± 8.17	18.70 ± 11.06
Flavones	4.34 ± 3.51	2.44 ± 1.69	3.4 ± 2.92
Total flavonoids	376.69 ± 237.41	249.47 ± 164.98	313.26 ± 213.75

4.2 Functional food

Nowadays, there are growing interests in the study of food that can promote and maintain optimal health. Food is any substance (animal or vegetal) that can be consumed to provide energy as essential nutrients (carbohydrates, fats and proteins) and vitamins or minerals as other essential substances (Ahn-Jarvis et al., 2013). Scientific advances show that food can also be used as a vehicle for bioactive substances that alter biochemical or physiological processes, improve health and reduce the risk of disease (Ahn-Jarvis et al., 2013). In this sense, functional food contains bioactive compounds that have health properties. This food can be natural or with/without an extra component. Typically, the design of a new functional food starts from epidemiological findings that correlate diet and diseases. The analysis identifies the bioactive compounds of interest. And finally this substance needs to be tested in *in vitro* and *in vivo studies* to validate the data (Siro et al., 2008).

Regarding these data, epidemiological studies suggest that diets rich in flavonoids are associated with a preventive role against the development of chronic diseases (Garcia-Lafuente et al., 2009, Pan et al., 2010). This situation has led to an increased research interest in specific natural dietary sources that have been used for treating obesity and diabetes (de la Garza et al., 2011, Dulloo, 2011, Etxeberria et al., 2012, Panickar, 2013). In this sense, experimental studies *in vitro* and *in vivo* have been performed to identify possible flavonoids that can be considered in the development of functional food to the control and prevention of obesity and associated metabolic disorders.

For example, flavonoids act reducing food intake and increasing energy expenditure and efficiency (Panickar, 2013). On the other hand, researchers are focusing on discovering new effective digestive inhibitors from plants with fewer side effects (Mata et al., 2013). Additionaly, flavonoids are widely recognized for their biological and pharmacological effects including antioxidant and anti-inflammatory properties (Garcia-Lafuente et al., 2009, Pan et al., 2010, Savini et al., 2013).

4.3 Pharmacological effect of flavonoids on obesity and diabetes

4.3.1 *Food intake management*

Understanding the neural mechanisms on food intake (appetite) is becoming increasingly important in obesity research, because the neural system regulates feeding behavior and metabolic processes. There are some flavonoids which act on neuropeptides and neurohormones that may have an effect on the central nervous system in obesity (figure 11). Thus, apigenin decreased food intake in mice fed a high-fat diet by increasing POMC and CART gene expression (Myoung et al., 2010). Soy isoflavone increased plasma PYY in healthy postmenopausal women and improved insulin sensitivity (Weickert et al., 2006). Resveratrol, when administered to mice, significantly suppressed food intake up to 48 h after one-time injection of the compound (Kim et al., 2010b). Likewise, other plant extracts rich in polyphenols showed an anti-obesity effect and a concomitant reduction in NPY expression in the brain (Hamao et al., 2011).

4.3.2 *Natural inhibitors of digestion and absorption of nutrients*

Recent studies indicate that the inhibition of fat digestion is an interesting approach for the reduction of body weight (figure 11). Different enzymes, particularly pancreatic lipase (PL), are involved in fat digestion. In this sense, the search for new inhibitors of PL from different natural sources is being carried out (Appendix 1). Many flavonoids, including flavones and flavonols, have shown an inhibitory activity of enzymes related to fat metabolism including PL (Birari and Bhutani, 2007). Hesperidin, a flavanone obtained from the peels of *Citrus unshiu*, has PL inhibitory activity (Kawaguchi et al., 1997). Grapevine (*Vitis vinifera*) contains several active compounds including flavonoids, specifically two major flavanols (catechin and epicatechin) that have inhibitory activity on PL (Kim et al., 2014). Other examples of flavanols with inhibitory activity on PL are those from tea, especially (-)-catechin gallate and (-)-gallocatechin gallate (Bose et al., 2008, Ikeda et al., 2005) and luteolin, a flavone present in peanut (*Arachis hypogaea*) (Birari and Bhutani, 2007).

Daidzein, that belongs to the isoflavones group, shows effects on body weight, adipose tissue, blood and liver lipid levels in obese mice fed a high-fat diet through the inhibition of PL activity (Guo et al., 2009). *Ilex paraguariensis* (Yerba mate) contains different flavonols (i.e., quercetin and rutin) that act on lipid metabolism by inhibiting PL activity (Martins et al., 2010).

In order to retard or decrease glucose uptake in the intestine, the inhibition of the activity of the digestive enzymes is an interesting approach (Appendix 2). Some animal studies have shown the effect of flavonoids on postprandial blood glucose levels by inhibiting both α-amylase and α-

glucosidase enzyme activities, and by decreasing intestinal transporters mediated-glucose uptake (figure 11). Tiliroside (kaempferol 3-*O*-β-D-glucopyranoside) inhibited pancreatic α-amylase and SGLT1 and GLUT2 (Goto et al., 2012). Pereira et al., (2011) showed an *in vitro* inhibitory effect of kaempferol on α-glucosidase activity and Matsui et al., (2002) also reported the inhibition of α-glucosidase by kaempferol in *in vitro* and *in vivo* studies. Kim et al., (2000) studied the inhibitory activity on α-amylase and α-glucosidase of twenty one flavonoids. Luteolin and luteolin 7-*O*-glucoside (type of flavones) showed strong inhibition against α-glucosidase. Luteolin also inhibited porcine pancreatic α-amylase activity. Kaempferol 3-*O*-glucoside and luteolin 7-*O*-glucoside also showed strong inhibitory activity against α-amylase (Kim et al., 2000). Tadera et al., (2006) studied the inhibitory activity of six groups of flavonoids against yeast α-glucosidase and porcine pancreatic α-amylase. They found that yeast α-glucosidase was potently inhibited by the anthocyanidin, isoflavone and flavonol groups, whereas luteolin, myricetin and quercetin were potent inhibitors of pancreatic α-amylase. In another study, (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG) inhibited glucose transport, possibly through SGLT1 inhibition (Kobayashi et al., 2000). Also, quercetin 3-*O*-glucoside, fisetin and gossypin reduced glucose uptake by 30% (0.1 mM), followed by quercetin 3-*O*-β-galactoside, naringenin, naringin, hesperetin and catechin, with inhibitory effects of about 20% (Williamson, 2013).

4.3.3 *Thermogenic effects*

The sympathetic nervous system (SNS) plays an important role in the regulation of thermogenesis. Hence, stimulation of metabolism by different bioactive compounds that mimic the activity of the SNS and increase thermogenesis, has been reported as a possible mechanism of action in obesity treatment (Dulloo, 2011). In this context, some bioactive compounds can act on β3-adrenoceptors, which in turn activate thermogenesis and fat oxidation in peripheral tissues, including the activation of UCP1 that mediates thermogenesis in BAT. *Citrus aurantium* contains flavonoids like hesperidin, neohesperidin, naringin and tangeretin that increase thermogenesis (Preuss et al., 2002). Resveratrol and quercetin may possess thermogenic properties (Dulloo, 2011). Hesperidin suppressed diet-induced obesity associated with the up-regulation of gene markers of lipid oxidation in mouse white adipose tissue (Fukuchi et al., 2008). Similarly, kaempferol increased cellular energy expenditure (da-Silva et al., 2007). Green tea, that contains both tea catechins and caffeine, showed significant effects on metabolic targets such as thermogenesis and fat oxidation (Westerterp-Plantenga, 2010).

4.3.4 *Antioxidant and anti-inflammatory properties*

Numerous studies have shown that flavonoids may have anti-obesity and anti-diabetic properties through multiple mechanisms, such as the modulation of inflammation and redox state. In this sense, supplementation with EGCG reduced body weight gain, body fat and MCP1 levels (Bose et al., 2008). Likewise, genistein was reported to reduce high fat diet-induced steatohepatitis through decreasing plasma TNFα levels (Yalniz

et al., 2007). Furthermore, in other study, isoflavones (genistein, daidzein and glycitein) reduced serum TNFα and increased adiponectin levels in women (Charles et al., 2009). In addition, flavonoids showed high antioxidant activity in *in vitro* and *in vivo* studies through the inhibition of ROS-generating enzymes (Savini et al., 2013). Thus, quercetin showed anti-inflammatory and antioxidant properties, decreasing inflammation in obese mice (Anhe et al., 2012). Likewise, in overweight-obese subjects, quercetin supplementation (150 mg/day) during 6 weeks, significantly decreased plasma oxLDL and TNFα (Egert et al., 2010) (figure 11).

Different studies have demonstrated that several inflammatory cytokines affect both insulin secretion and insulin action, promoting pathogenesis of insulin resistance and subsequently reducing insulin-dependent signaling (Bastard et al., 2006). Oxidative and cellular stress may also influence normal insulin action by altering the insulin-signaling pathway, which can lead to the development of insulin resistance (Savini et al., 2013). In this sense, flavonoids have shown antioxidant effects on hyperglycemic status (Ramana et al., 2006). Flavonoids act by interfering with proinflammatory cytokine-induced β-cell dysfunction, up-regulating insulin-dependent signaling and improving glucose uptake in skeletal muscle and different cell types (Pan et al., 2010). Likewise, (Cai and Lin, 2009) showed that EGCG and rutin might be potential agents for the attenuation of T2DM through the protection of pancreatic β cells, improving insulin secretion. In addition, quercetin improved insulin secretion and protected pancreatic β-cells against oxidative damage induced by ERK1/2 pathway (Youl et al., 2010). Several studies have shown that MAPK pathway might also be involved in the regulation of glucose uptake (Ahn et al., 2008).

Flavonoids, can also improve glucose metabolism by stimulating insulin-mediated glucose uptake in peripheral tissues (figure 11). Insulin stimulates glucose uptake in skeletal muscle and adipose tissue. GLUT4 plays an important role in glucose transport as the main insulin sensitive glucose transporter (Huang and Czech, 2007). Other studies have focused on the phosphorylation of downstream insulin receptor substrates (IRS) and activation of several signaling enzymes, such as phosphatidylinositol-3 kinase (PI3K) and Akt-serine/threonine kinase (Liu et al., 2007b).

In this sense, two flavonols isolated from *Euonymus alatus*, quercetin and kaempferol, improved insulin-stimulated glucose uptake in 3T3-L1 cells (Fang et al., 2008). Liu et al., (2007a) showed beneficial effects of myricetin on insulin sensitivity by enhancing insulin signaling pathway (IRS-1 associated PI3-kinase and GLUT4 activity) in muscles of Zucker rats.

Flavonoids are also implicated in hypoglycemic effects on liver (figure 11). Thus, mice whose diet was supplemented with hesperidin and naringin improved hyperglycemia by regulating the activity of hepatic enzymes involved in glycolisis and gluconeogenesis (Jung et al., 2006). Quercetin inhibited both glucose degradation and production (Constantin et al., 2011). Fisetin also prevented hyperglycemia by decreasing glycogen breakdown or blocking the glycogenolytic action of hormones (Constantin et al., 2011).

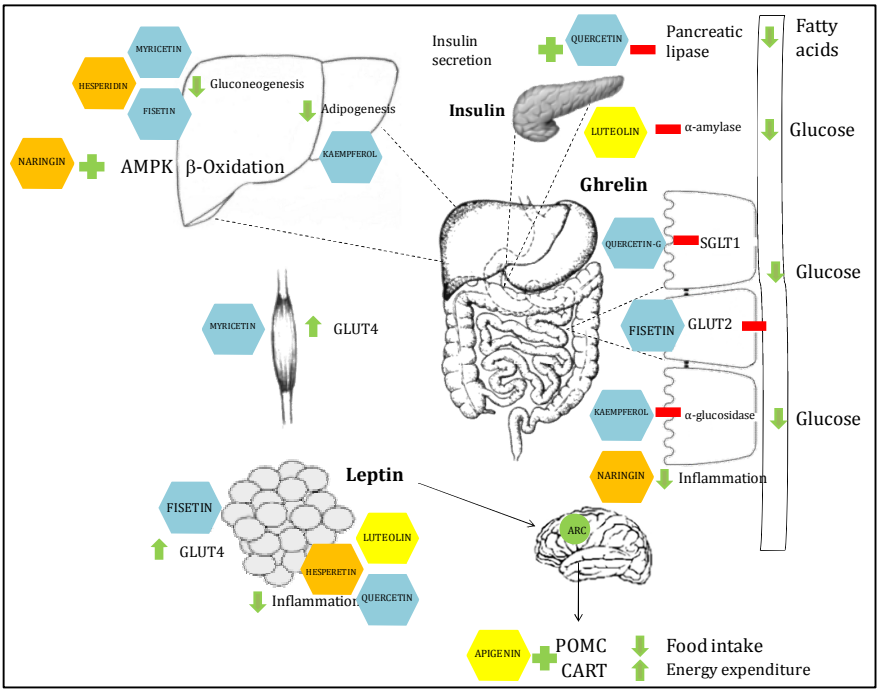

Figure 11. Physiological role of flavonoids in different target tissues related diseases. SGLT1, sodium-dependent glucose transporter-1; GLUT2, glucose transporter-2; GLUT4, glucose transporter-4; POMC, proopiomelanocortin; CART, cocaine- and amphetamine- regulated transcript; AMPK, AMP-activated protein kinase.

4.3.5 *Nutrigenomic and epigenetic effects*

Nutrigenomics is a modern science focused in the study of how diet (nutrients) regulates gene function (Kussmann et al., 2006). The mechanisms underlying the anti-obesity and anti-diabetic effects of flavonoids include that flavonoids can modulate the expression of genes involved in different metabolic pathways, such as glycogenolysis, gluconeogenesis, adipogenesis, inflammatory signaling and cellular function among others.

Table 7 shows some flavonoids and their modulatory role on genes involved in metabolic pathways related to obesity and diabetes.

Table 7. The anti-obesity and anti-diabetic properties of some representative flavonoids underlying mechanisms involved in gene expression.

Flavonoid	Gene	Effects	Model	Reference
Fisetin	PPARγ, FASN, G6Pase GLUT4 (WAT)	Enhanced hepatic lipid and glucose metabolism	Sprague-Dawley rats fed HFD	(Cho et al., 2013)
Myricetin	GLUT4	(+) glucose uptake in muscle	Zucker rats	(Liu et al., 2007a)
Quercetin	FNTA, PPARγ, ACACA, FASN	Body weight, lipid metabolism	Obese C57BL/6 mice	(Jung et al., 2013)
Kaempferol	PPARγ	(-) adipogenesis	3T3-L1 cells	(Park et al., 2012)
Naringin	PEPCK, G6Pase	(-) hyperglycemia	db/db mice	(Jung et al., 2004)
Hesperidin	GLUT4 (WAT)	Lipid metabolism	db/db mice	(Jung et al., 2006)
Bilberry anthocyanins	PEPCK, G6Pase, GLUT4 (WAT)	(-) hyperglycemia (+) insulin sensitivity	T2D mice	(Takikawa et al., 2010)
Luteolin	PPARγ TNFα, MCP1	(-) adipogenesis (-) inflammation	Adipocytes	(Park et al., 2009,Ding et al., 2010)
Apigenin	COX2	(-) inflammation	Immune cells	(Kang et al., 2009)
Genistein	COX2	(-) insulin resistance	human islets	(Corbett et al., 1996)

PPARγ, peroxisome proliferator-activated receptor-γ; FASN, fatty acid synthase; G6Pase, glucose 6-phosphatase; GLUT4, glucose transporter-4; FNTA, farnesyltransferase; ACACA, acetyl-coenzyme A carboxylase alpha; PEPCK, phosphoenolpyruvate carboxykinase; TNFα, tumor necrosis factor-α; MCP1, monocyte chemotactic protein-1; COX2, ciclooxigenase-2 WAT, white adipose tissue.

In this sense, EGCG down-regulated the expression of PPARγ and C/EBPα, that play a central role on preadipocyte proliferation (Chan et al., 2011). Axling et al., (2012) found a down regulation of proinflammatory markers (MCP1, TNFα) and lipogenic genes in mice fed a high fat diet supplemented with green tea catechins.

Fisetin enhanced GLUT4 gene expression in adipose tissue of Sprague Dawley rats fed a high fat diet (Cho et al., 2013). Naringin and hesperidin suppressed PEPCK and G6Pase expression in liver, and increased expression of GLUT4 in WAT of db/db mice (Jung et al., 2006). Cyanidin-3-glucoside increased expression of adiponectin and GLUT4 in human adipocytes (Scazzocchio et al., 2011). Kaempferol inhibited NADPH oxidase and proinflammatory gene expression through modulation of NFkB signaling pathway in mice (Kim et al., 2010a). Resveratrol inhibited adipogenic genes and proinflammatory cytokines in pre-adipocytes (Lasa et al., 2012), and enhanced UCP2 expression in rats fed a high fat diet (Poulsen et al., 2012).

Likewise, epigenetics has recently emerged as a new tool in the study of the mechanisms involved in the development of obesity (Milagro et al., 2012). This new area of science mainly focuses on the role of covalent modifications of DNA and histones, which, without changes in the sequence of nucleotides, have an effect on the gene expression (Junien and Nathanielsz, 2007).

The main epigenetic modifications are the methylation of DNA and the changes in the terminal tails of histone, mainly by methylation and acetylation (Campion et al., 2010). Thus, in recent years, nutrients are one of the factors that have been associated with epigenetic modification in

metabolic diseases. Two major mechanisms by which nutritional factors and diet may affect DNA methylation are (Campion et al., 2010):

1. Changes in the availability of methyl donors.
2. Changes in the activity of the enzymes involved in the process of DNA methylation (methyltransferases).

Nutrients involved in this mechanism are methyl donors and micronutrients that act as cofactors for the enzymes involved in the one-carbon metabolism, including folic acid, vitamin B_6, vitamin B_{12}, zinc, choline, and methionine. However, other nutrients and dietary components may affect the one-carbon metabolism indirectly (Milagro et al., 2013).

Moreover, changes in the histones can create or stabilize transcription factors or proteins involved in the binding sites. Among other modifications, histones undergo acetylation, phosphorylation and methylation. In this context, a number of nutrients and dietary compounds have also been correlated with changes in the methylation of histones (Campion et al., 2009).

Obesity and its related complications have been repeatedly associated with epigenetic alterations. In T2DM, the large number of genes presenting differential methylation status in skeletal muscle from normal glucose tolerance and diabetic subjects suggests that DNA methylation is an important contributor to the development of this pathology and associated complications (Barres et al., 2009). Some of the genes related to metabolic regulation that have been reported to be regulated by DNA methylation are shown in table 8.

Table 8. Some genes involved in the development of obesity and diabetes whose expression is controlled by epigenetic mechanisms. Based on Milagro et al., 2013.

Gene	Metabolic effect	Epigenetic mechanism	References
NPY	Appetite regulation	DNA methylation	(Kim et al., 2011)
POMC	Appetite regulation	DNA methylation and histone acetylation	(Begum et al., 2012,Stevens et al., 2010)
LEP	Appetite regulation	DNA methylation	(Cordero et al., 2011)
INS	Glucose metabolism	DNA methylation	(Kuroda et al., 2009)
InsR	Glucose metabolism	DNA methylation	(Plagemann et al., 2010)
GLUT4	Glucose metabolism	Histone acetylation	(Zheng et al., 2012)
AdipoQ	Adipogenesis	Histone acetylation	(Sakurai et al., 2009)
FASN	Lipid storage	DNA methylation	(Lomba et al., 2010)
TNFα	Inflammation	DNA methylation	(Cordero et al., 2011)
PPARα	Inflammation	DNA methylation	(Lillycrop et al., 2008)
TLR2	Microbiota T2DM	DNA methylation	(Remely et al., 2014)
TLR4	Microbiota Obesity	DNA methylation	(Remely et al., 2014)

NPY, neuropeptide Y; POMC, proopiomelanocortin; LEP, leptin; INS, insulin; InsR, insulin receptor; GLUT4, glucose transporter-4: AdipoQ, adiponectin; FASN, fatty acid synthase; TNFα, tumor necrosis factor-α; PPARα, peroxisome proliferator-activated receptor-α; NFkB, nuclear factor-kappaB; TLR2, toll like receptor-2; TLR4, toll like receptor-4.

Regarding obesity, there have been many efforts to study some cytokines, such as TNFα, in relation with epigenetic mechanisms. One of the epigenetic modifications of the TNFα gene is an increase in DNA methylation. For example, Cordero et al., (2011) suggested that the methylation levels of TNFα promoter could be used as an epigenetic biomarker concerning the response to a low-calorie diet. In other study,

Hermsdorff et al., (2013) associated the methylation levels of TNFα promoter with some metabolic features in young women.

Although there is evidence of the relationship between epigenetic mechanisms and metabolic diseases, recent studies have focused on the effects of diet (bioactive compounds) on the epigenetic patterns. In this sense, some of the flavonoids that act through this way are EGCG, genistein, curcumin or resveratrol (Milagro et al., 2013). However, so far only genistein has been directly linked to epigenetic effects on metabolic disorders. In this sense, Dolinoy et al., (2006) described that maternal supplementation with genistein (250 mg/kg) protected *Avy* mouse offspring from obesity. Other recent studies have demonstrated that genistein has anti-diabetic effects through epigenetic regulation of the cAMP/PKA signaling pathway (Gilbert and Liu, 2013). On the other hand, Kim et al., (2012) found that fisetin inhibited TNFα and IL6 expression in human cultured monocytes by the upregulation of HDAC activity and downregulation of HAT.

Dietary supplementation with apple extracts (700 mg/kg bw) rich in quercetin, catechin, epicatechin, procyanidin and rutin, prevented body weight gain and ameliorated hyperglycemia and hyperleptinemia in obese rats maybe by an increased methylation of aquaporin 7 promoter and decreased methylation in leptin promoter in adipocytes (Boque et al., 2013) (figure 12).

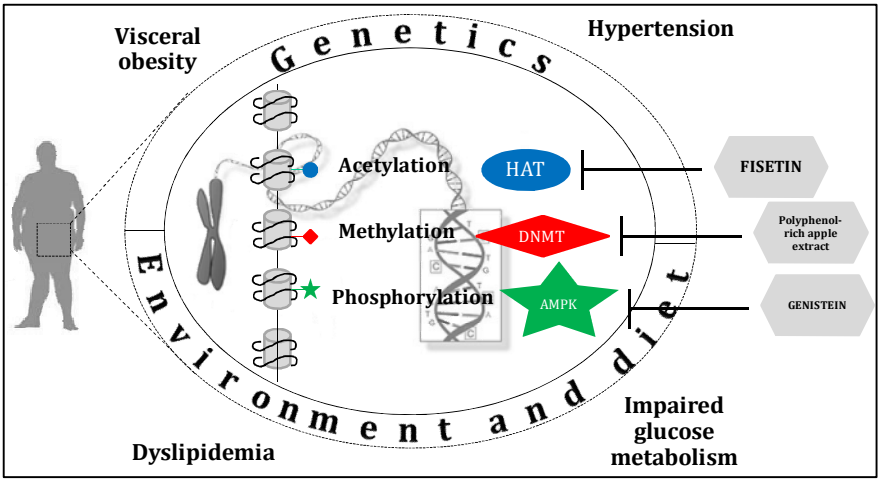

Figure 12. Epigenetic modulatory mechanisms of some flavonoids in obesity and diabetes models. HAT, histone acetyltransferase; DNMT, DNA methyltransferase; AMPK, AMP-activated protein kinase.

5. Helichrysum (*Helichrysum italicum*) and grapefruit (*Citrus x paradisi*) extracts

Helichrysum (*Helichrysum italicum*) is a flowering plant that belongs to the Asteraceae family (figure 13). There are more than 500 species of *Helichrysum* genus distributed around the world (Gouveia and Castilho, 2010). The name is derived from the Greek words *helisso* (to turn around) and *chrysos* (gold) (Aiyegoro and Okoh, 2010) probably due to the inflorescences of a bright yellow color (Perrini et al., 2009). Plants of this genus have been found to possess antimicrobial, anti-allergic, antioxidant and anti-inflammatory properties (Gouveia and Castilho, 2010, Suzgec et al., 2005).

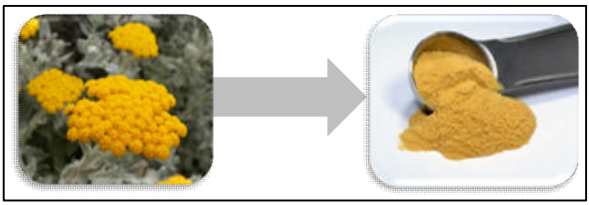

Figure 13. Helichrysum (*Helichrysum italicum*)

Helichrysum italicum grows around the Mediterranean area and is one of the most studied species of this genus because of its traditional therapeutic properties (Antunes Viegas et al., 2014). Previous studies reported conventional uses of *helichrysum italicum* in relation with respiratory, digestive and skin inflammatory conditions (Antunes Viegas et al., 2014). Recently scientific studies also showed anti-inflammatory and antioxidant activities (Sala et al., 2002) that have been attributed to several classes of flavonoids detected in different parts of the plant, such as kaempferol-3-*O*-glucoside and other flavanones (Aslan et al., 2007). However, the wide variety of extracts of *helichrysum italicum* results in different characterization of chemical compounds (Antunes Viegas et al., 2014). On the other hand, the composition can also change depending on the part of the plant used to obtain the extract. For example, different flavonoids, terpenes and steroids were obtained from the flowers of the plant (Antunes Viegas et al., 2014).

Kaempferol glucosides are very common in nature, having been identified in more than 400 plant species (Calderon-Montano et al., 2011). Regarding the biological activities of kaempferol, several *in vitro* and *in vivo* studies have shown that it has antioxidant, anti-inflammatory and anti-diabetic properties (Calderon-Montano et al., 2011) (table 9).

Table 9. Effect of kaempferol on lipid and glucose metabolism

Flavonoid	Metabolic effect	Model / doses	References
Kaempferol	(-) cellular apoptosis (+) insulin secretion	INS-1 E β-cells (0.01 – 10 μM)	(Zhang and Liu, 2011)
Kaempferol	(-) adipogenesis, PPARγ	3T3-L1 cells (40/80 μM)	(Park et al., 2012)
Kaempferol	(+) insulin-stimulated glucose uptake, PPARγ agonist	3T3-L1 cells (5-50 μM)	(Fang et al., 2008)
Kaempferol	Less body weight gained, visceral fat-pad weights and plasma lipid levels (+) hepatic PPARα	Wistar rats (75,150,300 mg/kg)	(Chang et al., 2011)
Kaempferol 3-neohesperidoside	(+) glucose uptake via the PI3K pathway	Rat soleus muscle (100 mg/kg)	(Zanatta et al., 2008)
Kaempferol 3-neohesperidoside	(+) glycogen synthesis	Rat soleus muscle (0.1 – 10 μM)	(Cazarolli et al., 2009)
Kaempferetrin	Hypoglycemic properties	Wistar rats (50, 100, 200 mg/kg)	(de Sousa et al., 2004)
Kaempferetrin	(+) glycogen synthesis	Rat soleus muscle (100 mg/kg)	(Cazarolli et al., 2013)
Kaempferetrin	(-) GLUT4	3T3-L1 (0-20 μM)	(Prasad et al., 2010)
Kaempferetrin	(+) insulin signaling pathway	3T3-L1 (15 μM)	(Lee et al., 2009)
Kaempferetrin	(-) α-glucosidase activity	Intestine Wistar rats (3.1-400 μM)	(Pereira et al., 2011)
Kaempferetrin	(+) glucose uptake	Wistar rats (100 mg/kg), *in vitro* rat soleus muscle (26-208 mM)	(Jorge et al., 2004)
Kaempferol-3-*O*-glucoside	(+) glucose tolerance and improve liver lipid levels	Mice (0.15%)	(Zang et al., 2011)
Kaempferol-3-*O*-α-rhanmnoside	(-) intestinal SGLT1 glucose transporter	Intestine from SD rats (5 mM)	(Rodriguez et al., 2010)

SD, Sprague-Dawley; PPARγ, peroxisome proliferator-activated receptor-γ; GLUT4, glucose transporter-4; PPARα, peroxisome proliferator-activated receptor-α; PI3K, phosphatidylinositol-3-kinase; SGLT1, sodium-dependent glucose transporter-1.

Grapefruit (*Citrus x paradisi*), from the Rutaceae family, is a hybrid fruit of recent origin, probably resulting from a cross between citrus fruits cultivated in the Mediterranean region (Uckoo et al., 2012) (figure 14). Likewise, there are different varieties besides the original grapefruit, thereby modifying the organoleptic properties and color between them (Owira and Ojewole, 2010). Medieval sources report pharmacological properties and uses of *citrus* species (Arias and Ramon-Laca, 2005). These properties may be attributed to several classes of phytochemicals that are present in the fruit depending on the variety, geographical location, time of harvesting and the method of processing the grapefruit (De Castro et al., 2006). The most common bioactive compounds are those corresponding to the family of flavonoids, such as flavanones, flavones, flavonols and anthocyanins (Benavente-Garcia and Castillo, 2008). The flavanones (naringin and hesperidin) are the molecules responsible for the bitter taste (Owira and Ojewole, 2010). Likewise, recently Park et al., (2013) have evidenced antioxidant, anti-diabetic, anti-inflammatory and cardioprotective activities from *citrus* flavonoids. On the other hand, other bioactive chemical compounds such as furanocoumarins have been also identified in grapefruit. These compounds inhibit intestinal cytochrome P450 (CYP3A4) activity through the degradation of the protein (Owira and Ojewole, 2010).

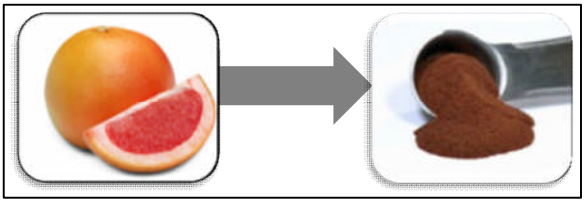

Figure 14. Grapefruit (*citrus x paradisi*)

Concerning the anti-diabetic effects of *citrus* flavonoids, Shen et al., (2012) have demonstrated their inhibitory effects on starch digestion, whereas Adeneye, (2008) reported the antihyperglycemic activity of grapefruit in an animal model. Likewise, *citrus* flavonoids prevented dyslipidemia and hepatic steatosis in obese *db/db* mice supplemented with *citrus* unshiu fruit (2 g/100 g diet) (Park et al., 2013).

To summarize the effects of *citrus* flavonoids (naringin, hesperidin, nobiletin and tangeretin), table 10 shows these compounds and their effects in relation to lipid and glucose metabolism.

Table 10. Effect on lipid and glucose metabolism by *citrus* flavonoids: flavanones (naringin and hesperidin) and flavones (nobiletin and tangeretin)

Flavonoid	Metabolic effect	Model / doses	References
Naringenin	(-) hepatic glucose production	Hepatocytes (25-100 μM)	(Purushotham et al., 2009)
Naringenin	(-) intestinal glucose uptake	Vesicles and everted intestine (500 μM)	(Li et al., 2006a)
Naringenin	Plasma and hepatic triglyceride and cholesterol	STZ rats (0.012%)	(Cho et al., 2011)
Naringenin	(-) α-glucosidase	T2D rats (25 mg/kg)	(Priscilla et al., 2014)
Naringenin	(-) adipogenesis	3T3L1 adipocytes	(Richard et al., 2013)
Naringenin	Anti-inflammatory activity	Rats induced liver damage (50 mg/kg)	(Jayaraman et al., 2012)
Naringenin	Antihyperglycemic and antioxidant	STZ rats (50 mg/kg bw)	(Annadurai et al., 2012)
Naringenin	Glucose uptake in muscle cell (AMPK)	L6 myotubes (75 μM)	(Zygmunt et al., 2010)
Naringenin	Improved insulin sensitivity	Rats fed high fructose diet (50 mg/kg bw)	(Kannappan and Anuradha, 2010)
Naringenin/hesperetin	Adiponectin, PPARγ	3T3L1 adipocytes (20-160 μM)	(Liu et al., 2008)
Naringenin/hesperetin	TNFα	Mouse adipocytes	(Yoshida et al., 2010)
Naringenin/hesperetin	ACAT2	HepG2 cells	(Wilcox et al., 2001)

Naringenin/hesperetin	(-) gluconeogenesis	Isolated rat liver	(Constantin et al., 2014)
Naringin	Hyperglycemia, IR, β-cell function, TNFα	T2D rats (100 mg/kg bw)	(Sharma et al., 2011)
Naringin	LDL-cholesterol	Hypercholesterolemic patients (400 mg/day)	(Jung et al., 2003)
Naringin	Plasma total and LDL-cholesterol	Rabbits fed high-cholesterol diet (0.05%)	(Jeon et al., 2004)
Naringin	Decreased serum TNFα	Mice (0.02%)	(Pu et al., 2012)
Naringin	Glucose intolerance, plasma lipid levels	Rats fed high fat diet (100 mg/kg/day)	(Alam et al., 2013)
Naringin Hesperidin	α1 acid glycoprotein	Obese cats (0.1%) Obese cats (0.05%)	(Leray et al., 2011)
Naringin / hesperidin	Hyperglycemia and oxidative stress	STZ diabetic rats (50 mg/kg bw)	(Mahmoud et al., 2012)
Naringin / hesperidin	Glucose, GCK activity, G6Pase and PEPCK	*db/db* mice (200 mg/kg bw)	(Jung et al., 2006)
Naringin / hesperidin	GLUT4 gene expression in WAT	*db/db* mice (200 mg/kg bw)	(Jung et al., 2004)
Nobiletin	Lipid accumulation	3T3L1 adipocytes (64μM)	(Miyata et al., 2011)
Nobiletin	COX2 and IL6 expression in WAT	Obese mice (100 mg/kg)	(Lee et al., 2013)
Nobiletin / tangeretin	MCP1	3T3L1 adipocytes (128μM)	(Miyata et al., 2011)
Nobiletin:tangeretin	Hepatic PPARα protein expression	Hamsters (125 mg/kg/day)	(Li et al., 2006b)

STZ, streptozotocin; T2D, type 2 diabetes; AMPK, AMP-activated protein kinase; PPARγ, peroxisome proliferator-activated receptor-γ; TNFα, tumor necrosis factor-α; ACAT2, acyl CoA: cholesterol acyltransferase; IR, insulin resistance; GCK, glucokinase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GLUT4, glucose transporter-4; WAT, white adipose tissue; COX2, ciclooxigenase-2; IL6, interleukin-6; FA, fatty acid; MCP1, monocyte chemotactic protein-1; PPARα, peroxisome proliferator-activated receptor-α.

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II *Hypothesis and aims*

1. *Hypothesis // 2. General aim // 3. Specific aims*

This section answers the following questions: why, what, and what for we conducted the present study?

1. Hypothesis

There is a growing interest in the research of natural products to be investigated and prescribed as therapeutical agents in some highly prevalent diseases such as obesity and diabetes. The European Food Safety Authority (EFSA) provides guidelines to define the functionality of foods, focusing on human health, based on the scientific knowledge and the risk assessment and adverse effects. Therefore, according to the EFSA fundaments, a number of studies have screened natural plant extracts, with possible beneficial health effects, that may be used for weight management, as well as for the prevention of obesity and diabetes.

The research of these bioactive compounds in a wide variety of natural extracts is interesting in order to identify potential substances with a beneficial health effect, as well as the possible biological mechanisms by which these bioactive compounds act in the human body to treat metabolic diseases.

2. General aim

The general goal of the present research was:

To research the possible beneficial anti-obesity and anti-diabetic effects of two natural extracts derived from helichrysum (*Helichysum italicum*) and grapefruit (*Citrus x paradisi*) through *in vitro* studies and animal models investigations.

3. Specific aims

Postprandial hyperglycemia is associated with an increased risk for diabetes and metabolic syndrome onset. In this sense, the postprandial glycemic control has been proposed as an important therapeutic strategy for diabetes and for the prevention of other complications associated with obesity.

Thereby, a therapeutic approach for reducing postprandial hyperglycemia can be based on delaying or reducing glucose absorption from the intestine. Therefore, the first specific aim of this investigation was:

1. *To evaluate the possible postprandial antihyperglycemic effect of helichrysum and grapefruit extracts through their inhibitory activities on α-amylase, α-glucosidase and SGLT-1 glucose transporter.* ***(Chapter 1)***

Furthermore, among the different molecular mechanisms that may be involved in the development of obesity and diabetes, new nutrigenomic and epigenetic processes have emerged in recent years as mechanisms that could significantly contribute to explain the basis of nutrient actions on human health. Therefore, the second aim was:

2. *To study the molecular mechanisms (nutrigenomics and epigenetics) by which helichrysum and grapefruit extracts may exert antihyperglycemic and anti-inflammatory effects in a mouse model of obesity and diabetes.* ***(Chapter 2)***

Finally, insulin plays an important role in the regulation of blood glucose levels and insulin resistance, which can be regarded as the physiopathological basis of metabolic syndrome and type 2 diabetes. An excessive accumulation of visceral fat can be a determinant factor to develop insulin resistance, which causes inflammation and increased production of proinflammatory cytokines, such as TNFα. Thus, the third aim was:

3. *To investigate the potential beneficial effects of dietary supplementation with helichrysum and grapefruit extracts on weight loss and the associated consequences in glucose metabolism, inflammation and oxidative stress-related states in overweight and insulin resistant rats.* ***(Chapter 3)***

III *Extracts and experimental design*

1. Extracts // 2. Experimental design // Chapter 1. Effects on digestion / absorption of nutrients // Chapter 2. Effects on a diabetes model: Nutrigenomic and epigenetic mechanisms // Chapter 3. Effects on weight loss: consequences in metabolic signaling.

This section describes the study design and the data analyses. All the measurement techniques used are mentioned. Methods of each technique are also explained in the section "Materials and Methods" for each chapter in the "Results".

1. Extracts

Helichrysum extract (*Helichrysum italicum*) was provided by Biosearch S.A. (Granada, Spain). They obtained the extract from the flowers of the plant and used two solvents (water and methanol) in the process with an accelerated solvent extraction (Dionex ASE200) in standard pressure conditions and temperature. Plant samples (1-5 g) were pulverized, mixed with washed sea sand and introduced into the extraction cells, where 30 ml of each solvent at 50 °C was added. The quantification of phenolic compounds was performed by ultraperformance liquid chromatography (UPLC) MS/MS as described elsewhere (Ortega et al., 2008), and is reported in table 1.

