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The contents in this manual are being continuously updated

RESEARCH MANUAL

O|N|R®

Metabolic Syndrome Solution

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SUMMARY

CLINICAL TRIALS AND TESTINGS

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1. INTRODUCTION

With about 315 million people world-wide estimated to fall into obesity category, overweight is now a major public health challenge worldwide. The primary causes of the rapid global rise in overweight rates lie in the profound environmental and societal changes now affecting large parts of the world and creating societies in which physical activity is low and the availability of high-fat, energy-dense foods has increased.

Excess body weight is implicated as a risk factor for many different health deseases, including diabetes, several types of cancer, cardiovascular diseases and stroke. Therefore, effective methods for weight reduction and for countering the metabolic syndrome are being required.

1.1. GENERAL INFORMATION

O|N|R® is a synergic bio-compound which has been formulated to help weight loss by reducing the digestion of diet carbohydrates. Its formula combines herbal bio actives from edible Mediterranean plants (olive and rosemary), known as powerful antioxidants, and black bean which is known to inhibit polysaccharides enzymatic digestion. The positive effect of O|N|R® on weight loss appears to be due to the synergic action of these three ingredients.

The antioxidant components of the complex from **Olive** (Olea europaea) and **Rosemary** (Rosmarinus officinalis) provided with significant antioxidant capacity to account for the protection of the gastrointestinal tract against oxidative stress.

Black beans (Phaseolus vulgaris) contain a family of plant defense proteins that includes phytohemagglutinins (PHA), arcelin, and α-amylase inhibitors. α-amylase inhibitors are claimed to help reducing carbohydrate absorption in humans and help to maintain or achieve a normal body weight which is considered to be beneficial to human health.

O|N|R® is formulated with ingredients which are 100% vegetal origin, cultivated in farms with low pollution environment and strict control of the quality. The combination between the 3 ingredients bio-actives maintains stability and increases the efficacy of the formula. Moreover, **O|N|R®** uses the ADS technology which helps delivering the actives directly to the target in the body: the cells mitochondria.

1.2. COMPOSITION

The efficacy of the product in the claimed effect is centered on the bio-active ingredients of the formula:

Ingredients	Quantity per day	Active Substances
Black bean extract	410 mg	4000 units of amylase inhibitory activity
Olive extract	45 mg	Oleuropein Hydroxytyrosol
Rosemary extract	45 mg	Carnosic Acid Carnosol Rosmarinic Acid

Characteristics of the food ingredient O|N|R®

The recommended dosage is 500 mg/day (250 mg before lunch and 250 mg before diner). Each dose (250mg) contains 40% (100mg) of bio actives and 60% (150mg) of excipient.

1.2.1 Olive

The Olive tree (*Olea europaea*) is an evergreen tree with gray-green leaves, native to the Mediterranean region and grown for over 5,000 years, which has given its actual shape to the landscape and culture of the region. The fruit, oil and leaves are some of the main components of the Mediterranean diet and have been you by folk medicine for thousands of years.

Olea Euopaea contains many bioactive compounds, including oleic acid, phenolic constituents, and squalene. Several reports demonstrated that olive possess antioxidant, anti-hypertensive, anti-inflammatory, hypoglycaemic and hypocholesterolemic properties, but aso antimicrobial properties against some microorganisms such as bacteria, fungi, and mycoplasma.

These potential health benefits of olive are mostly related to low molecular weight polyphenols such as oleuropein, hydroxytyrosol, and tyrosol.

1.2.2 Rosemary

Rosemary (*Rosmarinus officinalis*) belongs to the Lamiaceae family (also known as the mint family) and is also native to the Mediterranean area. Rosmarinus officinalis is an evergreen perennial shrub, well known for its needle-like evergreen leaves (dark green above and white hairy below) which have strong aromatic fragrance.

Rosemary is one of the most used culinary herbs on earth, and is especially popular in Mediterranean cuisine. It is used to savor meat, savory dishes, and salads. The essential oil of rosemary and powder extracts are used in cosmetics and in some pharmaceutical preparations.

The most important constituents of rosemary are caffeic acid and its derivatives such as rosmarinic acid, carnosic acid and carnosol. It is also considered one of the most important sources for the extraction of phenolic compounds with strong antioxidant activity.

Rosemary extracts have effective antioxidant capacity due to their phenolic hydroxyl groups but they also possess plenty of other beneficial effects like antimicrobial, antiviral, anti-inflammatory, anticarcinogenic activities and it is also known to be an effective chemopreventive agent.

1.2.3 Black Beans

Black beans *(Phaseolus vulgaris)* are well known for being an excellent source of fiber which aid to lower LDL cholesterol and stabilize blood sugar levels.

Soluble fiber helps in preventing the speedy increase of blood sugar levels after meal for the people who have insulin resistance and hypoglycaemia. The soluble fiber of black beans also prevents constipation and enhances the stool volume.

Black beans contain polyphenols which works as antioxidants in the bloodstream and averts the free radicals from oxidizing cholesterol. The high amount of antioxidants also helps to sustain cell damage and repair the damaged cells as well as build resistance by increasing immunity levels.

Polyphenols also proved beneficial for those with complains of elevated cholesterol. Polyphenols act as antioxidants in the bloodstream, preventing the free radicals from oxidizing cholesterol.

In addition to providing slow-burning complex carbohydrates, black beans can increase your energy by helping to replenish your iron stores.

2. BIOACTIVE COMPOUNDS

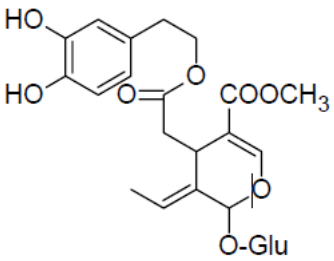
2.1. OLIVE

Olive (Olea europaea) contains several potentially bioactive compounds that may have various beneficial health properties. However, two of the main components of olive thought to be responsible for much of its beneficial effects are oleuropein and hydroxytyrosol. Studies have shown these components to exert a range of antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, and hypocholesterolemic properties.

2.1.1. Oleuropein

Oleuropein is the major phenolic constituent of the olive (Olea europaea) and is present throughout the different parts of the olive tree: fruit, leaves and bark.

Is is the most abundant polyphenol and the ester of elenoic acid with 3,4'-dihydroxyphenylethanol (hydroxytyrosol). This secondary metabolite responsible for the characteristic bitter, pungent taste of the olive oil.



Oleuropein Chemical structure

Molecular formula: $C_{25}H_{32}O_{13}$

Molecular weight: 540.51 g/mol

CAS Registry Number: 32619-42-4.

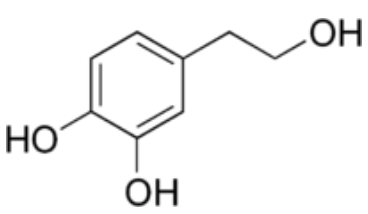
Oleuropein is represented by the chemical structure on the left.

Oleuropein has a powerful anti-bacterial and anti-viral effects. It has proven to be useful in cases of yeast and fungal infections, herpes, chronic fatigue, allergies, psoriasis and many other pathogens. In addition, it has been shown to lower blood sugar, normalize arrhythmias, inhibit oxidation of LDL (the bad cholesterol), and relax arterial walls, thereby helping to lower blood pressure. Other benefits are that it boosts energy and helps increase the body's immune response..

2.1.2. Hydroxytyrosol

Hydroxytyrosol (3, 4-dihydroxyphenylethanol; DOPET) is a phytochemical with antioxidant properties present naturally in olives. It is responsible together with other phenolic compounds as oleuropein for its bitter taste.

Hydroxytyrosol is a metabolite obtained from oleuropein hydrolysis. It is incorporated in the aglycon of oleuropein and is thought to be released from this glycoside owing to the action of cellular esterase or acidic catalysis.



Hydroxytyrosol Chemical structure

Molecular formula: $C_8H_{10}O_3$

Molecular weight: 154.16 g/mol

CAS Registry Number: 10597-60-1

Hydroxytyrosol is represented by the chemical structure on the left.

Hydroxytyrosol has a number of health benefits in humans which main is fighting harmful free radicals thanks to its action as potent inhibitor of metal-induced oxidation of low density lipoprotein. Metal-independent oxidation is also significantly retarded by hydroxytyrosol. The antioxidant activities of hydroxytyrosol, which has been proven to be more effective than BHT or vitamin E, were further confirmed, by the use of stable free radicals, such as DPPH

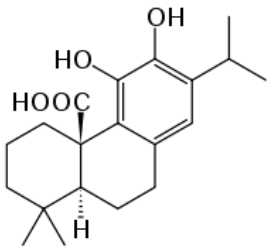
It also acts as reducing risk of cancer, reducing risk of diabetes, and slowing ageing process. It is also acts as an antibacterial and can strengthen the immune system.

The safety profile of hydroxytyrosol appears to be excellent: no untoward effects have been demonstrated even at very high doses.

2.2. ROSEMARY

2.2.1. Carnosic Acid & Carnosol

Rosemary extracts contain several compounds which have been shown to present antioxidative functions.



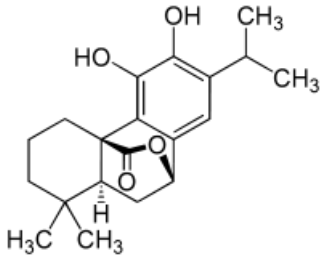
Carnosic Acid chemical structure

Molecular formula: $C_{20}H_{28}O_4$

Molecular weight: 332.42 g/mol

CAS Registry Number: 3650-09-7

Carnosic Acid is represented by the chemical structure on the left.