As helichrysum, grapefruit extract (*Citrus x paradisi*) was provided by Biosearch S.A. (Granada, Spain) using immature grapefruits to obtain the final product. All the process of extraction was similar to that used with the helichrysum extract, as well the quantification of phenolic compounds (Ortega et al., 2008). The quantification of phenolic compounds obtained from the grapefruit extract is also reported in table 1.

Table 1. Quantification (mg/kg extract) of Phenolic Compounds in Helichrysum and Grapefruit Extracts

phenolic compounds	helichrysum	Grapefruit
phenolic acids		
gallic acid	7.3	10.9
caffeic acid	67.3	20.2
chlorogenic acid	1039	109
chlorogenic acid-3-*O*-glucoside	2515	40.2
flavonoids		
flavanones		
Naringenin	230	1000
naringenin-7-*O*-glucoside	3892	219
naringenin diglycoside	1188	108
naringenin-7-*O*-rutinoside (narirutin)	37	5234
naringenin-4'-glucoside-7-rutinoside	0.03	83
Hesperidin	11	711
Flavonols		
kaempferol	7	nd[a]
kaempferol-3-*O*-glucoside	13375	6
kaempferol rutinoside	434	54193
myricetin glucoside	652	7
Flavones		
metoxiluteolin	0.8	0.03
Flavanols		
epigallocatechin	nd	nd
epigallocatechin-3-*O*-gallate	29	4

[a] nd, not detected.

2. Experimental design

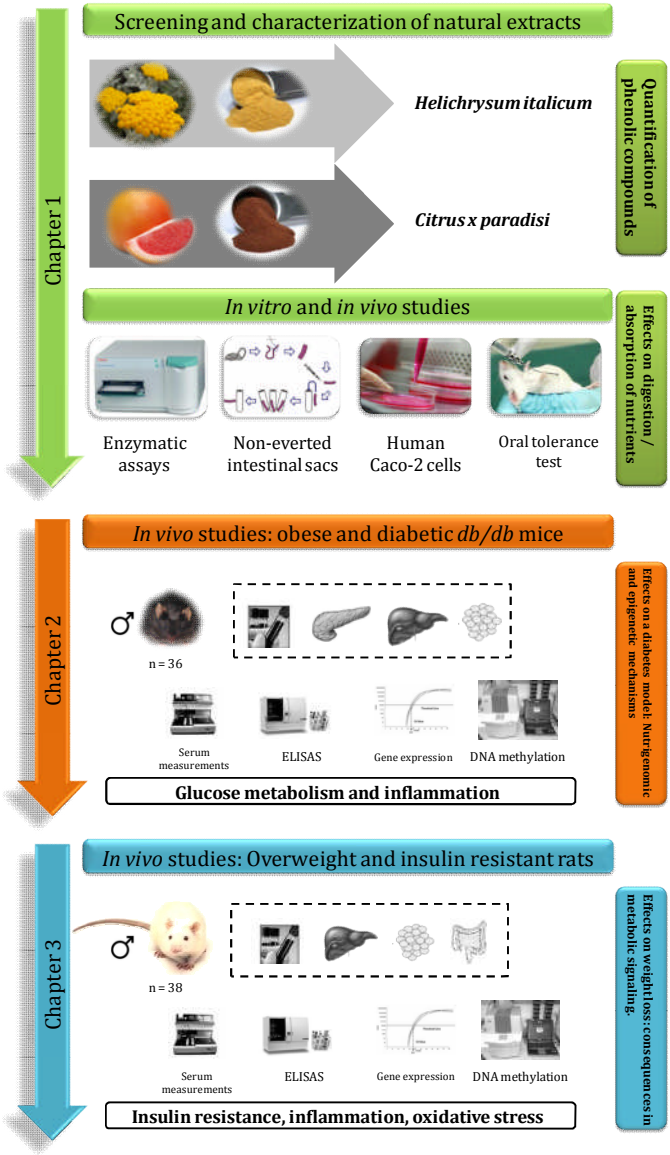

Figure 1. General experimental design.

The experimental procedure used in this work is described in the materials and methods of each article, which in turn corresponds to each chapter. This section briefly mentions the laboratory techniques employed with their respective equipment.

Chapter 1. Effects on digestion / absorption of nutrients

The following techniques were used in order to analyze: In vitro enzymatic assays, ex vivo absorption studies, human Caco-2 cells and postprandial studies in Wistar rats.

- Enzymatic assays: *α-amylase and α -glucosidase* activity assays. Spectrophotometry (Multiskan Spectrum, Thermo Scientific, USA) (Xiao et al., 2006, Elya et al., 2012).
- Intestinal digestion and absorption studies: Non-everted intestinal sacs and glucose measurements using the PENTRAC200 equipment (WILSON and WISEMAN, 1954).
- Methylglucoside (MG) uptake in human Caco-2 cells: ^{14}C MG, Radiactivity (Walak Oy, Turku, Finland) (Fanjul et al., 2012).
- Blood glucose levels *in vivo* (OSTT, OMTT, OGTT): glucometer and blood glucose test trips (Lomba et al., 2010).

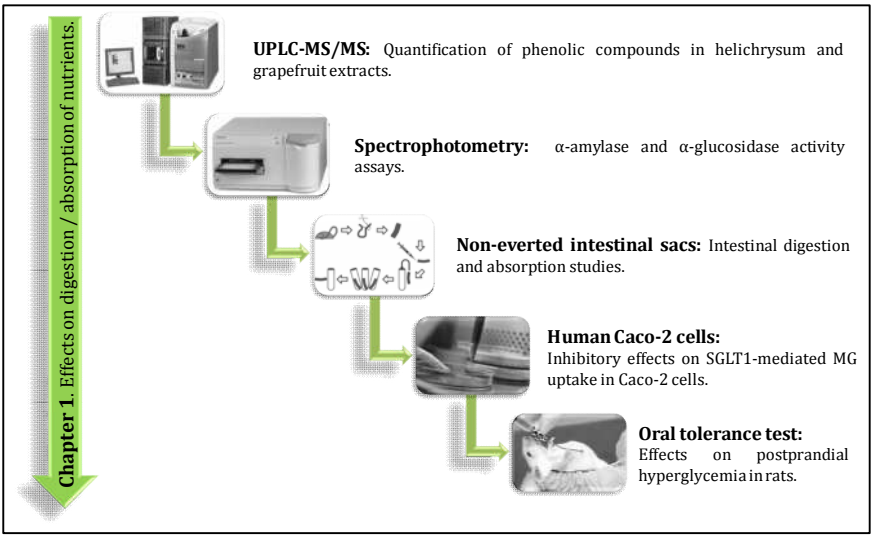

Figure 2. Experimental design. Characterization of natural extracts by UPLC-MS/MS analyses. Enzymatic assays were designed to study i*n vitro* α-amylase and α-glucosidase activity assays, the IC_{50} and to know the concentration to use in future studies. Non-everted intestinal sacs: intestinal digestion and absorption studies with starch and maltose. Human Caco-2 cells to analyze the inhibitory activity of extracts on SGLT1 glucose transporter. Oral tolerance test (*starch, maltose, glucose*): to study the effects of extracts on digestion and absorption of carbohydrates in an animal model.

Chapter 2. Effects on a diabetes model: Nutrigenomic and epigenetic mechanism.

Thirty six obese and diabetic male C57BL/6J *db/db* mice were randomly assigned into four experimental groups: non-treated control group, n = 8; acarbose group (5 mg/kg bw) n = 8; helichrysum group (1 g/kg bw), n = 10; and grapefruit group (0.5 g/kg bw), n = 10. The following techniques were used in order to analyze: body weight, food intake, body composition, glucose and lipid metabolism, respiratory quotient (RQ), energy expenditure (EE), gene expression and DNA methylation.

- Body weight and food intake: weight scale.
- Glucose levels *in vivo* (IPGTT, OSTT)*:* glucometer and blood glucose test trips (Lomba et al., 2010, Tai, 1994).
- Body composition: EchoMRI Analyzer system (Echo Medical Systems, Houston, TX, USA) (Nixon et al., 2010).
- Respiratory quotient (RQ) and energy expenditure (EE): Oxylet equipment (Panlab, Barcelona, Spain) (Garcia-Diaz et al., 2007).
- Serum measurements: *glucose, triglycerides, creatinine.* PENTRAC200 equipment.
- ELISA HbA1C: Serum and ELISA kit following the manufacturer's instructions. Spectrophotometry (Multiskan Spectrum, Thermo Scientific, USA).
- RNA extraction. Pancreas, liver and epididymal adipose tissue using TRIzol® reagent (Invitrogen, CA, USA) following the manufacturer's instructions. Nanodrop Spectrophotometer 1000 (Thermo Scientific, Delaware, USA).
- RT-PCR. Using ABI PRISM 7000 HT Sequence Detection System and predesigned TaqMan® Assays (GCK, G6Pase, PEPCK, Ins1, InsR, GLUT4, Betatrophin TNFα, COX2, MCP1, NFkB) (Applied Biosystems, Texas,USA).
- DNA extraction. From epididymal adipose tissue using QIAamp DNA Mini Kit (Qiagen).
- DNA methylation. Bisulfite-converted DNA by EpiTect Bisulfite Kit (Qiagen). Pico100 (Picodrop Limited, Hinxton, UK). Pyrosequencing using PyroMark Q24 System (Qiagen) (Switzeny et al., 2012).

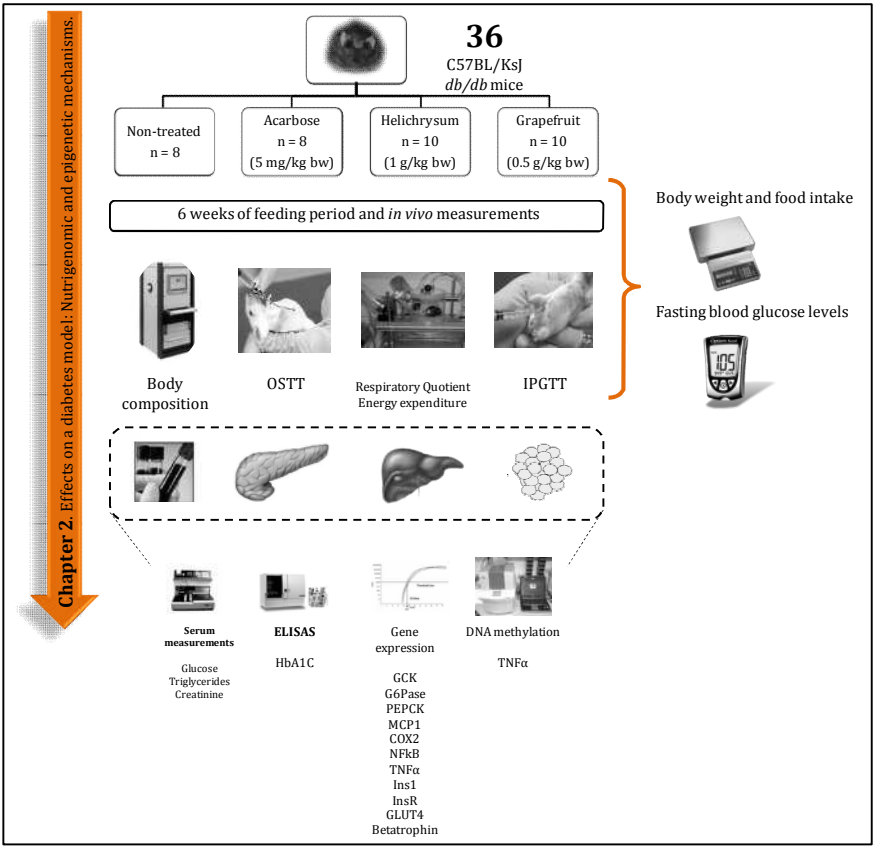

Figure 3. Experimental design. Thirty six obese and diabetic male C57BL/6J db/db mice were randomly assigned into four experimental groups: non-treated control group, n = 8; acarbose group (5 mg/kg bw), n = 8; helichrysum group (1 g/kg bw), n = 10, and grapefruit group (0.5 g/kg bw), n = 10. Body weight and food intake were recorded once a week. Body composition was measured by EchoMRI analyzer. OSTT and IPGTT were carried out and glucose was measured by a drop of blood from a tail vein. Respiratory quotient (RQ) and energy expenditure (EE) measurements were performed by using an Oxylet equipment. Glucose, triglycerides and creatinine were quantified in PENTRA C200 equipment. HbA1C was determined by ELISA using an automatized TRITURUS equipment. GCK, G6Pase, PEPCK, MCP1, COX2, NFkB, TNFα, Ins1, InsR, GLUT4, betatrophin gene expression were performed by RT-PCR (ABI PRISM 7000 HT Sequence Detection System). TNFα DNA methylation levels were determined by pyrosequencing using a PyroMark Q24 (Qiagen).

Chapter 3. Effects on weight loss: consequences in metabolic signaling.

Thirty eight male Wistar rats were divided in two groups: control (n=8) and HFS (n=30). After 22 days, the rats fed HFS diet changed to standard diet and were re-assigned into three groups: non-supplemented (n=10), helichrysum (2 g/kg bw) (n=10) and grapefruit (1 g/kg bw) (n=10) for 5 weeks. The following techniques were used in order to analyze: body weight, food intake, body composition, glucose and lipid metabolism, oxidative stress, gene expression and DNA methylation.

- Body weight and food intake: weight bascule.
- Glucose levels *in vivo*: glucometer and blood glucose test trips (Lomba et al., 2010,Tai, 1994).
- Serum measurements: *glucose, cholesterol, triglycerides, free fatty acids.* PENTRAC200 equipment.
- ELISA Insulin, leptin, adiponectin, TNFα, TBARS: serum and liver, and ELISA kits following each protocol described by the manufacturers (TRITURUS equipment. Grifols Internatinal S.A.).
- RNA extraction. Intestinal mucosa and epididymal adipose tissue. All-Prep® DNA/RNA/protein mini kit following the manufacturer's instructions (Qiagen). Nanodrop Spectrophotometer 1000 (Thermo Scientific, Delaware, USA).
- RT-PCR. Using ABI PRISM 7000 HT Sequence Detection System and predesigned TaqMan® Assays (TNFα and TLR2) (Applied Biosystems, Texas,USA).
- DNA methylation. Bisulfite-converted DNA by EpiTect Bisulfite Kit (Qiagen). Pico100 (Picodrop Limited, Hinxton, UK). Pyrosequencing using PyroMark Q24 System (Qiagen) (Switzeny et al., 2012).

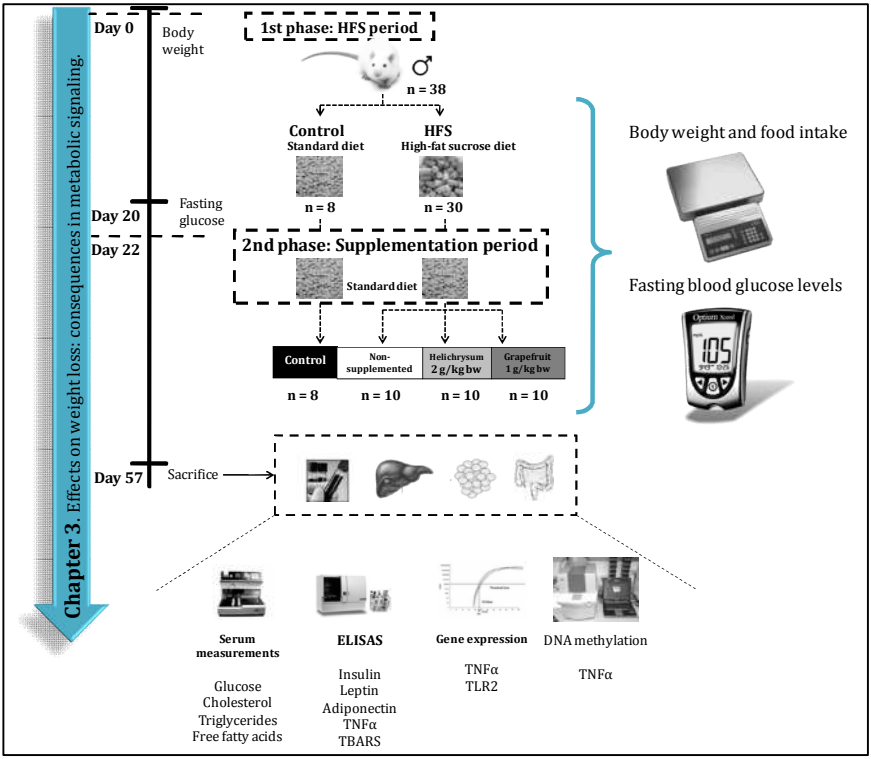

Figure 4. Experimental design. Thirty eight male Wistar rats divided in two groups: control (n = 8) and HFS (n = 30). After 22 days, the rats fed HFS diet changed to standard diet and were re-assigned into three groups: non-supplemented (n=10), helichrysum (2g/kg bw) (n=10) and grapefruit (1g/kg bw) (n=10) for 5 weeks. Body weight and food intake were recorded. At day 20th and 41th, fasting blood glucose levels were measured from the tail vein. Glucose, cholesterol, triglycerides and free fatty acids were quantified in PENTRA C200 equipment. Insulin, leptin, adiponectin, TNFα and TBARS were determined by ELISA using an automatized TRITURUS equipment. TNFα and TLR2 gene expression were performed by RT-PCR (ABI PRISM 7000 HT Sequence Detection System). TNFα DNA methylation levels were determined by pyrosequencing using a PyroMark Q24 (Qiagen).

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IV Results

The main findings of the present study were summarized in three chapters:

1. *Helichrysum and Grapefruit extracts inhibit carbohydrate digestion and absorption, improving postprandial glucose levels and hyperinsulinemia in rats.*
   de la Garza AL, Etxeberria U, Lostao MP, San Román B, Barrenetxe J, Martínez JA, Milagro FI.

   J Agric Food Chem. 2013 Dec 11;61: 12012-12019. doi; 10.1021/jf4021569.

2. *Modulation of hyperglycemia and TNFα-mediated inflammation by helichrysum and grapefruit extracts in diabetic db/db mice.*
   de la Garza AL, Etxeberria U, Palacios-Ortega S, Haslberger AG, Aumueller E, Milagro FI, Martínez JA.

   Under Review: Food Funct. 2014 Feb 25 (R.1)

3. *Helichrysum and grapefruit extracts boost weight loss in overweight rats reducing oxidative stress and inflammation.*
   de la Garza AL, Etxeberria U, Haslberger AG, Aumueller E, Martínez JA, Milagro FI.

   Submitted to: J Med Food 2014 May 26 (Under Review)

Chapter 1

Helichrysum and Grapefruit Extracts Inhibit Carbohydrate Digestion and Absorption, Improving Postprandial Glucose Levels and Hyperinsulinemia in Rats

Short title: Anti-Diabetic Effects of Helichrysum and Grapefruit Extracts

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ABSTRACT

Several plant extracts rich in flavonoids have been reported to improve hyperglycemia by inhibiting digestive enzyme activities and SGLT1-mediated glucose uptake. In this study, helichrysum (*Helichrysum italicum)* and grapefruit (*Citrus x paradise*) extracts inhibited *in vitro* enzyme activities. The helichrysum extract showed higher inhibitory activity of α-glucosidase (IC_{50}=0.19 mg/mL) than α-amylase (IC_{50}=0.83 mg/mL), whereas the grapefruit extract presented similar α-amylase and α-glucosidase inhibitory activities (IC_{50}=0.42 mg/mL and IC_{50}=0.41 mg/mL, respectively). Both extracts reduced maltose digestion in non-everted intestinal sacs (58% with helichrysum and 37% with grapefruit). Likewise, both extracts inhibited SGLT1-mediated methylglucoside uptake in Caco-2 cells in the presence of Na^+ (54% of inhibition with helichrysum and 29% with grapefruit). *In vivo* studies demonstrated that helichrysum decreased blood glucose levels after an oral maltose tolerance test (OMTT), and both extracts reduced postprandial glucose levels after the oral starch tolerance test (OSTT). Finally, both extracts improved hyperinsulinemia (31% with helichrysum and 50% with grapefruit) and HOMA index (47% with helichrysum and 54% with grapefruit) in a dietary model of insulin resistance in rats. Summarizing, helichrysum and grapefruit extracts improve postprandial glycemic control in rats, possibly by inhibiting α-glucosidase and α-amylase enzyme activities and decreasing SGLT1-mediated glucose uptake.

Keywords: α-amylase, α-glucosidase, caco-2 cells, flavonoids, SGLT1

1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder that affects different organs and tissues, and is characterized by chronically elevated levels of blood glucose due to insulin resistance. [1] The increasing incidence of T2DM, one of the world's most common chronic diseases, is primarily associated to lifestyle-related obesity and sedentarism and is modulated by the genetic background. [2,3] Considering that postprandial hyperglycemia is an early detected symptom in T2DM related to overconsumption of carbohydrate rich-foods, consumption of phytochemicals occurring in natural products could be a possible strategy to control T2DM. [4]

Phytochemicals are non-nutritive plant compounds that have health protective properties. One of the most relevant families of phytochemicals with proven health benefits are polyphenols. They are found in many plants including vegetables, fruits and grains, [5] being flavonoids the most prevalent phenolic compounds. Epidemiological data strongly suggest that diets rich in flavonoids are generally associated with a preventive role against the development of chronic diseases. [6-8]

This situation has led to an increased interest in finding specific natural dietary sources that could be used for treating diabetes. In this sense, numerous studies have investigated flavonoid-rich plant extracts that could reduce or suppress intestinal glucose uptake through the inhibition of digestive enzyme activities. [9-16]

In mammals, dietary carbohydrates are hydrolyzed by pancreatic α-amylase and intestinal α-glucosidase enzymes. Hence, the inhibition of these enzymes is an interesting strategy for the control of postprandial

hyperglycemia. [8,17,18] Other approaches are directed to develop agents that inhibit intestinal glucose uptake. [17] Thus, in the intestine, glucose is absorbed mainly by two transporters, depending on the luminal glucose concentration. At low concentrations, glucose is transported across the brush border membrane against a concentration gradient by the sodium-dependent glucose transporter 1 (SGLT1). At higher concentrations, glucose is transported mainly by the low-affinity facilitated transporter, glucose transporter 2 (GLUT2). [19] Dietary flavonoids, given their relative safety and low incidence of adverse gastrointestinal side effects, [15] are candidate agents for managing postprandial hyperglycemia due to their interactions with the intestinal α-glucosidase and pancreatic α-amylase and the inhibition of glucose uptake. [17]

In this context, there are more than 500 species of *Helichrysum* genus distributed around the world. [20] Plants of this genus have been found to possess antimicrobial, anti-allergic, antioxidant and anti-inflammatory properties. [20,21] The biological activities of *Helichrysum* plants have been attributed to several classes of flavonoids detected in different parts of the plant, such as kaempferol-3-*O*-glucoside and other flavanones. [22] Although several reports have been published on helichrysum and its flavonoid content, there is little information about its potential antihyperglycemic properties. [22,23] On the other hand, grapefruit is an excellent source of many nutrients and phytochemicals that contribute to a healthy diet. [24] *Citrus* flavonoids have evidenced antioxidant, anticancer, anti-inflammatory, chemopreventive and cardioprotective activities. [25] Many of the flavonoids present in grapefruit, such as hesperidin, naringenin and kaempferol, exhibit anti-diabetic activities. In fact, Shen *et al* [14] have demonstrated the inhibitory effects of *Citrus* flavonoids on starch digestion, whereas Pu *et al* [26]

reported the antihyperglycemic activity of naringenin isolated from *Citrus sinensis* in an animal model.

Thus, the aim of this study was to evaluate the anti-diabetic potential of helichrysum and grapefruit extracts by determining their postprandial anti-hyperglycemic effect and their inhibitory activities on α-amylase, α-glucosidase and SGLT-1 glucose transporter.

2. Materials and methods

2.1 Chemicals. Rats were fed a standard pelleted chow diet from Harlan Ibérica (Teklad Global, Barcelona, Spain; ref. 2014) or a high-fat sucrose (HFS) diet from Research Diets (New Brunswick, NJ, USA; ref. D12451). Helichrysum (*Helichrysum italicum*) and grapefruit (*Citrus x paradisi*) extracts, as well as acarbose ®, were provided by "Biosearch S.A." (Granada, Spain). Porcine pancreatic α-amylase, α-glucosidase (*Saccharomyces cerevisiae*), *p*-nitrophenyl α-D-glucopyranoside (*p*NPG), maltose, glucose and phloridzin were purchased from Sigma Chemicals, USA. Starch (162.14 g/mol) was purchased from Panreac. The Caco-2 cell line PD7 clone was kindly provided by Dr. Edith Brot-Laroche. The radiolabelled product [^{14}C]-α-methyl-glucoside (303 mCi/mL) was purchased from Perkin Elmer (Life Sciences, Boston, MA, USA).

2.2 Plant extracts preparation. Initial enzymatic *in vitro* assays were performed with plant extracts produced by accelerated solvent extraction (ASE) using a Dionex ASE200 equipment (Dionex Corporation, USA). Plant samples (1 – 5 g) were pulverized, mixed with washed sea sand (Panreac Química S.A.U., Spain) and introduced into the extraction cells, where 30 mL of the appropriate solvent at 50 °C was added: methanol:water (3:1) and

methanol:water (1:1) for *Helichrysum italicum* and *Citrus x paradisi*, respectively. After 3 h of incubation, liquid extracts were filtered, concentrated and dried by spray-drying. Solid extracts were stored at room temperature.

2.3 UPLC-MS/MS analyses. The extracts were previously dissolved in methanol/water (50:50, v/v) solution at a concentration of 10 mg/mL. Analyses of helichrysum and grapefruit extracts were performed by ultra-performance liquid chromatography (UPLC) and positive ion electrospray ionization (ESI) source Z-spray with MS/MS. The MS was operated in negative mode to analyze the phenolic compounds. The data were acquired in selected reaction monitoring (SRM). The ionization source working conditions were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h; desolvation gas flow rate, 800 L/h; desolvation temperature, 400 °C. [27] ABEH C_{18} (100 mm, 2.1 mm x 1.7µm) column, thermostated at 30 °C, was used. The solvents were (A) 0.2% acetic acid and (B) acetonitrile. The absorbance was recorded at 278 and 339 nm.

2.4 *In vitro* α-amylase activity assay. The α-amylase activity was determined using porcine pancreatic α-amylase solution (EC 3.2.1.1) in the absence (control) and presence of acarbose, helichrysum and grapefruit extracts. The reaction mixture consisted of 40µL of extracts at different concentrations (range 0.02 mg/mL to 1.5 mg/mL), α-amylase solution (5 µg/mL in 0.1 M sodium phosphate buffer at pH 7.0) and 40 µL of 1% (w/v) starch solution and was incubated for 10 minutes at 37 °C. After the incubation period, the reaction was stopped with 20 µL 1 M HCl. The reaction mixture was then diluted after adding 100 µL iodine solution (5 mM I_2, 5 mM KI) and absorbance was measured at 580 nm (Multiskan

Spectrum, Thermo Scientific, USA). Inhibitory activity was expressed as the relative absorbance difference (%) of the test natural extracts to the absorbance change of the control sample. [28]

2.5 *In vitro* α-glucosidase activity assay. The α-glucosidase activity was determined by measuring the release of *p*-nitrophenol from *p*NPG, as described elsewhere. [29] Briefly, 1 mg of α-glucosidase was dissolved in 100 mL of 0.1 M sodium phosphate buffer at pH 6.8, containing 200 mg of bovine serum albumin. The reaction mixture consisted of extracts at different concentrations of 10 µL (range 0.01 mg/mL to 0.5 mg/mL), premixed with 490 µL of sodium phosphate buffer and 250 µL of 0.5 mM *p*NPG and incubated at 37 °C for 5 min. After incubation, 250 µL of α-glucosidase solution was added and incubated at 37 °C for 15 min. The reaction was stopped with 200 µL of 2 M Na_2CO_3. The absorbance was measured at 400 nm on a Multiskan Spectrum spectrophotometer (Thermo Scientific, USA) and compared to the control, which contained 250 µL of PBS instead of natural extracts. Acarbose®, a marketed anti-diabetic drug that inhibits glycoside hydrolases resulting in a decrease of postprandial hyperglycemia, [30] was used as a positive control of α-glucosidase inhibition.

2.6 Intestinal digestion and absorption studies. Male Wistar rats (weight 220-250 g) were obtained from the Applied Pharmacology Research Center (CIFA) of the University of Navarra, Pamplona, Spain. After 15 h overnight fasting, rats were anesthetized by intraperitoneal injection of a mixture (4:1) of ketamine chlorohydrate (Ketolar; Merial SA, Barcelona, Spain) and medetomidine chlorohydrate (Domtor; Pfizer Orion, Espoo, Finland), at a dose of 0.25 mL/100 g bw. After anesthesia, laparotomy was performed

and a segment (20 cm) of jejunum was quickly excised, rinsed with ice cold saline solution (NaCl 0.9%) and cut into eight segments of 2.5 cm each.

One end of the intestinal segment was tied and filled with 0.4 mL of Krebs-Ringer-Tris buffer (KRT) composed by:1 M NaCl, 1 M KCl, 1 M Cl_2Ca, 0.5 M PO_4H_2K, 0.5 M SO_4Mg, 1 M Tris, 1 M HCl and containing 200 mM of starch or and 100 mM of maltose. Different conditions were tested: without α-amylase or glucosidase enzyme (as blanks), with the respective enzymes (0.5%), and with the enzymes plus acarbose ® (0.5%), plus helichrysum extract (2%) or plus grapefruit extract (1%). After tying the other end of the sacs, they were placed in an erlenmeyer containing 2.5 mL of the same KRT buffer. Sacs were incubated at 37 °C under continuous shaking and gassed with O_2; those with starch for 30 min and those with maltose for 7.5 min. After the incubation period, KRT buffer inside and outside of the intestinal sacs was collected to measure glucose concentration using the HK-CP kit (ABX Pentra, Montpellier, France) adapted for Pentra C200 analyser (HORIBA ABX, Montpellier, France). The experiment was approved by the Animal Research Ethics Committee of the University of Navarra (04/2011).

2.7 Methylglucoside uptake in human Caco-2 cells. Caco-2 cells were maintained in a humidified atmosphere of 5% CO_2-95% at 37 °C and grown in Dulbecco's Modified Eagles medium (DMEM, Gibco Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% penicillin (1000 U/mL), 1% streptomycin (1000 µg/mL) and 1% amphoterycin (250 U/mL). Once the cells reached 80% confluence, they were dissociated with 0.05% trypsin-EDTA and subcultured on 2.5 or 7.5 cm^2 plastic flasks at a density of 2.5 x 10^4 cells cm^{-2}. For uptake studies, the cells were seeded at 6 x 10^4 cells cm^{-2} density in 24-well culture plates.

Culture medium was replaced every 2 days. Cell confluence was confirmed by microscopic observance. Experiments were performed 17-21 days post seeding. [31]

For the glucose uptake measurements, the Caco-2 cells were pre-incubated in serum and glucose-free DMEM, 2 h before the beginning of the experiment. After washing with PBS, 0.5 mL of buffer containing 0.1mM α-methylglucoside (MG) with traces of ^{14}C MG (0.2 µCi/mL) were added to the cells. Substrate uptake was measured for 15 minutes in the presence and in the absence of helichrysum (0.6 mg), grapefruit (0.24 mg) or the SGLT1 inhibitor phloridzin (0.5 mM).

Methylglucoside uptake was stopped with ice-cold free-substrate buffer followed by aspiration. Cells were again washed twice with ice-cold buffer and finally solubilized in 500 µL 1% Triton X-100 in 0.1 N NaOH. Samples (100 µL) were taken to measure radioactivity on a Wallac 1409 liquid scintillation counter (Wallac Oy, Turku, Finland). Methylglucoside uptake values were corrected for protein concentration, as determined by the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

2.8 Oral tolerance tests. Twenty five male Wistar rats from CIFA of the University of Navarra (Pamplona, Spain), with an initial average weight of 210 g $\pm$ 12, were randomly distributed into five groups (n=5/group) and fasted for 15 h, with free access to water. These rats were orally administered by gastric intubation (5 ml/kg bw) either with water, vehicle or natural extracts as follows: (1) Control: water; (2) Vehicle: starch, maltose or glucose: 2 g/kg bw in a 30% w/v solution; (3) Acarbose ®: vehicle in a 30% w/v solution and 5 mg/kg bw of acarbose; (4)

Helichrysum: vehicle in a 30% w/v solution and 1 g/kg bw of helichrysum; and (5) Grapefruit: vehicle in a 30% w/v solution and 0.5 g/kg bw of grapefruit. Glycemia was measured before (0') and after the oral administration (30', 60', 90', 120', 180') by venous tail puncture using a glucometer and blood glucose test strips (Optium Plus, Abbott® Diabetes Care, Witney Oxon, UK). The glucose content was expressed as mg/dL, and the areas under the curve (AUC) were calculated according to the formula:

$AUC_{0\text{-}180\ min} = 30 \times [G_{30} + G_{60} + G_{90} + G_{120} + (G_0 + (G_{180} \times 2)/2]$

2.9 Glucose and insulin levels in insulin resistant rats. Thirty eight male Wistar rats from CIFA of the University of Navarra (Pamplona, Spain), with an initial average weight of 260 g $\pm$ 11, were kept in an isolated room exposed to a temperature between 21 and 23 °C, controlled humidity (50$\pm$10%) and a 12h:12h artificial light/dark cycle with water and food *ad libitum*. The experimental protocol was approved by the Animal Research Ethics Committee of the University of Navarra (04/2011).

The animals were randomized into two groups: Control (n=8) and HFS (n=30). During 22 days, rats had *ad libitum* water and food access (standard chow diet and HFS diet, respectively). Subsequently, the rats fed the HFS diet were divided into three groups: HFS non-supplemented (n=10), HFS supplemented with helichrysum extract (2g/kg bw) (n=10) and HFS supplemented with grapefruit extract (1g/kg bw) (n=10). After 35 days (5 weeks) of supplementation, rats were sacrificed, trunk blood collected and serum obtained for analysis of glucose and insulin. Glucose was measured using the HK-CP kit (ABX Pentra, Montpellier, France) adapted for the Pentra C200 analyser (HORIBA ABX, Montpellier, France), whereas insulin was quantified with a specific ELISA kit following the protocol described by

the manufacturer (Linco Research, Missouri, USA). Insulin resistance was evaluated by the homeostasis model of insulin resistance (HOMA-IR) formula:

[Serum glucose level (mmol/L) x insulin level (μU/mL) / 22.5]

2.10 Statistical analysis. All the results are expressed as mean ± standard deviation of the mean (SD). Statistical significance of differences among the groups were evaluated using One-Way ANOVA test followed by Dunnett's post hoc test or the non-parametric Kruskal Wallis test followed by the U Mann-Whitney test. A level of probability of $p<0.05$ was set as statistically significant. All analyses were performed using SPSS 15.0 packages of Windows (Chicago, IL).

The concentration giving 50% inhibition (IC_{50}) was calculated by non-linear regression. The dose-response curve was obtained by plotting the percentage inhibition versus concentration.

3. Results

3.1 Composition of helichrysum and grapefruit extracts. The quantification of the phenolic compounds in the two extracts (helichrysum and grapefruit) was performed by UPLC-MS/MS and is reported in **Table 1**. The major phenolic compound in helichrysum extract was kaempferol-3-*o*-glucoside (13.4 g/kg dried weight), but also high concentrations of chlorogenic acid-3-*o*-glucoside (2.5 g/kg dried weight), naringenin-7-*o*-glucoside (3.9 g/kg dried weight) and naringenin diglycoside (1.2 g/kg dried weight) were found. The most abundant phenolic compounds found in grapefruit extract were kaempferol rutinoside (54.2 g/kg dried weight),

which is a flavonol, and naringenin-7-*o*-rutinoside (5.2 g/kg dried weight), a flavanone.

3.2 α-amylase and α-glucosidase inhibitory activities. Helichrysum and grapefruit extracts showed *in vitro* α-amylase and α-glucosidase inhibitory activities. For example, the IC_{50} values in α-glucosidase inhibition assays were 0.19 mg/mL for helichrysum and 0.41 mg/mL for grapefruit (**Table 2**). Helichrysum reduced α-glucosidase activity by 9.3% and grapefruit by 15.5% at a concentration of 0.1 mg/mL, while acarbose-induced inhibition was 92.7%. As shown in **Table 2**, both extracts also inhibited α-amylase activity *in vitro. However,* helichrysum extract showed higher IC_{50} values for α-amylase than for α-glucosidase.

3.3 Effects of extracts on *in vitro* intestinal digestion and absorption of carbohydrates. The starch and maltose digestion was evaluated by using non-everted intestinal sacs and measuring glucose levels at both sides of the sacs. In these conditions, the activity of α-amylase was not inhibited in the presence of helichrysum or grapefruit extracts (**Figure 1A**). However, when the glucose levels were measured in the basolateral side of the sacs (glucose liberated from the starch that was transported from the intestinal lumen to the basolateral side), lower levels of glucose were detected in the presence of both extracts ($p < 0.05$, **Figure 1C**), suggesting that helichrysum and grapefruit extracts may have inhibitory activities on the intestinal glucose transporters, such as SGLT1. Regarding the intestinal α-glucosidase inhibitory activity, both extracts reduced the amount of glucose liberated from maltose inside the sacs, which means that the two extracts significantly inhibited the activity of intestinal maltase-glucoamylase (**Figure 1B**). Likewise, in the basolateral side, helichrysum and grapefruit

extracts significantly reduced glucose uptake (57% and 46%, respectively) up to similar levels to those found for acarbose (**Figure 1D**).