Carnosol chemical structure

Molecular formula: $C_{20}H_{26}O_4$

Molecular weight: 330.42 g/mol

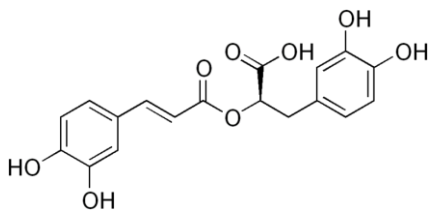
CAS Registry Number: 5957-80-2

Carnosol is represented by the chemical structure on the left.

These compounds belong mainly to the classes of phenolic acids, flavonoids, diterpenoids and triterpenes. The principal antioxidative components of the extracts are the phenolic diterpenes carnosol and carnosic acid.

2.2.2. Rosmarinic Acid

Rosmarinic acid is a natural phenol antioxidant carboxylic acid and a fundamental compound of *Rosmarinus officinalis*. Chemically, rosmarinic acid is an ester of caffeic acid with 3,4-dihydroxyphenyl lactic acid.



Rosmarinic acid chemical structure

Molecular formula: $C_{18}H_{16}O_8$

Molecular weight: 360.31 g/mol

CAS Registry Number: 20283-92-5

Rosemarinic Acid is represented by the chemical structure on the left.

The biosynthesis of rosmarinic acid starts with the amino acids l-phenylalanine and l-tyrosine. All eight enzymes involved in the biosynthesis are known and characterised and cDNAs of several of the involved genes have been isolated.

Rosmarinic acid has a number of interesting biological activities: anti-microbial, anti-inflammatory and antioxidant. The anti-inflammatory properties of rosmarinic acid are based on the inhibition of lipoxygenase and cyclooxygenases, and on the interference of rosmarinic acid with the expression of inflammatory cytokines. Rosmarinic acid has also antioxidant properties and can act as scavenger of free radicals in biological systems.

2.3. BLACK BEANS

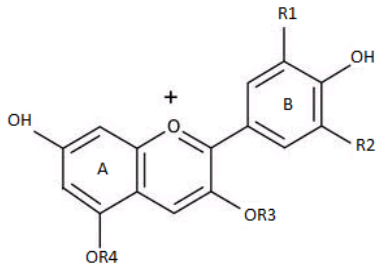
Black beans contain polyphenolic compounds: flavonoids such as flavonol glycosides, anthocyanins, and condensed tannins (proanthocyanidins), which exhibit natural antioxidant properties. That is useful for those with elevated cholesterol because they act as antioxidants in the bloodstream, preventing cholesterol from oxidation by free radicals. It is demonstrated that phenolic phytochemicals compounds inhibit the formation of free radicals, thereby minimizing the deterioration of significant biomolecules in the human body.

Factors that influence the levels of total phenolics in bean include genotype, environment, maturity at harvest, seed size, seed weight and seed age.

Black bean contains a higher concentration of phenolics than did beans with light-colored seed coats, such as white bean. The phenolic content in a extraction a 150°C of temperature and ethanol as solvent was 3.04 ± 0.05 mg/g bean for black bean whereas in same conditions it was 1.81 ± 0.01 mg/g bean for navi specie.

2.3.1. Anthocyanins

Anthocyanins are flavonoids, a type of polyphenols bioactive compounds. It is a natural pigment responsible for red, purple, and blue coloration in plants and the largest and most important group of polar-soluble pigments in nature.



Anthocyanin

In black bean extract there are three anthocyanin flavonoids in high quantity: delphinidin, petunidin, and malvidin, but also cyaniding, Peonidin and Pelargonidin.

These anthocyanins are primarily responsible for the rich black color that we see on the bean surface. Acids including, ferulic, sinapic, and chlorogenic acid, as well as numerous triterpenoids.

Flavonoids extracted from beans, mainly anthocyanins and proanthocyanidins, have shown antioxidant and antimutagenic activities. Recently, red beans were identified as having one of the highest antioxidant capacities (as measured in the ORAC assay) among over 100 common dietary fruits and vegetables examined.

2.3.2. Carbohydrates

Dry beans are about 70% carbohydrate. Starch (43/45%), non-starch polysaccharides or fiber (18/20%), α-galactosides (starchyose, verbascose, and raffinose; 3/5%), and sucrose (3/ 5%) are the major types of carbohydrates.

Carbohydrates constitute the main fraction of beans, accounting up to 55-65% of the dry matter. Of these, starch and non-starch polysaccharides (dietary fiber) are the major constituents, with smaller but significant amounts of mono, di and oligosaccharides. These leguminous contain SDC and high proportion of NDC that might be fermented in the large intestine. NDC reaching the colon include mainly resistant starch, soluble and insoluble dietary fiber, and non-digestible oligosaccharides.

2.3.3. Fibers

Black beans are rich in dietary fiber. This means that blood glucose (blood sugar) does not rise as high after eating beans as it does when compared to white bread.

Soluble fiber absorbs water in the stomach forming a gel that slows down the metabolism of the bean's carbohydrates. The presence of fiber is also the primary factor in the cholesterol-lowering power of beans. Fiber binds with the bile acids that are used to make cholesterol. Fiber isn't absorbed, so when it exits the body in the feces, it takes the bile acids with it. As a result, the body may end up with less cholesterol.

3. MECHANISMS OF ACTION

3.1. BLOOD SUGAR BALANCE

3.1.1. Alpha-amylase inhibitor

Before crossing the intestinal wall, all complex carbohydrates (starches) must be hydrolyzed to their monosaccharide units, in most cases glucose.

There are several enzymes involved in this process: α-amylase present in saliva and pancreatic juice, which converts complex carbohydrates into oligosaccharides, and various other enzymes (maltase, lactase, etc.) present in the brush border of the small intestine that convert these oligosaccharides to monosaccharides that can then be absorbed. We believe the mechanism behind the weight loss relies on the reported α-amylase-inhibiting activity of the Phaseolus vulgaris extract. It has been shown in vitro to inhibit the activity of α-amylase and may help promote weight loss by interfering with the digestion of complex carbohydrates to simple, absorbable sugars, potentially reducing carbohydrate-derived calories.

Black beans have three isoforms of a-amylase inhibitor (isoform 1 (a-AI1); isoform 2 (a-AI2); a-amylase inhibitor like (a-AIL)).

The a-AI1 isoform with anti-amylase activity in humans is found in most of the common bean. In the bean plant, a-AI1 is only found in the seeds and is concentrated in the axis. It acts as a starch blockers, increasing the resistance of starch to digestion and increased activity of colorectal bacteria.

3.1.2. Insulin Resistance

Insulin is a hormone central to regulating carbohydrate and fat metabolism in the body. Insulin causes cells in the liver, muscle, and fat tissue to take up glucose from the blood, storing it as glycogen in the liver and muscle.

Insulin stops the use of fat as an energy source by inhibiting the release of glucagon. With the exception of the metabolic disorder diabetes mellitus and Metabolic syndrome, insulin is provided within the body in a constant proportion to remove excess glucose from the blood, which otherwise would be toxic. When blood glucose levels fall below a certain level, the body begins to use stored sugar as an energy source through glycogenolysis, which breaks down the glycogen stored in the liver and muscles into glucose, which can then be utilized as an energy source. As its level is a central metabolic control mechanism, its status is also used as a control signal

to other body systems (such as amino acid uptake by body cells). In addition, it has several other anabolic effects throughout the body.

Epidemiological data have consistently demonstrated a positive relation between increased body size (obesity) and colorectal malignancy. Obesity induced insulin resistance leads to elevated levels of plasma insulin, glucose and fatty acids. Exposure of colonocytes to heightened concentrations of insulin may induce a mitogenic effect within these cells, producing hyperproliferation; whereas exposure to glucose and fatty acids may induce metabolic perturbations, alterations in cell signaling pathways and oxidative stress

Epidemiological evidence also suggests that long-term consumption of high glycemic index/load diets may increase the risk of developing NIDDM[1]. Six prospective studies have reported on the relationship of GI or GL to risk of NIDDM. Only two studies further evaluated dietary intake among different food categories, and included an analysis on beans. Collectively, these studies indicate a protective role for low glycemic index diets on risk of incident NIDDM.

Slowing of the rapid absorption of carbohydrates would favorably influence the insulin system that could, in turn, lead to lesser fat accumulation. We have previously shown in a rat model the ability of so-called "carbohydrate blockers" to prevent early absorption of rice starch and sucrose and prevent insulin resistance.

Black beans contain slow digested carbohydrates and high proportion of non-digested carbohydrates that might be fermented in the large intestine. Non-digested carbohydrates (NDC) reaching the colon include mainly resistant starch, soluble and insoluble dietary fiber, and non-digestible oligosaccharides.

The non-digested carbohydrates are associated with a low glycemic response, low serum cholesterol levels, and a decrease of colon cancer risk factors. The physiological effects of NDC from common beans may be related to colonic fermentation end products, short chain fatty acids (SCFA), such as acetic, propionic and butyric acids, and the content and distribution of SCFA are dependent on the microflora and the carbohydrate substrate at the intestinal tract.

Dietary factors that promote excess glucose in the blood (hyperglycemia), excess insulin in the blood (hyperinsulinemia), and excess body fat also promote development of several chronic diseases including type-2 diabetes, cardiovascular diseases, and cancer at several sites in the body. Hyperglycemia, hyperinsulinemia, and excess body fat are simply markers for a milieu of changes – hormones, growth factors, inflammatory products, and oxidative stress, to name a few – that contribute to development of chronic diseases.