3.4 Inhibition of SGLT1-mediated methylglucoside uptake in Caco-2 cells. In response to the above results, it was investigated whether helichrysum and grapefruit extracts could inhibit the uptake of 0.1 mM MG, a specific substrate of SGLT1. In this case, phloridzin (an SGLT1 inhibitor) was used as a positive control. As shown in **Figure 2**, both phloridzin and helichrysum inhibited MG uptake (56% and 54% respectively), whereas the inhibitory effect of the grapefruit extract was lower (29%). These findings strongly suggest that helichrysum and grapefruit extracts inhibited SGLT1-mediated methylglucoside uptake in enterocytes.

3.5 Effects of the extracts on postprandial hyperglycemia in rats. Oral carbohydrate (starch, maltose and glucose) tolerance tests analyzed the effects of the extracts on postprandial hyperglycemia in an *in vivo* animal model (**Figure 3**). In the oral starch tolerance test, the administration of both extracts and acarbose induced a significant reduction of blood glucose levels (at different times of the test) when compared with the vehicle group (**Figure 3A**). In the oral maltose tolerance test, helichrysum induced a significant decrease in postprandial glucose levels when measuring the AUC (**Figure 3B**), but no significant differences were found in the grapefruit group. Finally, in the oral glucose tolerance test, the administration of acarbose, as expected, had no effect when compared with the vehicle group (**Figure 3C**). However, helichrysum and grapefruit extract administration slightly decreased plasma glucose levels 30 min after oral administration, being statistically significant ($p < 0.05$) in the grapefruit group (**Figure 3C**).

3.6 Effects of the extracts on serum glucose and insulin levels in insulin resistant rats. The dietary supplementation with helichrysum and grapefruit extracts during 5 weeks resulted in a decrease of serum insulin levels in rats with diet-induced insulin resistance (31% and 50%, respectively, **Table 3**). Furthermore, the HOMA index, which is an indicator of insulin resistance, was significantly lower after the treatment with helichrysum ($p < 0.05$) and grapefruit ($p < 0.01$) extracts (47% and 54%, respectively, **Table 3**).

4. Discussion

The results of this study demonstrate that helichrysum and grapefruit extracts are able to improve postprandial glycemic control in rats by reducing glucose absorption in the gastrointestinal tract. Helichrysum extract inhibited the activity of α-glucosidase in *in vitro* studies, being less effective for α-amylase, and also inhibited α-glucosidase activity in intestinal sacs. Grapefruit extract inhibited both digestive enzymes in *in vitro* studies (with a similar IC_{50}) and also α-glucosidase activity in intestinal sacs. In rats, both extracts induced a reduction of postprandial hyperglycemia after an oral starch tolerance test and a decrease of hyperinsulinemia in insulin-resistant animals.

To precisely understand the mechanism of the digestive enzyme inhibitory activity, we analyzed by HPLC the phenolic compounds present in the extracts, particularly flavonoids and glycosidic flavonoids (**Table 1**). In previous investigations, several authors have reported that different natural sources containing flavonoids and glycosidic flavonoids are able to inhibit digestive enzyme activities *in vitro*. [12] In this sense, Goto *et al* investigated the effects of tiliroside, a glycosidic flavonoid found in strawberries, and

observed an α-amylase inhibitory activity that delayed carbohydrate digestion, as well as a reduction of SGLT1 and GLUT2-mediated glucose uptake in enterocytes. [9] The most abundant flavonoid found in our helichrysum extract was kaempferol 3-*o*-glucoside. In this sense, Pereira *et al* [32] showed an *in vitro* inhibitory effect of kaempferol on α-glucosidase activity, and Matsui *et al* [33] also reported *in vitro* and *in vivo* the inhibition of α-glucosidase by kaempferol. However, there are few studies about the inhibitory effect of *Citrus* flavonoids on digestive enzyme activities. In this context, Shen *et al* [14] found that *Citrus* flavonoids may not be effective as α-amylase and α-glucosidase digestive enzyme inhibitors because of the low percentage of inhibitory activity.

Based on these results, we speculate that the presence of flavonoid glycosides might have contributed to the inhibitory effect on digestive enzymes, although the role of other compounds cannot be discarded. Our results showed different responses to natural extracts depending on the assay model. *In vitro,* helichrysum and grapefruit extracts significantly inhibited α-glucosidase activity, which is in agreement with other results showing that extracts rich in flavonoids can inhibit the activity of this enzyme. [11,13,34] Moreover, other flavonoid-rich extracts can also contribute to reduce glucose uptake by modifying the activity of other carbohydrate-digestive enzymes. For instance, Grussu *et al* [10] showed that extracts from berries had α-amylase inhibitory activity *in vitro*. In contrast, our results showed that both extracts (helichrysum and grapefruit) are more effective inhibiting α-glucosidase than α-amylase *in vitro*, which is in accordance with the results reported by Rubilar *et al*. [35] It could be possible that interactions between some compounds occurring in the extracts, such as flavonol and flavanone derivatives, potentiated the inhibitory activity on digestive

enzymes. Therefore, the identification of those natural compounds with high α-glucosidase but lower α-amylase inhibitory activity could help to prevent certain side-effects resulted by the non-specific inhibition of α-amylase, and that are mediated by the excessive accumulation of undigested carbohydrates in the large intestine. [11]

To further study the mechanisms responsible for the health properties of our extracts, *ex vivo* intestinal digestion and absorption studies were performed. We have taken into account three reasons in order to choose the doses. First, the translation of the doses to human nutrition, that required to keep within certain limits. Secondly, we have studied the doses that other authors have used with similar extracts. And third, we have tested *in vitro* different concentrations of 10 µL extracts that served to find the amylase inhibitory activity (range 0.02 mg/mL to 1.5 mg/mL) and the glucosidase inhibitory activity (range 0.01 mg/mL to 0.5 mg/mL) and, as the amylase inhibitory activity was lower for helicrysum extract (**Table 2**) and the total amount of flavonoids was lower in helichrysum (**Table 1**), we decided to use higher doses of helichysum extract in the determinations in non-everted intestinal sacs, Caco-2 cells and rats. The results in non-everted intestinal sacs suggest that the extracts induce a reduction of maltose digestion, which is in accordance with the maltase inhibitory activity shown by both extracts *in vitro*. On the other hand, a clear inhibition of starch digestion was not observed in the sacs. However, a decrease in starch-derived glucose uptake was found in the same experiment. The effect was lower on maltose than on starch, which could be partially explained because maltase-glucoamylase is an enzyme located in the gut mucosa and because maltose digestion and absorption processes are faster than those of starch. These results suggest that other mechanisms could be involved. In this sense, the results in Caco-2

cells suggest that helichrysum and grapefruit extracts can inhibit SGLT1-mediated glucose uptake. SGLT1 is a low-capacity and high-affinity transporter that can transport glucose against a concentration gradient. Manzano *et al* [36] found that apple and strawberry extracts rich in different flavonoids inhibited glucose uptake in Caco-2 cells. Furthermore, Rodríguez *et al* [37] reported that kaempferol 3-*o*-α-rhamnoside purified from *Bauhinia megalandra* leaves inhibited glucose absorption in rat isolated intestinal segments, and suggested that this flavonol is a competitive inhibitor of intestinal SGLT1 co-transporter.

From our results, it could be hypothesized that both extracts not only inhibit carbohydrate digestive enzymes, but also reduce glucose uptake by decreasing SGLT1-mediated uptake (as demonstrated in Caco-2 cells). Previous studies have shown that phloridzin, a plant derivative glycoside, is a specific and competitive inhibitor of SGLT1 in the intestine. [38,39] In this sense, Lostao *et al* [40] reported that, besides phloridzin, other phenylglucosides can act as inhibitors of SGLT1, although it was unclear whether the monosaccharide moiety or the aglycons were responsible of the phenylglucoside interaction with SGLT1. In further studies, Díez-Sampedro *et al* [41] demonstrated that the structure of the aglycon determined whether or not a glucoside was a transport substrate, an inhibitor or a noninteracting sugar. In fact, our results suggest that the high content of glycosilated flavonoids in the extracts would contribute, at least in part, to the inhibition of glucose uptake.

In the *in vivo* model, helichrysum and grapefruit extracts induced a reduction of postprandial glycemia after a starch overload, but only the helichrysum extract was able to reduce glycemia after oral maltose

administration. Thus, although grapefruit showed strong α-glucosidase inhibitory activity *in vitro*, some interactions occurring in the intestinal tract might explain its lower inhibitory effect *in vivo*. Perhaps, as α-glucosidase is sensible to pH, changes induced by grapefruit extract on the intestinal pH may affect the enzyme activity. [11]

The effect of the dietary supplementation of these extracts in a model of insulin resistance in rats revealed an improvement in serum insulin levels and insulin resistance after five weeks of treatment. These results are in agreement with Aslan *et al* [22] who demonstrated the blood glucose-lowering effect of helichrysum in diabetic rats. Concerning grapefruit, Jung e*t al* [42] observed a decrease in blood glucose levels in *db/db* mice treated with *Citrus* flavonoids (0.2 g/kg), whereas Molvihill *et al* [43] found that naringenin (3% w/w) prevented hyperinsulinemia in mice with insulin resistance.

Overall, these results suggest that helichrysum and grapefruit extracts have a moderate effect on improving insulin resistance by affecting carbohydrate digestion and absorption. Although these results are positive in an animal model, it is important to study the effects of both extracts in humans, where other factors, such as genetics and microbiota, may be involved.

In the present study, some differences in the inhibitory activities of digestive enzymes and glucose transporters were observed. Variations in extract composition, likewise, the susceptibility of the α-amylase and α-glucosidase enzymes and SGLT1 glucose transporter to different conditions, can explain the differences observed. However, these results could indicate a synergistic action of different compounds occurring in the extracts (including the flavonoids), as was observed when a combination of roselle, chrysanthemum, and butterfly pea extracts with mulberry extract showed

additive interaction on intestinal maltase inhibition. [44] Nevertheless, as reported in the same study, [44] the interactions between different phenolic compounds can also reduce inhibition. These differences can also be due to the presence in the extracts of other components that may interact with the glycosylated flavonoids present in greater proportion. Thus, by comparing with other works, it can be speculated that the effects may be due to flavonoids present in the extracts, although the involvement of other compounds cannot be ruled out.

In summary, both extracts (helichrysum and grapefruit) have potential antihyperglycemic properties by acting on carbohydrate-digestive enzymes and SGLT1 glucose transporter. They also improve insulin resistance in rats. Considering that we have found positive effects of helichrysum and grapefruit extracts *in vitro* and *in vivo*, it can be concluded that the combination of natural compounds of the extracts can interact among them, enrich health properties of natural foods, and provide an opportunity to develop a novel class of natural agents to help manage glucose metabolism, that could be included in the diet as a supplement.

ABBREVIATIONS USED

HFS, high-fat sucrose diet; HOMA-IR, homeostatic model assessment – estimated insulin resistance; IC_{50}, concentratrion estimated to give 50% inhibition; KRT, Krebs-Ringer-Tris; OMTT, oral maltose tolerance test; OSTT, oral starch tolerance test; *p*NPG, *p*-nitrophenyl α-D-glucopyranoside; SGLT1, sodium dependent glucose transporter 1; T2DM, type 2 diabetes mellitus; UPLC, ultra-performance liquid chromatography

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Table 1. Quantification of phenolic compounds in helichrysum and grapefruit extracts.

Phenolic compounds (mg/kg extract)	Helichrysum	Grapefruit
PHENOLIC ACIDS		
Gallic acid	7.3	10.9
Caffeic acid	67.3	20.2
Chlorogenic acid	1039	109
Chlorogenic acid-3-o-glucoside	2515	40.2
FLAVONOIDS		
Flavanones		
Naringenin	230	1000
Naringenin-7-o-glucoside	3892	219
Naringenin diglycoside	1188	108
Naringenin-7-o-rutinoside (narirutin)	37	5234
Naringenin-4'-glucoside-7-rutinoside	0.03	83
Hesperidin	11	711
Flavonols		
Kaempferol	7	N.D.
Kaempferol-3-o-glucoside	13375	6
Kaempferol rutinoside	434	54193
Myricetin glucoside	652	7
Flavones		
Metoxiluteolin	0.8	0.03
Flavanols		
Epigallocatechin	N.D	N.D
Epigallocatechin-3-o-gallate	29	4

N.D. Not detected

Table 2. Inhibitory activity (IC_{50}) of helichrysum and grapefruit extracts against α-amylase and α-glucosidase, using starch and *p*NPG as substrates, respectively (n=2).

	α-amylase (IC_{50})	α-glucosidase (IC_{50})
Acarbose (μg/mL)	4.17 ± 0.01	6.91 ± 0.01
Helichrysum (mg/mL)	0.83 ± 0.05	0.19 ± 0.01
Grapefruit (mg/mL)	0.42 ± 0.06	0.41 ± 0.01

Table 3. Serum glucose and insulin levels after five weeks of supplementation with helichrysum and grapefruit extracts in a rat model of insulin resistance.

	GROUPS			
	Control (standard diet)	HFS non-supplemented	HFS + Helichrysum	HFS + Grapefruit
Serum values				
Glucose (mmol/L)	5.5 ± 0.3	6.1 ± 0.8	5.6 ± 0.5	5.6 ± 0.8
Insulin (μU/mL)	12.1 ± 5.2 *	24.1 ± 15.2	14.4 ± 4.4 *	12.2 ± 4.9 *
HOMA Index	2.7 ± 1.0 **	6.8 ± 4.6	3.6 ± 1.3 *	3.0 ± 1.2 **

Results are expressed as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with the non-supplemented group. * $p<0.05$; ** $p<0.01$.

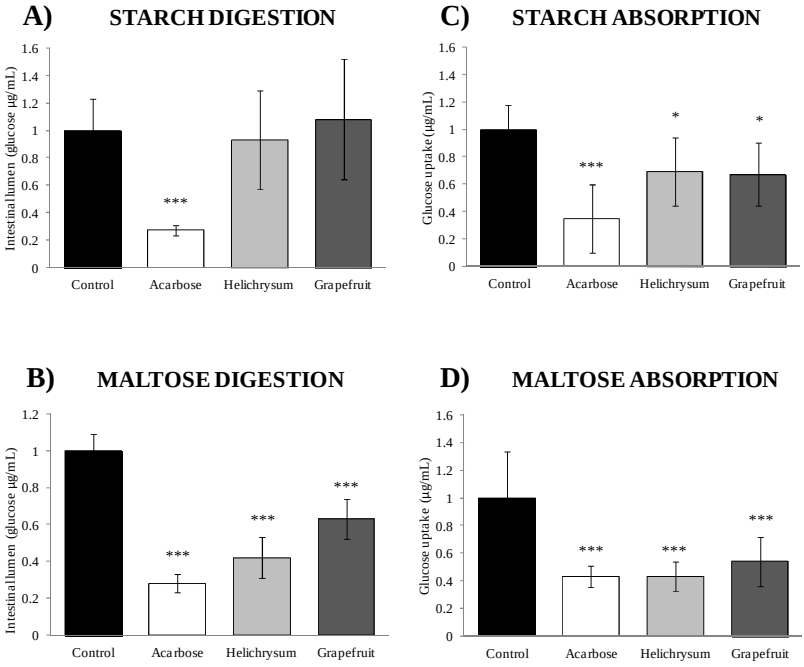

Figure 1. *In vitro* digestion and absorption studies using non-everted intestinal sacs with 200 mM starch (A and C) and with 100 mM maltose (B and D) as substrates, in the presence and in the absence of helichrysum extract (2%), grapefruit extract (1%) and acarbose (0.5%). Results are expressed as mean $\pm$ SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with control group. (A and C) n = 12 (B and D) n = 6. * $p < 0.05$, *** $p < 0.001$.

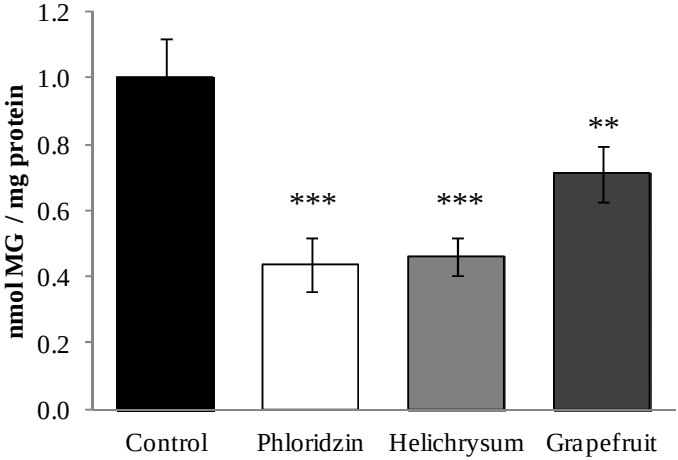

Figure 2. Inhibitory effects of helichrysum (0.6 mg) and grapefruit (0.24 mg) extracts on SGLT1-mediated MG uptake in Caco-2 cells. Results are expressed as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with control group. Control and phloridzin n = 6; Helichrysum and grapefruit n = 15. ** $p < 0.01$, *** $p < 0.001$.

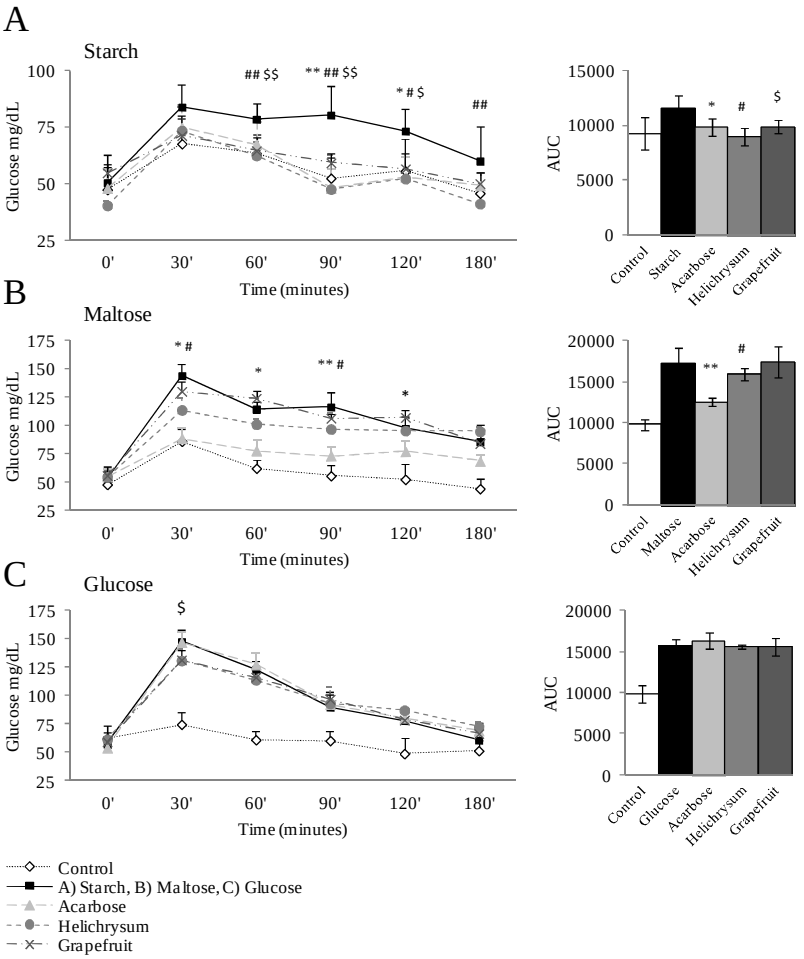

Figure 3. Effects of helichrysum and grapefruit extracts on blood glucose levels after oral (A) starch, (B) maltose, and (C) glucose administration in male Wistar rats. Results are expressed as mean ± SD. Statistical analysis was performed using non-parametric variable (Kruskal Wallis) followed by U Mann-Whitney to test differences in the mean of each group (starch, maltose and glucose) with vehicle (water) group. n = 5. * $p < 0.05$, ** $p < 0.01$ (acarbose); # $p < 0.05$, ## $p < 0.01$ (helichrysum); $ $p < 0.05$, $$ $p < 0.01$ (grapefruit).

Summary Chapter 1

	Helichrysum	**Grapefruit**
Composition (major phenolic compounds)	Kaempferol-3-*O*-glucoside Chlorogenic acid-3-*O*-glucoside Naringenin-7-*O*-glucoside Naringenin diglycoside	Kaempferol rutinoside Naringenin-7-*O*-rutinoside Naringenin
α-amylase inhibitory activity (IC_{50})	0.83 ± 0.05	0.42 ± 0.06
α-glucosidase inhibitory activity (IC_{50})	0.19 ± 0.01	0.41 ± 0.01
α-glucosidase in noneverted intestinal sacs	↓ 58%	↓ 37%
SGLT1-mediated MG uptake in Caco-2 cells	↓ 54%	↓ 29%
In vivo studies. Maltose digestion (α-glucosidase)	↓ AUC	= AUC
In vivo studies. Starch digestion (α-amylase)	↓ AUC	↓ AUC

Chapter 2

Modulation of hyperglycemia and TNFα-mediated inflammation by helichrysum and grapefruit extracts in diabetic db/db mice

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Keywords: helichrysum, grapefruit, diabetes, hyperglycemia, DNA methylation, TNFα

ABSTRACT

Type-2 diabetes is associated with a chronic low-grade systemic inflammation accompanying an increased production of adipokines/cytokines by obese adipose tissue. The search of new anti-diabetic drugs with different mechanisms of action, such as insulin sensitizers, insulin secretagogues and α-glucosidase inhibitors, have opened the focus for the potential use of flavonoids for the management of type-2 diabetes. Thirty six diabetic male C57BL/6J db/db mice were fed a standard diet and randomly assigned into four experimental groups: non-treated control, (n=8); acarbose (5 mg/kg bw, n=8); helichrysum (1 g/kg bw, n=10) and grapefruit (0.5 g/kg bw, n=10) for 6 weeks of treatment. mRNA expression in pancreas, liver and epididymal adipose tissue was determined by RT-PCR. DNA methylation was quantified in epididymal fat using pyrosequencing. Mice supplemented with helichrysum and grapefruit extracts showed a significant decrease in fasting glucose levels ($p<0.05$). A possible mechanism of action could be the up-regulation of liver glucokinase ($p< 0.05$). The antihyperglycemic effect of both extracts was accompanied by decreased mRNA expression of some proinflammatory genes (monocyte chemotactic protein-1, tumor necrosis factor-α, cyclooxygenase-2, nuclear factor-kappaB) in liver and epididymal adipose tissue. The site CpG3 of TNFα, located 5 bp downstream of the transcription start site, showed increased DNA methylation in the grapefruit group compared with the non-treated group ($p<0.01$). In conclusion, helichrysum and grapefruit extracts improved hyperglycemia through the regulation of glucose metabolism in liver and the reduction of the expression of proinflammatory genes in liver and visceral fat. The hypermethylation of TNFα in adipose tissue may contribute to reduce the inflammation associated to diabetes and obesity.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by chronic hyperglycemia as a result of impairments in insulin secretion and insulin action in target tissues.[1] Insulin resistance (IR) is produced as soon as the pancreatic β-cells cannot compensate a reduced insulin function, leading to elevated circulating glucose levels.[2] Insulin inhibits gluconeogenesis in liver and reduces lipolysis in adipose tissue.[3] Likewise, adipose tissue in diabetes and obesity is characterized by hypertrophy, relative hypoxia, low-grade chronic inflammation and endocrine dysfunctions.[4] In this context, the proinflammatory cytokines, many of them secreted by the hypertrophied adipocytes, are controlled through transcription nuclear factor-kappaB (NFkB), whereby the inflammatory response can be down-regulated.[5] In addition, this transcription factor represents a link between inflammation and IR, as it is activated by factors known to promote IR and T2DM.[6] One important downstream target of NFkB is cyclooxygenase 2 (COX2), which catalyzes the production of prostaglandins, the key molecules in inflammation processes of the body.[7] Moreover, NFkB is involved in the expression of many cytokines, including TNFα.[5] On the other hand, epigenetic changes are heritable yet reversible modifications that occur without alterations in the primary DNA sequence. These modifications may provide a link between the environment (i.e. nutrition) and T2DM.[8] Recently, epigenetic modifications have also been implicated in disease-associated changes influencing gene expression.[9]

Targeting the reduction of chronic inflammation is a beneficial strategy to combat several metabolic diseases, including T2DM.[10] Thus, numerous studies have underlined the interest in finding nutritional factors that may help to prevent or treat these diseases.[10, 11] In this sense, flavonoids can act

through a variety of mechanisms to prevent and attenuate inflammatory responses.[12] These bioactive compounds can also improve glucose metabolism by stimulating peripheral glucose uptake in different tissues.[11] In relation to this, grapefruit extract is rich in flavanones (i.e., naringenin-7-*O*-rutinoside) and flavonols (i.e., kaempferol rutinoside).[13] Previous studies have reported that *citrus* flavonoids have many pharmacological activities, including anti-inflammatory properties.[14] Thus, an improvement in hyperglycemia by the hepatic enzymes involved in glucose metabolism was reported in groups of mice, whose diet was supplemented with naringin.[15] Furthermore, a recent study reported that orange juice appears to mediate the inflammatory response, both gene expression and plasma level.[16]

Helichrysum (*helichrysum italicum*) is a flowering plant that grows around the Mediterranean area and contains naringenin-7-*O*-glucoside, kaempferol-3-*O*-glucoside and other flavonoids.[13] Likewise, *helichrysum* genus has been found to have several biological activities, such as anti-inflammatory properties, which have been attributed to different flavonoids.[17] Thus, beneficial roles of kaempferol have been reported in inflammation, hyperglycemia and diabetes in different *in vitro* and *in vivo* models.[18] Additionally, some investigations have concluded that the anti-inflammatory activity of *Helichrysum italicum* may be explained by enzyme inhibition, free-radical scavenging activity and corticoid-like effects.[19] In this sense, our group previously demonstrated that helichrysum and grapefruit extracts ameliorated hyperglycemia by inhibiting α-glucosidase (a similar mechanism as acarbose) and α-amylase enzyme activities and by decreasing SGLT1-mediated glucose uptake in the gut.[13]

Since inflammation in the adipose tissue plays a central role in obesity-related IR and T2DM, our research was conducted in a recognized model of

obesity and diabetes, *db/db* mice, displaying characteristics such as overweight, hyperglycemia and hyperinsulinemia due to leptin receptor mutations.[20] Therefore, the aim of this study was to investigate the antihyperglycemic and anti-inflammatory effects of helichrysum and grapefruit extracts, studying the possible involvement of epigenetic mechanisms in db/db mice. The effects of both extracts were compared with those of acarbose, an oral anti-diabetic agent whose main mechanism of action is the inhibition of α-glucosidase.

2. Material and methods

2.1 Chemicals. Mice were fed a standard pelleted chow diet from Harlan Ibérica (Teklad Global, Barcelona, Spain; ref. 2014). Helichrysum (*Helichrysum italicum*) and grapefruit (*Citrus x paradisi*) extracts, as well as acarbose ®, were provided by "Biosearch S.A." (Granada, Spain). Plant samples (1-5 g) were pulverized, mixed with washed sea sand and introduced into the extraction cells, where 30 ml of each solvent at 50 °C was added: methanol/water (3:1) and methanol/water (1:1) for helichrysum and grapefruit, respectively. The quantification of the phenolic compounds was performed by UPLC-MS/MS.[13] Helichrysum extract contained phenolic acids and flavonoids as flavanones and flavonols subclasses, as previously described.[13] The flavanones found in higher proportion were naringenin-7-*O*-glucoside (3.9 mg/g extract) and naringenin diglycoside (1.2 mg/g extract). Kaempferol-3-*O*-glucoside (13.4 mg/g extract) is the flavonol that was found as a greater proportion. Likewise, grapefruit extract mainly contained naringenin-7-*O*-rutinoside (5.2 mg/g extract) and naringenin (1 mg/g extract) as flavanone, and kaempferol-rutinoside (54.2 mg/kg extract) as flavonol.[13] Glucose was

purchased from Sigma Chemicals (St. Louis, MO, USA) and starch (162.14 g/mol) from Panreac (Barcelona, Spain).

2.2 Experimental animals. Thirty six obese and diabetic male C57BL/6J db/db mice (Charles River, Barcelona, Spain) were randomly assigned into four experimental groups: non-treated control group, n = 8; acarbose group (5 mg/kg bw), n = 8; helichrysum group (1 g/kg bw), n = 10, and grapefruit group (0.5 g/kg bw), n = 10. The doses used were calculated comparing with the acarbose effect and based on the IC_{50} of the extracts, as described elsewhere.[13] For 6 weeks, all mice were fed a standard pelleted chow diet from Harlan Ibérica (ref. 2014 S, Barcelona, Spain) containing 20% of energy as proteins (corn and wheat), 67% as carbohydrates (5% sucrose, 62% starch), and 13% as fat by dry weight (2.9 kcal/g). Animals were kept in an isolated room under a constantly regulated temperature between 21 and 23 °C, and controlled humidity (50$\pm$10%) in a 12h:12h artificial light/dark cycle. Body weight and food intake were recorded once a week. Body composition was measured at the beginning and at the end of the feeding period. On the 1st, 3rd and 6th weeks, fasting glucose was measured by a drop of blood from a tail vein. On the 5th week, respiratory quotient (RQ) and energy expenditure (EE) (kg/day/bw^{¾}) measurements were performed by using an Oxylet equipment (Panlab, Barcelona, Spain), as previously reported.[21] This procedure was carried out in groups of four mice daily, introducing each mouse in a box with water and food during 24 hours. At weeks 3 and 6, oral starch tolerance test (OSTT) and intraperitoneal glucose tolerance test (IPGTT) were carried out, respectively. After 6 weeks of experimental treatment, mice were killed by decapitation and trunk blood was collected to obtain serum for the biochemical measurements. Liver, pancreas, spleen and different adipose

depots, such as subcutaneous, retroperitoneal, epididymal and mesenteric, were carefully dissected and weighed. Tissue samples and serum were immediately frozen in liquid nitrogen and stored at -80 °C for further analyses. All the procedures were performed according to the Animal Research Ethics Committee of the University of Navarra (04/2011).

2.3 Oral starch tolerance test (OSTT) and Intraperitoneal glucose tolerance test (IPGTT). The OSTT was performed at the 3rd week. After a 15-h fast, animals were orally administered by gastric intubation (5 ml/kg bw) with starch (2 g/kg bw in a 30% w/v solution) and acarbose (5 mg/kg bw), helichrysum (1 g/kg bw) and grapefruit (0.5 g/kg bw), respectively. Glycemia was measured before (0') and after the oral administration (30', 60', 120', 180', 240') by venous tail puncture using a glucometer and blood glucose test trips (Optium Plus, Abbott® Diabetes Care, Witney Oxon, UK). The IPGTT was performed at the 6th week. After a 15-h fast, mice were injected intraperitoneally with glucose (2 g/kg bw in 30% w/v solution). Blood glucose levels were determined from the tail vein before (0') and after glucose injection (180', 240', 360', 420'). The glucose content was expressed as mmol/L, and the areas under the curve (AUC) were determined by the trapezoidal rule approach.[22]

2.4 Biochemical measurements. Fasting glucose levels were measured with the HK-CP kit (ABX diagnostic, Montpellier, France), creatinine was determined with the Creatinine-CP kit (ABX Pentra), and triglycerides with the RANDOX triglycerides kit (Randox Laboratories, Crumlin, UK), adapted for the PENTRA C200 equipment (HORIBA Medical, Montpellier, France). Levels of glycated hemoglobin (HbA1C) were determined at the end of the feeding period and measured with the mouse GHbA1C ELISA kit (Cat. No. CSB-E08141m, Cusabio Biotech Co.,Ltd., China).

The pancreatic insulin content was determined by acid-ethanol extraction. Briefly, the pancreas was placed into 5 ml acid-ethanol (1.5% HCl in 70% EtOH) overnight at -20 °C, homogenized and incubated overnight at -20 °C. Samples were centrifuged at 2000 rpm 15 minutes at 4 °C. The complete liquid was transferred to clean tubes and was neutralized with 100 µl 1 M Tris pH 7.5. The pancreatic insulin content was analyzed by enzyme-linked-immunosorbent assay (ELISA) following the protocol described by the manufacturer (Mercodia AB, Uppsala, Sweden). The absorbance was calculated with the appropriate dilution factor. Pancreatic insulin values were corrected for protein concentration, as determined by Bradford assay with bovine serum albumin as a standard (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). Finally, insulin content (ng/mL) was normalized by the protein content (µg/mL).

2.5 RNA extraction, reverse transcription and quantitative real-time polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from pancreas, liver and epididymal adipose tissue using TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA concentration and quality were measured with a Nanodrop Spectrophotometer 1000 (Thermo Scientific, Delaware, USA). Then, RNA (2 µg) was reverse-transcribed to cDNA using MMLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen). RT-PCR assays were performed following the manufacturer's recommendations using an ABI PRISM 7000 HT Sequence Detection System and predesigned TaqMan® Assays-on-Demand by Applied Biosystems (Texas, USA). Glucokinase (GCK), Mm00439129_m1; Glucose 6-phosphatase (G6Pase), Mm00839363_m1; Phosphoenolpyruvate carboxykinase (PEPCK), Mm01247058_m1; Monocyte chemotactic protein 1 (MCP1), Mm00656886_g1; Nuclear factor-

kappaB (NFkB), Mm00476361_m1; Cyclooxygenase 2 (COX2), Mm00478374_m1; Tumor necrosis factor α (TNFα), Mm00443260_g1; Betatrophin, Mm01175863_g1; Insulin (Ins1), Mm019550294_s1; Insulin receptor (InsR) Mm01211875_m1; Glucose transporter 4 (GLUT4), Mm00436615_m1 and Taqman Universal Master Mix were also provided by Applied Biosystems. mRNA levels were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mm 99999915_g1, and Beta actin (ActB), Mm 00607939_s1, as housekeeping genes. All samples were analyzed in triplicate. The relative expression level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6 DNA extraction and bisulfate conversion. Genomic DNA was isolated from epididymal adipose tissue using the DNA purification protocol for tissues of the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA). DNA concentration and quality were measured by Nanodrop Spectrophotometer 1000 (Thermo Scientific, DE, SA). The stock solution of DNA samples was stored at -80 °C until use. For epigenetic analysis, all DNA samples were bisulfite-treated using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), resulting in the deamination of unmethylated cytosine to uracil. The concentration of DNA was measured on a Pico100 (Picodrop Limited, Hinxton, UK). All procedures were carried out according to the manufacturer's protocols.

2.7 PCR and methylation analysis by DNA pyrosequencing. Quantitative methylation analyses were performed by pyrosequencing of bisulfite-converted DNA using the PyroMark Q24 (Qiagen). PCR was carried out in 25 µl reaction mixtures with 12.5 µl PyroMark 2x PCR master mix, 0.15 nM of primers for TNFα, 5'-GGAAGTTTTTAGAGGGTTGAATGAGA- 3' (forward), 5'-CTAACTAATCCCTTACTATCCT-3' (reverse), 2.5 µl CoralLoad Concentrate

10x (Qiagen) and 1 µl of DNA samples after bisulfite conversion, at concentration of 10 ng/µl. PCR conditions were 95°C for 15 minutes; 45 cycles of 94 °C for 30 s, 55.5 °C for 45 s, 72 °C for 45 s; and a final elongation at 72 °C for 10 minutes. PCR products were checked by 2% agarose gel electrophoresis. A total of 22 µl of the PCR product was used for subsequent pyrosequencing using a PyroMark Q24 System (Qiagen). All procedures of quantification of CpG methylation levels were performed based on a protocol described elsewhere.[23] For quality control, each experiment included non-CpG cytosines as internal controls to verify efficient bisulfite DNA conversion.

2.8 Statistical analysis. All the results are expressed as mean ± standard deviation (SD) of the mean. Statistical significance of differences among the groups was evaluated using One-Way ANOVA test followed by Dunnett's post hoc test. The two-tailed Pearson test was used to assess selected correlations among variables. A level of probability of $p<0.05$ was set as statistically significant. All analyses were performed using SPSS 15.0 packages of Windows (Chicago, IL).

3. Results

3.1 Food intake, body weight gain and body fat mass. After the end of the supplementation period, the grapefruit group gained more body weight ($p < 0.05$) than the non-treated group (**Table 1**). Although not statistically significant, the percentage of total adipose tissue (WAT) was slightly higher in the treated groups (**Table 1**). Furthermore, significant differences were found in spleen weight between the acarbose ($p < 0.05$) and helichrysum ($p < 0.01$) groups when compared to the non-treated group, whereas liver weights were similar in all groups (**Table 1)**.

Regarding food efficiency, the average daily food intake throughout the experimental period remained unaltered in the acarbose group and after helichrysum and grapefruit extract administration (**Table 1**).

3.2 Respiratory quotient and energy expenditure. The respiratory quotient (RQ) assessment, which is used to evaluate the relative oxidation of substrates, evidenced that the grapefruit group ($p < 0.05$) improved carbohydrate oxidation when compared with the non-treated group (**Table 1**). Otherwise, there were no differences among groups with respect to energy expenditure (EE), suggesting that the possible effect of helichrysum and grapefruit extracts in glucose metabolism did not significantly affect thermogenesis (**Table 1**).

3.3 Blood glucose and serum parameters. Glycemia levels at baseline and at the end of the supplementation period are shown in **Table 1**. All mice were diabetic when the experiment began ($x = 10 \pm 3$ mmol/L). Although no significant differences were found in the acarbose group, both supplemented groups showed significantly lower levels of glycemia ($p < 0.05$) at the end of the 6-week treatment when compared with the non-treated group (**Table 1**). The grapefruit group decreased the glucose AUC in the OSTT ($p < 0.05$) (**Fig. 1A**). Likewise, both supplemented groups showed lower AUC than the non-treated group in the IPGTT ($p < 0.05$) (**Fig. 1B**).

No statistical differences between groups were found in fasting triglyceride levels. Conversely, creatinine serum levels were slightly lower in the acarbose and grapefruit groups, but did not reach statistical significance in comparison with the non-treated group (**Table 1**).

The long-term glucose control was also evaluated by measuring HbA1C (**Table 1**), but no relevant differences were found among the experimental groups.