[1]NIDDM: noninsulin-dependent diabetes mellitus

The extent to which different foods or meals raise blood glucose depends on the Glycemic index of the consumed foods and the quantity of carbohydrate. The GI of a food is a ratio of how much the blood glucose rises after consuming a standard amount of available carbohydrate compared to a standard. The Glycemic load is calculated by multiplying the glycemic index of a food by the quantity of available carbohydrate eaten. The glycemic load of a meal is computed by summing the glycemic loads of all foods consumed. The following will discuss how the type of carbohydrate and the amount of the carbohydrate consumed impacts hyperglycemia, hyperinsulinemia, and body weight and thus indirectly to the development of chronic diseases

The ability of low GI carbohydrates to decrease risk of NIDDM may be related to lower post-prandial excursions in glucose and insulin coupled to improvements in insulin sensitivity.

High glycemic index foods are known to cause rapid elevations in blood glucose and insulin following a meal. Chronic consumption of high glycemic index diets may in turn lead to down-regulation or desensitization of receptors for insulin, eventually contributing to insulin resistance. The body initially adjusts to higher circulating glucose by increasing insulin secretion from the pancreas. However, in susceptible individuals over time insulin resistance combined with exhaustion of insulin producing cells will eventually lead to type-2 diabetes.

3.1.3. Lowering HbA1c

Glucose in the blood sticks to haemoglobin in red blood cells, making glycosolated haemoglobin, called haemoglobin A1c or HbA1c. The more glucose in your blood, the more HbA1c will be present, so the level reported will be higher. The HbA1c gives a measure of what your average blood glucose level has been in the previous 2–3 months.

Normally about 5% of our red cells are glycosolated. If you have greater than 6.5% you are diabetic. It is not affected by fasting, and since our RBC's live 90-120 days, it reflects an average over that time.

In an example of olive hypoglycaemic properties, in a recent randomized clinical trial, the subjects treated with olive extract exhibited significantly lower HbA1c and fasting plasma insulin levels. The authors concluded that olive extract may represent an effective adjunct therapy that normalizes glucose homeostasis in individuals with diabetes. To add to this, olive extract has been shown to attenuate pain associated with diabetic neuropathy.

3.2. LOWERING BLOOD PRESSURE

In an example of olive extract's antihypertensive properties, it was shown to be similarly effective in lowering systolic and diastolic blood pressures in subjects with stage-1 hypertension as Captopril -a conventional hypertension treatment. Further to this, in a study of 40 borderline hypertensive monozygotic twins, the twin receiving the higher dose of olive extract (i.e. 1000mg vs 500mg) showed a significant decrease in mean blood pressure and cholesterol after 8 weeks. Lastly, an earlier study in France found that administration of olive extract to 30 patients with essential hypertension for 3 months resulted in a significant drop in blood pressure.

A series of studies have also provided good evidence that supplementation with olive extract can help reduce skin damage and risk of melanoma associated with ultraviolet B exposure. Other studies have suggested olive extract has analgesic properties and may be useful in the treatment and/or management of painful conditions. Yet other studies have suggested olive extract may be effective for enhancing the healing of cartilaginous injuries and slowing/reducing the pathogenesis of degenerative joint diseases in humans.

3.3. ANTI-INFLAMMATORY EFFECT

The pro-inflammatory cytokines, prostaglandins, and NO produced by activated macrophages play critical roles in inflammatory diseases. Hence, the inhibition of pro-inflammatory cytokines or iNOS and COX-2 expressions in inflammatory cells, offers a new therapeutic strategy for the treatment of inflammation.

Hydroxytyrosol inhibition effect on COX-2 and iNOS

It was found that hydroxytyrosol inhibits COX-2 and iNOS expressions in THP-1 cells, and that it probably acts at the transcriptional level, as evidenced by dose-dependent reductions in their mRNA levels. The inhibition of the LPS-stimulated expressions of these molecules in THP-1 cells by hydroxytyrosol was not due to hydroxytyrosol cytotoxicity, as assessed by MTT assay (data not shown) and the expression of the housekeeping gene, β-actin.

TNF-α is the primary cytokine induced in LPS induced THP-1 cells and the cytokine responsible for the perpetuation of the inflammatory response in monocytes. Thus, drugs that inhibit TNF-α production may play an important role in the control of inflammation. Studies have indicated that other simple and polyphenol-inhibited cytokines TNF-α production in LPS-stimulated cells. In this study, hydroxytyrosol showed inhibitory effect on TNF-α expression in the same cells induced by LPS. This finding further supports that HT possesses potent anti-inflammatory activity.

In conclusion, HT possesses potent anti-inflammatory activity. It prevented the cytokines formation, NO generation, and iNOS and COX-2 expression in LPS-treated THP-1 cells. These findings suggest that hydroxytyrosol exerts anti-inflammatory effects probably through the suppression of COX-2 and iNOS expression.

Furthermore, Visioli et al. showed that oleuropein increases nitric oxide (NO) production in macrophages challenged with lipopolysaccharide through induction of the inducible form of the enzyme nitric oxide synthase, thus increasing the functional activity of these immunocompetent cells. It is well known that oleuropein elicits anti-inflammatory effects by inhibiting lypoxygenase activity and the production of leukotriene B4.

Carnosic acid and carnosal activation effect of peroxisome proliferator-activated receptor gamma

Carnosic acid and carnosol are phenolic diterpenes present in several labiate herbs like Rosmarinus officinalis (Rosemary). Extracts of these plants also exhibit anti-inflammatory properties.

Recently, scientists found that carnosic acid and carnosol activate the peroxisome proliferator-activated receptor gamma, implying an anti-inflammatory potential on the level of gene regulation. Here we address short-term effects of carnosic acid and carnosol on typical functions of human polymorphonuclear leukocytes (PMNL). It has been found that carnosic acid and carnosol inhibit the formation of pro-inflammatory leukotrienes in intact PMNL as well as purified recombinant 5-lipoxygenase. Both carnosic acid and carnosol potently antagonise intracellular Ca(2+) mobilisation induced by a chemotactic stimulus. Thirdly, carnosic acid and carnosol attenuate formation of reactive oxygen species and the secretion of human leukocyte elastase.

Together, these findings provide a pharmacological basis for the anti-inflammatory properties reported for CS- and CA-containing extracts: inhibition of human 5-lipoxygenase and suppression of pro-inflammatory responses of stimulated human polymorphonuclear leukocytes.

Anthocyanins chemoprevention of inflammatory diseases

Epidemiological investigations and animal experiments indicate that anthocyanins may contribute to chemopreventive activities of various chronic inflammatory diseases.
Anthocyanins-rich extracts were demonstrated to possess a broad spectrum of biological properties, including antioxidant, cadioprotective, neuroprotective, anti-inflammatory and anticancer.
In an animal study, cyanidin was reported to reduce PGE2 levels in paw tissues and TNF-a levels in serum in adjuvant-induced arthritis. Damage and apoptosis of vascular endothelial cells is frequently observed in

atheromatous plaques and contributes to pathology of atherosclerosis. It has been shown that cyanidin inhibited TNF-a-induced endothelial cell apoptosis, elevated expression of eNOS and thioredoxin may improve vascular endothelial cell function and vasculopathy. VEGF is known as a major pro-angiogenic and pro-atherosclerotic factor. Both cyanidin and delphinidin, other major anthocyanidins present in pigmented fruits and vegetables, inhibit PDGF-induced VEGF expression through down-regulation of MAPK and JNK signalings in vascular smooth muscle cells.

Delphinidin also shows protective effects against cardiovascular disease. It is suggested that proliferation of vascular endothelial cells is important in the pathogenesis of atherosclerosis.

Delphinidin treatment inhibits serum and VEGFinduced bovine aortic endothelial cell proliferation through modulation of ERK and also results in cell cycle arrest. Also, delphinidin increased eNOS expression by mediating the MAP kinase pathway, thus preventing bovine aortic endothelial cell apoptosis.In addition, delphinidin was found to fight against ox-LDL-induced damage in HUVECs and regulate apoptotic molecule expression.

3.4. ANTIOXIDATION EFFECT

Oleuropein and hydroxytyrol reduce LDL oxidation

Several compounds from olive leaves, oleuropein and hydroxytyrosol among them, have shown a variety of biological activities as an antioxidant.

Oleuropein and hydroxytyrosol imparts some important antioxidant benefits to the user including reduction of LDL oxidation**.** Polyphenols potently and dose-dependently inhibits copper sulphate-induced oxidation of low-density lipoproteins (LDL). According to De la Puerta et al., Oleuropein has both the ability to scavenge nitric oxide and to cause an increase in the inducible nitric oxide synthase (iNOS) expression in the cell. A scavenging effect of oleuropein was demonstrated with respect to hypochlorous acid (HOCl). HOCl is an oxidative substance produced in vivo by neutrophil myeloperoxidase at the site of inflammation and can cause damage to proteins including enzymes.

Hydroxytyrosol is believed to be one of the most powerful antioxidants. Its oxygen radical absorbance capacity is 40,000umolTE/g, which is ten times higher than green tea. The antioxidant polyphenol is known for its activity in preventing or reducing the deleterious effects of oxygen-derived free radicals associated with numerous inflammatory and stress-related human and animal diseases. It is also effective at inhibiting LDL oxidation.

Rosemary, protective agent against oxidative protein damage

A large number of reports have shown rosemary constituents to be an efficient antioxidant against lipid peroxidation and DNA damage induced by radical oxygen species. Rosemarinic Acid demonstrated its ability to protect tissues and cells against oxidative stresses (Bradley, 2006).