3.4 Determinations in pancreas. Pancreatic insulin content was analyzed to determine whether the use of both extracts might have beneficial effects on glucose metabolism via the insulin secretory capacity of the pancreas. There were no differences in the pancreatic insulin content among the experimental groups (**Table 1**). However, the mRNA expression of Ins1 was decreased in the pancreas from the acarbose group when compared with the non-treated group (**data not shown**). No statistical differences were found between groups in the mRNA expression of GCK in pancreas (**data not shown**).

3.5 Glucose metabolism. In order to investigate the mechanisms through which flavonoid-rich extracts ameliorate hyperglycemia in *db/db* mice, the mRNA expression of different genes that regulate glucose homeostasis in liver was examined (**Table 2**). GCK expression levels were statistically higher in the acarbose group ($p < 0.001$) and both supplemented groups ($p < 0.05$) when compared to the non-treated group. No statistical differences were found in G6Pase, PEPCK and betatrophin mRNA levels in liver (**Table 2**). Interestingly, mRNA expression levels of GCK in liver showed a negative correlation ($r = -0.692$, $p < 0.001$) with final blood glucose levels (mmol/L) (**Fig. 2**).

Moreover, mRNA expression levels of betatrophin, InsR and GLUT4 were measured in epididymal adipose tissue, although no differences were found among the experimental groups (**Table 3**).

3.6 Inflammatory markers. The expression of several proinflammatory markers was analyzed in liver and epididymal adipose tissue. Thus, the hepatic mRNA levels of TNFα, MCP1, COX2 and NFkB decreased in the acarbose group and after the supplementation with both helichrysum and grapefruit extracts (**Table 2**). Statistical differences in the mRNA expression

of TNFα, MCP1 and COX2 were also found in epididymal adipose tissue, but only in the groups supplemented with the natural extracts (**Table 3**).

3.7 DNA methylation analysis. The methylation pattern of TNFα was measured in epididymal adipose tissue (**Fig. 3**). Interestingly, a hypermethylation (Δ of methylation: 2.5%) was detected in the CpG 3 (CpG site + 5 bp) after supplementation with grapefruit extract ($p < 0.01$) (**Fig. 3B**). Moreover, TNFα CpG3 methylation levels (%) showed a positive correlation with body weight gain (g) ($r = 0.562$, $p < 0.05$) and WAT (%) ($r = 0.706$, $p < 0.01$) (**Fig. 3C**), suggesting a link between DNA methylation, inflammation and adipose tissue mass.

4. Discussion

Persistent efforts to identify potential compounds that can be useful in the control and treatment of T2DM have been devoted. In this sense, flavonoids are attractive candidates because of a widespread presence in nature and their potential pharmacological effects.[11] Flavonoids are bioactive constituents abundant in the grapefruit and helichrysum extracts. Different *in vitro* and *in vivo* studies have shown beneficial roles of flavonoids in inflammation,[6,10] hyperlipidemia[24, 25] and diabetes.[11] With regard to the anti-diabetic effects of the 6-week supplementation with grapefruit and helichrysum extracts, lower fasting blood glucose levels were found when compared to the non-treated *db/db* mice. At the end of the experimental period, we noted that the mice were already in a state of diabetes with symptoms that caused severe metabolic disturbances. However, the grapefruit extract administration apparently delayed cachexia associated with diabetes and showed slightly higher levels of RQ, suggesting a better management of the carbohydrate metabolism. This improvement in metabolic glucose utilization as an energy source was significantly

correlated with the results obtained from the OSTT. Concerning the molecular mechanisms implicated, previous studies have shown that flavonoids can improve glucose metabolism by stimulating peripheral glucose uptake in the adipose tissue.[26, 27] GLUT4, an insulin sensitive glucose transporter, plays an important role in glucose transport in peripheral tissues.[28] Thus, hesperidin and naringin enhanced GLUT4 expression in WAT in type-2 diabetic mice.[29] Likewise, naringenin improved insulin-stimulated glucose uptake in 3T3-L1 cells.[30] Kaempferol and kaempferol 3-neohesperidoside (the flavonoid glycoside) showed insulinomimetic effects and stimulation of glucose uptake in differentiated 3T3-L1 adipocytes.[31, 32] Conversely, in our study no significant differences among the experimental groups were found in the expression of GLUT4 and InsR in adipose tissue.

However, although no statistically significant, a slight increase in betatrophin gene expression of supplemented groups was found. The expression of betatrophin in adipose tissue may be an indicator of the action of pancreatic β-cells,[33] but the mechanisms involved in the control of the proliferation of pancreatic β-cells are still unclear.[33]

Furthermore, it has been reported that flavonoids may directly act on pancreatic β-cells.[34] In an *in vitro* study, naringenin downregulated the expression of GCK and Ins1, suggesting an enhancement of glucose-stimulated insulin secretion and glucose sensitivity in INS-1E cells.[35] In the present study, no significant differences were found in the expression of GCK and Ins1 in pancreas, which might be due to different factors like the dose used, the time or the period of supplementation.

In liver, glucose is phosphorylated by glucokinase (GCK) and, depending on the cell's requirements, can be stored via glycogenesis activation (PEPCK) or oxidized to generate ATP (glycolysis). In this sense, previous studies showed

that dietary supplementation with hesperidin and naringin improved hyperglycemia by altering the expression of genes involved in glycolysis and gluconeogenesis in liver.[14,15] Jung et al. [29] showed increased liver expression of GCK after administering hesperidin and naringin in *db/db* mice, whereas naringin reduced the expression of PEPCK and G6Pase. Moreover, the inhibition of PEPCK decreased the hepatic glycogen content and finally improved the glucose metabolism. Park et al. [14] found a significantly lower expression of PEPCK in the liver of *db/db* mice supplemented with *citrus* extract. However, they did not find significant differences in G6Pase expression. In our study, no significant differences were obtained in the expression of PEPCK and G6Pase in the liver. Meanwhile, liver GCK expression was significantly higher in the mice supplemented with grapefruit and helichrysum extracts, suggesting that the anti-diabetic effects may occur in the liver by affecting the enzymes involved in glycolysis and gluconeogenesis. Thus, there is a negative correlation between blood glucose levels and liver GCK expression ($p < 0.001$), proposing that the decrease of glucose levels may be related to an increase of liver glucose sensitivity.

Several studies reported that down-regulation of inflammatory cytokine genes, including TNFα or MCP1, protect against the development of insulin resistance and hyperglycemia in obese mice.[36-38] Flavonoids might also act by interfering with the secretion of proinflammatory cytokines, improving thus the state of T2DM and obesity.[10] In this sense, mice supplemented with kaempferol showed an inhibition of proinflammatory gene expression by modulating the NF-kB signaling cascade.[39] Likewise, Park et al.[40] showed that kaempferol also inhibited COX2, iNOS and MCP1 gene expression in the kidney of aged Sprague-Dawley rats. Our data indicates that the

supplementation with grapefruit and helichrysum extracts seems to have a favorable effect on the inflammatory status in *db/db* mice. In cultured cells, lipopolysaccharide (LPS)-stimulated macrophages treated with naringenin presented lower expression of TNFα and IL-6.[41] Several studies in animals analyzing the effects of *citrus* flavonoids have also shown a preventive effect on obesity- and diabetes-associated inflammation.[11,24,25] Thus, mice treated with naringin showed lower serum TNFα levels,[42] whereas naringenin and naringin suppressed the activation of NFkB.[43] Although the inflammatory pathways regulated by these flavonoids have not been fully elucidated, a recent study suggested that local upregulation of TNFα in intestine was more sensitive than circulating cytokine levels.[44] Recent studies have found that TNFα is a key player in adipose tissue chronic inflammation, inducing the activation/inhibition of signaling cascades that perpetuate the inflammatory status and cause insulin resistance and hyperlipidemia by activating NFkB.[45] TNFα is usually overexpressed in the adipose tissue of different animal models of obesity and insulin resistance.[46]

Concerning epigenetic modifications, DNA methylation may influence the pathogenesis of T2DM and inflammation[1,47] and dietary factors are a major aspect of the environment that may influence DNA methylation.[48] One of the epigenetic modifications of the TNFα gene is an increase in DNA methylation.[49] In this sense, we measured the methylation pattern of the promoter and first exon of TNFα. The results suggest that the DNA methylation levels of TNFα were higher in the *db/db* mice supplemented with grapefruit extract. Interestingly, we have found correlations between DNA methylation in the CpG3 and body weight gain and the percentage of WAT. Previous studies of our group have evidenced a role of dietary factors on the modulation of TNFα DNA methylation[50] and have reported that the

promoter methylation levels of TNFα could be used as an epigenetic biomarker concerning the response to a low-calorie diet in obese women.[50]

To date, no study with *citrus* flavonoids and kaempferol have analyzed their effects on DNA methylation. However, other bioflavonoids, such as quercetin, fisetin, myricetin and tea catechins, have been reported to exert an effect on this epigenetic mechanism.[9,51]

These results suggest that epigenetic changes in TNFα could subsequently contribute to ameliorate inflammation and finally improve insulin resistance-induced hyperglycemia. The supplementation with helichrysum and grapefruit extracts shows beneficial effects against diabetes and obesity associated inflammation associated to diabetes and obesity in *db/db* mice. These changes may be due, at least in part, to small epigenetic modifications that can be induced by the flavonoids and other compounds found in the natural extracts. Regarding the implication of inflammation in DNA methylation patterns,[52] flavonoids could be an interesting therapeutic tool in the management of this situation. Thus, defining the role of epigenetic regulation of TNFα may lead to new therapeutic strategies for these metabolic diseases through the modulation of the inflammatory status.[53] However, more detailed studies at the molecular and cellular levels are needed to determine how both extracts exert their anti-diabetic activity as well as the individual compounds with more effect.

In summary, helichrysum and grapefruit extracts modulate hyperglycemia and TNFα-mediated inflammation in a diabetic model. Advances in this area may open the door to recognize the epigenetic regulatory role of different bioactive compounds involved in the metabolic control and the conditions that facilitate DNA methylation.

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Abbreviations

ActB, beta actin; AUC, area under curve; COX2, ciclooxigenase-2; EE, energy expenditure; ELISA, enzyme-linked-immunosorbent assay; G6Pase, glucose 6-phosphatase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GCK, glucokinase; GLUT4, glucose transporter-4; InsR, insulin receptor; IPGTT, intraperitoneal glucose tolerance test; IR, insulin resistance; MCP1, monocyte chemotactic protein-1; NFkB, nuclear factor-kappaB; OSTT, oral starch torelance test; PEPCK, phosphoenolpyruvate carboxykinase; RQ, respiratory quotient; RT-PCR, reverse transcription and quantitative real-time polymerase chain reaction; SGLT1, sodium-dependent glucose transporter-1; T2DM, type 2 diabetes mellitus; TNFα, tumor necrosis factor-α; WAT, white adipose tissue.

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Table 1. Effects of flavonoid-containing extracts from helichrysum and grapefruit on body weight, tissues, and biochemical measurements.

	Non-treated	Acarbose	Helichrysum	Grapefruit
Weight gain (g)	9.6 ± 3.5	10.6 ± 2.2	11.6 ± 2.0	13.6 ± 2.8 *
Food efficiency (g/100 kcal)	0.75 ± 0.04	0.72± 0.01	0.68 ± 0.02	0.80 ± 0.01
Total WAT (%)	51 ± 0.9	50 ± 1.1	52 ± 0.6	53 ± 0.4
Liver (g/bw)	4.4 ± 0.5	4.5 ± 0.2	4.3 ± 0.2	4.2 ± 0.2
RQ 24 h	0.78 ± 0.02	0.75 ± 0.03	0.79 ± 0.02	0.81 ± 0.03 *
EE 24 h (kg/day/bw3/4)	122 ± 16	112 ± 4	111 ± 9	114 ± 15
Blood glucose (mmol/L)				
Initial	10.5 ± 2.0	10.2 ± 1.1	9.4 ± 0.7	9.4 ± 1.3
Final	27.3 ± 1.5	24.5 ± 1.7	20.0 ± 1.4 *	20.1 ± 1.8 *
Pancreatic insulin (µg/mL * mg protein)	0.78 ± 0.00	0.79 ± 0.01	0.80 ± 0.01	0.80 ± 0.01
HbA1C (ng/mL)	2.31 ± 0.12	2.23 ± 0.14	2.13 ± 0.15	2.17 ± 0.13
Triglycerides (mg/dL)	136 ± 10	127 ± 10	145 ± 9	139 ± 7
Creatinine (mg/dL)	0.42 ± 0.08	0.33 ± 0.11	0.41 ± 0.08	0.34 ± 0.06

Results are expressed as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with the non-treated group. Non-treated and acarbose groups (n = 6); helichrysum and grapefruit groups (n = 8). * $p<0.05$; ** $p<0.01$.

Table 2. Effects of flavonoid-containing extracts from helichrysum and grapefruit on mRNA expression in liver. Genes related to glucose metabolism and inflammation.

			FOLD CHANGE		
Metabolism	**Gene description**	**Non-treated**	**Acarbose**	**Helichrysum**	**Grapefruit**
Glucose	GCK	1.0 ± 0.2	2.8 ± 0.2 ***	1.8 ± 0.2 *	1.8 ± 0.1 *
	G6Pase	1.0 ± 0.3	1.3 ± 0.3	1.2 ± 0.2	1.3 ± 0.2
	PEPCK	1.0 ± 0.2	1.5 ± 0.4	1.3 ± 0.3	0.9 ± 0.2
	Betatrophin	1.0 ± 0.2	1.4 ± 0.4	1.2 ± 0.5	0.9 ± 0.2
Inflammation	TNFα	1.0 ± 0.2	0.3 ± 0.1 *	0.6 ± 0.2	0.4 ± 0.1 *
	MCP1	1.0 ± 0.1	0.4 ± 0.3 *	0.2 ± 0.1 **	0.3 ± 0.2 *
	COX2	1.0 ± 0.2	0.1 ± 0.1 ***	0.1 ± 0.1 ***	0.1 ± 0.1 ***
	NFkB	1.0 ± 0.2	0.5 ± 0.2 *	0.4 ± 0.1 **	0.4 ± 0.1 **

Results are expressed as fold changes compared to housekeeping (GAPDH), and shown as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-treated group (normalized to 1). (n = 6) * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 3. Effects of flavonoid-containing extracts from helichrysum and grapefruit on mRNA expression in epididymal adipose tissue. Genes related to glucose metabolism and inflammation.

			FOLD CHANGE		
Metabolism	**Gene description**	**Non-treated**	**Acarbose**	**Helichrysum**	**Grapefruit**
Glucose	InsR	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
	GLUT4	1.0 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
	Betatrophin	1.0 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	1.4 ± 0.1
Inflammation	TNFα	1.0 ± 0.2	0.8 ± 0.2	0.5 ± 0.1 ***	0.7 ± 0.2 **
	MCP1	1.0 ± 0.1	0.8 ± 0.4	0.5 ± 0.2 *	0.6 ± 0.4
	COX2	1.0 ± 0.3	0.7 ± 0.1	0.5 ± 0.3 **	0.5 ± 0.2 **
	NFkB	1.0 ± 0.3	0.9 ± 0.2	0.7 ± 0.3	0.8 ± 0.3

Results are expressed as fold changes compared to housekeeping (GAPDH), and shown as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-treated group (normalized to 1). (n = 6) * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

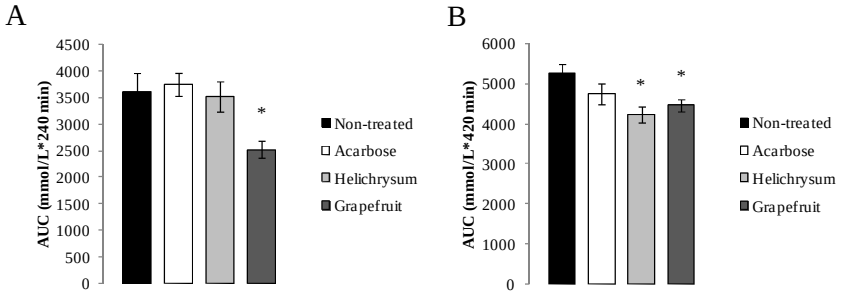

Fig. 1 Area under the curve (AUC) after the oral starch tolerance test - OSTT (A) and the intraperitoneal glucose tolerance test - IPGTT (B) in *db/db* mice. Results are expressed as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-treated group. Non-treated and acarbose groups (n = 6); helichrysum and grapefruit groups (n = 8). * $p < 0.05$.

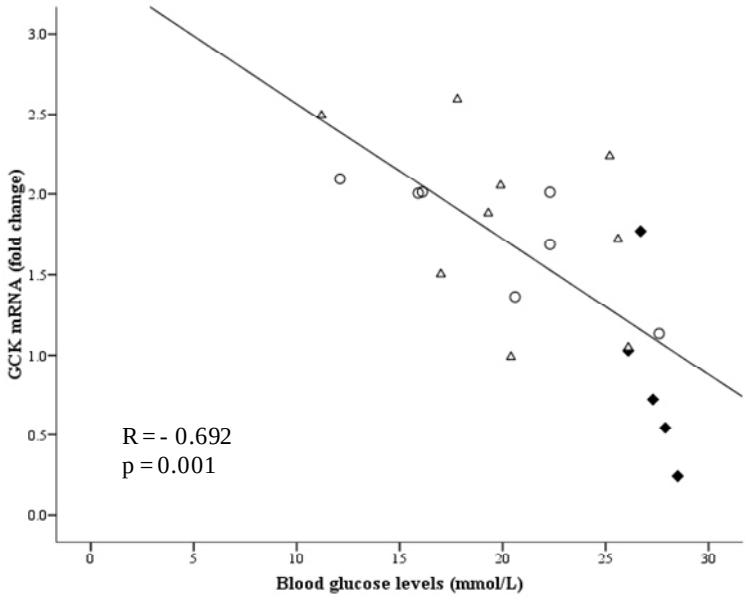

Fig. 2 Correlation analyses between GCK gene expression in liver (fold change) and final blood glucose (mmol/L). R, Pearson's correlation coefficient. Results are expressed as mean. Non-treated (n = 5); helichrysum and grapefruit groups (n = 8). (♦ non-treated group, Δ helichrysum group and ○ grapefruit group)

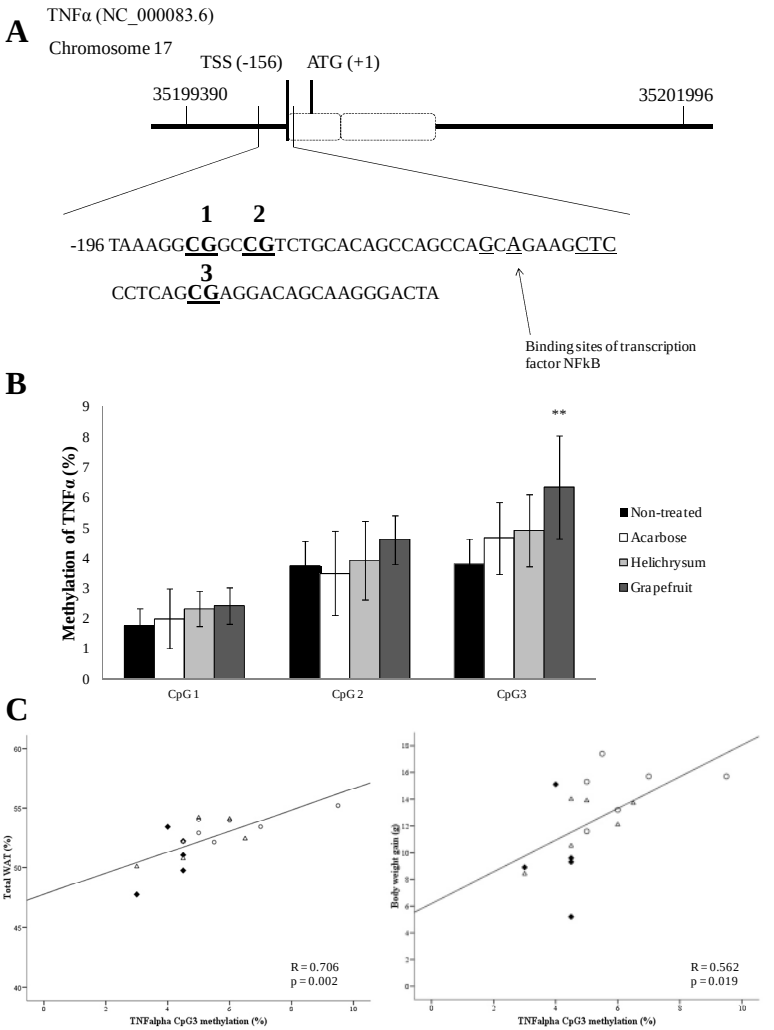

Fig. 3 Nucleotide sequence of the CpG island in the TNFα promoter and exon regions showing individual CpG dinucleotides **(A)**. Effect of helichrysum and grapefruit extracts in the methylation levels of individual CpG dinucleotides in the TNFα promoter in adipose tissue **(B)**. Correlation analyses between percentage of DNA methylation and **(C)** Total WAT (%) and **(D)** body weight gain (g). Results are expressed as mean ± SD. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-treated group. R, Pearson's correlation coefficient. (n = 6). ** $p < 0.01$. (♦ non-treated group, Δ helichrysum group and ○ grapefruit group).

Summary Chapter 2

	Helichrysum	Grapefruit
Weight gain (g)		↑
Fasting blood glucose levels	↓	↓
Pancreatic insulin content	=	=
Glucose metabolism in liver (GCK)	↑	↑
Inflammation in liver:		
MCP1	↓	↓
COX2	↓	↓
NFkB	↓	↓
TNFα	↓	↓
Inflammation in adipose tissue:		
MCP1	↓	
COX2	↓	↓
TNFα	↓	↓
TNFα methylation levels		↑

Chapter 3

Helichrysum and grapefruit extracts boost weight loss in overweight rats reducing oxidative stress and inflammation

Running title: Helichrysum and grapefruit boosted weight loss

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Keywords: flavonoids, grapefruit, helichrysum, insulin resistance, TLR2, TNFα

ABSTRACT

Background: Obesity is characterized by an increased production of inflammatory markers. High levels of circulating free fatty acids and chronic inflammation lead to increased oxidative stress, contributing to the development of insulin resistance. Recent studies have focused on the potential use of flavonoids for obesity management due to their antioxidant and anti-inflammatory properties. This study was designed to investigate the antioxidant and anti-inflammatory effects of helichrysum and grapefruit extracts in overweight insulin resistant rats.

Design and methods: Thirty eight male Wistar rats were randomly distributed in two groups: control group (n=8) and HFS group (n=30). After 22 days of *ad libitum* water and food access, the rats fed HFS diet changed to standard diet and were re-assigned into three groups (n=10 each group): non-supplemented, helichrysum (2g/kg bw) and grapefruit (1g/kg bw) for 5 weeks.

Results: Rats supplemented with both extracts gained less body weight during the 5 week period of treatment, showed lower serum insulin levels and liver TBARS levels. Leptin / adiponectin ratio, as an indicator of insulin resistance, was lower in both extract-administered groups. These results were accompanied by a reduction in TNFα gene expression in epididymal adipose tissue and intestinal mucosa, and TLR2 expression in intestinal mucosa.

Conclusions: Helichrysum and grapefruit extracts might be used as a complement hypocaloric diets in the weight loss treatment. Both extracts helped to reduce weight gain, hyperinsulinemia and insulin resistance, improved inflammation markers and decreased the HFS diet-induced oxidative stress in insulin resistant rats.

INTRODUCTION

High-fat diets may play an important role in the increase prevalence of obesity.[1] Obesity is a multifactorial disease usually accompanied by insulin resistance (IR) and an increase in oxidative stress and inflammatory markers, including tumor necrosis factor-α (TNFα) and toll- like receptors (TLRs).[2] TNFα is an important mediator of insulin resistance acting as a link between obesity and inflammation.[3] Adipose tissue does not act only as fat storage, but also as a major endocrine and secretor organ that produces different adipokines such as leptin and adiponectin.[4] Thus, leptin is a hormone secreted by adipocytes that regulates energy homeostasis,[5] while adiponectin is an anti-inflammatory adipokine which improves insulin sensitivity.[6] Toll-like receptors (TLRs) play a crucial role in the innate immune response, being their expression elevated in obese adipose tissue.[7] There is increasing evidence concerning the involvement of TLRs in obesity-induced inflammation and insulin resistance.[8] In this sense, TLR2 has been shown to be stimulated by free fatty acids, and the activation of these receptors has been found to induce insulin resistance.[9] Likewise, oxidative stress is a mediator mechanism implicated in these pathological conditions and contributes to the release of cytokines and inflammation.[10] Oxidative stress induces tissue damage, which is responsible for the lower insulin sensitivity in different tissues.[11]

Numerous strategies have been developed to treat chronic diseases. Regard natural extracts, have been studied as dietary supplements for body weight management. Helichrysum and grapefruit extracts contain different kind of flavonoids,[12] and are attractive candidates as antioxidants and anti-inflammatory agents because they are abundant in nature and may have fewer side-effects.[13-15] Some reports have shown that *citrus* flavonoids

improved lipid metabolism,[16] insulin sensitivity,[17] decreased serum TNFα and ameliorated serum and liver MDA.[18] Beneficial roles of *helichrysum* flavonoids, such as kaempferol-3-*O*-glucoside have been reported in inflammation, hyperglycemia and hyperlipidemia.[19] Based on these protective properties the aim of this study was to analyze the beneficial effects of flavonoids present in helichrysum and grapefruit on inflammation and oxidative stress-related states induced by high-fat sucrose (HFS) diet in insulin resistant rats.

MATERIALS AND METHODS

Natural extracts and diets.

A standard pelleted chow diet containing 20% of energy as proteins, 67% as carbohydrates (5% sucrose and 62% starch) and 13% as fat by dry weight (290 kcal/100 g diet), was purchased from Harlan Ibérica (Teklad Global, Barcelona, Spain; ref. 2014). High-fat sucrose (HFS) diet contains 20% of energy as protein, 35% as carbohydrates (18% sucrose, 10% maltodextrin and 7% starch) and 45% as lipids by dry weight (473 kcal/100 g diet) as detailed by the supplier (Research Diets, New Brunswick, NJ, USA; ref D12451). Helichrysum (*Helichrysum italicum*) and grapefruit (*Citrus x paradisi*) extracts were provided by "Biosearch S.A." (Granada, Spain). Plant samples (1-5 g) were pulverized and mixed with washed sea sand and introduced into the extraction cells, where 30 ml of each solvent at 50 °C was added: methanol/water (3:1) and methanol/water (1:1) for helichrysum and grapefruit, respectively. The quantification of the phenolic compounds in both extracts (helichrysum and grapefruit) was performed by UPLC-MS/MS and is shown in Supplementary Table S1.

Experimental design.

Thirty eight male Wistar rats from CIFA of the University of Navarra (Pamplona, Spain), with an initial average weight of 260 g ± 11, were kept in an isolated room maintaining to a temperature between 21 and 23 °C, controlled humidity (50 ± 10%), and a 12h:12h artificial light/dark cycle with water and food ad libitum. The experimental protocol was approved by the Animal Research Ethics Committee of the University of Navarra (04/2011).

Development of the overweight, glucose intolerant animal model

The animals were randomly assigned into two groups: control group (n = 8) and HFS group (n = 30). During 22 days, rats had *ad libitum* water and food access. Body weight and food intake were recorded 3 times per week. At day 20, glycemia was measured by venous tail puncture using a glucometer and blood glucose test strips (Optium Plus, Abbott Diabetes Care, Witney Oxon, UK). The glucose content was expressed as milligrams per deciliter (mg/dL).

Second phase: treatment with the natural extracts

The thirty rats fed the HFS diet were randomly divided into three dietary groups: non-supplemented (n=10), helichrysum (2 g/kg bw) (n=10), and grapefruit (1 g/kg bw) (n=10). From this period, all the rats were fed a standard diet. Body weight and food intake were recorded three times per week. Glycemia was measured at day 41 of the total experimental period. After 35 days (5 weeks) of supplementation, rats were sacrificed by decapitation without anesthesia, where blood was collected from the trunk. Liver, different adipose depots (subcutaneous, retroperitoneal, epididymal and mesenteric) and intestinal mucosa were carefully dissected and

weighed. Tissue samples and serum were immediately stored and frozen (-80 °C) for further analyses.

Serum measurements.

Glucose levels were determined with the HK-CP kit; total cholesterol, with the Cholesterol-CP kit; HDL Cholesterol, with the HDL direct-CP kit (ABX diagnostic, Montpellier, France); triglycerides with the RANDOX triglycerides kit (Randox Laboratories, Crumlin, UK); and free fatty acids with the free fatty acid reagent (NEFA) adapted for the PENTRA C200 equipment (HORIBA Medical, Montpellier, France). Serum insulin (Mercodia AB, Uppsala, Sweden), leptin (Linco Research, St Charles, MO, USA), adiponectin (Linco Research, St Charles, MO, USA), and TNFα (Diaclone SAS, Besancon Cedex, France) were determined by enzyme-linked-immunosorbent assay (ELISA) using an automatized TRITURUS equipment (Grifols International S.A., Barcelona, Spain). Serum thiobarbituric acid reactive substrates (TBARS) were measured colorimetrically following the manufacturer's protocols (Cayman Chemical Company, MI, USA). The homeostasis model assessment (HOMA) was estimated as fasting serum glucose (mM) multiplied by fasting serum insulin (μU/mL) divided by 22.5. L / A ratio and A / HOMA ratio were also calculated to estimate insulin resistance, as described elsewhere.[20,21]

Liver determinations.

TBARS in liver were measured colorimetrically following the manufacturer's protocols for tissue homogenates (Cayman Chemical Company, MI, USA).

RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis.

Total RNA was extracted from intestinal mucosa and epididymal adipose tissue using All-Prep® DNA/RNA/protein mini kit as described in the manufacturer's instructions (Qiagen, Germantown, Md., USA). RNA concentration and quality were measured by Nanodrop Spectrophotometer 1000 (Thermo Scientific, DE, SA). Then, RNA (2 μg) was reverse-transcribed to cDNA using MMLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) as described by the suppliers. RT-PCR assays were performed following the manufacturer's recommendations using an ABI PRISM 7000 HT Sequence Detection System and predesigned TaqMan® Assays-on-Demand by Applied Biosystems (Austin, Tex., USA). Tumor necrosis factor α (TNFα), Rn01525859_g1; Toll like receptor 2 (TLR2), Rn02133647_s1; and Taqman Universal Master Mix were provided by Applied Biosystems. mRNA levels were normalized with Beta actin (ActB), Rn00667869_m1 as housekeepting gene (Applied Biosystems). All samples were analyzed in triplicate. The relative expression level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

DNA extraction and bisulfite conversion.

Genomic DNA was isolated from intestinal mucosa and epididymal adipose tissue using the All-Prep® DNA/RNA/protein mini kit as described in the manufacturer's instructions (Qiagen, Germantown, Md., USA).DNA concentration and quality were measured by Nanodrop Spectrophotometer 1000 (Thermo Scientific, DE, SA). The stock solution of DNA samples was stored at -80 °C until use. For epigenetic analysis, all DNA samples were bisulfite treated using the EpiTectBisulfite Kit (Qiagen, Hilden, Germany), resulting in the deamination of unmethylated cytosine to uracil. DNA

concentration was quantified with a Pico100 (Picodrop Limited, Hinxton, UK). All procedures were carried out according to the supplier's protocols.

PCR and methylation analysis by DNA pyrosequencing.

DNA methylation levels in intestinal mucosa and epididymal adipose tissue were determined by pyrosequencing using a PyroMark Q24 (Qiagen). PCR was carried out in 25 µl reaction mixtures with 12.5 µl PyroMark 2x PCR master mix, 0.15 nM of primers for TNFα, 5' – TGAGAGAGGTGTAGGGTTATT – 3' (forward), 5' – TCTCCCTCCTAACTAATCC – 3' (reverse), 2.5 µl coralLoad Concentrate 10x (Qiagen) and 1 µl of DNA samples after bisulfite conversion, at concentration of 10 ng/µl. PCR conditions were 95 °C for 15 minutes; 45 cycles of 94 °C for 30 s, 51.5 °C for 45 s, 72 °C for 45 s; and a final elongation at 72 °C for 10 minutes. PCR products were checked by 2% agarose gel electrophoresis. A total of 22 µl of the PCR product was used for subsequent pyrosequencing using a PyroMark Q24 System (Qiagen). All procedures of quantification of CpG methylation levels were performed based on a protocol described elsewhere.[22] For quality control, each experiment included non-CpG cytosines as internal controls to verify efficient bisulfite DNA conversion.

Statistical analysis.

All the results are expressed as the mean $\pm$ standard error (SE). Normality was tested with the *Shapiro*-Wilk test. Statistical significance of differences among the groups was evaluated using One-Way ANOVA test followed by Dunnett's post hoc test, and dependent samples t-test used to test differences in the mean of each group. The two-tailed Pearson test was used to assess correlations between L/A ratio and different measures. A level of

probability of $p<0.05$ was set as statistically significant. All analyses were performed using SPSS 15.0 packages of Windows (Chicago, IL).

RESULTS

Development of the overweight insulin resistant animal model.

In the first phase, the HFS group (n=30) was significantly heavier in body weight as compared to the control group (n=8) (Table 1). In addition, the HFS group showed significantly higher levels of glycemia at the end of this period (Fig. 2). In our study, after the 3-weeks, the rats fed HFS diet gained more body weight ($p < 0.001$) and showed hyperglycemia ($p < 0.001$), suggesting a model of insulin resistance.

Body weight gain and body fat mass after supplementation period.

The rats treated with helichrysum and grapefruit extracts for 5 weeks gained less body weight ($p < 0.01$, Table 1) than the non-supplemented animals. The body fat mass was significantly lower in the grapefruit extract (Table 1) than in the non-supplemented group. However, helichrysum group showed no change as compared to the non-supplemented group.

Improvement of insulin resistance status induced by high-fat diet after supplementation period.

At day 41th, the rats supplemented with the grapefruit extract showed significantly lower fasting glucose levels in comparison with non-supplemented group (Fig. 2). However, after 5-weeks of supplementation period, differences between groups were not statistically significant (Table 2). Both extracts significantly decreased insulin serum levels (Table 2). The grapefruit extract also normalized serum leptin and adiponectin levels (Table 2). In the non-supplemented group the HOMA index, commonly used in the assessment of insulin resistance status, was higher than in the helichrysum and grapefruit groups (Fig. 3A). L / A and A / HOMA ratios, also

recovered to normal levels as a result of the supplementation with helichrysum and grapefruit extracts (Fig. 3B and 3C). Regarding lipid metabolism, at the end of the study, there were no differences between the experimental groups (Table 2).

Antioxidant effect after the supplementation period with helichrysum and grapefruit extracts.

The effect of the HFS diet on the rat redox status is reported in Fig. 4. While in serum there were no differences between groups concerning TBARS levels (Fig. 4A), the hepatic content of TBARS was significantly higher in the non-supplemented group (Fig. 4B). Interestingly, the administration of helichrysum and grapefruit extracts produced a positive effect on oxidative damage induced by HFS diet in liver.

Anti-inflammatory effect after supplementation period with helichrysum and grapefruit extracts.

Helichrysum extract reduced the circulating levels of TNFα (Fig. 5A). The expression of this proinflammatory cytokine in intestinal mucosa was down-regulated after supplementation with both natural extracts (Fig. 5C), while in epididymal adipose tissue, the levels of this cytokine were significantly ameliorated only by grapefruit (Fig. 5B). In addition, the expression levels of TLR2 in intestinal mucosa significantly decreased in both supplemented groups (Fig. 5D)

Finally, the methylation pattern of TNFα was measured in intestinal mucosa and epididymal adipose tissue (Fig. S2). There were no differences between groups with the exception of a slight hypermethylation (Δ of methylation: 1%) in the CpG 1 (CpG site -178 bp) after supplementation with helichrysum extract in intestinal mucosa (Fig. S2B).

DISCUSSION

Flavonoids are widely recognized for biological and pharmacological effects including antioxidant and anti-inflammatory properties.[23] Epidemiological studies suggest that the prevalence of some diseases, such as obesity, is inversely correlated with the consumption of food rich in flavonoids.[24] Thereby, in this report we evidenced that both extracts might have beneficial effects on obesity-related insulin resistance, inflammation and oxidative stress. The experimental design included two phases. In the first one, HFS diet initiated a state of insulin resistance and significantly increased body weight gain and fasting blood glucose levels. The objective was to create an animal model mimicking some of the metabolic characteristics of human obesity. In a second phase, the four groups received a standard diet, but two of the HFS groups were supplemented with helichrysum and grapefruit extracts. In this sense, the body weight gained during the 5-week supplementation period was significantly lower with both extracts compared to the non-supplemented group. In this experimental design, the animals were treated with natural extracts but at the same time the HFS diet was replaced by a standard diet (caloric restriction) in order to initiate a conventional weight loss treatment as usually performed in humans.[25] Likewise, at the end of the supplementation period, glucose levels were decreased in all groups due to the change of diet, but during the second phase (day 41) hyperglycemia induced by HFS diet diminished after supplementation with grapefruit extract. Naringin is the most abundant flavanone in grapefruit, and is usually converted to its corresponding aglycone (naringenin) and sugars by intestinal bacteria following ingestion. In this sense, in a streptozotocin (STZ)-induced model of diabetes in rats, the oral administration of naringin (50 mg/kg) for 4

weeks ameliorated hyperglycemia and oxidative stress.[18] In another study, supplementation with naringin (200 mg/ kg diet) during 5 weeks, decreased blood glucose levels as well as plasma insulin and leptin concentrations in *db*/*db* mice.[26] In the current study, the non-supplemented group exhibited significantly higher HOMA-IR than groups supplemented with both extracts. However, a recent study highlighted that the L / A ratio may be more powerful than HOMA-IR for evaluating insulin resistance.[20] In this context, there was a positive correlation ($p < 0.001$) between HOMA-IR and L / A ratio. Adiponectin has emerged as a protective adipokine in insulin resistance.[27] Thus, Makni et al.[21] demonstrated that A / HOMA ratio is the most sensitive predictor of insulin resistance in obese children. Thus, in our study, this ratio also reached normal levels after dietary supplementation. In accordance with adiponectin, it has been reported that visceral fat accumulation or adipocyte hypertrophy induce insulin resistance and the mechanism or action involves decreased secretion of adiponectin and increased secretion of TNFα.[28] Our results suggest that the grapefruit extract increase serum adiponectin levels.