On the other hand, it is well-known that several antioxidants exhibited pro-oxidant effect producing protein damage under certain conditions as in the presence of transition metals such as Fe and Cu.
The protection of rosemary compounds against protein damage in comparison with ascorbate and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), were examinated through hydroxyl radical-mediated oxidation experiments, carried out using a metalcatalyzed reaction. Results showed that the plant extract used reduced significantly protein damage compared with ascorbate and trolox.

Black beans anthocyanin pigments prevent the generation of free radicals

There are several explanations for the antioxidant mechanisms of black beans. Firstly, anthocyanin with 2-benzopyran core structure is a kind of conjugate structure. The unpaired electron is not fixed in the oxygen atom, but is close to the benzene ring, thus weakening the hydrogen bond. Thus the activity of the hydrogen atom of the hydroxyl is increased, and is easily lost to become a hydrogen donor.

Anthocyanins are able to reduce capillary permeability and fragility, so they could be the key component in red wine that protects against cardiovascular disease. The anthocyanin pigment prevented the generation of free oxygen radicals, and decreased the peroxidation of lipids.

The biological activity of flavonoid compounds from beans has been reported in vitro as well as in vivo. The antioxidant activity has been evaluated using different methods and different common beans. The cyanidin 3-0-P-D-glucoside extract showed strong antioxidant activity in the linoleic acid system at neutral condition (pH 7.01), while the pelargonidin 3-0-P-D-glucoside and the delphinidin 3-0-P-D- glucoside extracts exhibited no antioxidant activity at pH 7.0. However, pelargonidin and delphinidin showed a strong antioxidant activity in acidic conditions (pH 3.0 and 5.0, respectively), suggesting that the antioxidant capacity is chemical-structure dependent. Using a fluorescence assay with liposomes and 3-[4-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid, showed that pure flavonoid compounds such as anthocyanins, quercetin glycosides and protoanthocyanidins (condensed tannins), present in the seed coat methanol extract and tannin fractions from 10 colored genotypes of common bean Phaseolus vulgaris, all displayed antioxidant activity, while the highest activity was obtained with extracts rich in condensed tannins.

4. ADS (ADVANCED DELIVERY SYSTEM)

Bio availibility is a very important factor to allow bioactives to cross the cells double membrane and act at the cellular level. A green carrier allowed us to create a complex with the ability to cross hydrophilic and hydrophobic barriers.

4.1. CONTROLLED DELIVERY OF BIOACTIVE COMPOUNDS

Formulate an active ingredients is important, but to deliver the actives to the researched target has the same importance; otherwise the ingredients are useless to the body.

The main idea of the protection is to create a carrier for the actives so they won't be in contact with the external environment until the release phase. The shell will act as a vessel and will navigate to the researched locations in the body.

We have developed and created ADS® – Advanced Delivery System for a targeted, controlled delivery of bioactive compounds.

Background

The active ingredients are given into the body, go through various membranes and arrive to the points of action. The movement of active ingredients depends on the efficiency of amount of the ingredient and time, which is Bioavailability.

Since 1995, our researchers began research on Fenugreek plant.

Why Fenugreek?

The main research was focusing on the anti-diabetic activity of Fenugreek. While trying to understand the mechanism of action of this plant, our researchers noticed that the Fenugreek has the particularity to present an exceptional system of delivery of molecules.

Fenugreek properties have been analysed and our scientist team discovered that the plant has the ability to facilitate and guide the circulation of actives for acting on defined points of the skin and cell.

Thanks to this discovery, we decided to use similar Fenugreek receptors to deliver the active ingredients and created a carrier encapsulation.

Created by amino phospholipid, ADS® carrier allows a targeted and innovative controlled delivery of actives. ADS® protects the activity of the ingredients, and allows their delivery on zones never reached with classical ingredient.

Advantages:

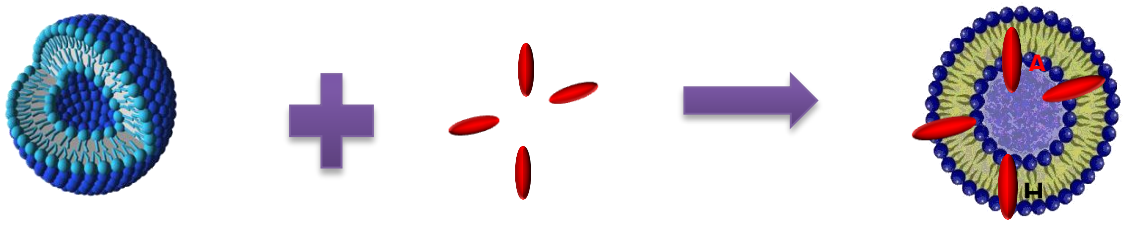
- Quick and visible effect of the treatment (both cosmetics & nutraceuticals)
- Little quantity of ingredient is enough, as no waste
- No need to intake big amount of actives to feel the results, thus reducing side effect of overtaking of actives
- Reducing the size of end products (capsules)
- 100% safe, no animal origin ingredients, only natural vegetable origin

4.2. LOCALISATION OF O|N|R® IN CELLULAR SYSTEM

Intracellular localisation of O|N|R® by fluorescence microscopy using lipophilic probes in mitochondria.

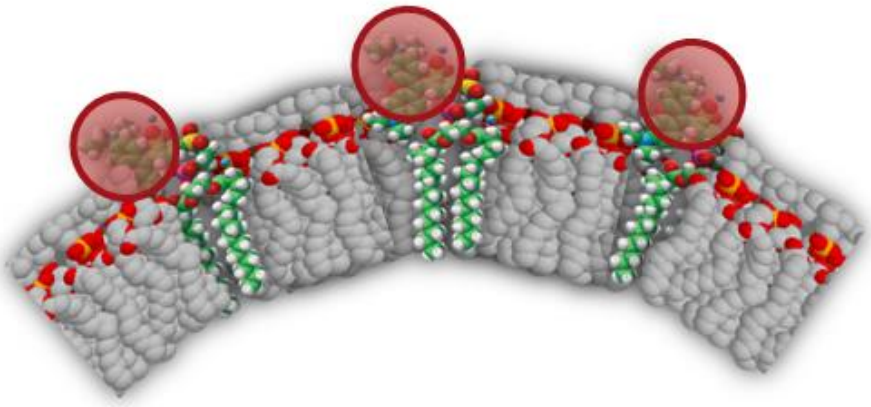
Labelling of O|N|R® with a fluorescent lipophilic probe

Labelling of O|N|R® carrier

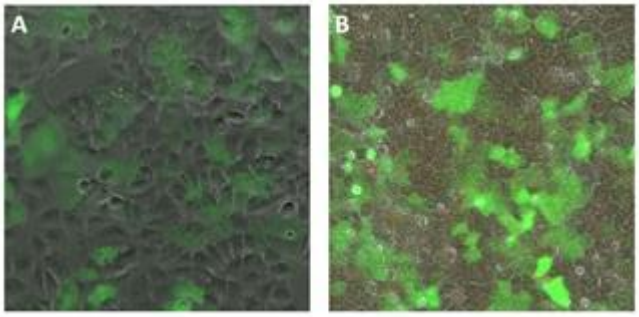


Separation of labelled O|N|R® from free probe

Labelling of O|N|R® with a fluorescent lipophilic probe



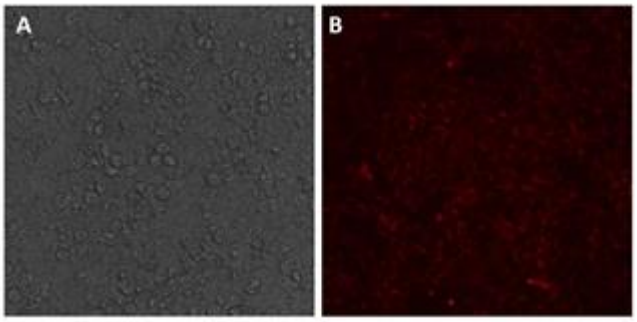
Incubation of human cells with labeled O|N|R® (fluorescence microscopy)



A - Non-labeled MCF-7 cells (green cells express GFP, green fluorescent protein)

B - MCF-7 cells incubated with labeled O|N|R® (red labelling is O|N|R®)

Incubation of human cells with labeled O|N|R® (fluorescence microscopy)



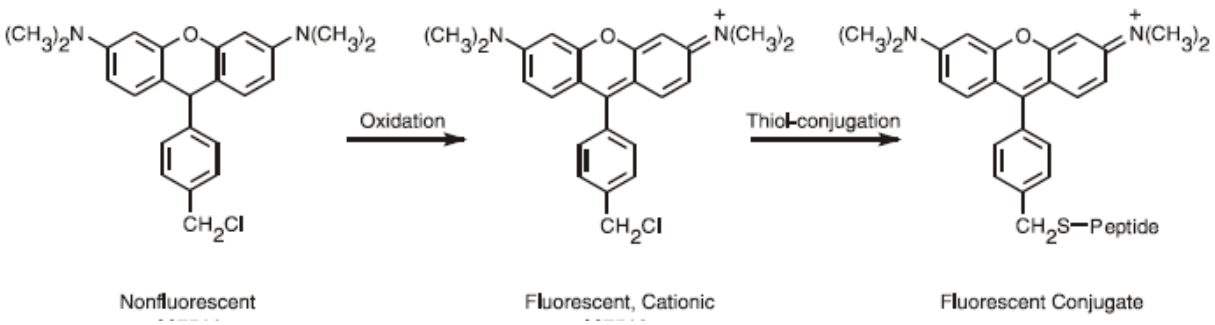
A- Non-labeled MCF-7 cells observed by phase contrast microscocopy.