Furthermore, it is known that diet-induced obesity is associated with an inflammatory state, which promotes oxidative stress and insulin resistance. Accordingly, the rats supplemented with helichrysum extract showed lower serum TNFα levels than the non-supplemented group. Regarding the characterization of helichrysum extract, we found that the most abundant flavonoid is kaempferol-3-*O*-glucoside. In this sense, kaempferol was reported to antagonize TNFα and to exert beneficial roles in inflammation.[24] On the other hand, some animal studies carried out with the supplementation of *citrus* flavonoids have also shown anti-inflammatory effects associated with obesity.[29]

Furthermore, in addition to linking adipose tissue as an organ associated with inflammation-related diseases, recently are focusing in the gastrointestinal tract as a target of early inflammation associated with diet-induced obesity.[30] In this regard, in some studies which no changes in plasma TNFα were found, correlations with ileal TNFα expression and the body weight gain were detected.[30] This may suggest that local inflammation in intestine could be more sensitive than circulating cytokine levels.[30] Indeed, supplementation with both extracts significantly inhibited the mRNA expression of TNFα and TLR2 in intestinal mucosa. Thus, the supplementation with helichrysum and grapefruit extracts inhibited the diet-induced intestinal inflammation that may represent an early state that precedes and predisposes to obesity and insulin resistance and impairs energy metabolism. Yoshida et al.[7] showed for the first time that citrus flavonoid naringenin inhibited TLR2 expression and suppressed TNFα-induced TLR2 expression by inhibiting the NF-kB and JNK pathways in *in vitro* differentiated adipocytes. Finally, they also confirmed that naringenin decreased TLR2 expression in the adipose tissue of HFD fed obese mice.[7]

The methylation pattern in the promoter area of TNFα was measured in intestinal mucosa, in order to understand a possible epigenetic effect of the extracts rich in flavonids. Our finding suggests that DNA methylation at least in the region analyzed, does not regulate TNFα gene expression in intestinal mucosa.

Oxidative stress is also implicated in the development of obesity complications such as insulin resistance.[31] A correlative and causative association has been reported between inflammation, oxidative stress and insulin resistance.[31] On the other hand, obesity exhibit high oxidative stress due to persistent and chronic hyperglycemia.[10] In this sense, in our study we

observed significant correlations between L / A ratio, as an indicator of insulin resistance, and serum TNFα levels and liver TBARS levels. TBARS have been previously observed to be exacerbated in the liver of the HFD-fed obese rats[32] as well as in serum and liver of diabetic rats.[33] In this sense, both extracts induced a significant reduction of liver TBARS levels. Furthermore, Mahmoud et al.[18] found lower concentrations of MDA in serum and liver after treatment with naringin in diabetic rats. Thus, drugs with antioxidant potential may attenuate obesity and insulin resistance related-states.

CONCLUSIONS

Taking together, these results suggest that helichrysum and grapefruit extracts downregulate the expression of several proinflammatory cytokines and improve their circulating levels in insulin-resistant rats, decreasing also the HFS diet induced oxidative stress in liver and ameliorating insulin resistance.

In summary, supplementation with helichrysum and grapefruit extracts boosted the beneficial metabolic effects of caloric restriction, as indicated by the reduced weight gain and lower levels of blood glucose, insulin, leptin and adiponectin. The anti-inflammatory and antioxidant effects of the extracts may be responsible for these results, although further studies are required to determine the mechanisms underlying these observations as well as the applicability of these findings to humans.

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Table 1. Effect of helichrysum and grapefruit extracts on weight and adiposity-related parameters.

	Control	Non-supplemented	Helichrysum (2 g/kg bw)	Grapefruit (1 g/kg bw)
1st phase: HFS period				
Initial body weight (g)	256 ± 15	259 ± 9	259 ± 8	258 ± 14
Body weight gain (g)	78 ± 13 ***	113 ± 19	117 ± 13	111 ± 22
Food efficiency (g/100 kcal)	5.7 ± 0.9	6.4 ± 0.9	6.2 ± 0.7	6.1 ± 0.9
2nd phase: Supplemention period				
Final body weight (g)	410 ± 38 **	459 ± 34	436 ± 23	430 ± 34
Body weight gain (g)	76 ± 20	86 ± 19	60 ± 14 **	61 ± 16 **
Food efficiency (g/100 kcal)	3.9 ± 0.8 *	4.8 ± 0.7	4.3 ± 0.4	4.1 ± 0.6
Subcutaneous fat (g)	5.8 ´2.0 **	8.8 ± 2.0	8.2 ± 1.6	6.9 ± 1.4 *
Visceral fat (g)	21.0 ± 7.4 **	30.7 ± 5.6	28.7 ± 3.6	24.2 ± 4.6 *
Retroperitoneal fat (g)	8.5 ± 3.5 **	13.1 ± 2.6	12.6 ± 2.2	10.5 ± 2.2
Epididymal fat (g)	8.0 ± 2.6 **	11.4 ± 2.4	10.2 ± 1.0	8.9 ± 1.8 *
Mesenteric fat (g)	4.6 ± 1.5 *	6.3 ± 1.0	5.9 ± 1.4	4.9 ± 1.5
Fat mass (g)	26.8 ± 9.1 **	39.6 ± 7.3	36.9 ± 5.0	31.1± 5.5 *

Results are expressed as mean ± SE. Fat mass indicates the sum of the subcutaneous fat and visceral fat. Visceral fat indicates the sum of the retroperitoneal, epididymal and mesenteric fats. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with the non-supplemented group. * $p<0.05$; ** $p<0.01$.

Table 2. Effect of helichrysum and grapefruit extracts on serum biochemical and hormonal parameters after the 5-week supplementation period.

	Control	Non-supplemented	Helichrysum (2 g/kg bw)	Grapefruit (1 g/kg bw)
Glucose (mmol/L)	5.5 ± 0.3	6.1 ± 0.8	5.6 ± 0.5	5.6 ± 0.8
Insulin (uU/mL)	12.1 ± 5.2 *	24.1 ± 15.1	14.4 ± 4.4 *	12.2 ± 4.9 *
Leptin (ng/dL)	11.7 ± 9.9 *	22.2 ± 7.4	16.1 ± 7.1	12.0 ± 8.1 *
Triglycerides (mg/dL)	100 ± 32	114 ± 38	120 ± 33	126 ± 43
Total cholesterol (mg/dL)	65.6 ± 9.7	74.8 ± 14.5	66.3 ± 5.2	78.7 ± 10.1
HDL cholesterol (mg/dL)	20.6 ± 1.6	23.4 ± 3.6	21.3 ± 1.5	24.3 ± 2.5
Free fatty acids (mg/dL)	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Adiponectin (ng/mL)	76.8 ± 9.8 *	57.1 ± 4.9	71.6 ± 17.5	75.3 ± 13.5 *

Results are expressed as mean ± SE. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with the non-supplemented group. * $p<0.05$; ** $p<0.01$.

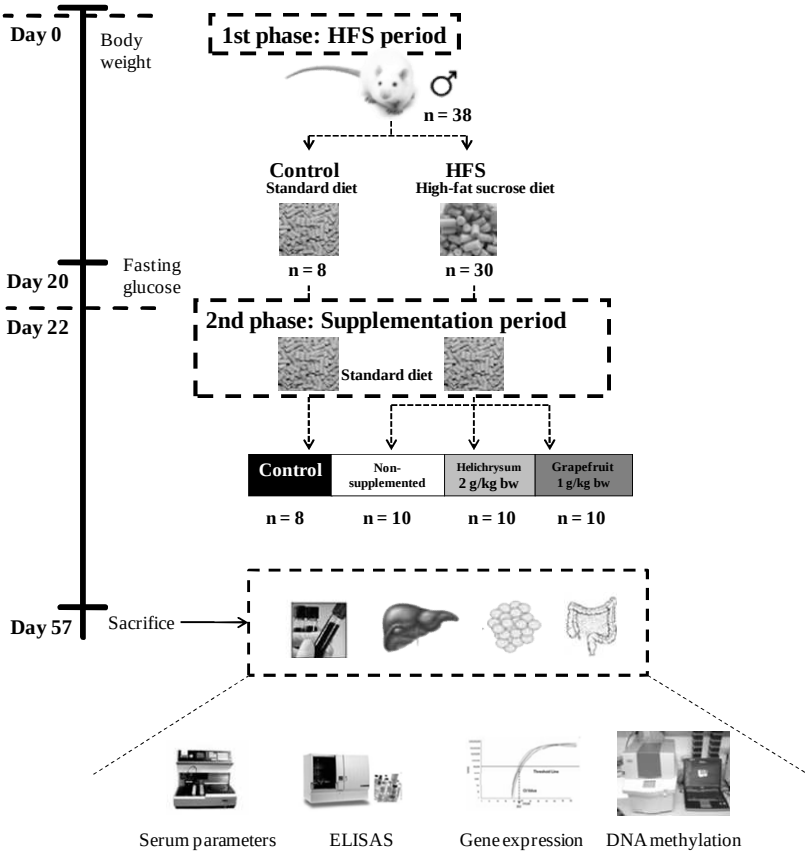

Fig. 1 Experimental design

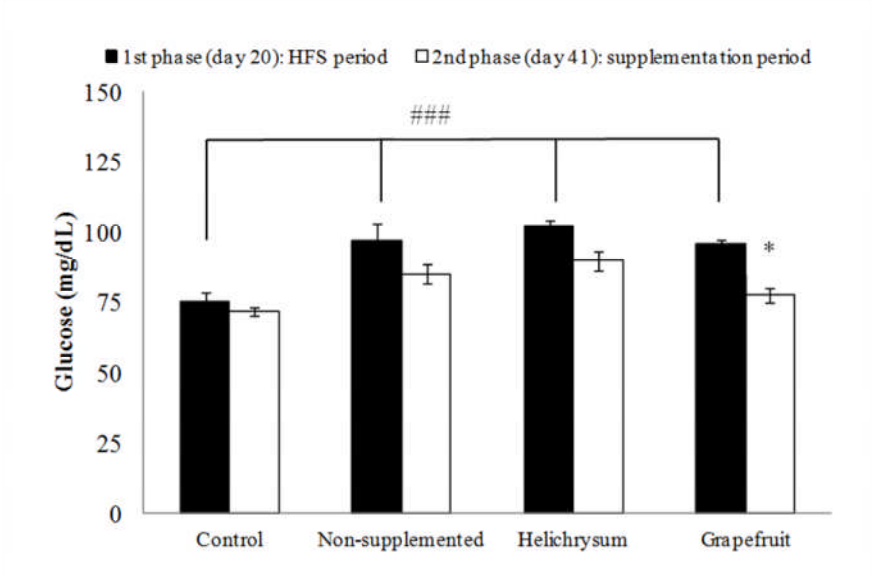

Fig. 2 Effect of helichrysum and grapefruit extracts on serum glucose levels. Results are expressed as mean ± SE. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with control group ### $p < 0.001$ and dependent samples t-test used to test differences in the mean of each group * $p < 0.05$. Control group (n = 8); non-supplemented, helichrysum and grapefruit groups (n = 10). [black columns, final of the 1st phase (day 20); white columns, 2nd phase (day 41)]

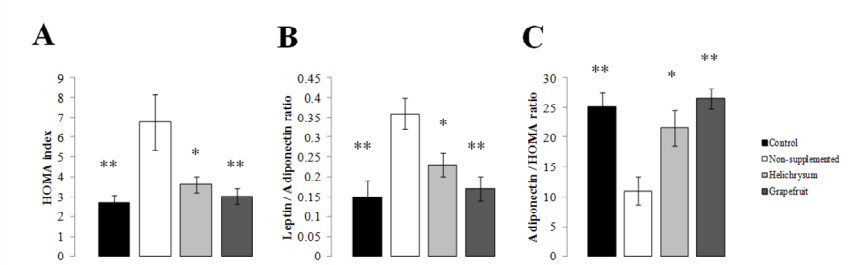

Fig. 3. Effect of helichrysum and grapefruit extracts on different markers of insulin resistance. HOMA index (A), Leptin / Adiponectin ratio (B) and Adiponectin / HOMA ratio (C). Results are expressed as mean ± SE. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-supplemented group. Control group (n = 8); non-supplemented, helichrysum and grapefruit groups (n = 10).* $p < 0.05$, ** $p < 0.01$.

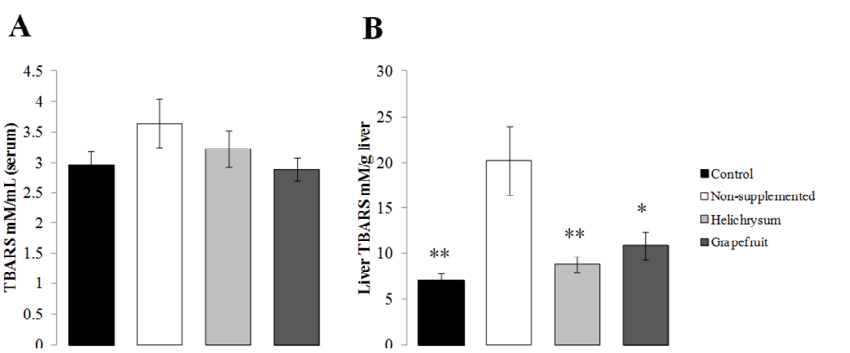

Fig. 4. Effect of helichrysum and grapefruit extracts on serum TBARS (A) and liver TBARS levels (B) after the 5-week supplementation period. Results are expressed as mean ± SE. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-supplemented group. Control group (n = 8); non-supplemented, helichrysum and grapefruit groups (n = 10).* $p < 0.05$, ** $p < 0.01$.

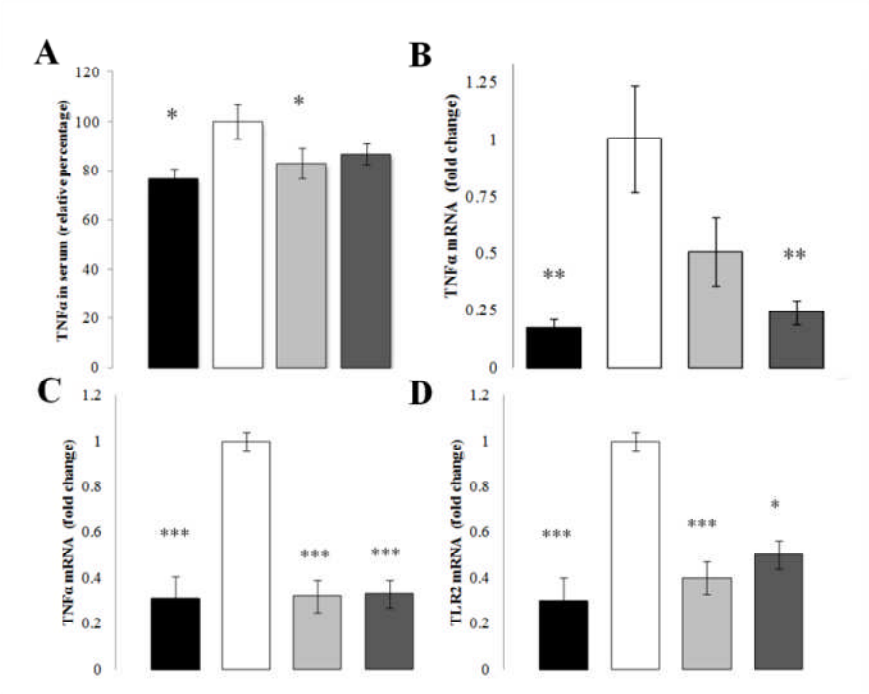

Fig. 5. Effect of helichrysum and grapefruit extracts on different inflammation-related parameters after the 5-week supplementation period. Serum TNFα (A), gene expression of TNFα in epididymal adipose tissue (B), gene expression of TNFα (C) and TLR2 (D) in intestinal mucosa in insulin resistant rats. Results are expressed as mean ± SE (A) or as fold changes compared to housekeeping (Act B) (B, C and D). Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-supplemented group (normalized to 100% or 1). Control group (n = 8); non-supplemented, helichrysum and grapefruit groups (n = 10).* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Table S1. Quantification (mg/kg extract) of Phenolic Compounds in Helichrysum and Grapefruit Extracts

phenolic compounds	helichrysum	grapefruit
phenolic acids		
gallic acid	7.3	10.9
caffeic acid	67.3	20.2
chlorogenic acid	1039	109
chlorogenic acid-3-*O*-glucoside	2515	40.2
flavonoids		
flavanones		
naringenin	230	1000
naringenin-7-*O*-glucoside	3892	219
naringenin diglycoside	1188	108
naringenin-7-*O*-rutinoside (narirutin)	37	5234
naringenin-4'-glucoside-7-rutinoside	0.03	83
hesperidin	11	711
flavonols		
kaempferol	7	nd[a]
kaempferol-3-*O*-glucoside	13375	6
kaempferol rutinoside	434	54193
myricetin glucoside	652	7
flavones		
metoxiluteolin	0.8	0.03
flavanols		
epigallocatechin	nd	nd
epigallocatechin-3-*O*-gallate	29	4

[a] nd, not detected.

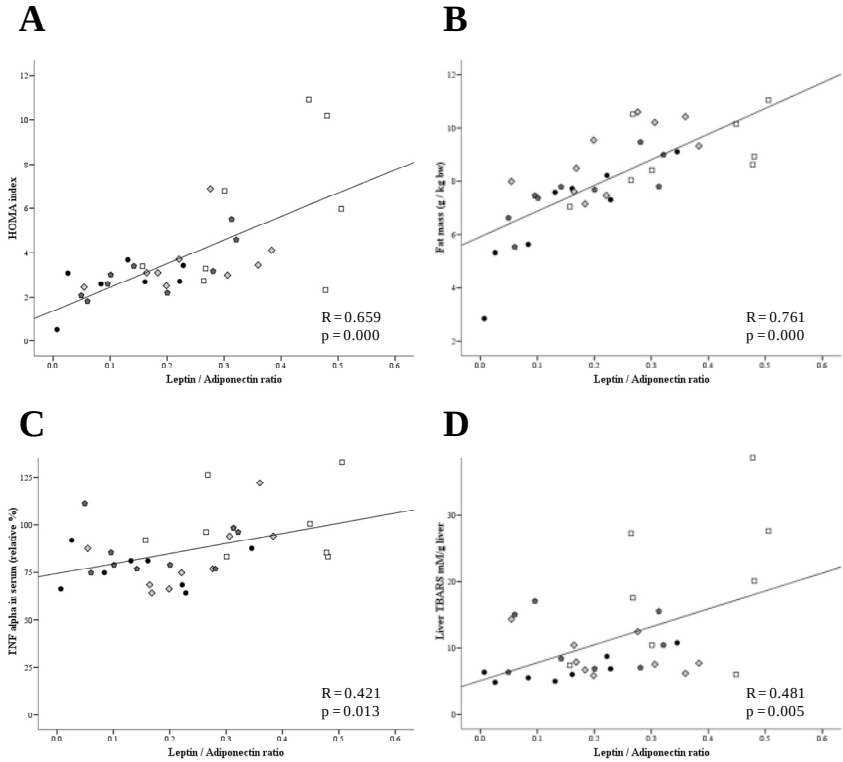

Fig. S1. Correlation analyses between HOMA index and L / A ratio (A), L / A ratio and fat mass (B), L / A ratio and serum TNFα (C) and L / A ratio and liver TBARS (D). R, Pearson's correlation coefficient. (n = 6). (• control group, □ non-supplemented group, ◇ helichrysum group and ⬟ grapefruit group)

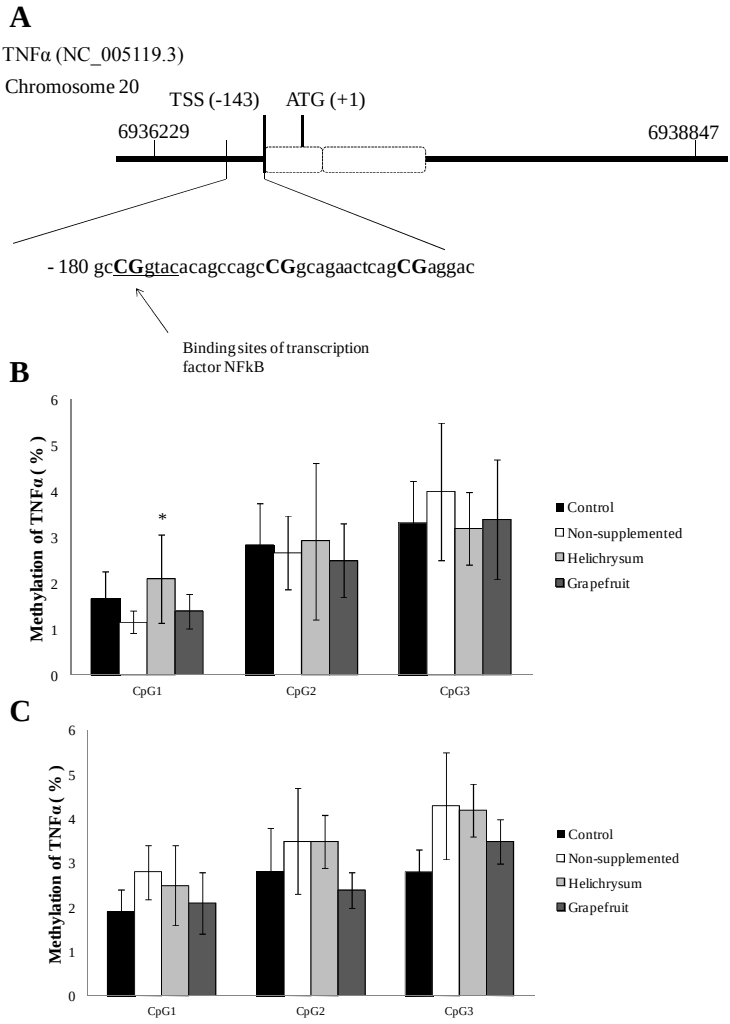

Fig. S2. Nucleotide sequence of the CpG island in the TNFα promoter showing individual CpG dinucleotides (A). Effect of helichrysum and grapefruit extracts in the methylation levels of individual CpG dinucleotides in the TNFα promoter in intestinal mucosa (B) and epididymal adipose tissue (C). Results are expressed as mean ± SE. Statistical analysis was performed using ANOVA test and Dunnett's test was used to analyze differences in the mean of each group with non-supplemented group. (n = 6). * $p < 0.05$.

Summary Chapter 3

	Helichrysum	Grapefruit
Weight gain (g)	↓	↓
Fat mass (g)		↓
Fasting blood glucose levels		↓
Serum insulin levels	↓	↓
Serum leptin levels		↓
Serum adiponectin levels		↑
HOMA index	↓	↓
L / A ratio	↓	↓
Liver TBARS levels	↓	↓
Serum TNFα levels	↓	
TNFα mRNA in adipose tissue		↓
TNFα mRNA in intestinal mucosa	↓	↓
TLR2 mRNA in intestinal mucosa	↓	↓
TNFα methylation levels : Intestinal mucosa Epididymal adipose tissue	= =	= =

V Discussion

What did the study show? In the previous section (results) we present the detailed discussions of each chapter. In this section, we show the results analyzed together.

What we found? Comparing with prior knowledge, what is new? Future research.

General discussion

Obesity and diabetes are two of the biggest emerging health problems worldwide, not only in developed countries, but also in transition countries (WHO, 2014). While obesity can start as a physiological adaptive condition, it ends up becoming a physiopathology that, according to WHO, (2014), is the fifth leading risk factor for death in the world. Likewise, obesity is generally associated with insulin resistance, oxidative stress, inflammation and dyslipidemia (Kushner, 2014, Bondia-Pons et al., 2012), increases the risk of developing T2DM, being almost half of the cases of T2DM attributable to obesity (WHO, 2014).

In this context, several therapeutic approaches are being used to fight against obesity, considering the diet and exercise as the main pillars, followed by pharmacological treatment (Chatzigeorgiou et al., 2014). However, considering that some of the drugs targeting obesity have been discontinued from sale due to their side effects, there is growing interest in new strategies to manage and combat the progression of obesity and diabetes, such as for example the development of functional foods (Arch, 2008).

Therefore, there is renewed interest in the study of natural products as sources of bioactive compounds to be used in a new generation of functional foods (Dulloo, 2011). These bioactive compounds occur in different vegetable parts (leaves, stems, flowers, roots, tubers or fruits) and include a group of substances characterized by the presence of a phenol group per molecule, the polyphenols (Zamora-Ros et al., 2010). Flavonoids are the most common polyphenols and can be subclassified into six

subgroups (flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins) as described elsewhere (Pan et al., 2010).

Scientific evidence shows that flavonoids have beneficial health effects (Pan et al., 2010, Babu et al., 2013). Some of these bioactive compounds exhibit pharmacological properties as antioxidants (Savini et al., 2013), anti-inflammatory (Pan et al., 2010), anti-obesity (de la Garza et al., 2011), and anti-diabetic (Etxeberria et al., 2012), among others. Furthermore, previous studies have found that various natural extracts and compounds may have beneficial effects in the gastrointestinal tract (Kazeem et al., 2013, Liu et al., 2013), such as the inhibition of various enzymes involved in the digestion of fat, like pancreatic lipase (de la Garza et al., 2011), or in the digestion of carbohydrates, such as pancreatic α-amylase and intestinal α-glucosidase (Tundis et al., 2010, Etxeberria et al., 2012).

In this context, there are commercially available drugs focused on inhibiting the digestion of different macronutrients such as Orlistat®, an inhibitor of pancreatic lipase, used for the treatment of obesity (Bray and Ryan, 2014); and acarbose®, which is an anti-diabetic drug that inhibits the activity of the α-glucosidase (Grover and Utreja, 2014). Also, it has been found that grape seed and tea extracts are potent inhibitors of α-amylase and α-glucosidase activities (Yilmazer-Musa et al., 2012), while *Moringa oleifera* leaf extract is an α-glucosidase inhibitor (Adisakwattana and Chanathong, 2011). Likewise, onion extracts (Kim et al., 2011b) and mulberry leaf extracts (Kim et al., 2011a) are inhibitors of α-glucosidase. Bean (Preuss, 2009), peanut seed skin (Tsujita et al., 2014), *Salvia virgata* (Nickavar and Abolhasani, 2013) and other extracts (Tundis et al., 2010) have shown α-amylase inhibitory activities.

Regarding this scientific evidence, a food complement that acts on the carbohydrate metabolism by inhibiting the absorption of this macronutrient has been developed (Excess 500 Control®), whose effect is attributed to the presence of *Phaseolus vulgaris* that, as clinical studies show, has an inhibitory effect on α-amylase activity, thus contributing to weight loss and reducing postprandial hyperglycemia (Barrett and Udani, 2011).

In our research, the two extracts (helichrysum and grapefruit) have demonstrated *in vitro* effects on the activity of digestive enzymes and consequently improved postprandial blood glucose levels in rats by diminishing carbohydrate digestion/absorption in the gastrointestinal tract (de la Garza et al., 2013). Previous studies have shown that different helichrysum species had anti-diabetic properties (Aslan et al., 2007). However, none of these studies have directly attributed the anti-diabetic effect to an inhibition of digestive enzyme activity. Furthermore, extracts from *Citrus x paradisi* have also been investigated in relation with their antihyperglycemic properties (Adeneye, 2008), but without specifying the mechanism responsible for this outcome. In this regard, Shen et al., (2012) found that *citrus* flavonoids had an inhibitory effect on starch digestion, thereby producing lower glucose uptake on HepG2 cells.

Consequently, before focusing on the study of the mechanisms involved in the experimental effects of helichrysum and grapefruit extracts, the quantification of phenolic compounds was carried out, which in accordance with the available scientific literature (Gouveia and Castilho, 2010, Uckoo et al., 2012), showed that both extracts contained phenolic acids and flavonoids as flavanones and flavonols subclasses. In helichrysum extract, the flavanones found in higher proportions were naringenin-7-*O*-

glucoside and naringenin diglycoside. Kaempferol-3-*O*-glucoside is the flavonol that was found in the greatest proportion. Likewise, grapefruit extract mainly contained naringenin-7-*O*-rutinoside and naringenin as flavanones, and kaempferol-rutinoside as flavonol.

Interestingly, some studies evidenced that kaempferol, *in vitro*, has effects on the α-glucosidase activity (Matsui et al., 2002, Pereira et al., 2011, Habtemariam, 2011), whereas lower effects were found on α-amylase activity in another study (Tadera et al., 2006). On the other hand, a few studies have reported the effect of naringenin on digestive enzymes (Priscilla et al., 2014), while some trials have investigated the effects of the flavonoids found in the *citrus* fruits. Thus, Girones-Vilaplana et al., (2014) have recently reported that *citrus* fruits can be useful in the treatment of diseases such as obesity and diabetes due to their inhibitory effect on α-glucosidase activity. In our assays, both extracts had an inhibitory effect on α-glucosidase *in vitro* and only grapefruit extract showed similar results for the inhibition of α-amylase, also *in vitro*. Furthermore, helichrysum and grapefruit extracts inhibited α-glucosidase activity in intestinal sacs. And finally, both extracts decreased postprandial glycemia after the oral starch administration (α-amylase and α-glucosidase) in the *in vivo* results. In this way, our results suggested different responses depending on the assay model. For example, although grapefruit showed α-glucosidase inhibitory activity *in vitro* and in intestinal sacs, *in vivo* studies did not produce any effect on the activity of this enzyme. Some factors can affect the activity of digestive enzymes, such as pH, temperature and incubation time (Obiro et al., 2008). In all these procedures we used acarbose as a positive control for studying the inhibitory effect of both extracts on digestive enzyme activity. Thus, according with the *in vitro* results (IC_{50}), the amount of each extract

that was required to match the action of acarbose was calculated. Thereby, as the amylase inhibitory activity was lower for the helichrysum extract, higher doses of helichrysum extract were used in the *ex vivo* (noneverted intestinal sacs) and in the *in vivo* studies.

Moreover, in the intestinal sacs, an inhibition of starch digestion was not observed; however, a decrease in starch-derived glucose uptake was found. These findings led us to support that other mechanisms might be involved in the results obtained *in vivo*. Furthermore, previous studies have shown that the antihyperglycemic effect of flavonoids was not only due to the inhibition of enzyme activities, but also to an inhibition of glucose transporters found in the intestine (Williamson, 2013). In this respect, it is well known that phloridzin, a plant derivative glycoside, is a specific and competitive inhibitor of SGLT1 glucose transporter in the intestine (Alvarado, 1967). Therefore, we could hypothesize that both extracts might have inhibitory effects on the glucose transporter SGLT1 similar to phloridzin. Thus, an *in vitro* study with Caco-2 cells using phloridzin - as a positive control - was conducted, calculating the concentration of each extract according to the amount of phloridzin (0.5 mM) used by Goto et al., (2012) in a similar study. In this context, the results in Caco-2 cells suggest an inhibition of SGLT1- mediated glucose uptake by helichrysum and grapefruit extracts. The lactase phloridzin hydrolase (LPH) is located in the brush border of the small intestine epithelial cells. The absorption of flavonoid glycosides is associated with the released aglycone, as a result of the action of LPH, which may then enter the epithelial cells by passive diffusion (Del Rio, D., 2013). SGLT1 is a glucose transporter found in the intestinal mucosa of the small intestine. With the cloning of this intestinal brush border Na^+/glucose cotransporter, the specificity and kinetics of

SGLT1 for phenylglucopyranosides were investigated (Lostao et al., 1994). These authors showed that phenylglycosides may be categorized into three groups: transported substrates for SGLT1, blockers of SGLT1 and noninteracting molecules. Thereby, these results suggest that the high content of glycosilated flavonoids in both extracts would contribute, at least in part, to the inhibition of glucose uptake.

To understand the way that helichrysum and grapefruit extracts modulate postprandial blood glucose levels, we designed an *in vivo* intervention study with genetically diabetic mice (C57BL/6J *db/db* mice) during 6 weeks. This research was conducted in a recognized model of obesity and diabetes, *db/db* mice, displaying characteristics such as overweight, hyperglycemia and hyperinsulinemia due to leptin receptor mutations (Herberg and Coleman, 1977). On the other hand, according to the literature, some examples that used acarbose - as a positive control - for studying the effects of various natural extracts or compounds on postprandial glucose levels were found (Chang, et al., 2006, Youn, et al., 2004). Therefore, in this study, a reduction in fasting blood glucose levels that may be attributed to the inhibition of digestive enzyme activities was shown. Moreover, changes in body weight and the RQ were observed in the group of mice that were supplemented with grapefruit extract, suggesting a better management of the carbohydrate utilization. Previous authors, such as Preuss et al., (2002) showed that *citrus aurantium* (rich in naringin) increased thermogenesis whereas da-Silva et al., (2007) showed that kaempferol increased cellular EE. However, our results do not show differences between groups in relation with the possible impact of the flavonoids found in both extracts on increasing energy expenditure.

Hence, to precisely understand the mechanism of both extracts at the molecular level, we analyzed the expression of genes involved in the glucose metabolism in different tissues and observed that liver GCK expression was significantly higher in these mice supplemented with grapefruit and helichrysum extracts. In this sense, we found a negative correlation between blood glucose levels and liver GCK expression suggesting better glucose sensitivity in liver. Similarly to our results, Jung et al., (2006) showed increased liver expression of GCK after administering hesperidin and naringin in *db/db* mice.

In T2DM, inflammation may play an important role in the disease progression. In this sense, some studies explored whether inflammatory mediators envisage the development of T2DM. Thus, Duncan et al., (2003) showed that some inflammatory markers predicted the future occurrence of T2DM in adults. Chronic inflammation in peripheral tissues, such as adipose tissue and liver, provokes insulin resistance and induces β-cell dysfunction in the pancreas (Schuster, 2010). In this sense, some researchers (Park et al., 2013) have found not only that *citrus* unshiu peel extract ameliorated hyperglycemia by altering hepatic glucose regulating enzymes, but also that these effects might be related with anti-inflammatory effects in the liver. Therefore, according to the results obtained in our previous studies, we wondered if our extracts could also have anti-inflammatory properties. Other studies, such as those of Sala et al., (2002) and Antunes Viegas et al., (2014), reported anti-inflammatory properties in helichrysum extract. Furthermore, Dallas et al., (2014), that studied the effect of different *citrus* fruits, showed positive results regarding inflammation and healthy weight management in overweight individuals. The authors attributed these results to the presence of polyphenols in the extract they administered. Similarly, in

our study the supplementation with helichrysum and grapefruit extracts seems to have a favorable effect on the inflammatory status (TNFα, COX2, MCP1 and NFkB gene expression) in liver and adipose tissue of *db/db* mice. Similar results of flavonoid-rich extracts or isolated flavonoids as anti-inflammatory agents have been previously reported (Pan et al., 2010). In this context, mice treated with naringin showed lower serum TNFα levels (Pu et al., 2012), naringenin (2%) suppressed the activation of NFkB in kidney (Tsai et al., 2012), naringin improved glucose intolerance and metabolic disorders by reducing inflammatory cell infiltration in rats (Alam et al., 2013), naringin ameliorated insulin resistance and β-cell dysfunction in T2DM rats partly by regulating inflammation (Sharma et al., 2011), and Pan et al., (2010) reported beneficial effects of kaempferol on inflammation.

These results lead us to consider that helichrysum and grapefruit extracts could improve glucose metabolism in a disturbed metabolic situation, *i.e.*, in a state of chronic T2DM. Given that generally diet-induced obesity leads to T2DM, that is usually accompanied by an impairment in insulin action leading to insulin resistance, and is also associated with a state of low-grade chronic inflammation and oxidative stress (Tanti and Jager, 2009), we sought to study whether the antihyperglycemic and anti-inflammatory properties of the extracts, could potentiate possible outcomes following a strategy focused on weight loss (caloric restriction), as well as the mechanisms involved in this beneficial effect.

Thus, we aimed to analyze this hypothesis by studying the effect of the supplementation with helichrysum and grapefruit extracts on overweight insulin-resistant rats. Moreover, all the measurements that we have studied in relation with inflammation so far were performed in

adipose tissue and liver. However, the gastrointestinal tract has been also considered as a target of early inflammation associated with diet-induced obesity (Ding and Lund, 2011), which also suggests that it may be more sensitive that changes in serum cytokine levels (Ding and Lund, 2011). In this sense, the expression of different inflammatory markers were measured and analyzed in intestinal mucosa, and according to the results, we could assume that the supplementation with helichrysum and grapefruit extracts ameliorated the diet-induced intestinal inflammation that may represent an early state in the development of insulin resistance and obesity (Ding and Lund, 2011). In this sense, Yoshida et al., (2013) showed for the first time that the *citrus* flavonoid naringenin inhibited TLR2 expression and suppressed TNFα-induced TLR2 expression by inhibiting NFkB in adipocytes. Our results suggest that intestinal inflammation may be an early indicator of other diseases and that the diet can influence the development of the early inflammation.

Analyzing the data, the supplemented groups had better results on the response to the diet, consequently reflected in higher weight loss, decreased levels of insulin and leptin and increased adiponectin levels in serum. Furthermore, inflammation and oxidative stress-related states were improved in the insulin resistant HFS-fed rats. These results cannot be directly attributed to the lower caloric intake compared to the non-supplemented group, because no differences were found in food efficiency between groups. This finding means that supplementation with both extracts did not decrease the food intake in our study, as well as Andrade and Burgess, (2007) showed no influence of naringenin (30-120 mg/kg of diet) in food consumption.

In this sense, we can hypothesize that the positive effects found in our study may be due to supplementation with both extracts. Reviewing the literature, there are few comparable studies and not all of them show similar results. For example, Wang et al., (2013) conducted a study with obese mice that were fed a high-fat diet for 22 weeks. After this period, energy intake was reduced (10%) and two groups were supplemented with curcumin or piperine. All the groups showed positive outcomes after caloric restriction in relation to body weight, glucose and insulin levels and inflammation. However, the presence of curcumin and/or piperine did not add any extra effect. On the other hand, in our study the groups supplemented with helichrysum and grapefruit boosted the beneficial effects of caloric restriction. In this sense, the anti-inflammatory properties of both extracts may contribute to attenuate obesity and insulin resistance-related conditions.

Therefore, in both animal models (*db/db* mice and overweight rats) the supplementation with helichrysum and grapefruit extracts was accompanied by anti-inflammatory effects. Thus, in both *in vivo* trials, an improvement was found in both supplemented groups in the circulating levels and gene expression of TNFα in adipose tissue, liver and intestinal mucosa. In this sense, it has been reported that TNFα plays an important role in the pathogenesis of some diseases (Kawaguchi et al., 2011) and that the intake of flavonoids, such as naringin, is able to modulate chronic inflammatory diseases (Kawaguchi et al., 2011). Furthermore, Kim et al., (2007) administered kaempferol in rats and showed a downregulation of TNFα expression via NFkB inactivation. Moreover, it has also been observed that kaempferol may inhibit oxidative stress via the inhibition of TNFα expression in aged gingival tissues (Kim et al., 2007). In this context,

regarding oxidative stress, although antioxidant effects were found in the liver of overweight and insulin-resistant rats supplemented with both extracts, there were no positive effects in serum. Thus, while Sharma et al., (2011) reported the attenuation of oxidative stress in T2DM rats after supplementation with naringin, the most abundant glycosidic flavonoid in our extracts, new studies would be required to test the antioxidant properties of helichrysum and grapefruit.

After analyzing the anti-inflammatory properties of both extracts, and observing positive effects on glucose metabolism and weight loss, we sought to discover if epigenetic mechanisms could be involved in the regulation of gene expression (Milagro and Martinez, 2013) and, in this sense, if nutriepigenetics could be implicated in the prevention of inflammation-related obesity and diabetes. In this context, according with the scientific literature, there is evidence about the epigenetic regulation of genes involved in diseases such as obesity and diabetes, and the possible modifications of their expression due to the influence of nutritional factors (Milagro et al., 2013). Indeed, several studies have demonstrated the role of polyphenols in the modulation of DNA methylation linking to various diseases such as cancer (Vanden Berghe, 2012). However, few studies have shown a direct relationship between the bioactive compounds and the epigenetic modulation of genes involved in metabolic diseases such as obesity and diabetes. A previous study from our group showed that apple polyphenols modulated changes in two CpG sites in the leptin promoter in rat epididymal adipocytes (Boque et al., 2013). In relation with the nature of helichrysum and grapefruit extracts, one study have demonstrated that broccoli extracts, containing kaempferol, regulated the progression of cancer through anti-inflammatory and epigenetic mechanisms (Ferguson

and Schlothauer, 2012). Otherwise, although there is scientific evidence of the effect of naringin on gene expression, there is no study showing that this effect may be due to an epigenetic modulation.

Thus, taking into account our previous results, the role of flavonoid-rich extracts on DNA methylation in *in vivo* animal models of obesity and diabetes was investigated. In addition, previous studies from our group have recorded the methylation levels of TNFα as a potential biomarker of inflammation and predictor of weight loss in overweight patients following a hypocaloric diet (Campion et al., 2009). Furthermore, Cordero et al., (2011) also studied the role of TNFα as a possible predictor of response to a low calorie diet.

Accordingly, when we analyzed the methylation of TNFα, a hypermethylation in one CpG site (CpG site+5 bp) was found after the supplementation with grapefruit extract in *db/db* mice. These methylation levels showed a positive correlation with body weight gain and percentage of WAT, suggesting a link between DNA methylation, inflammation and adipose tissue mass. However, no differences in the TNFα methylation pattern were found in the study with overweight insulin-resistant rats.

Therefore, these results suggest that changes in the methylation levels of TNFα may sometimes contribute to improve the inflammatory status associated with an unbalanced metabolic state. Although these advances open up new opportunities to study and understand the role of different bioactive compounds in epigenetic regulation, more studies are needed.

Since the composition of both extracts, such as other natural extracts rich in flavonoids, is complex, different substances can be detected

and may be responsible for the possible beneficial health effects. Therefore, the identification and interpretation of the results can be difficult. In this context, the study of isolated compounds has been considered in order to analyze the possible effect of each one, but also the combination of various compounds or extracts to study the synergistic or antagonistic effects they both may have. As an example, Adisakwattana et al., (2012) observed that the combination of roselle, chrysanthemum and butterfly pea extracts with mulberry extract had a synergistic action on intestinal maltase inhibition. However, the interactions between different phenolic compounds can also reduce the positive effects found in an isolated form (Adisakwattana et al., 2012).

Likewise, it is important to consider the dose, assay models and results obtained for the possible therapeutic applications. For example, in our studies we have used doses according to the results obtained in *in vitro* studies to avoid overdose and therefore adverse effects. Other studies have used doses of 50 mg/kg bw of *citrus* extract in STZ diabetic rats (Mahmoud et al., 2012), 200 mg/kg bw, in *db/db* mice (Jung et al., 2006) and 1200 mg /kg bw, in obese Zucker rats (Raasmaja et al., 2013). Other investigators (de Sousa et al., 2004), have used kaempferol-3,7-*O*-α-dirhamnoside, a major flavonoid found in *Bauhinia forficata* leaves, at different doses (50, 100 and 200 mg/kg). In another study, kaempferol glycoside-rich fraction from unripe soybean leaves, at a concentration of 0.15% in the diet, was used in KK-A^{y} mice. This concentration was calculated to provide an amount of kaempferol of 0.095% (Zang et al., 2011).

Although our results are promising, further studies are needed on humans to examine the bioavailability of these bioactive compounds in the human body. The recent technological advances allow us to discover that

the glycosydic flavonoids are present in most plant products and, when they are ingested, some of them may be absorbed in the small intestine or be partially metabolized in the colon. If they are absorbed, most of them lose the glycoside and are modified in different metabolites that are found in fluids or tissues of the body (Escudero-Lopez et al., 2013). Therefore, to further study these bioactive compounds, more human studies are needed to verify the intestinal uptake and fermentation, as well as the mechanism of action once the responsible agent or agents (*i.e.,* synergistic effects) are identified and their biovailability is known. On the other hand, additional human trials should be conducted to test the "dose-response" and the pharmacokinetic profile of the bioactive compounds occurring in the natural extracts. Thus, following the FDA guidelines (FDA, 2005) for the selection of the initial dose in humans, the non-observed adverse effect level (NOAEL) in animal models is commonly used to calculate the human equivalent doses (HED). In this context, the NOAELs (mg/kg) are multiplied by the factor 0.08 for studies in mice, and 0.16 in rats. The lowest of these results (HED) are divided by the correction factor (/10) and finally multiplied by the body weight in humans (*i.e.*, 70 kg). Therefore, according to the dose used in our mice model, the human equivalent dose of helichrysum extract would be 8 mg/kg bw or 560 mg for an adult weighing 70 kg, whereas the dose of grapefruit extract would be 4 mg/kg bw or 280 mg per day.

The two most abuntant flavonoids in both natural extracts studied in this project are kaempferol (flavonol) and naringin (flavanone). Both of them are glycosidic flavonoids and are fairly common in the plant kingdom. Other natural extracts with beneficial effects that have been reported to contain big proportions of these two flavonoids are those of *Nymphaea mexicana* Zucc. (Hsu et al., 2013), *Sambucus nigra* L. (Bhattacharya et al.,

2013) and *Phaleria macrocarpa* (Hendra et al., 2011). According to the composition of our extracts, the amounts of these flavonoids used in our mice studies were about 13 mg/kg bw per day of kaempferol and 4 mg/kg bw per day of naringin for helichrysum extract, and 27 mg/kg bw per day of kaempferol and 2.5 mg/kg per day of naringin for grapefruit extract.

Furthermore, Zamora-Ros, et al., (2010) showed that the mean of the estimated total flavonoid intake in Spanish population was 313.26 mg/day, of which 17% corresponds to the subgroup of flavanones and 6% to flavonols. According to the FDA guidelines (FDA, 2005) the doses of flavonoids used in these studies correspond to: 7.3 mg of kaempferol and 2.2 mg of naringin for an adult per day (helichrysum extract); and 15 mg of kaempferol and 1.4 mg of naringin per day (grapefruit extract). Nevertheless, there are other flavonoids and components in the extracts, including vitamins and minerals, which could also be responsible, at least in part, for the effects found in these studies.

A recent review that included articles from 2000 to 2012, Coelho et al., (2013) analyzed the effects of orange juice (rich in naringin) on inflammatory markers. The authors concluded that the flavanones found in the orange juice were probably responsible for some of the healthy properties. However, further studies are needed in order to identify the effects of isolated flavonoids in humans. Thus, the study of each flavonoid should be considered in an isolated form or in combination, in order to analyze the synergistic or inhibitory effect in different *in vitro* and *in vivo* assay models before testing them in controlled and randomized human intervention studies.

Summarizing all these data, the current work has analyzed the potential role of helichrysum and grapefruit extracts rich in flavonoids as anti-obesity and anti-diabetic agents. According to the FDA guidelines (FDA, 2005), the amount of each extract used in these studies is reasonably suited to daily use in humans. In our *in vitro* and animal studies, we observed that helichrysum and grapefruit extracts improved postprandial glycemic control in rats, possibly by inhibiting the activities of carbohydrate-digestive enzymes and decreasing SGLT1-mediated glucose uptake that may be relevant for the treatment of obesity and T2DM. Furthermore, in a diabetic animal model, helichrysum and grapefruit extracts improved glucose metabolism in liver and the inflammatory status in liver and adipose tissue. Finally, in a model of energy restriction in overweight insulin-resistant rats, anti-inflammatory and antioxidant effects were found which may be associated to the beneficial metabolic effects.

In this context and for oncoming studies, once the effectiveness and safety of the helichrysum and grapefruit extracts has been demonstrated, their use might be considered as a supplement and/or food complement or for the design of a functional food focused on weight loss and diabetes management.

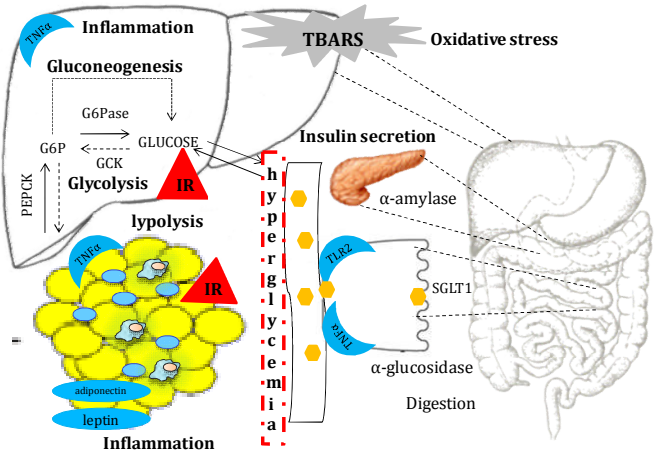

Figure 1. Physiophatology of obesity and diabetes. Hyperglycemia. Impaired insulin secretion (pancreas), leads to insulin resistance in peripheral tissues. Associate with a state of low-grade chronic inflammation and oxidative stress. Lypolysis.

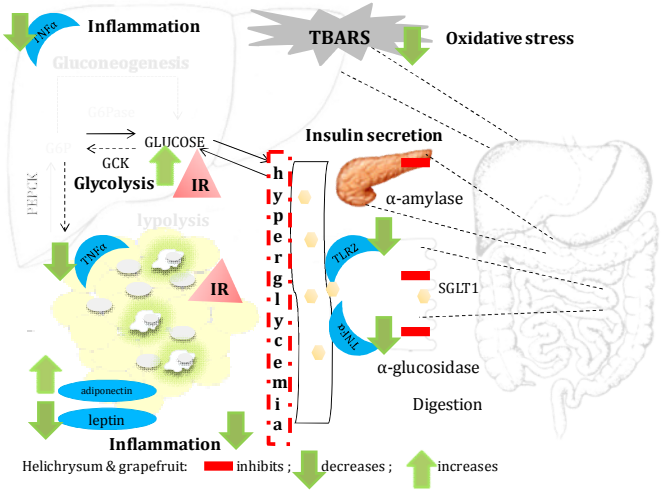

Figure 2. Effects of helichrysum and grapefruit on digestion / absorption of nutrients, modulate hyperglycemia, improve glucose metabolism in liver and insulin resistance, ameliorate inflammation and decrease oxidative stress.

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VI Conclusions

What the results mean?

Conclusions

1. Helichrysum and grapefruit flavonoid-rich extracts inhibited the activity of the α-amylase and α-glucosidase digestive enzymes in both *in vitro* and *in vivo* assays.

2. Helichrysum and grapefruit extracts decreased intestinal glucose uptake by inhibiting glucose transporter SGLT1 in Caco-2 cells, where glycosidic flavonoids content may be involved.

3. Helichrysum and grapefruit extracts showed beneficial effects on diabetes in a genetic animal model by retarding weight loss, and modulating hyperglycemia, which may be due to an improvement of glucose metabolism pathways (glycolysis) in liver.

4. Gene expression of several proinflammatory markers (TNFα, MCP1, COX2 and NFkB), in liver and adipose tissue of *db/db* mice, decreased after the supplementation with helichrysum and grapefruit extracts.

5. Epigenetic changes in TNFα after supplementation with grapefruit extract rich in flavonoids could subsequently contribute to explain and ameliorate inflammation processes (in adipose tissue) and improve insulin resistance-induced hyperglycemia.

6. Supplementation with a grapefruit extract in overweight rats fed a HFS diet boosted the beneficial effects of caloric restriction, caused higher weight loss, contributed to reduce the visceral and total fat depots, and further decreased blood leptin levels.

7. The administration of helichrysum and grapefruit extracts reduced fasting blood glucose levels and improved insulin sensitivity in overweight rats.

8. Helichrysum and grapefruit extracts downregulated the expression of several proinflammatory cytokines in adipose tissue (TNFα) and intestinal mucosa (TNFα and TLR2), and improved their circulating levels in overweight insulin-resistant rats, decreasing also the HFS diet induced oxidative stress in liver.

General conclusion

Helichrysum and grapefruit extracts have potential antihyperglycemic properties by acting on carbohydrate-digestive enzymes and SGLT1-mediated glucose uptake. Likewise, both extracts also improved hyperglycemia through the regulation of glucose metabolism in liver (GCK) and the reduction of inflammation in liver and fat depots (TNFα, MCP1, COX2 and NFkB) in a diabetic animal model.

Furthermore, helichrysum and grapefruit extracts also elicited anti-inflammatory (TNFα and TLR2) and antioxidant effects (liver TBARS) in overweight insulin-resistant rats, boosting the beneficial metabolic effects associated to energy restriction.

VII *Appendix*

Natural inhibitors of pancreatic lipase as a key players in obesity treatment // Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase // Prenatal stress increases the obesogenic effects of a high-fat-sucrose diet in adult rats in a sex-specific manner // Transcriptomic and epigenetic changes in the hypothalamus are involved in an increased susceptibility to a high-fat-sucrose diet in prenatally stressed female rats // Screening of polyphenolic plant extracts for anti-obesity properties in Wistar rats // Diet-induced hyperinsulinemia differentially affects glucose and protein metabolism: a high-throughput metabolomic approach in rats // Prevention of diet-induced obesity by apple polyphenols in Wistar rats through regulation of adipocyte gene expression and DNA methylation patterns.

Natural inhibitors of Pancreatic Lipase as new players in obesity treatment

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Abstract

Obesity is a multifactorial disease characterized by an excessive weight for height due to an enlarged fat deposition as adipose tissue, which is attributed to a higher calorie intake than the energy expenditure. The key strategy to combat obesity is to prevent chronic positive impairments in the energy equation. However, it is often difficult to maintain energy balance, because many available foods are high energy-yielding, which is usually accompanied by low levels of physical activity.

The pharmaceutical industry has invested many efforts in producing antiobesity drugs; but only a lipid digestion inhibitor obtained from an actinobacterium is currently approved and authorized in Europe for obesity treatment. This compound inhibits the activity of pancreatic lipase, which is one of the enzymes involved in fat digestion.

In a similar way, hundreds of extracts are currently being isolated from plants, fungi, algae or bacteria and screened for their potential inhibition of pancreatic lipase activity. Among them, extracts isolated from common foodstuffs such as tea, soybean, ginseng, yerba mate, peanut, apple or grapevine have been reported. Some of them are polyphenols and saponins with an inhibitory effect on pancreatic lipase activity, which could be applied in the management of the obesity epidemic.

Keywords: Orlistat, high fat diet, polyphenols, saponins, obesity, fat digestion

Introduction

Obesity is becoming one of the greatest threats to global health in this century, with more than 1.5 billion overweight adults and at least 400 million of clinically obese subjects [1]. Due to these increasing obesity rates, the World Health Organization (WHO) has prompted to consider it as the epidemic of XXI century and to promote strategies to prevent and control its progress [2].

The development of obesity is characterized by a chronic imbalance between energy intake and energy expenditure [3-5] and it is often ascribed to changing lifestyles and inadequate dietary habits [3]. Also, decreased energy expenditure is often associated with an inherited low basal metabolic rate, low energy cost of physical activity and low capacity for fat oxidation [6]. To reduce body weight and adiposity, a change in lifestyle habits is still the crucial cornerstone [7]. Physical activity might be helpful in the prevention of obesity by elevating average daily metabolic rate and increased energy expenditure [3]. Unfortunately, this clinical approach is not-long term lasting and weight regain is often seen. Drugs that prevent weight regain appear necessary in obesity treatment [7]. Thus, the development of natural products for the treatment of obesity is a challenging task, which can be launched faster and cheaper than conventional single-entity pharmaceuticals [8]. Many medicinal plants may provide safe, natural, and cost-effective alternatives to synthetic drugs [9,10]. Currently, one of the most important strategies in the treatment of obesity includes development of inhibitors of nutrient digestion and absorption. For example, acarbose is an anti-diabetic drug that inhibits glycoside hydrolases, preventing thus the digestion of complex carbohydrates and decreasing postprandial hyperglycemia [11,12]. Similar compounds with alpha-amylase inhibiting activity that can be used for diabetes control are being isolated from different plants. The list includes valoneaic acid dilactone [13], obtained

from banaba (*Lagerstroemia speciosa*), the ethanol extract obtained from chestnut astringent skin [14] or the purified pancreatic alpha-amylase inhibitor isolated from white beans (*Phaseolus vulgaris*), which is able to reduce glycemia in both non-diabetic and diabetic rats [15].

In this context, since dietary lipids represent the major source of unwanted calories, the inhibition of fat digestion is an interesting approach for reducing fat absorption [16]. Orlistat is the only authorised anti-obesity drug in Europe and has been shown to act through inhibition of pancreatic lipase (PL), which is a key enzyme for the digestion of dietary triglycerides [17]. Orlistat is the saturated derivative of lipstatin, an inhibitor of PL isolated from the bacterium *Streptomyces toxytricini* [18]. This molecule exerts a modest weight lowering effect when accompanying a suitable dietary advice. Thus, in a recent meta-analysis [19], the mean BMI change with Orlistat (120 mg three times daily) was a reduction of 0.83 kg m^{-2} (95% CI: 0.47–1.19) compared with placebo. Accompanying this anti-obesity action, Orlistat is also able to modestly reduce blood pressure, improve oral glucose tolerance and prevent the onset of type 2 diabetes [20,21].

Now, extracts from hundreds of species of medicinal plants, vegetables and fruits [22] as well as products from microorganisms [9], fungi [23] and marine algae [24] are being screened for potential lipase inhibitory activity. Ideally, these treatments will be viewed as adjuncts to behavioural and lifestyle changes aimed at maintenance of weight loss and improved health [8].

Obesity and high fat diets

Epidemiological studies have shown a direct relation between the incidence of overweight/obesity and dietary fat consumption [3,6,25].

Humans are frequently exposed to fat rich foods, which are usually associated to a high energy intake [6,26]. Thus, those foods with a high energy and dietary fat content are considered to promote body fat storage and weight gain in humans [8]. One explanation is that, in commercially available food items, the percentage of energy derived from fat is highly correlated with energy density. Given that fat contains 9 kcal/g compared with 4 kcal/g for carbohydrates and proteins, foods rich in fat are often high in energy density. Thus, when a similar volume of food is consumed, energy intake will be higher in high fat diets compared with low fat diets [3].

On the other hand, independently of an increased energy intake, specific dietary constituents may promote the development of obesity. This statement means that even consuming an equal amount of energy, the diet composition is important, especially the balance between nutrients [27,28].

Thus, macronutrient profile (high-protein, high-carbohydrate and high-lipid diets) can affect diet-induced thermogenesis, the oxidation pathway, energy intake, gene expression or the level of some hormones [29]. Following a high-fat diet, the diet-induced thermogenesis is lower than following high protein and carbohydrate diets, and also fat is more effectively absorbed from the gastrointestinal tract than are carbohydrates, which translates into lower energy expenditure when following a high fat diet [26]. So, high fat diets produce a metabolically more efficient state, at least in part because of the lower postprandial thermogenic effect of lipids in comparison with carbohydrates [30].

Furthermore, the consumption of a high fat diet has the capacity to modulate the gastrointestinal responses to ingested fat and, thereby, may lead to impairments in appetite regulation that favour the development of obesity. Dietary fat usually implies

an increase in energy consumption because it has a lower potential for inducing satiety than carbohydrate and protein [6,31].

Hence, high fat diets may play an important role in the increased prevalence of obesity and can be a triggering factor in the development of hyperglycemia and hyperinsulinemia [3,32]. Moreover, the intake of dietary fats is usually accompanied by a higher intake of refined sweet carbohydrates (fast food, desserts), where the high intake of sucrose promotes weight gain, visceral adiposity and the development of diseases that are related with obesity, such as diabetes and cardiovascular diseases [33]. Therefore, low fat diets often are prescribed in the prevention and treatment of overweight and obesity because a reduction in dietary lipids without restriction of total energy intake could cause weight loss [26].

Fat digestion

Recent studies indicate that fat digestion is a prerequisite for the effects of fat on gastric emptying, gastrointestinal hormone secretion, appetite, and energy intake [6]. An increasing number of gastrointestinal enzymes involved in nutrient digestion are being identified and characterized, representing a rich pool of potential therapeutic targets for obesity and other metabolic disorders [9]. Especially interesting are those enzymes that are related with dietary fat, which includes pre-duodenal lipases (lingual and gastric lipases), pancreatic lipase (PL), cholesterol-ester lipase, and bile-salt stimulated lipase [34].

Most dietary fat is ingested as triglycerides (90-95%) and their hydrolysis starts in the mouth, then through the stomach by an acid stable gastric lipase, continues in the duodenum, through the synergistic actions of gastric and colipase-dependent pancreatic lipases (PL), leading to the formation of monoglycerides and free fatty acids (FFA)

(figure 1). FFA are absorbed by the enterocyte to synthesize new triglyceride molecules, which are transported to the different organs via lipoproteins, especially chylomicrons, after a meal [34].

Pancreatic lipase (PL), encoded by the PNLIP gene in humans, plays a key role in the efficient digestion of triglycerides [35]. It is secreted into the duodenum through the duct system of the pancreas and is responsible for the hydrolysis of 50 – 70% of total dietary fats [9]. This enzyme has been widely used for the determination of the potential efficacy of natural products as antiobesity agents [36].

Orlistat is currently the only clinically approved drug for obesity management in Europe. This molecule acts by the inhibition of PL activity and the reduction of triglyceride absorption, and its long-term administration accompaning an energy restricted diet, results in weight loss [37]. Reduction on intestinal lipid digestion has been related to a decrease in the intra-abdominal fat content [7]. Thus, this compound is associated with a small, but statistically significant weight loss of about 3% more than diet alone in overweight and obese people [1]. In addition to losing weight, Orlistat within a prescribed diet has been shown to be safe and more effective than diet alone in modifying some of the risk of coronary artery disease and other obesity-related co-morbidities. The most commonly reported adverse effects of Orlistat are a range of gastrointestinal side effects, including steathorrhea, bloating, oily spotting, faecal urgency and faecal incontinence, as well as hepatic adverse effects [19,38]. These adverse effects are similar to those observed for other lipase inhibitors tasted in phase II studies, such as Cetilistat (ATL-962) [39].

On the other hand the inhibition of fat absorption could be accompanied by liposoluble vitamin deficiencies, which could be prevented by the vitamin supplementation

strategy, as other authors have recommended when vitamin deficiency occur in patients undergoing Orlistat therapy [40].

Hence the interest in the search for new natural substances that show potent inhibitory activity against PL and have fewer side effects than the current ones.

Natural inhibitors of pancreatic lipase

In the continued search of effective antiobesity agents, several bacterial, fungal and marine species have been screened to find new compounds with PL inhibitory activity.

Many metabolic products from microorganisms, such as different kinds of *Streptomyces (toxytricini, sp.NR 0619, albolongus, aburaviensis and lavendulae)* have a potent inhibitory activity of PL [9]. Lipstatin was isolated from an actinobacterium, *Streptomyces toxytricini*, and the catalytic hydrogenation product of lipstatin is the approved antiobesity drug tetrahydrolipstatin (Orlistat; marketed by Roche as Xenical^{TM}) [18]. Panclicins, analogs of tetrahydrolipstatin isolated from *Streptomyces sp.* NR0619, also present strong anti-lipase activity [41]. Other compounds which act also as potent inhibitors of PL, at least *in vitro*, are ebelactones A and B, isolated from *Streptomyces aburaviensis* [42], and vibralactone, isolated from the culture broth of the polypore *Boreostereum vibrans* [43]. Finally, other examples of lipase inhibitors have been obtained from yeasts and fungi such as *Candida antarctica, Candida rugosa, Gestrichum candidum, Humicola lanuginose,* and *Pseudomonas glumae*, which have received special attention and are widely used in the pharmaceutical industry [44].

Due to the biodiversity and unexplored resources, the fungal kingdom has been particularly searched to find new substances with lipase inhibitory activity. In a thorough screening of lipase inhibitors of fungal origin in Slovenia [23], extracts obtained from three species, *Laetiporus sulphureus, Tylopilus felleus* and *Hygrocybe*

conica, exhibited very high lipase inhibitory activities (83% ± 5%, 96% ± 3% and 97% ± 5%, respectively), even higher than Orlistat. *Pleurotus eryngii* water extract also shows a significant inhibitory activity against PL, preventing postprandial hyperlipidemia through low intestinal absorption of dietary fat [45]. Finally, the water and ethanol extracts from fruiting bodies of *Phellinus linteus* show a potent lipase inhibitory and anti-obesity effect [46]. A special case is that of monascus pigments from *Monascus sp*., which have been used for many years as natural colorants and as a healthy food in East Asia, being used in the production of certain fermented foods. Various monascus derivatives with incorporated unnatural amino acids show inhibitory activities against lipase [47].

In the same way, marine products are an especially rich source on bioactive compounds [48]. In a milestone study, Bitou et al. [24] screened the lipase inhibitory activities of methanol and ethyl acetate extracts from 54 species of marine algae. These investigations observed a very high activity (almost 100% inhibition) in the methanol extracts from *Caulerpa taxifolia* and *Asparagopsis tociformis*, although the methanolic extracts of other Chlorophyta (i.e., *Caulerpa okamurae* or *Codium latum*) Rhodophyta (i.e., *Gloiopeltis tenax* or *Hypnea charoides*) and Phaeophyta (i.e., *Sargassum muticum*, *Dictyopteris latiuscula* or *Cutleria cylindrica*), were also very promising. In this sense, Phaeophyta generally contains large amounts of polyphenols such as tannins, with lipase-inhibiting activity. In fact, most compounds with a porphyrin structure are able to inhibit lipase activity [49]. Two algae whose extract inhibits gastric and pancreatic lipases are *Caulerpa prolifera,* which may be a source of a potential antiobesity agent [50], and *Caulerpa taxifolia* that synthesizes the toxin caulerpenyne [24]. On the other hand, carotenoids from *Undaria pinnatifida* and *Sargassum fulvellum*, specifically

fucoxanthin that is *in vivo* metabolised to fucoxanthinol, suppress triglyceride absorption via the inhibition of PL in the intestinal lumen [51].

Medicinal plants have been used as dietary supplements for body weight management and control in many countries. In this sense, presence of PL inhibitors has been demonstrated in different plant species **(table 1)**, although more research is needed for identifying and characterizing effective lipase inhibitors [52]. Lipase inhibitors of plant origin include certain proteins, such as those from soybean [53] and from wheat bran and germ [54]. Other proteins that strongly inhibit hydrolysis of triglycerides are the basic protein protamine [55] and ε-polylysine [56], which could act, as several amphiphilic proteins like ovoalbumin and β-lactoglobulin [57], by the desorption of lipase from its substrate due to a change in interfacial quality [58].

Other lipase inhibitors from plant origin are basic polysaccharides, especially chitosan oligosaccharides, water-soluble chitosan (46 kDa) and polydextrose when a basic group is introduced [59,60], phytic acid and other myo-inositol phosphate esters [61], phenylboronic acid, a potent inhibitor of lipase from *Oryza sativa* [62] and carnosic acid, a diterpene isolated from the methanolic extract of the leaves of sage (*Salvia officinalis)* and rosemary [63]. Korean and Chinese researchers have been very active in the search of new lipase inhibitors of herbal origin. Among the most promising compounds there are platycodin D, isolated from the fresh roots of *Platycodon grandiflorum* [64,65], dioscin, from *Dioscorea nipponica* [66], licochalcone A, from the roots of *Glycyrrhiza uralensis* [67], phenolic constituents from the leaves of *Nelumbo nucifera* [68], the aqueous ethanol extracts of *Juniperus communis* or common juniper (bark) and *Illicium religiosum* (wood) [69], the ethanol extract from stem bark and leaves from mango tree (*Mangifera indica*), which is able to prevent weight gain induced by feeding a high-fat diet to Wistar rats [70], a pomegranate leaf extract rich in

ellagic acid and tannins [71], *Rhei rhizoma* (rhubarb) and the combinatorial drug Chunghyuldan [72], *Prunella vulgaris*, *Rheum palmatum* and other herbs [73]. Most of the common compounds that are found in different plant species are polyphenols, saponins and terpenes **(table 2)**.

In the following chapters we will present those compounds with more information available classified according to the biochemical structure.

Polyphenols

A number of studies have revealed various health benefits of plant polyphenols and their importance in foods, beverages and natural medicine. In this context, polyphenols have some potential efficacy for preventing obesity. They inhibit enzymes related to fat metabolism including PL, lipoprotein lipase, and glycerophosphate dehydrogenase [74]. Polyphenol extracts are able to decrease the blood levels of glucose, triglycerides and LDL cholesterol, increase energy expenditure and fat oxidation, and reduce body weight and adiposity [75,76]. In fact, many polyphenols, including flavones, flavonols, tannins and chalcones, have shown an inhibitory activity of PL [9,22].

Flavonoids are a type of plant secondary metabolites which are characterized as containing two or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge [76]. Some of them are polymerized into large molecules, either by the plants themselves or as a result of food processing. These polymers are called tannins and three subclasses (condensed tannins, derived tannins and hydrolysable tannins) exhibit a variety of beneficial effects on health [76]. For example, hesperidin, a flavonoid obtained from the peels of *Citrus unshiu* shows a PL inhibitory activity [77].

Proanthocyanidins (PA), also known as condensed tannins, are the most common group of flavonoids in the Western diet. They consist of monomeric units of flavans linked through carbon-carbon and ether linkages, which are considered the second most abundant group of natural phenolics after lignins [78]. PA can be found in such common foodstuffs as cereals, legumes, fruits, vegetables and beverages (red wine and tea in particular) [75,79]. They have a putative role as antioxidants, showing beneficial effects on inflammatory processes, cardiovascular diseases and other pathological conditions [80,81]. For example, these compounds actively reduce plasma triglycerides by inhibiting the absorption of dietary lipids [79] and possess inhibitory effects on different digestive enzymes, such as trypsin, amylase, and lipase [36].

Some examples of polyphenols with inhibitory action on PL are proanthocyanidins from edible herbs, such as those from *Cassia mimosoides* [82], and tea catechins, especially (−)-catechin gallate and (-)-gallocatechin gallate, [83]. Some of the most thoroughly studied polyphenol extracts in relation to PL inhibition are the following:

1. *Arachis hypogaea*
2. Peanut (*Arachis hypogaea*) shells (hulls, seed coats), which are by-products of the peanut industry, provide several compounds showing PL inhibitory activity in a dose dependent manner (1 mg/ml = 42% inhibitory effect)that are able to reduce body weight gain in rats fed a high-fat diet [84]. This plant contains several bioactive molecules, such as luteolin **(figure 2)**, certain fatty acids, caffeic, ferulic and benzoic acids, all of which are able to inhibit lipases [9]. Coumarin derivates and phenolic acids were assumed to be the major active constituents. However the authors have not examined the individual effects of each compound. *Camellia sinensis*

Camellia sinensis or tea plant (green tea, black tea or oolong tea) contains over 60 polyphenols, some of them with a potent PL inhibitory activity. It is likely the plant whose extracts have been more thoroughly used for searching new PL inhibitors. The major polyphenols are catechins **(figure2)**, which constitute about one third of its total dry weight. A serving of tea is moderate to high in flavonoid and/or tannin content [85-89]. Nakai et al. [90] found that the polyphenols with more potent PL inhibitory effect were flavan-3-ol digallate esters isolated from oolong tea, such as (-)-epigallocatechin-3,5-digallate. Oolong tea-polymerized polyphenols reduced postprandial hypertriglyceridemia in olive oil loaded in rats and mice [91]. Also (-)-epigallocatechin, abundant in the green tea extract, is a weak inhibitor of PL and is able to decrease the postprandial hypertriglyceridemia in rodents [92].

The administration of black-tea polyphenols suppressed postprandial hypertriglyceridemia in a dose-dependent manner in rats, with theaflavin-3,3'-digallate as the most effective PL inhibitor [93], whereas other authors point out to thearubigins [94]. These extracts are able to prevent increases in body weight and adiposity in mice fed a high-fat diet [95]. The PL inhibitory and hypotriglyceridemic effects of tea extracts were corroborated by Tanaka et al. [96], who orally administered mixed fermented tea extracts and Loquat tea extracts to rats with a 10% soybean oil emulsion.

Finally, cocoa tea extract (*Camellia sinensis var. ptilophylla*) is rich in polyphenols with PL inhibitory effect. A single oral administration of this extract produces an inhibition of plasma triglyceride levels in olive oil-loaded ICR mice and triolein-loaded rats [97].

3. *Glycine max*

Daidzein **(figure2)** belongs to the group of isoflavones and is produced almost exclusively by the members of the fabaceae/leguminosae (bean) family such as soybean. In one study, Guo et al. [98] investigated the effects of daidzein on body weight,

adipose tissue, blood and liver lipid levels in obese mice fed a high-fat diet, finding that daidzein reduced body and white adipose tissue weights in obese mice and ameliorated the hyperlipidemia induced by the high fat diet. The authors attributed this effect to the inhibition of PL activity and fat digestion.

4. *Ilex paraguariensis*

Yerba mate (MT) is a plant from the subtropical region of South America that is widely consumed in Brazil, Argentina, Paraguay, and Uruguay. Yerba mate contains polyphenols, such as flavonoids (quercetin and rutin) **(figure2)** and phenolic acids (chlorogenic and caffeic acids) and is also rich in caffeine and saponins [99]. These substances act on the lipid metabolism by inhibiting PL activity in a concentration value of 1.5 mg/mL [99]. Several triterpene saponins and monoterpene oligoglycosides from the leaves of Yerba mate were found to exhibit potent inhibitory activity on porcine PL [100].

5. *Malus domestica*

Apples *(Malus domestica)* belong to the Rosaceae family whose fruits contain several phenolic substances (cholorogenic acid, catechin, epicatechin, phloridzin and procyanins). Procyanidins in apples are mainly composed of various polymerized catechins, with some of them showing a PL inhibitory activity and reducing triglyceride absorption [36]. In corn oil-loaded mice, a single oral administration of apple polyphenols reduced plasma triglyceride levels, and a test diet containing 600 mg of apple polyphenols significantly inhibited triglyceride elevation at 6 h after ingestion, indicating an inhibition of triglyceride absorption [36].

6. *Salacia reticulata*

Salacia reticulata contains a high concentration of polyphenols, including catechins and condensed tannins. In hot water-soluble extract from the roots of *Salacia reticulata*

(SRHW) the concentration is about 24% polyphenols [74]. The polyphenols from *Salacia reticulata* inhibit enzymes related to fat metabolism including PL, lipoprotein lipase, and glycerophosphate dehydrogenase, and are effective in preventing obesity [101]. In fact, *Salacia* extract markedly improved metabolic syndrome symptoms (including body weight, adiposity, glucose intolerance, hypertension and peripheral neuropathy) in TSOD mice [102].

7. *Taraxacum officinale*

Dandelion *(Taraxacum officinale)* is a perennial herbaceous plant of the family Asteraceae that has been used as a phytomedicine due to its choleretic, antirhemetic, diuretic, and anti-inflammatory properties [103]. Extracts from this plant have shown hypolipidemic effects and an inhibitory activity of PL, decreasing AUC (area under curve) for the postprandial triglyceride response curve [103].

8. *Vitis vinifera*

Grapevine *(Vitis vinifera)* has become a model plant for studying proanthocyanidin biosynthesis. Grapevine proanthocyanidins **(figure2)** consist of two major flavan 3-ol monomers, catechin and epicatechin, that have inhibitory activity on PL [79,104].

Polyphenol-rich extracts from a range of berries, particularly cloudberry, are able to inhibit PL activity *in vitro*, which has been attributed to their content in ellagitannins and proanthocyanidins [105].

Saponins

Saponins are a major family of secondary metabolites that occur in a wide range of plants species [106]. These compounds have been isolated from different parts of the plants, including the roots, rhizomes, stems, bark, leaves, seeds and fruits. Occasionally, the whole plant has been used [107].