B- MCF-7 cells incubated with labeled O|N|R® observed by fluorescence microscopy (red labelling is O|N|R®)

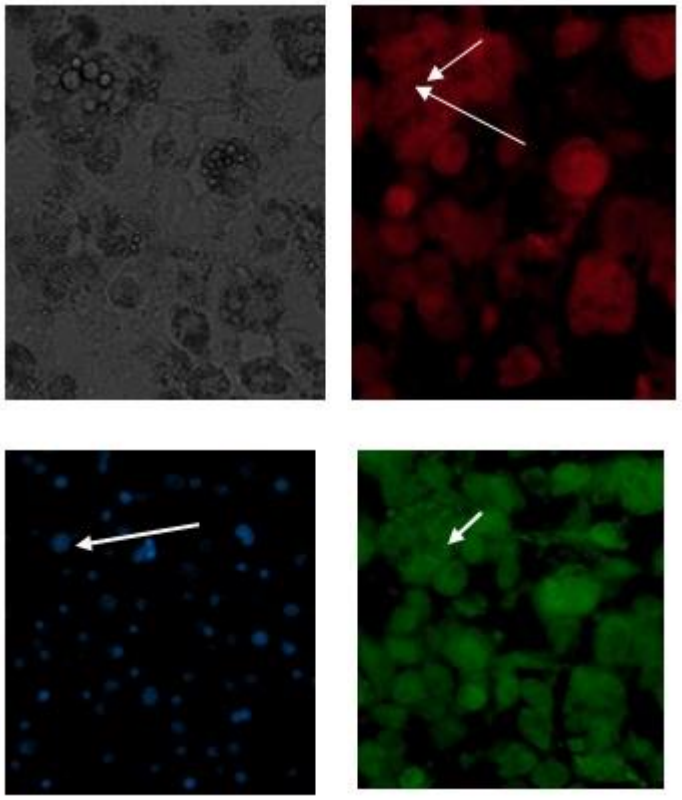
Colocalization of O|N|R® and mitochondria in adipocytes

MitoTracker Green FM probe is essentially nonfluorescent in aqueous solutions and only becomes fluorescent once it accumulates in the lipid environment of mitochondria. (InvitroGen).

MitoTracker Green FM probe preferentially accumulates in mitochondria regardless of mitochondrial membrane potential (InvitroGen).



Co-staining of O|N|R® phospholipids and adipocyte mitochondria



Red: Phospholipid (O|N|R®) / **Green:** Mitotracker Green / **Blue:** Hoesch dye (nuclei)

5. CLINICAL TRIALS

5.1. ANTIOXIDANT CAPACITY

Customer: SANKI MAYOR

Laborator: INSTITUTO DE BIOLOGÍA MOLECULAR Y CELULAR Universidad Miguel Hernández.

Avda. del Ferrocarril s/n, E-03202 Elche (Alicante), Spain

Corresponding author: Dr. Vicente Micol

Objective: Evaluate the antioxidant activity of the dietary supplement O|N|R® (ORAC test).

Introduction

The antioxidant capacity of O|N|R® was determined by the ORAC (Oxygen Radical Absorbance Capacity) test with measures both the time and degree of free-radical inhibition.

Method

Evaluation of the effects of O|N|R® as compared to placebo on total antioxidant capacity (TAC) as measured through ORAC assay (accurate +/- 5%). The antioxidant capacity was estimated by Ferric Reducing Power, and expressed as micromole Trolox equivalent (TE) per 100 grams (µTE/100 g).

Results

Results are shown for antioxidant capacity of subjects before and after 30-day administration of O|N|R® or placebo. As can be seen from Figure 1, at Day 0, there are no differences between groups in antioxidant capacity. After O|N|R® ingestion for 30 days, there is an increment change in antioxidant capacity.

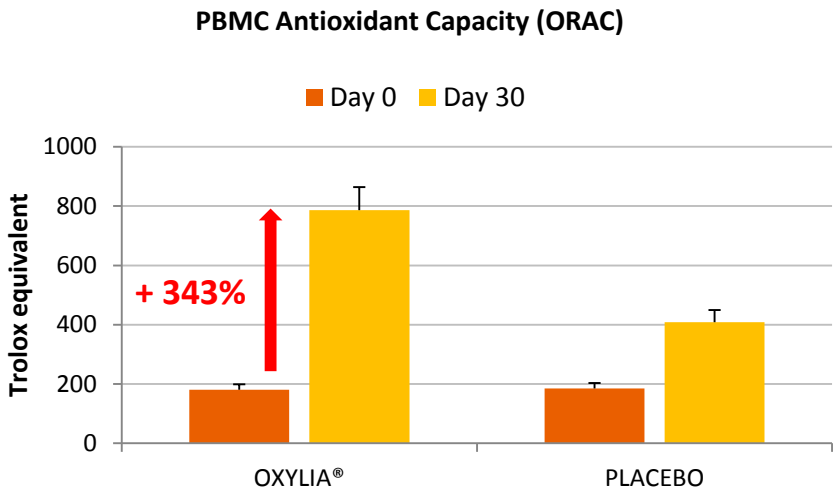


Figure1. Analysis of the Antioxidant Capacity of PBMCs across Study Group over Time

Conclusion

The results of this investigation show that O|N|R® enhanced consistently the antioxidant capacity of the subjects after 30 days (+343%).

5.2. ANTHROPOMETRIC MEASUREMENTS

5.2.1. Clinical evaluation of efficacy of O|N|R® on weight loss

Customer: SANKI MAYOR

Scientific supervisors: Dr Masato TANAKA - Dr Hirata KANEMOTO - Dr Asako FUJIWARA

Setting: Tokyo Hospital – Nutrition & Health Dept, Tokyo, Japan

Study protocol number: OXT-JP-06/2006

Objective: Investigate the efficacy of O|N|R® – Food supplement (derived from black beans, olive and rosemary extracts) on weight loss.

Design: Randomized, double blind, placebo controlled clinical trial.

Subjects: 60 healthy, overweight 25 ≤ BMI ≤30, men and women, between 24 to 65 years.

Interventions: 2 tablets of 125 mg before lunch and 2 tablets of 125 mg before diner for 60 days. Each tablet contains 40% (50mg) of bio actives and 60% (85mg) of excipient.

Main outcome measures: Body weight and impedance measurements and waist, hip and thigh circumferences.

Results: 60 subjects completed the study. O|N|R® was well tolerated. At the end of the study, subjects receiving O|N|R® showed significant reduction of body weight, fat mass, adipose tissue, and waist/hip/ thigh circumferences.

Conclusion: The results show that O|N|R® produces significant reductions in body weight of overweight individuals and this reduction is caused by loss in fat mass rather than lean mass.

Final report date: September, 2006

INTRODUCTION

Overweight is defined as an excessive accumulation of *fat* in adipose tissue, which can affect human health and lead to serious diseases, as diabetes, and cardiovascular diseases. Overweight already became a global public health problem, with a rapid global rise linked to societal changes, affecting indifferently any part of the world. The World Health Organization (WHO) estimates that more than 1.4 billion adults were overweight (with BMI ≥ 25) in 2008, which represents 35% of world population.

The principal causes of overweight include low physical activity and high fat and calorie foods intake increase. Being overweight increases the risk of heart diseases and strokes, diabetes, musculoskeletal disorders, some cancers, etc...

Strategies aimed at losing weight and preventing overweight has not been successful to date. Follow up on longer periods are rare and only a few people achieve to maintain weight loss in the long term. Moreover, repeated weight loss followed by weight regain cycles may be unhealthy as they have been associated with increased risk of heart disease and bone loss. However, there are some strong evidence that even a small weight loss (5% of body weight) can significantly decreases the risk of diabetes and cardiovascular diseases.

Various approaches and treatments have been used for management of overweight. These include lifestyle and behavioural modifications as well as use of prescription and non-prescription drugs. Lifestyle and dieting approaches include restriction of caloric intake, and increased physical activity. These regimens are difficult to follow, may cause adverse effects and often result in regaining of lost weight when the intervention is stopped.

Food extracts, herbs and botanicals that have been used for centuries have a potential in managing overweight. An approach for treating overweight is the inhibition of polysaccharides during the digestion process through amylase inhibitors which can be found in the black beans. Alpha amylase inhibitors are known as starch blockers and are effective agents for controlling overweight and associated diseases. Alpha-amylase inhibitors have the capacity to interfere with the breakdown of starch, which will prolong the digestion time, reduce the energy derived from the starch and reduce the body glucose absorption.

The black beans alpha amylase inhibitors have been shown through several animal and human studies to significantly reduce postprandial hyperglycemia and to cause weight loss without any observable side effects on.

In humans, uncooked black beans consumption can be associated with gastrointestinal disorders, mainly due to the potentially toxic substance phytohemagglutinens (PHA) present in raw beans. However, PHA levels can be reduced considerably by cooking, and commercial preparations have been shown to be safe to use.

Olea europaea (olive tree), belonging to the family Oleaceae is a small evergreen tree native to the Mediterranean region. Olive preparations have been used in folk medicine in European Mediterranean area as diuretic, hypotensive, emollient and for urinary and bladder infections for a very long time. The Mediterranean diet is characterized by a high consumption of olive oil, whose intake is greatly growing worldwide, since it influences on health outcomes have been investigated.