Saponins are categorized into two major classes, the triterpenoid and the steroid saponins, which are both derived from the 30 carbon atoms-containing precursor oxidosqualene [107,108]. Some of the triterpene-rich plant materials are common foodstuffs consumed in large amounts in Mediterranean countries. Therefore, the correlation of a triterpene-rich diet and the beneficial effects of consuming a Mediterranean diet should be investigated in more detail [32]. These types of plant secondary metabolites are found to inhibit PL and, thus, may represent potential effective treatments for obesity and related disorders [9,22]. One example are different saponins isolated from tea [85] or ginseng [109].

1. *Aesculus turbinata*

The Japanese horse chestnut (*Aesculus turbinata*) is a medicinal plant widely used in East Asia. The saponin mixture extracted from the seeds is called escins and has a strong inhibitory activity on PL [110]. In mice fed a high-fat diet, total escins suppressed the increase in body weight, adiposity and liver fat, and increased triglyceride level in the feces, whereas it decreased plasma triglycerides after the oral administration of a lipid emulsion [111,112].

2. *Dioscorea nipponica*

The methanol extract of *Dioscorea nipponica* Makino powder has a potent inhibitory activity against porcine PL, with an IC_{50} value of 5-10 μg/mL [66]. In fact, the saponin dioscin and its aglycone, diosgenin, both suppressed the increase of blood triacylglycerols when orally injected with corn oil to mice. Rats fed a high-fat diet containing 5% *Dioscorea nipponica* Makino gained significantly less body weight and adipose tissue than control animals [66], and a similar result has been observed after administering the aqueous extract of this rhizome to mice fed a high-fat diet [113].

3. *Eleutherococcus senticosus*

Eleutherococcus senticosus is a shrub, belonging to the family Araliaceae, which is commonly distributed in north-eastern Asia. It is used as a traditional Chinese medicine against ischemic heart diseases, neurasthenia, hypertension, arthritis, and tumors [114]. At least fifteen triterpenoid saponins **(figure3)** with *in* vitro PL inhibitory activity have been isolated from the fruits of *Eleutherococcus senticosus* [115]. The total saponin fraction obtained from the fruits of *Eleutherococcus senticosus* exhibits inhibitory activity on PL with an IC_{50} value of 3.63 mg/mL [114].

4. *Eleutherococcus sessiliflorus*

Different lupine-type triterpene triglycosides isolated from a hot water extract of *Eleutherococcus sessiliflorus* leaves are able to inhibit PL activity *in vitro*, and to suppress the body weight gain of mice fed a high-fat diet [116].

5. *Gardenia jasminoides*

Crocin is a glycosylated carotenoid extracted from the fructus of *Gardenia jasminoides* **(figure3)**. Gardeniae fructus is used in Asian countries as a natural colorant, and in Chinese traditional medicine for its antioxidant, cytotoxic, antitumor and neuroprotective effects. Crocin and crocetin are effective hypolipidemic agents that act by reducing the absorption of fat and cholesterol through inhibition of PL activity [117]. Sheng et al. demonstrated that crocin selectively inhibited the activity of PL as a competitive inhibitor [118].

6. *Gypsophila oldhamiana*

Gypsophila oldhamiana (Caryophyllaceae) is a plant distributed in the north of China whose roots have high amounts of saponins, sterols and fatty acids. The extract from this plant shows a potent inhibitory activity of PL with an IC_{50} value of 0.54 mg/ml [118,119], with different triterpenoid saponins, gypsosaponins A-C as the more efficient compounds [119].

7. Panax ginseng

Ginseng is one of the most popular medicinal herbs and is commonly consumed as powder, a beverage or a food supplement. Roots of *Panax ginseng* contain high levels of ginsenosides **(figure3)**, which are steroidal saponins that show beneficial effects on lipid metabolism. Saponins from ginseng roots suppress the expected increase in body weight and plasma triacylglycerols in mice following a high-fat diet, which was probably mediated by inhibiting PL with an IC_{50} value of 500 μg/mL [109].

8. Panax japonicus

The rhizomes of *Panax japonicus* (Japanese ginseng) are used in folk medicine for the treatment of arteriosclerosis, hyperlipidemia, hypertension and diabetes mellitus. Chikusetsusaponins prevent the increase in body weight and parametrial adipose tissue weight induced by a high-fat diet and inhibited the elevation of postprandial plasma triacylglycerols due to their inhibitory action of PL on dietary fat [120]. The delay in intestinal fat absorption was also behind the antiobesity effects observed for Korean white ginseng extract in high-fat diet-induced obese mice [121].

9. Panax quinquefolium

American ginseng (*Panax quinquefolium*) is a native plant from North America. The saponins isolated from stems and leaves of *Panax quinquefolium* may prevent fat storage in adipose tissue and postprandial elevations of plasma triacylglycerols by inhibiting the intestinal absorption of dietary fat through the inhibition of PL activity [122].

10. Platycodi grandiflorum

Platycodi radix, widely used in traditional Oriental medicines as a remedy for respiratory disorders, is rich in saponins, which are responsible for a diversity of effects including antiinflammation, antiallergy, antitumor, and immunostimulation [64]. Given

its inhibitory action on PL [123], with platycodin D as the most efficient compound [124], it ameliorated high fat-induced obesity in mice [125] and rats [64]. SK1 is an edible saponin-rich compound from Platycodi radix that is able to reduce body weight and fat accumulation by increasing fecal lipid outputs in high-fat-fed mice [126].

11. Sapindus rarak

The methanolic extract from the pericarps of *Sapindus rarak* (Lerak) shows a PL inhibitory activity that is probably due to diverse saponins and sesquiterpene glycosides [127].

12. Scabiosa tschiliensis

Different triterpenoid saponins isolated form the Mongol and Chinese traditional medicinal herb *Scabiosa tschiliensis* have shown strong inhibition of PL *in vitro* [128]. Due to the difficult task of isolating scabiosaponins and the scarceness of this type of saponins in nature, some of them have been successfully synthesized in the laboratory [129].

13. Teasaponins

At least three kinds of tea (oolong, green and black) have been used as healthy drinks. Teasaponins suppress the increases in body and parametrial adipose tissue weights and adipocyte diameters induced by a high-fat diet in mice by inhibiting PL, and also reduce the elevation in plasma triacylglycerol levels after oral administration of a lipid emulsion. The *Ki* value of teasaponins was determined to be 0.25 mg/mL [85]. Thus, the crude saponin fraction from the flower buds of Chinese tea plant exhibits accelerating effects on gastrointestinal transit in mice and inhibitory effects against porcine PL, and thee floratheasaponins (A-C) showed inhibitory effects on serum triglyceride elevation [130].

Triterpenes

Terpenes are the primary constituents of the essential oils of many types of plants and are classified by the number of terpene units in the molecule (diterpenes, triterpenes, among others). The pharmacological relevance of triterpenes has increased during the last two decades demonstrating multi-target properties such as wound healing, anti-inflammatory, anti-bacterial, antiviral, hepatoprotective and anti-tumoral effects, combined with low toxicity [32]. Triterpene extracts are safe and provide high potential for further pharmaceutical and pharmacological research [131], some of them inhibiting PL activity:

1. *Betula alba*

Bark of birch *(Betula alba)* contains pentacyclic triterpenes **(figure3)**. This triterpene extract is safe and provides high potential for further pharmaceutical and pharmacological research [32,131], playing an inhibitory activity on PL [22].

Clinical studies about pancreatic lipase inhibitors

A number of plants and natural products have been screened for their PL inhibitory activity but just some of them have gone up to clinical studies. In this line, only one product derived from natural compounds (Orlistat) is currently in clinical use, although others are under investigation. Some of them are *Panax ginseng* [132], *Camellia sinensis* [133], *Eleutherococcus senticosus* [134], *Malus domestica* [135] and *Arachis hypogaea* [136].

In one study [132], the administration of an extract of *Panax ginseng* in humans for 8 weeks decreased circulating cholesterol, triglyceride and low density lipoprotein levels (LDL). Each subject ingested 2 g of *Panax ginseng* extract three times a day.

Lee et al. [134] reported that healthy postmenopausal women treated for 6 months with E*leutherococcus senticosus* supplementation showed significant decreases in serum LDL levels and LDL/HDL ratios.

In other study, Sugiyama et al. [135] assessed six healthy male volunteers that followed a high fat diet with 40 g of fat with 10 control or 10 apple polyphenol (*Malus domestica*) capsules (600 or 1500 mg, respectively). In this study, they demonstrated that apple polyphenols may prevent obesity in humans by a PL inhibitory mechanism.

Green tea (*Camellia sinensis*) has been extensively studied in relation to obesity and other metabolic disorders. Thus, Chantre et al. [133] showed that green tea consumption may be useful to treat obesity by both, increasing thermogenesis and inhibiting PL. Thus, a green tea extract showed a direct *in vitro* inhibition of gastric and pancreatic lipases [133]. In moderately obese patients, green tea lowered body weight by stimulating thermogenesis and increasing energy expenditure when each subject received 2 times/d a green tea extract (2 capsules morning, 2 capsules midday). Ingestion of 4 capsules containing AR25 (Exolise) provided a daily total intake of 375 mg catechins, of which 270 mg was epigallocatechin gallate. Also, He et al. [137] administered daily 8 g of oolong tea for 6 weeks to 102 obese subjects. As a result, 70 % of the obese subjects decreased more than 1 kg in body weight. In *in vitro* studies suggested that the effect of oolong tea on body weight could be partially attributed to the inhibition of PL [68].

According to these data, a number of common herbal products that are being studied in animal **(table 3)** and human models for obesity treatment contain different metabolites that act on lipid digestion and absorption. However, it is very difficult to establish in *in vivo* studies whether these antiobesity effects are only or mainly due to PL activity

inhibition. The clinical implications of this therapeutic approach have yet to be determined.

Conclusions

Orlistat is the only drug authorized and presented in Europe for the treatment of obesity within an adequate energy intake, which acts by inhibiting the lipolytic activity of PL. With the aim of finding new compounds more potent or with less secondary effects than Orlistat, new natural products are being identified and screened for their PL inhibitory potential. Some of these extracts are obtained from plants that are rich in polyphenols and saponins and show inhibitory effects on fat digestion, whereas other extracts come from algae, fungi and microorganisms. Thus, natural products provide an exciting opportunity and promise for the development of new therapeutic approaches to the treatment of obesity by blocking the digestion and absorption of dietary lipids, and constitute a valuable alternative to other pharmacological agents. Some of the products reviewed in this article show potentially promising effects for weight control. In particular apple, green tea, soybean and ginseng seem to have great potential as sources of molecules with PL inhibitory activity. For all of them more data are needed to define effects, optimal dose required, and mechanism of action, as well as their possible side or toxic effects.

Thus, there is an urgent need to update the knowledge on the numerous natural sources that could act as inhibitors of PL in order to screen them as new potential therapeutic antiobesity agents with low secondary effects.

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Table 1 Plant extracts that showed over 40% inhibitory activity *in vitro* of pancreatic lipase and part of the plant from which the extract has been isolated.

Family	Scientific name	Common name	Part of plant	Ref	Family	Scientific name	Common name	Part of plant	Ref
Aeraceae	*Acer pseudosieboldianum*	Korean maple	Whole	[138]	Lamiaceae	*Spirodela polyrhiza*	Common duckmeat	Whole	[138]
Anacardiaceae	*Pistacia vera*	Pistachio	Fruits hull	[52]	Lamiaceae	*Thymus pulegoides*	Lemon thyme	Whole	[22]
Apiaceae	*Levisticum officinale*	Garden lovage	Whole	[52]	Lauraceae	*Cinnamomum zeylanicum*	Cinnamon	Derm	[52]
Apiaceae	*Sanicula chinensis*	Bian Dou Cai	Whole	[138]	Lauraceae	*Lindera glauca*	Grayblue spicebush	Whole	[138]
Araliaceae	*Eleutherococcus senticosus*	Siberian ginseng	Leaves	[114]	Liliaceae	*Asparagus cochinchinesis*	Shiny asparagus	Radix	[138]
Aspidiaceaes	*Cyrtomium falcatum*	Japanese holly fern	Whole	[138]	Liliaceae	*Scilla scilloides*	Chinese scilla	Whole	[138]
Asteraceae	*Artemisia scoparia*	Redstem wormwood	Whole	[138]	Linaceae	*Linum usitatissimum*	Oil flax	Seed	[139]
Asteraceae	*Helianthus annus*	Common sunflower	Seed	[139]	Lythraceae	*Lythrum salicaria*	Purple loosestrife	Whole	[138]
Brassicaceae	*Brassica nigra*	Black mustard	Radix	[22]	Musaeae	*Musa sapientum*	French plantain	Fructus	[22]
Brassicaceae	*Brassica oleracea capitata*	Cabbage	Folium	[22]	Myricaceae	*Myrika spp*	Bayberry	Bark	[140]
Brassicaceae	*Raphanus sativus*	Radish	Radix	[22]	Myrtaceae	*Myrtus communis*	True myrtle	Leaves	[52]
Caprifoliaceae	*Lonicera japonica*	Japanese honeysuckle	Whole	[138]	Myrtaceae	*Solanum tuberosum*	Potato	Flowers	[22]
Celastraceae	*Euonymus sachalinensis*	Spindletree	Whole	[138]	Oleaceae	*Olea europeae*	Olive	Folium	[22]
Crassulaceae	*Rhodiola rosea*	Roseroot stonecrop	Whole	[141]	Orchidaceae	*Gastrodia elata*	Tien Ma	Whole	[138]
Cucurbitaceae	*Cucurbita pepo*	Field pumpkin	Whole	[138]	Oxalidaceae	*Oxalis corniculata*	Sleeping beauty	Whole	[138]
Cucurbitaceae	*Momordica cochinchinensis*	Spiny bittergourd	Whole	[138]	Poaceae	*Eriochloa villosa*	Hairy cupgrass	Whole	[138]
Cyperaceae	*Bulbostylis barbata*	Watergrass	Whole	[138]	Poaceae	*Hemarthria sibirica*	Weed	Whole	[138]
Cyperaceae	*Carex kobomugi*	Japanese sedge	Whole	[138]	Poaceae	*Panicum dichotomiflorum*	Fall panicgrass	Whole	[138]
Cyperaceae	*Cyperus amuricus*	Asian flatsedge	Whole	[138]	Poaceae	*Setaria italica*	Foxtail bristlegrass	Whole	[138]
Eleagnaceae	*Elaeagnus macrophylla*	Oleaster	Whole	[138]	Polygalaceae	*Polygala tenuifolia*	Yuan Zhi	Whole	[138]
Ericaceae	*Arctostaphylos uva-ursi*	Bear berry	Folium	[22]	Polygonaceae	*Reynoutria elliptica*	Black bindweed	Whole	[138]
Ericaceae	*Vaccinium myrtillus*	Bilberry	Fructus	[22]	Polygonaceae	*Rheum ribes*	Rhubarb	Rhizomes	[52]
Eriocaulaceae	*Eriocaulon sieboldianum*	Flattened pipewort	Whole	[138]	Potamogetonacea	*Potamogeton distinctus*	Pondweed	Whole	[138]
Fabaceae	*Alhagi camelorum*	Camelthorn	Aerial parts	[52]	Rosaceae	*Rosa damascene*	Damask rose	Floret	[52]
Fabaceae	*Glycyrrhiza uralensis*	Gan Cao	Whole	[138]	Rosaceae	*Rubus idaeus*	Raspberry	Fructus	[22]
Fabaceae	*Lespedeza cuneata*	Chinese bush clover	Whole	[138]	Rosaceae	*Malus domestica*	Apple	Fructus	[22]
Fabaceae	*Phaseolus vulgaris*	Common bean	Whole	[22]	Rubiaceae	*Gardenia jasminoides*	Cape jasmine	Whole	[138]
Fabaceae	*Pisum sativum*	Garden pea	Fructus	[22]	Rubiaceae	*Rubia akane*	Asian madder	Whole	[138]
Fabaceae	*Pueraria thunbergiana*	Kudzu	Whole	[138]	Rutaceae	*Citrus aurantifolium*	Lime	Whole	[138]
Fabaceae	*Quercus infectoria*	Aleppo oak	Galls	[52]	Rutaceae	*Murraya koeninggi*	Curryleaf tree	Leaves	[142]
Juncaceae	*Juncus effusus*	Soft rush	Whole	[138]	Rutaceae	*Orixa japonica*	Pearl frost	Whole	[138]
Lamiaceae	*Agastache rugosa*	Purple giant hyssop	Whole	[138]	Saxifragaceae	*Chrysosplenium grayanum*	Golden saxifrage	Whole	[138]
Lamiaceae	*Origanum vulgare*	Oregano	Herba	[22]	Simaroubaceae	*Ailanthus altissima*	Tree of heaven	Whole	[138]
Lamiaceae	*Prunella vulgaris*	Common selfheal	Whole	[73]	Tiliaceae	*Tilia platyphyllos*	Largeleaf linden	Whole	[22]
Lamiaceae	*Rosmarinus officinalis*	Rosemary	Folium	[22]	Urticaceae	*Urtica urens*	Dwarf nettle	Aerial parts	[52]
Lamiaceae	*Salvia officinalis*	Salvia	Folium	[22]	Zingiberaceae	*Afromomum meleguetta*	Meleguetta pepper	Seed	[143]

Table 2 Some classes of natural compounds that have been reported to *in vitro* inhibit pancreatic lipase activity and species from which the compound has been obtained.

Metabolites	Scientific name	Common name	Family	References
Flavonoids	*Alpinia officinarum*	Lesser galangal	Zingiberaceae	[144,145]
Flavonoids	*Taraxacum officinale*	Dandelion	Asteraceae	[103]
Flavonoids, Triterpenes	*Actinidia arguta*	Kiwi	Actinidiaceae	[146]
Polyphenols	*Arachis hypogaea*	Peanut	Fabaceae	[9]
Polyphenols	*Mangifera indica*	Mango	Anacardiaceae	[9]
Polyphenols	*Medicago sativa*	Alfalfa	Fabaceae	[78]
Polyphenols	*Nelumbo nucifera*	Sacred lotus	Nelumbonaceae	[9]
Polyphenols	*Salacia reticulata*	Kotala himbutu	Celastraceae	[101]
Polyphenols	*Salix matsudana*	Corkscrew willow	Salicaceae	[147]
Polyphenols, Proanthocyanidins, Catechins	*Camellia sinensis*	Green, black, oolong tea	Theaceae	[89]
Polyphenols, Saponins	*Ilex paraguariensis*	Yerba mate	Aquifoliaceae	[99]
Proanthocyanidins	*Cassia mimosoides*	Nomame herba	Fabaceae	[148]
Proanthocyanidins	*Cinnamomum sieboldii*	Cinnamon	Lauraceae	[86]
Proanthocyanidins	*Theobroma cacao*	Cocoa	Malvaceae	[86]
Proanthocyanidins, Saponins	*Vitis vinifera*	Grape vine	Vitaceae	[79,104]
Saponins	*Aesculus hippocastanum*	Horse chestnut	Sapindaceae	[32]
Saponins	*Aesculus turbinate*	Japanese horse chestnut	Hippocastanaceae	[110]
Saponins	*Arctostaphylos uva-ursi*	Bearberry	Ericaceae	[32]
Saponins	*Ardisia japonica*	Marlberry	Myrsinaceae	[152]
Saponins	*Avena sativa*	Oat	Poaceae	[149]
Saponins	*Coffea arabica*	Coffee	Rubiaceae	[32]
Saponins	*Cyclocarya paliurus*	Wheel wingnut	Juglandaceae	[9]
Saponins	*Dioscorea nipponica*	Yam	Dioscoreaceae	[9]
Saponins	*Eleutherococcus senticosus*	Siberian ginseng	Araliaceae	[114]
Saponins	*Eleutherococcus sessiliflorus*	Sessiloside	Araliaceae	[9]
Saponins	*Gardenia jasminoides*	Cape jasmine	Rubiaceae	[118]
Saponins	*Gypsophila oldhamiana*	Oldham's baby's-breath	Caryophyllaceae	[119]
Saponins	*Kochia scoparia*	Burningbush	Chenopodiaceae	[150]
Saponins	*Malus domestica*	Apple	Rosaceae	[32]
Saponins	*Momordica charantia*	Balsampear	Cucurbitaceae	[151]
Saponins	*Olea europeae*	Olive	Oleaceae	[32]
Saponins	*Panax ginseng*	Ginseng	Araliaceae	[109]
Saponins	*Panax japonicus*	Japanese ginseng	Araliaceae	[120]
Saponins	*Panax quinquefolium*	American ginseng	Araliaceae	[122]
Saponins	*Platycodi radix*	Doraji	Campanulaceae	[64]
Saponins	*Platycodon grandiflorum*	Balloon flower	Campanulaceae	[103]
Saponins	*Sapindus rarak*	Soapberry	Sapindaceae	[127]
Saponins	*Scabiosa tschiliensis*	Pincushions	Dipsacaceae	[9]
Saponins	*Solanum lycopersicum*	Tomato	Solanaceae	[32]
Terpenes	*Salvia officinalis*	Salvia	Lamiaceae	[32]
Triterpenes	*Aloe vera*	Aloe vera	Asphodelaceae	[32]
Triterpenes	*Betula alba*	Birch	Betulaceae	[32]
Triterpenes	*Calendula officinalis*	Pot marigold	Asteraceae	[32]
Triterpenes	*Melissa officinalis*	Lemon balm	Lamiaceae	[32]
Triterpenes	*Origanum vulgare*	Oregano	Lamiaceae	[32]

Table 3 Plant extracts that showed *in vivo* inhibitory activity of pancreatic lipase, doses and effects.

Scientific name	Common name	Doses	Model	Effects	References
Aesculus turbinate	Japanese horse chestnut	0.1 – 0.5% of diet	DIO mice	↓ TG plasma levels and body weight gain	[153]
Arachis hypogaea	Peanut	1% of diet	DIO rats	↓ Body weight gain	[136]
Camellia sinensis	Green, black, oolong tea	3% of HFD	Rats	↓ Body weight gain and visceral fat	[89]
Cassia mimosoides	Nomame herba	1 – 3.5% of diet	DIO rats	↓ Body weight gain	[154]
Coffea arabica	Coffee	0.5% of standard diet	Mice	↓ Body weight gain	[155]
Cyclocarya paliurus	Wheel wingnut	250 mg/kg; VO	Mice	↓ TG plasma levels and blood glucose levels	[156]
Dioscorea nipponica	Yam	5% of HFD	Rats	↓ TG plasma levels and body weight gain	[157]
Eleutherococcus senticosus	Siberian ginseng	12 mg/kg	DIO rats	↓ Abdominal fat, TG in liver and serum and LDL in serum	[158]
Eleutherococcus sessiliflorus	Sessiloside	100-300 mg/kg; VO	Mice	↓ TG plasma levels	[159]
Gardenia jasminoides	Cape jasmine	50 mg/kg/d	Mice	↓ Body weight gain	[118]
Humulus lupulus	Common hop	0.2 – 1.2% (w/w) of extract	Mice	↓ Body weight gain and blood glucose levels	[160]
Ilex paraguariensis	Yerba mate	0.24% of HFD	Rats	↓ Body weight gain	[99]
Kochia scoparia	Burningbush	3% of HFD	Mice	↓ Body weight gain	[150]
Malus domestica	Apple	200 mg/kg; VO	Mice	↓ TG plasma levels	[161]
Myrica spp	Bayberry	------	------	↓ TG plasma levels	[140]
Nelumbo nucifera	Sacred lotus	5% of diet	Mice	↓ TG plasma levels and body weight gain	[162]
Panax ginseng	Ginseng	200 mg/kg with HFD	Rats	↓ Body weight gain	[109]
Panax japonicus	Japanese ginseng	1 – 3% of diet	DIO mice	↓ Body weight gain	[120]
Platycodi radix	Doraji	70 mg/kg, with HFD	Sprague Dawly rats	↓ Body weight gain	[64]
Rhodiola rosea	Roseroot stonecrop	150 mg/kg	Mice	↓ TG plasma levels	[141]
Rosmarinus officinalis	Rosemary	200 mg/kg HFD	Mice	↓ Body weight and fat mass	[163]
Salacia reticulata	Kotala himbutu	125 mg/kg; VO HFD	Rats	↓ Body weight gain	[101]
Salix matsudana	Corkscrew willow	5% of HFD	Wistar rats	↓ Body weight gain	[147]

DIO: Diet-induced obesity; HFD: High-fat diet; VO: Via oral. (Daily food intake is approximately rats: 20 g/day; mice: 4.5 g/day)

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Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase

Abstract

Importance of the field: The increasing prevalence of type 2 diabetes mellitus and the negative clinical outcomes observed with the commercially available anti-diabetic drugs have led to the investigation of new therapeutic approaches focused on controlling postprandrial glucose levels. The use of carbohydrate digestive enzyme inhibitors from natural resources could be a possible strategy to block dietary carbohydrate absorption with less adverse effects than synthetic drugs.

Areas covered in this review: This review covers the latest evidence regarding *in vitro* and *in vivo* studies in relation to pancreatic alpha-amylase inhibitors of plant origin, and presents bioactive compounds of phenolic nature that exhibit anti-amylase activity.

What the reader will gain: The state of the art of the search for new pancreatic alpha amylase inhibitors of plant origin for the treatment of type 2 diabetes mellitus.

Take home message: Pancreatic alpha-amylase inhibitors from traditional plant extracts are a promising tool for diabetes treatment. Many studies have confirmed the alpha-amylase inhibitory activity of plants and their bioactive compounds *in vitro*, but few studies corroborate these findings in rodents and very few in humans. Thus, despite some encouraging results, more research is required for developing a valuable anti-diabetic therapy using pancreatic alpha-amylase inhibitors of plant origin.

Keywords: alpha-amylase; flavonoids; proanthocyanidins; tannins; type 2 diabetes mellitus

1. Introduction

Diabetes mellitus is one of the world´s major diseases, with an estimation of 347 million adults affected in 2011 (1). Type 2 diabetes mellitus, by far the most common type, is a metabolic disorder of multiple etiology characterized by carbohydrate, lipid and protein metabolic disorders that includes defects in insulin secretion, almost always with a major contribution of insulin resistance (2). These abnormalities could lead to lesions such as retinopathy, nephropathy, neuropathy, and angiopathy (3). In this context, the inhibition of carbohydrate digestive enzymes is considered a therapeutic tool for the treatment of type 2 diabetes (4). The most important digestive enzyme is pancreatic alpha-amylase (EC 3.2.1.1), a calcium metalloenzyme that catalyzes the hydrolysis of the alpha-1,4 glycosidic linkages of the starch, amylose, amylopectin, glycogen, and various maltodextrins and is responsible of most of starch digestion in humans. A second enzyme named alpha-glucosidase or maltase (EC 3.2.1.20) catalyzes the final step of the digestive process of carbohydrates acting upon 1,4-alpha bonds and giving as a result glucose (4).

A positive correlation between human pancreatic alpha-amylase (HPA) activity and the increase in postprandial glucose levels has been shown, demonstrating the relevance of suppressing postprandial hyperglycemia (PPHG) in the treatment of type 2 diabetes (5). The ability of the alpha-amylase enzyme inhibitors to avoid dietary starch to be digested and absorbed in the organism has allowed to designateing these compounds as starch blockers. However, only a mild pancreatic alpha-amylase inhibition activity is recommended in order to prevent the abnormal bacterial fermentation of undigested carbohydrates in the colon as a result of an excessive inhibition of this enzyme, which results in flatulence and diarrhea (6). Currently, there are some antidiabetic drugs that act mainly by inhibiting carbohydrate digestion and absorption. Acarbose (BAY g 5421) was the first alpha-glucosidase inhibitor available for diabetes treatment. This inhibitor of microbial origin inhibits the activities of alpha-amylase, sucrase and maltase. Voglibose is a newer alpha-glucosidase inhibitor of bacterial origin that inhibits the activities of isomaltase, sucrase and maltase, whereas miglitol is a 1-deoxynojirimycin derivative that strongly inhibits glucoamylase, sucrase and isomaltase activities (7,

8). Although efficient in maintaining postprandial blood glucose levels under control in many patients, the administration of these drugs is often associated to serious gastrointestinal adverse effects (7). Moreover, the adverse clinical outcomes associated with the commercially available anti-hyperglycemic compounds are in one part responsible for the prevalent medication nonadherence that occurs in diabetic patients (9). The prominent side effects of such drugs have driven for seeking alternative therapies with less severe or no side effects. In this sense, herbal compounds appear to offer gentler means of managing metabolic disorders and, since ancient times, have been used in traditional medicine systems like Chinese, Indian ayurveda and Arabic unani (10). Therefore, evidence for a putative beneficial effect of a wide range of medicinal plants exists in relation to type 2 diabetes therapy (11). In addition to their effectiveness, herbal remedies seem to produce hardly adverse effects and present an economical alternative to the oral synthetic hypoglycemic agents. In 1990, World Health Organization (WHO) recommended that rigorous research on these plant beneficial effects should be performed (12). In this sense, the present review summarizes the studies concerning the alpha-amylase inhibitory activity of natural plants and their bioactive compounds that could be useful in the treatment of diabetes. For this purpose, a quasi-systematic review was conducted using Medline (PubMed) and ISI Web of Knowledge. Search terms included the combination of phrases like “amylase inhibitor”, “antidiabetic properties” “plant extracts” “pancreatic amylase” and all recent publications concerning pancreatic amylase (porcine and human) inhibitory activity of natural plant extracts were incorporated.

2. Antidiabetic plants used in traditional medicine

There is increasing interest to report evidences about the efficacy and safety of specific herbs and natural dietary supplements that have been used for treating diabetes in traditional medicine. In fact, many of the currently available drugs have been directly or indirectly derived from plants. Nevertheless, the evaluation of alpha-amylase inhibitory activity is not only limited to traditional herbs (13-16) or spices (17, 18), but also to diverse food extracts (19, 20).

Within the large number of plants have been used in traditional Chinese medicine for diabetes therapy (21), some of them have been approved by the Chinese health regulatory agency for their commercial use in China (22). That is the case of the total flavonoids from *Polygonatum odoratum*, that have been reported to exert an anti-diabetic effect in streptozotocin (STZ)-induced diabetic mice and alloxan-induced diabetic rats by inhibiting alpha-amylase activity (23).

In Japan and Korea there is also an ancient tradition in the use of herbal therapies for the treatment of diabetes , as has been demonstrated with the oral administration of the phlorotannins isolated from the brown alga *Ecklonia cava*, particularly dieckol, in streptozotocin-induced diabetic mice (24). Also different Malaysian and Indonesian plants traditionally used in the treatment of diabetes have been analyzed in relation to their inhibitory effect on amylase, with *Phyllanthus amarus* hexane extract (25) and *Phyllanthus urinaria* methanol extract (26) as the most promising.

In Ayurvedic Indian tradition, over 800 plants have been reported to be potential antidiabetic drug sources (27). Some of them exert an inhibitory action on pancreatic alpha-amylase, such as some of the 126 extracts isolated from 17 Indian medicinal plants that were analyzed by Ponnusamy *et al* (28), who concluded that isopropanol extracts from *Linum usitatisumum*, *Morus alba* and *Ocimum tenuiflorum* were the most potent (≥50% inhibition). When the same research group studied 91 extracts isolated from 11 Ayurvedic Indian medicinal plants, 10 of them (isolated from *Curcuma longa, Cinnamonum verum, Ficus bengalensis*, *Syzygium cumini*, *Bixa orellana*, *Murraya koenigii* and *Tribulus terrestris*) inhibited HPA (29). In other study that analyzed the effects of six ethnobotanical plants used to treat diabetes in Ayurveda, *Murraya koenigii* and *Ocimum tenuiflorum* were identified as the best inhibitors of pancreatic and glucosidase enzymes (30).

The traditional Arabic medicine is still practiced and may provide effective new compounds for treating diabetes and other diseases, as the 69 plant species that are reported in a review concerning diabetes treatment in Jordan (11). Some of these plants have been demonstrated to inhibit amylase and

glucosidase activities *in vitro* and *in vivo*, including *Geranium graveolens* and *Varthemia iphionoides* (31), *Pistacia atlantica*, *Rheum ribes* and *Sarcopoterium spinosum* (32).

In contrast to Chinese, Indian or Arabic living "great traditions", in other parts of the world the indigenous systems of medicine have become "little traditions "(33). Despite that, some of the plants used in Africa to treat diabetes are able to inhibit intestinal alpha-amylase and glucosidase activities, such as *Euclea undulata*, *Pteronia divaricata* and *Elaeodendron transvaalense* (34) or *Spondias mombin* (35).

3. Pancreatic alpha amylase inhibitory activity *in vitro*

In the following section the potential pancreatic alpha- amylase inhibitory activity of the most commonly used plants is mentioned. A summary of the plant extracts reported to display a pancreatic alpha-amylase inhibitory activity is provided in **tables 1** and **2**.

3.1. *Olea europaea* L. (Oleaceae)

Olea europaea L. (olive) is abundant in phenolic compounds, especially the leaves (36, 37) and the extra virgin olive oil (VOO) whose beneficial properties are attributed in part to the phenolic fraction (38). In fact, olive oil has been used for the treatment of diabetes since ancient times (37). Some of the phenolic compounds occurring in olives could act by inhibiting carbohydrate-hydrolyzing enzymes. In this sense, the *in vitro* pancreatic alpha-amylase inhibitory activity of olive leaves and their potential hypoglycemic effect were evaluated by Komaki *et al*. (39), showing that the hot water extract (OWE) and the ethanol extract (OEE) presented IC_{50} values against HPA of 70.2 mg/ml and 0.02 mg/ml, respectively. When different doses of olive leaf powder and different quantities of the isolated compounds were administered to GK/Jcl diabetic rats, a significant reduction in blood glucose levels was observed (39). Moreover, a significant decrease in glycemic values was achieved after cooked rice was given together with olive leaves to prediabetic volunteers, which was not observed in normoglycemic subjects. With the aim of identifying the major antidiabetic compounds, the OEE was

further studied and the IC_{50} values of luteolin glucosides (luteolin-7-*O*-β-glucoside and luteolin-4´-*O*-β-glucoside) and oleanolic acid were determined (39). In addition, the inhibitory activity of oleuropein´s aglycon moiety also showed a strong inhibitory capacity (IC_{50}= 0.03 mg/ml). In another trial conducted by Loizzo *et al* (40) pancreatic alpha-amylase inhibitory activity was detected in eight extracts from virgin olive oil. However, in all cases, the effects on alpha-amylase were weaker than on alpha-glucosidase enzyme.

3.2. *Castanea sativa* Mill. (Fagaceae)

Different health benefits have been attributed to *Castanea sativa* (chestnut) intake, including a preventive role in cardiovascular diseases and a reduction in the risk of type 2 diabetes mellitus and metabolic syndrome (41). The positive effects achieved with increased chestnut consumption could be associated to its high content in organic acids and phenolic compounds (42). Concerning the relevance of chestnut for diabetes treatment, chesnut astringent skin (CAS) extract has been studied as an amylase inhibitor (43). In this study, CAS was able to retard carbohydrate absorption and to reduce PPHG in diabetic rats (GK/jcl) by specifically inhibiting alpha-amylase activity. Moreover, it was suggested that polyphenols could be the main active compounds of the CAS extract. Thus in db/db mice, this research group observed that CAS extract not only delayed carbohydrate absorption and reduced PPHG by inhibiting the alpha-amylase enzyme, but also prevented the increase of body weight and fat mass (44).

3.3. *Allium* species: *sativum, cepa, Akaka* and *porrum* (Alliaceae)

The antidiabetic effects of *Allium* species (including garlic, *A. sativum* L. and onion, *A. cepa* L.) have been repeatedly studied *in vivo* by many authors (45). In this sense, Nickavar and Yousefian (15) reported alpha-amylase inhibitory potential of four *Allium* species (*A. akaka*, *A. sativum*, *A. porrum* and *A. cepa*), which showed IC_{50} values of 16.74 mg/ml, 17.95 mg/ml, 15.73 mg/ml and 16.36 mg/ml, respectively. In a recent study (46) the porcine pancreatic alpha- amylase (PPA) inhibitory activity of the ethyl alcohol extract (EOS) of a Korean onion skin was determined (IC_{50} of > 3mg/ml) and

compared to that of the major phenolic compound present in onion, quercetin, which showed an IC_{50} value of > 0.60 mg/ml. Nevertheless, both extracts were more effective in the inhibition of alpha-glucosidase, especially sucrase, and the inhibitory potential was further confirmed *in vivo*, suggesting the relevant contribution of *Allium* phenolic phytochemicals to achieve the anti-hyperglycemic effect.

3.4. *Salvia acetabulosa* L. (Lamiaceae)

Various species of the genus *Salvia* (sages) have been used as folk medicine to treat diabetes- induced hyperglycemia (47). The ability of nine plant species to inhibit carbohydrate digestive enzymes was analyzed by Loizzo *et al*. (48), who reported that the methanol extract of *S. acetabulosa* contained 80 mg/ml of phenolic compounds and strongly inhibited PPA (IC_{50} value of 91.2 µg/ml). A weaker activity was found in the n-hexane extract against the same enzyme (IC_{50}= 212.0 µg/ml).

3.5. *Ocimum basilicum* L. and *Ocimum tenuiflorum* L. (Lamiaceae)

Ocimum basilicum (basil) is a common culinary herb widely used in many traditional medicines. Health beneficial effects include bactericidal, anti-inflammatory, antioxidative, antiulcer, hypolipidemic, radiation protective and hypoglycemic (49, 50). In relation to the latter, different *in vitro* studies have analyzed the PPA inhibitory activity of *Ocimum basilicum* extracts. Thus, aqueous leaf extracts were reported to have a strong alpha-amylase inhibitory action with an IC_{50} value of 42.50 mg/ml (14). This inhibitory action was attributed to the high total polyphenol (146 mg catechin/g dry extract) and flavonoid (44 rutin/g dry extract) content of the extract. In another trial (17), the PPA inhibitory activity of aqueous and methanol extracts of different spices, including basil, was measured. *O. basilicum* exerted 20% inhibition (1 mg dry spice/100µl of aqueous extract), with aqueous extract being more effective than the methanolic one apparently due to its higher content in tannins. Furthermore, the isopropanol extract of *O. tenuiflorum* L., a species of *Ocimum* commonly used in Ayurveda, has been reported to produce a strong PPA inhibitory activity (53.4%) at a concentration of 0.0094 mg/ml. Alkaloids, tannins and flavonoids were identified in the extract as putative candidates for this effect (28).