Among the different components, Oleuropein is the most important active compound of the Olea europaea, responsible for the known antioxidant action of the extracts of the olive plant. In-vitro and in-vivo experiments have demonstrated the antioxidant activity of olive extracts to reduce free radical production. Several studies have also showed hypoglycemic and hypolipidemic activity of olive. The main active constituent reported was oleuropein, which is involved into the potentiation of glucose-induced insulin release process. Another important ingredient is hydroxytyrosol which also has antoxidant properties.

Rosemary (Rosmarinus officinalis L.) is a well know aromatic plant which has been cultivated and used for a long time in folk medicine and cooking. It has been found that rosemary herbs were used as medicinal, culinary and cosmetic virtues in the ancient Egypt, China and India. Its wide usage as a culinary herb as well as clinical studies has proven its safety. Rosemary has strong antioxidant properties. There are several reports that identify the antioxidant activity of these extracts is mainly due to the content of phenolic carnosic acid (and its derivative products).

In this article we will now describe a clinical study on O|N|R®, which is a new weight loss product derived from black beans, olive and rosemary extracts.

MATERIALS AND METHODS

Study design

A randomized, double-blinded, placebo-controlled study of 60 days duration, in accordance with the Helsinki Declaration and other applicable laws related to the protection of study subjects, was carried out.

Volunteers were recruited from a group of individuals who expressed a willingness to participate in such evaluations. Sixty subjects, aged from 24-65 years, found to be overweight were selected.

Overweight was measured using the following formula: body weight – ideal weight. The ideal weight calculation was (kg) = 100/(100 - % normal body fat) x lean mass.

The inclusion criteria included age between 20 and 65 years, overweight, 25 ≤ BMI ≤30, general good health, stable weight for past two months, no ongoing drug treatments, commitment to eating as prescribed by

nutritionist, commitment to avoid any changes in lifestyle throughout test period, commitment to avoid use of other weight loss products during study.

The exclusion criteria included pregnant or breast feeding females, weight reduction treatments prior to study, within 6 months postpartum, planning to become pregnant, patients of diabetes, heart disease, renal, liver or thyroid disease, concurrent medications, psychiatric patients , those taking more than 3 alcohol drinks per day and any condition contrary to those indicated in enrollment criteria.

The chosen participants reported to the Center at 0, 30 days and 60 days to have their body weight and other measurements checked and recorded. The two groups took the assigned tablets, two tablets before lunch and two tablets before dinner. Volunteers were asked to eat daily complex carbohydrates during one of the principal meals, with no additional alterations in daily habits (job, sports etc).

TEST PRODUCTS

The active substance to be tested was a 2 x 125 mg tablets, twice a day, of O|N|R®. Active and placebo tablets were supplied in opaque white plastic bottles containing a known number of tablets. Subjects were directed to take 2 tablets before lunch and 2 tablets before diner. The placebo was an identical appearing tablet containing inert ingredients. The control substance was an identical tablet which was visually indistinguishable from the tablet containing active ingredient. It contained no pharmacologically active substances.

MEASUREMENTS

Body weight and impedance measurements, and waist, hip, and thigh circumferences tests were measured at the beginning and at the interim (30-day treatment phase) and at the end of 60 days.

Baseline measurements are showed in table 1.

Baseline measurements	Test (N=30)	Placebo (N=30)
Age	39.2±6.7	40.5±7.2
Gender	23F – 7M	25F – 5 M
BMI (kg/m²)	26.6±1.7	26.2±1.6
Weight (kg)	73.8±5.9	72.9±5.3
Waist circumference (cm)	83.8±4.2	84.2±3.9
Hip circumference (cm)	106.2±2.3	105.8±1.8
Thigh circumference (cm)	67.6±1.9	67.2±1.6

Table 1. Baseline outcomes at the beginning of the clinical trial.

Body Weight and Composition

Body weights, performed on individuals wearing only undergarments, were measured using a calibrated balance beam scales to the nearest 0.1kg. BMI was calculated as weight in kilograms divided by height in meters squared.

Waist. Hip. and Thigh Circumferences

The respective circumference of the waist, hips, and right thigh was measured using a standard non-stretchable flexible measuring tape. Temporary tattoos were used to identify the area of reference from one reading to the next. Waist circumference was measured at the level midway between the lowest rib margin and the iliac crest, and hip circumference was measured at the widest level over the greater trochanters. Thigh circumference was measured on the left leg directly below the gluteal fold. The mean value of two measurements was used in the analyses.

Adverse/Side Effects

The subjects were monitored throughout the investigation for the occurrence of any adverse or side effects.

Statistical Analysis

To minimize differences in values between subjects, they were stratified into two groups very similar in size, age, gender, and body weight distribution. At completion, data from subjects receiving the Test supplement and subjects receiving the Placebo supplement were available for statistical analysis. For each subject, the differences between pre-treatment (baseline) and post-treatment (30-days and 60 days) values for each parameter (body weight, BMI, etc.) were calculated. The differences were always obtained by subtracting the 30 or 60 days values from the baseline values. A negative difference indicates a reduction in the parameter after 30 and 60 days. A positive difference indicates an increase in that parameter. The difference between baseline and 30 and 60 days values are analysed using Student's t-test.

RESULTS

The Test and Placebo groups were comparable in age, gender, weight, BMI and various body circumferences. All the subjects completed the study. No significant adverse effects were reported. The results are showed in table 2 and 3.

At 30 days	Test (N=30)	Placebo (N=30)	P Value
BMI (kg/m²)	-0.8±1.4	0.1±0.5	<0.001
Weight (kg)	-3.18±0.7	0.1±0.8	<0.001
Waist circumference (cm)	-3.18±0.6	1.3±0.9	<0.001
Hip circumference (cm)	-5.02±0.8	1.8±0.8	<0.001
Thigh circumference (cm)	-3.01±0.8	0.8±0.7	<0.001

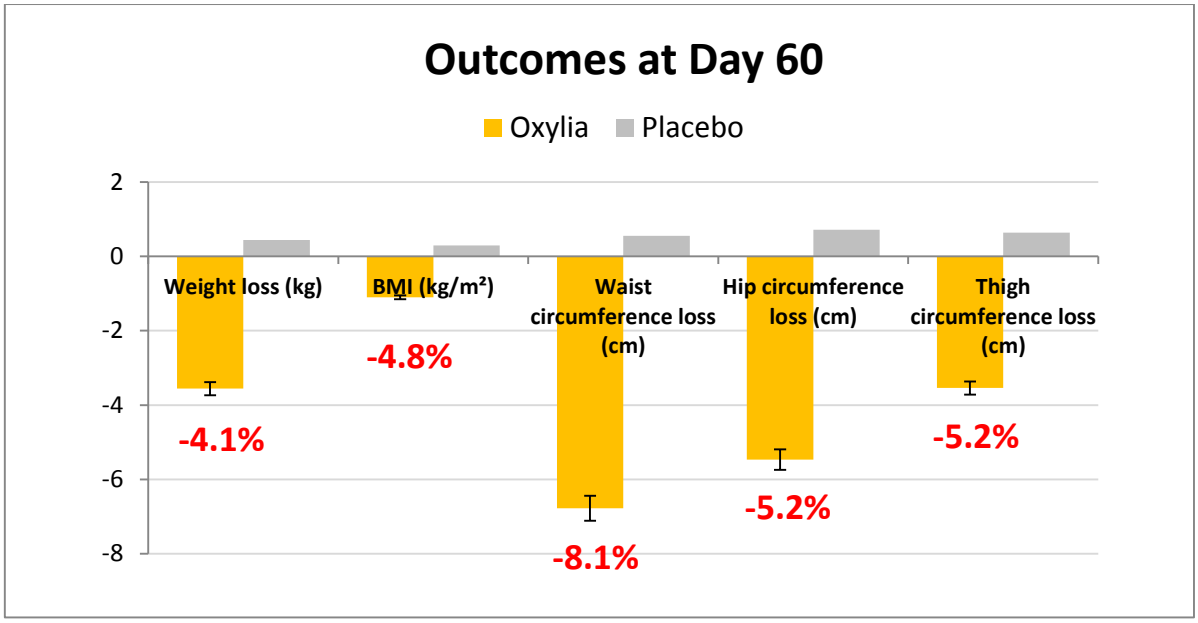
Table 2. Results of active group and placebo group measures after 30 days of treatment

At 60 days	Test (N=30)	Placebo (N=30)	P Value
BMI (kg/m²)	-1.1±1.2	0.3±0.9	<0.001
Weight (kg)	-3.56±0.9	0.44±0.8	<0.001
Waist circumference (cm)	-6.78±0.8	0.56±0.6	<0.001
Hip circumference (cm)	-5.47±0.8	0.72.±0.7	<0.001
Thigh circumference (cm)	-3.54±0.7	0.64±0.8	<0.001

Table 3. Results of active group and placebo group measures after 60 days of treatment

All the subjects receiving active ingredient experienced weight loss. The average reduction in body weight in Test group was 3.56 Kg., average reduction in waist size was 6.78 cm and average reduction in body fat was 9.5 %. The average gain in Placebo group during this period was 0.44 Kg. The best results that were achieved were 4.62 Kg. in weight reduction and 8.45 cm in waist measurement.

The study results after 30 days showed a reduction in waist measurement of 3.18 cm, reduction in thigh circumference of 3.01 cm and in hip circumference of 5.02 cm. After 60 days of treatment, subjects in the Test group had lost an average of 6.78 cm in waist measurement and 3.54 cm and 5.47 cm in thigh and hip circumference respectively (see graphic 1).



Graphic1. Placebo and test group outcomes at the 60th day of the trial.

The participants found the compliance easy. There were secondary findings of increased energy, feeling of well-being, toning of body etc .which, however, didn't reach significant levels.

DISCUSSION

The results of this study indicate that a dietary supplement O|N|R® is effective at reducing body weight and body fat mass when taken daily by overweight human subjects.

Indications by Bodystat measurements showed that most of the weight changes are induced by fat loss rather than diminution in lean body mass. Many dietary ingredients which aim is to combat overweight provide only scale weight loss, but not based on true fat loss.

O|N|R® appears to influence weight loss through multiple mechanisms. The main mechanism is the inhibitory action of bean extract that has been shown to inhibit the activity of a-amylase and interfere with the digestion of carbohydrates. This inhibition results in influencing the insulin system, which lead to less fat accumulation and promote weight loss.

In other hand, if we consider that overweight is defined as a state of chronic oxidative stress, then antioxidant supplementation should help in controlling overweight. All the ingredients contained in O|N|R® have strong antioxidant activity. Thus, supplementation with O|N|R® should not only confer all other known benefits of reducing oxidative stress but should also increase weight loss being induced by other ingredients of O|N|R®.

All the ingredients of O|N|R® are common dietary agents and have been used by humans for centuries. The quantity contained in O|N|R® is within acceptable limits.

CONCLUSION

The results of this study show that O|N|R® produced significant decreases in body fat while essentially maintaining lean body mass when taken daily during 60 days by overweight subjects. O|N|R® appears to be a safe and effective aid to consider in weight loss and maintenance programs.

5.2.2. Evaluating the efficiency of O|N|R® in enhancing and maintaining weight loss in humans

Customer: SANKI MAYOR

Laborator: INSTITUTO DE BIOLOGÍA MOLECULAR Y CELULAR Universidad Miguel Hernández. Avda. del Ferrocarril s/n, E-03202 Elche (Alicante), Spain

Corresponding author: Dr. Vicente Micol

Title: Evaluating the efficiency of O|N|R® in enhancing and maintaining weight loss in humans

Number of participants	30 healthy human (15 females, 15 males) between 5-25 kg overweight
Age of participants	Between 25 and 65 years old
Period of the study	90 days (Control each month)
Products tested	O|N|R® (250mg capsule) and Placebo. Each O|N|R® capsule contains 100mg of bio active compounds and 150mg of excipient.
Dosage	2 capsules per day

Summary

O|N|R® is a nutritional supplement which has been developed to help weight loss by reducing the digestion of diet carbohydrates. Its formula combines a mixture of herbal extracts from edible Mediterranean plants (olive and rosemary), known as powerful antioxidants, and black bean extract which is known to inhibit polysaccharides enzymatic digestion. The antioxidant components of the complex provided with significant antioxidant capacity to account for the protection of the gastrointestinal (GI) tract against oxidative stress. A 90 days double-blind and placebo-controlled study was done with O|N|R®. To highlight the activity claimed by O|N|R®, 30 healthy human volunteers between 25 and 65 years old (15 females, 15 males), with 5-25 kg overweight, were asked to take a capsule of O|N|R® complex (250 mg) before each meal (lunch & dinner). At t0, t30, t60 and at the end of the study t90, weight of each volunteer was measured. Waistline, hips and thigh circumferences were measured. The results show that O|N|R® group maintained a regular weight loss of approximately 3.2 Kg during the 90 days of the treatment. O|N|R® group demonstrated no over weight gain during the 90 days either. A remarkable decrease of the waist circumference of almost 4% was observed throughout the treatment in the O|N|R® group, and a minor decrease in hip and thigh circumferences was also observed (approximately 1.5% each). All these results suggest that O|N|R® can be taken as a safe therapy for weight loss management and to protect the GI against the dietary-induced oxidative stress.

Introduction

Overweight is a main risk factor for many different serious disorders, including heart disease, diabetes, several types of cancer, cardiovascular diseases and stroke. Therefore, effective methods for weight reduction are constantly being required. Although there are many different weight control methods and slimming diets in use today, it is very difficult for most people to maintain a regular weight loss. Many researches have shown that almost all individuals regain the weight they lost after some time. Long-term maintenance is rare, and repeated weight loss followed by weight regain cycles may be unhealthy, as it has been associated with increased cardiovascular risks.

Caloric restriction is the main goal of most weight reduction slimming approaches. A basic principle is that if food intake is less than energy consumption, stored calories, mainly in the form of lipids, will be consumed. Other slimming approaches are based on the principle of increasing metabolic rate through burning calories, what lead to a decrease of body weight by calories. However, the majority of these treatments often cause side effects, particularly those involving the use of non-prescription and prescription drug products. Furthermore, these treatments often result in a rapid weight increase once treatment is concluded, unless a drastic modification of the behaviour that led to weight gain is undertaken.

Some of the most commonly used weight-loss remedies to treat obesity are based on a decrease of digestive nutrients absorption by non-digestible fiber (chitosan, wheat bran, psyllium or pectin). Alternatively, another approach consists of deducing the digestion of polysaccharides through the action of alpha amylase inhibitors present black beans. The claimed slimming capacity of these products is based on their ability to reduce the rate of starch digestion in the small intestine by the inhibition of alpha amylase, a pancreatic enzyme that hydrolyses starch. Alpha amylase inhibitors are lectin-like inhibitors that prevent starch digestion and can be extracted from several types of plants, especially those from the Leguminaceae family.

Currently available amylase inhibitors are extracted from either black bean or wheat. The common bean (Phaseolus vulgaris) contains a family of plant defense proteins that includes phytohemagglutinins (PHA), arcelin, and alpha amylase inhibitors.

Foods and beverages rich in phenolic compounds have often being associated with the decreased risk of developing several diseases. It is assumed that bioactive components of the human diet, such as flavonoids, may play a vital role in reducing the risk of "radical-related" oxidative damage. These effects may include scavenging of metals, reactive oxygen, chlorine and nitrogen species and the inhibition of inflammatory processes. However, many flavonoids have poor antioxidant effect due to poor absorption rate into the gastrointestinal tract.

Olive (Olea europaea) and rosemary (Rosemary officinalis) are vegetable species rich in phenolic compounds bearing strong antioxidative activity, and have been also a part of the Mediterranean diet for centuries. Hydroxytyrosol and oleuropein from olive leaves have been shown as potent radical scavengers. In addition, its presence in the human diet, among other factors, has been related to the prevention of coronary artery diseases and atherosclerosis. Rosemary is one of the most widely used and commercialized plant extracts, bearing antioxidative effect, not only as a culinary herb for flavouring but also used as antioxidant in processed food and cosmetics. There are several reports that identify the compounds that are chiefly responsible for antioxidant properties of rosemary extracts both in lipophilic and hydrophilic fractions. The antioxidant activity of these extracts is mainly due to the content of phenolic carnosic acid and its derivative carnosol. Moreover, the occurrence of other phenolic compounds such as flavonoids and phenolic acids, especially rosmarinic, also contributes to the bioactivity of this aromatic plant.

In the present article we describe a clinical study on O|N|R®, a new weight loss complex derived from the black bean and containing two powerful antioxidants extracts from Mediterranean edible plants (olive and rosemary) which provides with additional free radical scavenging properties for the GI tract. The clinical trial demonstrates the efficacy of O|N|R® on weight loss in a three months clinical study. The results also shows that O|N|R® group experienced significant hip (1.5%), thigh (1.6%) and waist (3.8%) circumference losses compared to placebo group. In addition, all these criteria were maintained after three months what demonstrates the effectiveness of O|N|R® to maintain a constant weight loss. O|N|R® was very well tolerated with no evidence of the side effects commonly experienced with other existing obesity drugs.

Methods

Clinical trial on weight loss

Thirty healthy 5-25 Kg overweight volunteers between 25 and 65 years old (15 females and 15 males) participated in the present study. Subjects were included in a randomized placebo-controlled and double-blind trial to highlight the activity claimed for O|N|R® complex. 30 days before starting the test, volunteers were controlled by a nutritionist. Weight of each volunteer was registered after 10, 20 and 30 days the experiment started and only volunteers whose weights remained stable in such a period were recruited for the study. A placebo group of 30 participants was also used for the study.

Subjects were assigned to receive either one 250 mg capsule of O|N|R® or indistinguishable placebo twice daily (one before lunch and dinner) during 90 consecutive days. Volunteers were asked to eat daily complex carbohydrates during one of the principal meals, with no additional alterations in daily habits (job, sports, etc.). At the starting date of the study (t0), day 30th, (t30), day 60th (t60), and at the end of the study (t90), weight, and waistline, hips and thigh circumferences were measured.

Informants were asked to remove all outer layers of clothing, shoes with heels, tight garments intended to alter the shape of the body, and belts before the measurements. The mean value of 2 measurements was used in the analyses. Volunteers did not report any side effects during the study.

None of the subjects were taking any drug or dietary supplement at the time of the study. All were briefed on the protocol and gave consent to the trial. Standard deviation was not higher than 1% in any case.

Results and discussion

An innovative black bean-derived complex called O|N|R® was developed and its slimming and antioxidant properties have been evaluated throughout this study.