3.6. *Theobroma cacao* L. and *Theobroma grandiflorum* L. (Malvaceae)

Theobroma cacao L. (Cocoa tree) is a polyphenol-rich food that contains monomeric flavanols ((-)-epicatechin and, in lower quantities, (+)-catechin, (+)- gallocatechin and (-)-epigallocatechin) which are found in a 10% of the total content and oligomeric and polymeric C4β-C8 linked B type procyanidins, as well as, anthocyanins and other flavanol glycosides, that could be found in a 90% of the total content (51).There are numerous studies describing the health benefits of cocoa in animal models, including antiobesity and antidiabetic actions (52, 53). The bioactive compounds occurring in cocoa have been reported to reduce blood glucose levels (54). In an effort to elucidate whether cocoa extracts and their procyanidins displayed an inhibitory action against carbohydrate digestive enzymes, three cocoa extracts (from regular, lavado, and Dutch-processed cocoa powder) were analyzed (55) and an inhibitory activity against alpha-amylase of 25, 20 and 10% was determined at a concentration of 200 μg/ml for each extract. The inhibitory potential of cocoa procyanidins was observed to be dependent on their degree of polymerization (DP), achieving higher inhibitory percentages (17-45.5%) with higher DPs (DP= 5-10) and lower inhibition (< 15%) with smaller DPs (DP < 5). In other study in which the *in vitro* alpha-amylase inhibitory activity of polyamide-purified phenolic extracts of different Brazilian plants were analyzed, *Theobroma grandiflorum*, commonly known as cupuaçu, exerted the most potent inhibition (2.5-9 times higher than other extracts) followed by cambuci fruit and pana frozen pulp (56).

3.7. *Curcuma longa* L. (Zingiberaceae)

The perennial herb *Curcuma longa*, commonly known as turmeric, is a medicinal plant widely cultivated in tropical regions of Asia. Most of the studies have been focused on its major active principle called curcumin and the yellow pigmented fraction of *C. longa*, which contains curcuminoids chemically related to curcumin (57). In this context, the antihyperglycemic effects produced by these compounds have been investigated (58) and the alpha-glucosidase inhibitory activity of curcuminoids and their analogs have been remarked (59) as well as the inhibitory potential of the water-soluble

protein fraction of turmeric rhizome (60). Isopropanol and acetone extracts of *Curcuma longa* L were among the eleven Ayurvedic Indian medicinal plants that were recently screened for their pancreatic alpha-amylase inhibitory activity (29), presenting an IC_{50} value of 0.16 µg/ml and 7.4 µg/ml, respectively. Podocarpic acid, curlone and cinnamic acid were considered as the major compounds within the isopropanol extract, whereas curlone, 3-cyano-7-hydroxyl-4-mehylcoumarin, and 5-amino-2-hydroxybenzoic acid were suggested as the major compounds in the acetone extract.

3.8. *Cinnamomum verum* Presl. and *Cinnamomum cassia* Nees (Lauraceae)

Cinnamon, ground from the bark of *Cinnamomum verum*, and *C. cassia* have been shown to present blood glucose lowering properties in animal models and diabetic patients (61). Different active constituents, such as the water-soluble polyphenol type-A polymers (62), cinnamaldehyde (63) and cinnamic acid (64) have been reported as candidates for these effects. The extracts of several *Cinnamomum* species have been reported to exert PPA inhibitory actions (65), including the leaves of *C. verum*, whose isopropanol and acetone extracts, containing 1,2,3,4-tetrahydro-1,1,6-trimethyl naphthalene, eugenol, and 4-acetoxycinnamic acid as major components, were observed to be the most efficient (IC_{50} value of 1.0 µg/ml) compounds (29).

3.9. *Camellia sinensis* (L.) Kuntze (Theaceae)

There are a great deal of publications on green, oolong, and black tea, but also on catechins and (-)-epigallocatechin gallate (EGCG), the most abundant catechin in tea, in relation to their protective role against cancer, obesity, diabetes, and cardiovascular diseases (66). Regarding diabetes, conflicting data exist about hypoglycemic potential of different tea extracts (67). However, many authors have reported not only a blood glucose lowering and insulin sensitizing effect of tea plant extracts (68-70), but also of its bioactive compounds (71, 72) that have been evidenced to inhibit alpha-amylase enzyme activity.

3.10. *Vaccinium corymbosum* L. (Ericaceae) and other berries

A relationship between berry phenolic phytochemicals and health beneficial effects has been demonstrated (73). In this context, the antidiabetic effect of various members of *Vaccinium* species has been reported (74). In some studies, the alpha-amylase inhibitory activity of berry extracts has been proposed as a possible mode of action for the glycaemic control. Thus, fifteen blueberry cultivars of *Vaccinium corombosum* (75) have shown *in vitro* alpha-amylase inhibitory activities ranging from 91.8 to 103.3 %, which correlates well with the total phenolic content of the cultivars (r= 0.85). In other study, polyphenol-rich extracts of diverse berries were tested against alpha-amylase enzyme showing that raspberries (*Rubus idaeus*) and rowanberries (*Sorbus aucuparia*) were the most effective ones (IC_{50} values of 4.5 and 21.0 μg of gallic acid equivalent/ml). From the phytochemical analysis performed in raspberry extracts, it was concluded that low levels of anthocyanins did not substantially affect the amylase inhibitory activity, but high levels of ellagitannins (ET) did either brighten the amylase inhibitory activity. In contrast, the amylase inhibitory activity of rowanberry extracts was shown in the proanthocyanidins-rich fraction (76) supporting previous studies where proanthocyanidins (PAs) were described as potent alpha-amylase inhibitors (77). Similar to these findings, a study of McDougall *et al*., (78) described the strawberry and raspberry extracts as the most potent alpha-amylase inhibitors and pointed out to a mixture of ETs and ellagic acids as the major inhibitory compounds.

3.11. *Aloe vera* (L.) Burm.f. (Asphodeloideae)

Aloe vera has been used for many centuries due to its curative properties (79). Type 2 diabetes mellitus and hyperlipidemia, for instance, have been orally treated by the administration of this plant (80). However, reports regarding the *in vivo* antidiabetic effects of *A. vera* preparations are conflicting, with several studies demonstrating blood glucose lowering effects (81) but other investigations achieving different outcomes depending on the plant species, part of the plant (leaf pulp, leaf skin, leaf gel, leaf juice), mode of preparation and the diabetic model used (82). Nevertheless, the results obtained in two

nonrandomized clinical trials (n= 40 and n= 76) showed an improvement in fasting blood glucose levels after 6 weeks of treatment with aloe gel juice (83, 84). In a recent study that investigated 126 extract derived from 17 plants (28), *A. vera* extract, especially the cold water and the cyclohexane extracts, showed PPA inhibitory activity (23.3 and 15.8% of PPA inhibition at concentrations of 2.5 mg/ml and 2.4 mg/ml, respectively).

3.12. *Origanum vulgare* L. (Lamiaceae)

Origanum vulgare (oregano) has been used in traditional medicine systems of many countries, and its properties have been attributed to the presence of different phenolic compounds (85), especially rosmarinic (RA) and caffeic acids (86). Some of these polyphenols, such as flavonoids and proanthocyanidins, have been described to possess amylase inhibitory activity, providing a chance to control hyperglycemia in diabetes (87, 88). In this context, in a research work conducted by McCue and Shetty (89), the PPA inhibitory activity of lemon balm, oregano extracts (containing 50% and 7% of RA) and purified RA (97%) was analyzed. Curiously, the oregano-based extract (7% of RA) showed an alpha-amylase inhibitory activity similar to that of the lemon balm extract, which contained fairly higher content of RA. As a possible explanation, mechanistic synergies among different phenolic compounds were hypothesized. In other study performed by the same group, eight of the eleven clonal lines of oregano extracts analyzed were reported to display approximately 33% of PPA inhibition, observing a positive relationship between some of the oregano extracts and their phenolic content. However, RA, which was the major compound identified within all samples, was not reported to correlate well with the anti-amylase activity of seven of the tested extracts (19).

3.13. *Rosmarinus officinalis* L. (Lamiaceae)

Rosmarinus officinalis (rosemary) is a spice Mediterranean plant with strong antioxidant activity (90) that has been reported to possess antidiabetic properties (91). In this sense, rosemary clonal extracts have been demonstrated to exert alpha-glucosidase inhibitory activity (92), although no alpha-amylase inhibitory capacity was found in the same herb extracts. In contrast, in a recent study (17) that

analyzed the pancreatic alpha-amylase inhibitory activity of different spice extracts, rosemary aqueous extract exerted the most effective inhibitory activity (approximately 30%). In addition, the aqueous extracts of Lamiaceae species in this study (rosemary, sage and basil) were the richest in phenolic compounds.

3.14. *Moringa oleifera* (Moringaceae)

Moringa oleifera (Drumstick tree) is a tropical tree with many potential pharmacological actions including the treatment of diabetes mellitus (93). In fact, aqueous extract from *M. oleifera* leaves have been reported to reduce blood glucose levels in normolglycemic and diabetic rats (94). In a research work in which the PPA inhibitory activities of 11 medicinal plants were measured, *M. oleifera* showed a 16% of inhibitory activity (95).

3.15. *Phaseolus vulgaris* (Fabaceae)

Proteinaceous alpha-amylase inhibitors are especially abundant in common bean (*Phaseolus vulgaris*) (96). From the three isoforms of alpha-amylase inhibitor (alpha-A1, alpha-A2 and alpha-A3) that have been described in beans, the alpha-AI, which is found in the cotyledons and embryonic axes, are able to inhibit HPA (97). Several *in vitro* studies have demonstrated the amylase inhibitory activity of different compounds that, as phaseolamin (specific for animal alpha-amylases), have been isolated from white kidney beans (98). In this context, the use of kidney bean extracts as alpha-amylase inhibitors for obesity and diabetes treatment has been discussed in different reviews (99, 100) and a great body of research has gone into the use of some extracts, specifically Phase 2®, which is a water extract of *P. vulgaris* that is commercialized as a dietary supplement (97).

3.16. *Triticum aestivum* (Poaceae) and other cereals

Alpha- amylase inhibitors have been found in cereals including wheat (*Triticum aestivum)*, barley (*Hordeum vulgareum),* sorghum (*Sorghum bicolor)*, rye (*Secale cereal)* and rice (*Oriza sativa)* (101).

Wheat (*Triticum aestivum*) amylase inhibitors which were isolated and characterized by Maeda *et al.* (102), have been the most studied inhibitors within cereals (103). Interestingly, a higher inhibitory activity has been reported for wheat amylase inhibitor against HPA compared to the white bean amylase ones, which could be related to a larger carbohydrate malabsorption (104). Three families (60, 24 and 12 kDa) of wheat amylase inhibitors have been distinguished (105). The 0.19 amylase inhibitor, a member of the 24 kDa family, has been extensively studied as an inhibitor of PPA, human saliva and pancreas amylase enzyme (98, 106).

4. Phenolic phytochemicals

Phenolic compounds or polyphenols are secondary metabolites widespread in the plant kingdom and found in diverse quantities in usually consumed fruits, vegetables, beverages and grains (107). These numerous phenolic phytochemicals are classified depending on their ring structure and the number of carbon atoms that substitute the ring. Phenolic structure of these compounds could chemically differ from being simple molecules (e.g phenolic acids with a unique ring structure), presenting 2-3 phenolic rings (biphenyls and flavonoids), or being polymers of 12-16 phenolic groups, such as proanthocyanidins (PAs) (108).

Phenolic phytochemicals, apart from presenting several industrial applications, have been revealed to exert health beneficial effects, especially in chronic-oxidation-linked disorders such as cardiovascular disease, obesity and diabetes (109, 110). One of the mechanisms by which these compounds may produce a blood glucose lowering response is their inhibitory effect on carbohydrate digestive enzymes, particularly on alpha-glucosidases (especially sucrase and maltase) and pancreatic alpha-amylase (89, 111) (see **Table 3**). Although some studies have reported an interesting alpha-amylase inhibitory activity in addition to the most common alpha-glucosidase inhibitory effect (112, 113), it is more common to find natural plants possessing stronger inhibitory activity against alpha-glucosidase than on alpha-amylase, which could be attributed to the different phytochemicals present (72, 114-120). In this sense, several studies have found a direct correlation between the amount of phenolic

compounds in plant extracts and their capacity to inhibit carbohydrate digestive enzymes (19, 25, 89, 92, 112, 121). However, not always plant extracts with the highest phenolic content have been demonstrated to exert the strongest inhibitory activity on alpha-amylase (122), which points out to the importance of the nature of the different molecules and the interactions among them. Furthermore, several studies have confirmed the relevance of the extraction method, finding differences in the inhibitory yield of the tested extracts (4, 123-125).

4.1. Flavonoids

Flavonoids are the most common group of polyphenolic compounds. They are present in considerable quantities in common food products, spices, and beverages (107) and have been used since ancient times to treat a great variety of human diseases including diabetes (126). Their structure consists of two moieties: benzopyran (A and C rings) and phenyl (B ring) groups. Depending on the C ring type and to the linkage between the benzopyran and phenyl groups, six groups of flavonoids have been catergorized: flavones, flavonols, flavanones, isoflavones, flavanols (or flavan-3-ols), and anthocyanidins (127).

Some of the mechanisms by which these compounds exert their antidiabetic effects have been reported by Kim *et al.* (87). In this work, twenty-two flavonoids were evaluated for their inhibitory activity against yeast alpha-glucosidase and PPA *in vitro*. Among all of them, isoflavones (genistein and daidzein) and luteolin showed the lower IC_{50}. Moreover, luteolin was more efficient than acarbose in inhibiting alpha-amylase (IC_{50} of about 0.5mg/ml). In contrast, in another study conducted by Matsui *et al*. (128) different results were elicited, concluding that the flavonoids they tested did not have enough power to delay or inhibit the release of glucose in the gastrointestinal track. Tadera *et al.* (129) investigated the inhibitory activity of six groups of flavonoids on digestive enzymes and observed the relationship between the structures of the A, B and C rings with their inhibitory potential. Regarding the inhibition of PPA, luteolin, myricetin and quercetin were the most powerful compounds, showing IC_{50} values of 0.36, 0.38 and 0.50 mM, respectively. This study established a relationship between the

inhibitory activity and the increasing number of hydroxyl groups on the B ring (Flavonols: myrecetin > quercetin > kaempferol. Flavones: luteolin > apigenin). Nevertheless, differences between the amino acids that form the diverse alpha-amylases, porcine and human, should be considered and more studies are required to investigate the effects of flavonoids on human alpha-amylase and the mechanisms of action. In a different study, water and ethanol extracts of *Varthemia iphionoides* (Asteraceae) were tested against PPA by two different methods named 2-chloro-4-nitrophenil alpha-maltotrioside degradation (CNP-G_3) and iodo-starch. The extracts from aerial parts of the plant showed a pronounced inhibitory effect (>70%) with the first method, whereas a weaker inhibitory effect (14.8 and 21.2%) was achieved with the second one (130). Seven 3-methoxyflavones and an eudesmane sesquiterpene were isolated and their inhibitory capacity evaluated. The authors concluded that those compounds possessing more than three hydroxyl groups presented the highest inhibition against PPA. Therefore, the amount of hydroxyl groups contained in the flavonol structure was demonstrated to affect the inhibitory potential of these compounds (130).

Another recent study (56) has reported that, in order to attain higher pancreatic alpha-amylase inhibitory activity, not only the amount of flavonoids is important, but also their molecular nature. In this study, a potent PPA inhibitory activity was found for the polyamide-purified phenolic extracts of *Campomanesia phaea* (Myrtaceae), *Theobroma grandiflorum* (Sterculiaceae) and *Annona crassifolia* (Annonaceae), which showed IC_{50} values of 1.0, 1.1 and 1.3 mg of sample dried weight/ml of reaction, respectively. In addition, rutin, quercetin, chlorogenic acid and catechins, occurring in these plants, were investigated with the purpose of characterizing their inhibitory potential against PPA. Quercetin was reported as the most potent compound (IC_{50}= 0.9 μM) while chlorogenic acid was the least efficient (IC_{50}= 3.9 μM) (56).

It is worth mentioning that the presence of phenolic compounds in *Camellia sinensis* (Theaceae) fractions, but particularly flavonoids, confers this herb the highest inhibitory potential against alpha-amylase when compared to other plants like *Trigonella foenum-graecum* (Leguminosae) and *Urtica dioica* (Urticaceae) (70). Fruits and leaves of *Schizandra chinensis* (Schisandraceae) contain high

levels of anthocyanins, which could be related with its favorable health properties (131). Thus, when water extracts of Omija pulp/skin and seed extracts were evaluated against PPA activity, both extracts were able to inhibit the enzyme (74% and 2%, respectively) at a concentration of 1mg/ml (132).

Catechins are a group of flavonoids belonging to the flavan-3-ol class that are present in fruits, vegetables and wine but especially in tea and cocoa (133, 134). They are able to reduce dietary carbohydrate bioavailability via reduction of glucose uptake, by inhibiting intestinal glucose transporters (135) or pancreatic alpha-amylase, as observed for the ethyl acetate fraction of the Nepalese medicinal herb *Bergenia ciliata* (Saxifragaceae) (136). This study identified (-)-3-*O*-galloylepicatechin and (-)-3-*O*-galloylcatechin (with IC_{50} values of 739 and 401 μM, respectively) as the compounds responsible for this inhibition.

4.2. Tannins: Proanthocyanidins and hydrolysable tannins

Hydrolysable tannins (gallotannins and ellagitannins) are a group of phenolic metabolites of relatively high molecular weight that, besides antioxidant properties, could present the ability to strongly complex with carbohydrates and proteins (137). These compounds are common in Grossulariaceae and Rosaceae plants (i.e., berries) and some of them are able to inhibit alpha-amylase enzyme activity, including casuarictin, tellimagranadin I and II, and rugosin A and D, all of them isolated from *Rosa gallica* petals (138). In a study performed by Li *et al*. (139), six ellagitannins designated as rubusuaviins A-F, and seven tannins called pedunculagin, 1(β)-O-galloyl pedunculagin, strictinin, sanguiin H-5, lambertianin A, sanguiin H-6 and 1-desgalloyl sanguiin H-6, all of them isolated from the dried leaves of *Rubus suavissimus* (Rosaceae), were able to inhibit alpha-amylase activity. The authors concluded that the potential of ETs to inhibit the alpha-amylase enzyme depended on the type of galloyl group (alpha-galloyl, β-galloyl or free hydroxyl group).

PAs, from fruits and berries, legume seeds, cocoa, wine and tea, are polymer chains of flavan-3-ols whose elementary units are linked by C-C and occasionally C-O-C bonds (140). Several beneficial properties have been assigned to these compounds, including a preventive role in some kinds of

cancer, cardiovascular diseases and diabetes development (141). Persimmon is one of the foods richer in PAs, especially in its peel (142). In this context, Lee *et al.* (143) tested PA polymers and oligomers from persimmon peel against alpha-amylase from *Bacillus licheniformis* and alpha-glucosidase from *Saccharomyces cerevisiae*. In this study, the inhibitory activity against alpha-amylase was linearly correlated with the DP, with polymers exerting stronger inhibitory activity against alpha-amylase than oligomers, whereas the opposite result was observed for alpha-glucosidase. Similarly, in a more recent study (144), the inhibitory action on PPA activity was higher when the polymerization degree of the oligomeric procyanidin fractions was also higher. So, the authors hypothesized that the most polymerized compounds were more reactive towards alpha-amylase due to the existence of more interaction sites. In this sense, in another research where polyphenols quantity and composition in persimmon leaves and persimmon leaf tea was determined, a varying polyphenol content and activity was found during the growth of the leaves, concluding that the best harvesting time for polyphenol-rich leaves with higher alpha-amylase inhibitory activity was June. In this study, PAs were reported as the main components responsible for the alpha-amylase inhibitory activity of persimmon leaves (145).

5. Pancreatic alpha amylase inhibitory activity of natural plant extracts *in vivo*

Several *in vivo* experiments have demonstrated the anti-hyperglycemic or hypoglycemic effects of different natural plant extracts that acted through various mechanisms (132, 146-148). Specific studies carried out in animal models have supported the hypothesis that pancreatic alpha-amylase enzyme inhibition of plant extracts might be the most promising mechanism for the observed antidiabetic effect. In these studies, the amylase inhibitory activity of plant extracts was firstly confirmed by *in vitro* bioassays and a verification of these findings was obtained in the subsequent *in vivo* tests. Some of these animal studies were focused on analyzing the reduction in blood glucose levels, after an oral starch tolerance test (OSTT) in which a decline in starch digestion and absorption was expected (31, 32, 44, 149). In some other studies, in contrast, an oral sucrose or glucose tolerance test was performed (150-152). Nevertheless, the *in vitro* amylase inhibitory activities of some extracts were not potent enough to produce an *in vivo* response (153).

The potential of *Phaseolus vulgaris* to inhibit the pancreatic alpha-amylase enzyme has been extensively studied in rodents (154) , Thus, the administration of *P. vulgaris* water extract produced a significant antihyperglycemic effect (dose of 200 mg/kg body weight) in STZ-induced diabetic rats after 30 and 45 days of treatment. Similar results were obtained with the administration for 22 days of a pancreatic alpha-amylase inhibitor isolated from *P. vulgaris* in non-diabetic (ND) and type 2 diabetic (neonatal diabetes models n0- STZ and n5- STZ) Wistar rats (155). In addition to the reduction of blood glucose levels, a significant decrease in body weight gain was observed in ND rats. These results confirmed a previous work performed in ND Wistar rats (154) in which the authors did not observe signs of malabsorption, diarrhea or an increase in stools. Regarding the use of wheat (*Triticum aestivum*) amylase inhibitors for diabetes and obesity treatment, the blood glucose lowering capacity has been also demonstrated *in vivo* in mongrel dogs (104).

Different bioactive constituents isolated from natural plant extracts have also been found to produce antihyperglycemic effects *in vivo*. Kobayashi *et al*. (156) reported the capacity of scirpusin B, an active component from the ethyl acetate extract of *Callistemon rigidus* (Myrtaceae), to suppress hyperglycemia in glycogen-loaded ddY mice. Also, the administration of andrographolide (10 mg/kg), the major active principle of *Andrographis paniculata* (Acanthaceae), significantly reduced postpandrial blood glucose levels after an OSTT (118). The *in vitro* capacity to inhibit pancreatic alpha-amylase enzyme of total flavonoids from *Polygonatum odoratum* was demonstrated by Shu *et al.* (23), who also observed a significant hypoglycemic effect at doses of 100 and 200 mg/kg in alloxan-induced diabetic rats. The alpha-amylase inhibitory effect of tea catechins has been widely studied (71, 129, 157, 158). Also tea polysaccharides have shown anti-amylase properties, as reported by Quan *et al*. (159), who studied two water-soluble polysaccharide fractions (TFP-1 and TFP-2) from *Camellia sinensis* flower. According to these authors, TFP-2 not only exerted *in vitro* alpha-amylase inhibitory activity but also produced a marked hypoglycemic effect when administered (75, 150 and 300 mg/kg/body weight) for 3 weeks to alloxan-induced diabetic mice.

Despite the interesting results of some of these studies (see **table 4**), very few *in vivo* studies have been carried out in order to corroborate the amylase inhibitory effects observed in *in vitro* assays. Therefore, further *in vivo* studies in different models of hyperglycemia are required.

6. Pancreatic alpha amylase inhibitory activity of natural plant extracts in humans

Clinical trials testing hypoglycemic effects of plant extract are not abundant in the literature (160), with the exception of wheat and bean alpha-amylase inhibitors. With respect to amylase inhibitors from wheat (*Triticum aestivum*), reductions in peak levels of postprandial glucose have been found in humans with minimal carbohydrate malabsorption signs (161, 162). Concerning the latter, a randomized, double blind, placebo-controlled trial was conducted in forty overweight and obese subjects that received Phase 2, an aqueous extract of white kidney bean, for twelve weeks (163). The treated group was dosed with two tablets of the product after the three main meals, each one containing 200 mg Phase 2, 200 mg inulin and 50 mg of *Garcinia cambogia* extract. At the end of the study a significant reduction in weight and fat mass were demonstrated. However, other human placebo-controlled trial observed a small but not statistically significant reduction in body fat and weight in body weight and fat after administering 1500 mg of Phase 2 with lunch and dinner for eight weeks (164). When the same research group administered 1000 mg Phase 2 twice a day for four weeks, but accompanied by a program of dietary modification, exercise and behavioral intervention, a significant reduction in body weight and waist girth was achieved (165). In other study, in which 445 mg of Phase 2 were administered to overweight subjects once a day for 30 days together with a meal rich in complex carbohydrates, a decrease in body weight, fat mass, and waist and hip circumferences was observed (166). When two doses (2 g and 2.9 g) were tested in eight healthy subjects that received a meal of 650 calories, a significant anti-hyperglycemic response was observed with the higher dose but not with the lower (167), unfortunately, in some cases, accompanied by diarrhea. More recently, two random double-blind, crossover human pilot studies analyzed the inhibition of starch absorption by administering two doses of Phase 2 (0.75 g and 1.5 g) in normoglycemic volunteers (168). A dose-

dependent response was found, with the highest dose causing a 66% inhibition while the lowest dose provoked a maximum inhibition of 41%.

The anti-obesity effect of the formula "Phaseolamin^{TM} 1600 diet", which is composed of the alpha-amylase inhibitor Phaseolamin (750mg), clove (a seasoning that increases body temperature, 200 mg), and three amino acids (lysine, arginine and alanine, 20 mg of each), was investigated in 10 subjects with a body mass index (BMI) between 23 and 30 kg/m^2. It was administered twice per day for eight weeks, 30 minutes before lunch and dinner, and at the end of the treatment, significant reductions in body weight, fat mass, waist circumference, BMI, and energy intake were observed (169).

The effectiveness of the pancreatic alpha-amylase inhibitory activity of other natural plant extracts has been evidenced in humans after *in vitro* or *in vivo* animal models. Thus, Tsujita *et al.*, (43) reported that the carbohydrate-hydrolyzing enzyme present in chestnut astringent skin (CAS) from *Castanea sativa* produced no adverse effects at doses of 2000 mg/kg body weight/d *in vivo*. When eleven healthy Japanese volunteers received two different doses of CAS extract (300 mg and 600 mg) together with 200 g of boiled rice after an overnight fasting, a dose-dependent reduction in plasma glucose levels (11 and 23%, respectively) was observed. In a similar study with *Olea europea* leaves, the glycemic response of 14 adult healthy volunteers who were previously classified as normal and borderline group for diabetes was studied. For this purpose two consecutive experiments were carried out; in the first one, the volunteers ate 300g of cooked rice after 12 hours of fasting whereas in the second one 1g of olive leaves (*Olea europea* L.) was added to the same amount of cooked rice (39). A significant reduction in blood glucose levels was observed in the borderline group 30 and 60 minutes after the administration of the olive leaf extract.

In brief, these clinical trials highlight the potential of these natural extracts to exert an anti-diabetic effect possibly by inhibiting the HPA. Nevertheless, more human trials are needed to demonstrate the safety and the antidiabetic properties of alpha-amylase inhibitors obtained from other natural plant extracts.

7. Side effects of natural alpha-amylase inhibitors

Several anti-diabetic drugs, such as acarbose, miglitol, voglibose, sitagliptin, nojirimycin and 1-deoxynojirimycin, target different glucosidases, especially sucrase, maltase and alpha-amylase, and produce favourable effects on glycemic values after food intake (170). Although their safety and tolerability has been widely evaluated due to the common clinical use of these drugs, their lack of specificity has been seen to produce several gastrointestinal side effects like abdominal cramping, flatulence and diarrhea (171-174). Natural alpha-glucosidase and alpha-amylase inhibitors are being investigated as new candidates to control hyperglycemia in diabetic patients, but few data are available regarding the negative effects they might produce. Most of the studies have been carried out with alpha-amylase inhibitors of bean origin. Thus, different acute and subchronic toxicity studies have been developed in animal models regarding consumption of Phase 2 with no side effects being reported (175, 176) and safety studies in humans have also been carried out. For instance, the safety of "Phaseolamin^{TM} 1600 diet", Phase 2 and Suco-Block$^{®}$ consumption was investigated and no significant side effects were found (163, 164, 169). In summary, it could be stated that the principle advantage of carbohydrate digestive enzyme inhibitors of plant origin consists in not causing severe side effects and may also be beneficial in weight reduction in individuals consuming large amounts of starch (168, 177).

8. Conclusions

Inhibition of carbohydrate hydrolyzing enzymes is emerging as a useful tool for type 2 diabetes treatment. Therefore this review has been focused on summarizing the existing data on pancreatic alpha- amylase inhibitory actions of natural plant extracts and related bioactive compounds. Pancreatic amylase enzyme inhibitors retard carbohydrates digestion and absorption in the diet reducing postprandial blood glucose levels. Several *in vitro* studies have confirmed the inhibitory potential of traditional plants and in some cases the bioactive compounds, which presumably are responsible of this mechanism of action, have been identified. However, studies conducted in animal models are few and

even less abundant are the studies performed in human subjects. Further investigations will provide useful information to set doses of compounds and natural extracts that will produce the seeking health benefits with no adverse effects.

9. Expert opinion

Significant progress has been achieved with the latest findings on the existence of different plant extracts with pancreatic alpha-amylase inhibitory activity that could lead to a slow rise of the postprandial blood glucose levels becoming an interesting therapeutic target for diabetes treatment. Moreover, clinical studies reporting a successful weight loss accompanied with a fat mass reduction, have suggested the possible antiobesity effect derived from this specific inhibition. However, although many plant extracts are able to inhibit HPA *in vitro,* their effect in diabetic animal models has been poorly investigated, even with less studies being conducted in humans. On the other hand, most of the reported data demonstrate the blood glucose lowering effect of natural plant extracts but the precise bioactive compounds underlying the mechanism have not been clearly identified. Thus, on one hand, the biggest goal in this field is the requirement of stronger evidence from preclinical studies regarding the response of diabetic patients to the natural pancreatic amylase inhibitors and the overall benefits obtained from this treatment. On the other hand, further research is required in order to assess the nature, isolation, purification and analysis of individual compounds responsible for the observed effects and information about potential synergistic effects of these compounds with other metabolites and different antidiabetic therapies that should be determined, with the aim of establishing the effective and safe doses in each case.

A huge challenge lies in developing new approaches for treating diabetes. Alternative therapies should be safer and produce any or hardly negative effects, while should give rise to efficient results in reducing glycemic values in diabetic patients. In addition, this approach will be economically less expensive than the currently available products, which is an important feature since diabetes is becoming a serious problem not only in industrialized countries but also in many developing countries.

Pancreatic alpha- amylase inhibitors might be used for the design of novel functional foods with blood glucose lowering potential, which could be useful as a complement of other andiabetic drugs, but could be also used for the industrial synthesis of analogous molecules to the existing antidiabetic ones.

Therefore, inhibition of pancreatic alpha- amylase obtained from natural resources seems to be a promising strategy for diabetes. Nevertheless, there is still a long way to go and research work should be focused mainly on the isolation of the principle active compounds and more clinical studies are essential to draw concise conclusions regarding the safety and efficacy of acute and long- term administration of the extracts and/or their bioactive compounds in type 2 diabetic patients.

Article highlights

- The control of postprandial blood glucose levels in diabetic patients is considered of relevance in the treatment of Type 2 Diabetes Mellitus. A mild inhibition of the pancreatic alpha-amylase is emerging as a potential therapeutic target.
- Studies reporting the antidiabetic effect of many herbs used in ancient traditions are extent.
- Potential of commonly used plant extracts to inhibit pancreatic alpha- amylase (porcine and human) has been demonstrated by *in vitro* studies.
- Phenolic phytochemicals, secondary metabolites found in plants, such as different flavonoids and proanthocyanidins have been reported to display a pancreatic alpha- amylase inhibitory activity.
- Few studies corroborate the amylase inhibitory potential of plant extracts and their bioactive compounds *in vivo*. Amylase inhibitors isolated from *Phaseolus vulgaris* have been the most extensively studied, finding positive blood glucose lowering effects in animal models.
- With the exception of human studies reporting the pancreatic alpha- amylase inhibitory activity from wheat and bean species, there are no many clinical trials analyzing the effects of natural

plant extracts and bioactive compounds previously demonstrated to exert an amylase inhibitory action.

- Safety and tolerability of natural amylase inhibitors have been demonstrated in various studies, and no significant adverse effects have been found till now.

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Other publications

1. **Prenatal stress increases the obesogenic effects of a high-fat-sucrose diet in adult rats in a sex-specific manner**

Laura Paternain, Ana Laura de la Garza, Angels Battle, Fermín I. Milagro, J. Alfredo Martínez, Javier Campión.

Stress, 2013

Impact factor: 3.252. Q2: 48/122 (Endocrinology and Metabolism, 2012)

2. **Transcriptomic and Epigenetic Changes in the Hypothalamus are involved in an increased susceptibility to a high-fat-sucrose diet in prenatally stressed female rats.**

Laura Paternain, Angels Battle, Ana Laura de la Garza, Fermín I. Milagro, J. Alfredo Martínez, Javier Campión

Neuroendocrinology, 2012

Impact factor: 3.537. Q2: 45/122 (Endocrinology and Metabolism, 2012)

3. **Screening of polyphenolic plant extracts for anti-obesity properties in Wistar rats.**

Noemí Boqué, Javier Campión, Rocío de la Iglesia, Ana Laura de la Garza, Fermín I. Milagro, Belén San Román, Oscar Bañuelos, J. Alfredo Martínez

Journal of the Science of Food and Agriculture, 2013

Impact factor: 1.759. Q1: 6/57 (Agriculture, multidisciplinary, 2012)

4. **Diet-induced hyperinsulinemia differentially affects glucose and protein metabolism: a high-throughput metabolomic approach in rats.**

Usune Etxeberria, Ana Laura de la Garza, J. Alfredo Martínez, Fermín I. Milagro

Journal of Physiology and Biochemistry, 2013

Impact factor: 1.654. Q3: 51/80 (Physiology, 2012)

5. **Prevention of diet-induced obesity by apple polyphenols in Wistar rats through regulation of adipocyte gene expression and DNA methylation patterns.**

Noemi Boqué, Rocío de la Iglesia, Ana Laura de la Garza, Fermín I. Milagro, Mónica Olivares, Oscar Bañuelos, Cristina Soria, Sonia Rodríguez-Sánchez, J. Alfredo Martínez, Javier Campión

Molecular Nutrition and Food Research, 2013

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6. **Therapeutic perspectives of epigenetically active nutrients**

Marlene Remely, Luca Lovrecic, Ana Laura de la Garza, Lucia Migliore, Borut Peterlin, Fermín I. Milagro, J. Alfredo Martínez, Alexander G. Haslberger

Under review (R.1): British Journal of Pharmacology

Congress contributions

1. Prenatal stress increases the obesogenic effects of a high-fat-sucrose diet in rats.

   Laura Paternain, Fermín I. Milagro, Ana Laura de la Garza, J. Alfredo Martínez, Javier Campión. 18th European Congress on Obesity (ECO 2011). May, 2011. Istambul. Published in: Obesity reviews 12 (suppl 1) (2011) 63-279.

2. Screening of polyphenolic plant extracts for anti-obesity properties in Wistar rats.

   Noemí Boqué, Javier Campión, Rocío de la Iglesia, Ana Laura de la Garza, Fermín I. Milagro, J. Alfredo Martínez. 18th European Congress on Obesity (ECO 2011). May, 2011. Istambul. Published in: Obesity reviews 12 (suppl 1) (2011) 63-279.

3. Antiobesity and antihyperglycemic effect of natural inhibitors of pancreatic α-amylase in Wistar rats

   Ana Laura de la Garza, Usune Etxeberría, Javier Campión, Mónica Olivares, Oscar Bañuelos, J. Alfredo Martínez, Fermín I. Milagro. Jornadas de experimentación. March, 2012. Spain.

4. Antiobesity and antihyperglycemic effect of natural inhibitors of pancreatic α-amylase in Wistar rats.

   Ana Laura de la Garza, Usune Etxeberria, Javier Campión, Mónica Olivares, Oscar Bañuelos, J. Alfredo Martínez, Fermín I. Milagro. 19th European Congress on Obesity (ECO 2012). May, 2012. France. Published in: Obes Facts 2012;5(suppl 1):1-280

5. Polyphenolic inhibitors of pancreatic alpha-amylase retard progression to diabetes in *db/db* mice

   Ana Laura de la Garza, Usune Etxeberria, Javier Campión, Mónica Olivares, Oscar Bañuelos, J. Alfredo Martínez, Fermín I. Milagro. September, 2012. Germany.

6. Búsqueda de extractos ricos en polifenoles con actividad inhibidora de la α-amilasa pancreática: efectos sobre la prueba oral de tolerancia al almidón en ratas

   Ana Laura de la Garza, Usune Etxeberria, Fermín I. Milagro, Mónica Olivares, Oscar Bañuelos, Javier Campión, J. Alfredo Martínez. Congreso de la Sociedad Española de Nutrición (SEN). September, 2012. Spain. Published in: Nutr Hosp. 2012; 27(5):3-66

Oral communications

1. Effects of natural inhibitors of Pancreatic lipase in the treatment of obesity.

   Ana Laura de la Garza, Noemí Boqué, Javier Campión, Mónica Olivares, Oscar Bañuelos, J. Alfredo Martínez, Fermín I. Milagro. 11th European Nutrition Conference (FENS). October, 2011. Spain. Published in: Ann Nutr Metab 2011;58(suppl 3): 20-21

2. Anti-inflammatory and antioxidant properties of flavonoid-rich extracts from helichrysum and grapefruit in insulin resistant rats.

   Ana Laura de la Garza, Usune Etxeberria, Fermín I. Milagro, Mónica Olivares, Oscar Bañuelos, J.Alfredo Martínez. XX International Congress of Nutrition. September, 2013. Spain. Published in: Ann Nutr Metab 2013;63(suppl 1):244

VIII *Thematic index*