A randomized, placebo-controlled and double-blind clinical trial was set in order to prove the efficacy of O|N|R® as a slimming complex using thirty healthy 5-25 Kg overweight volunteers between 25 and 65 years old. Volunteers' weight was measured before the study and at 30, 60 and 90 days after the beginning of the test. Figure 2 shows the evolution of the average weight loss for the participants' groups, O|N|R®-treated and placebo group. The placebo group did not show a significant weight loss during the time of treatment, only an average weight loss of 0.38 Kg. In contrast, the O|N|R® group, which had 250 mg of the complex twice a day, showed a significant weight loss, approximately 3 Kg, after 30 days of treatment. This weight loss increased slightly at the day 60th (3.25 Kg) and was maintained almost invariable until the end of the treatment (90 days). Although the weight loss in the O|N|R®-treated group did not reach values further than those obtained at day 60th, i.e. 3.2 Kg, it is a remarkable fact that the rate of weight loss was maintained throughout the treatment period. These results show an encouraging trend for expectations of longer-term dosing of the complex.

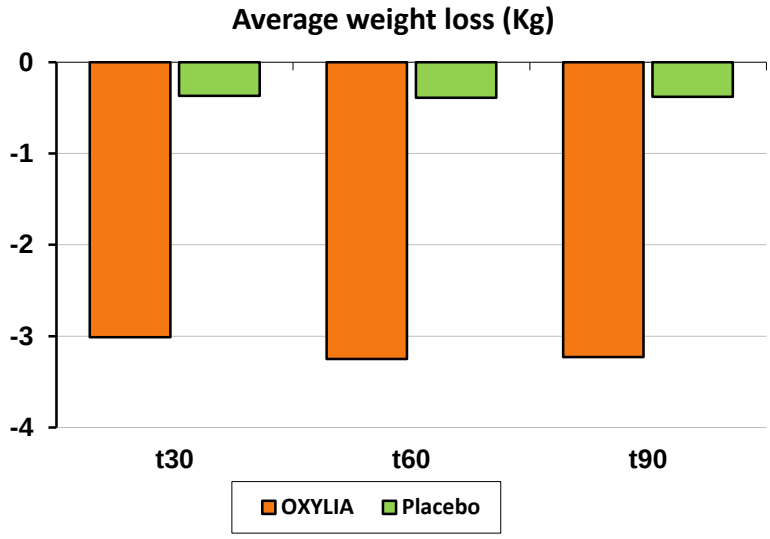
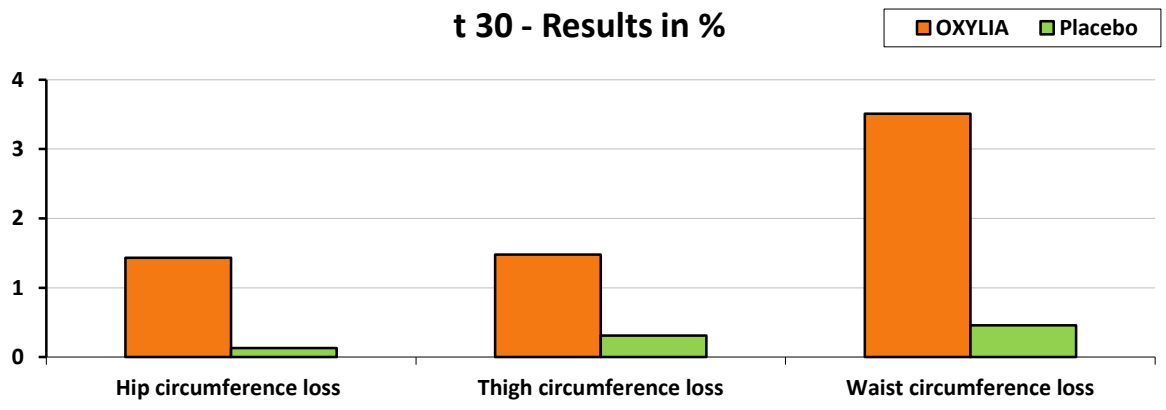


Figure 2. Average of the participants' weight loss measured at times t30, t60 and t90.

Additionally, hip, thigh and waist circumferences of volunteers were measured to obtain the evolution of the distribution of volunteers' fat during the treatment with O|N|R® or placebo. Figure 3 shows that individuals treated with O|N|R® presented an average reduction in their hips size of 1.43% at t30. This reduction was increased with the follow-up of the treatment, reaching values of 1.54% and 1.55% at t60 and t90 respectively. In a similar way, a thigh circumference decrease of 1.48% was observed at t30, which increased with the treatment to 1.53% and 1.57% at t60 and t90. The average waist circumference of the O|N|R® group exhibited the most significant reduction compared to placebo. O|N|R® participants experienced a waist circumference decrease of 3.51% at t30. In addition, waist circumference decrease showed higher values throughout the study, 3.78% and 3.82% at t60 and t90 respectively, meaning a continuous diminution of the fat around waist by the end of the treatment. Moreover, no adverse or side effects were observed in any of the volunteers during the study.

In summary, O|N|R® is a tested slimming complex that exhibits a significant antioxidant capacity against lipid peroxidation measured by the TBARS assay with a stronger potency than the antioxidant compound BHT. This antioxidant capacity may provide with important free radical scavenging properties to protect the gastrointestinal tract against reactive species derived from the diet or phagocytes activation in the gut. The results of the clinical study show that placebo group did not demonstrate any significant weight loss or hip, thigh and waist circumference losses, while O|N|R® group demonstrated a good efficacy for all these criteria. The study also demonstrated that O|N|R® group maintained a regular weight loss of approximately 3.2 Kg during the 90 days of the treatment. O|N|R® group also showed no over weight gain during the 90 days either. Therefore, the conclusions of this 12-week weight loss trial were associated with a decrease on weight and fat distribution at hip, thigh and waist of the participants and consequently with positive effects on health variables. A remarkable decrease of the waist circumference of almost 4% was observed throughout the treatment in the O|N|R® group. All these results suggest that O|N|R® can be taken as a safe therapy for weight loss management.



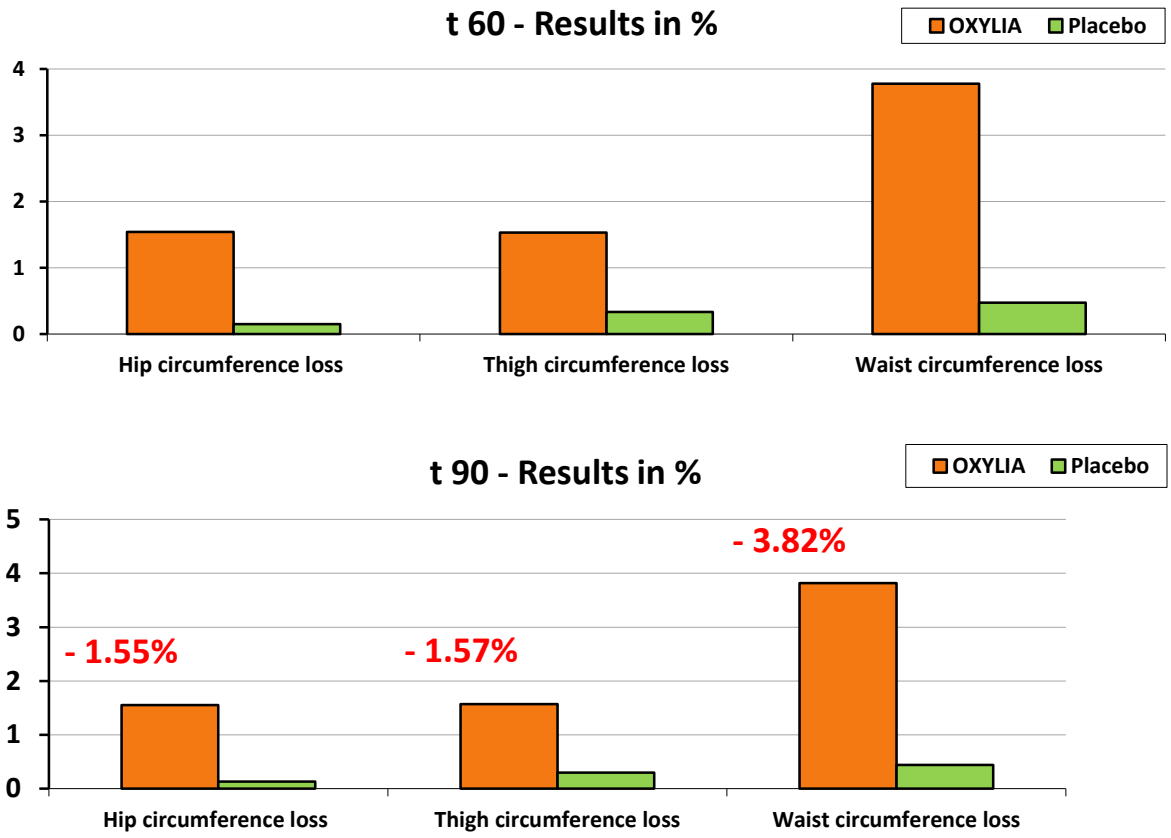


Figure 3 Average of the participants' hip, thigh and waist circumference losses measured at times t30, t60 and t90 of the study.

5.3. BLOOD SUGAR CHOLESTEROL

5.3.1. Efficacy of OXI-280 on blood lipid parameters

This study was designed to investigate the effects of OXY-280, a dietary supplement ingredient OXY-280 on the weight management of overweight (25<BMI<30) adult Mexicans, 25-55 years of age. OXY-280 has been developed to help weight loss by reducing the digestion of diet carbohydrates. Its formula combines a mixture of herbal extracts (olive and rosemary), known as powerful antioxidants and black bean extracts which is known to inhibit polysaccharides enzymatic digestion and purple potato which demonstrates alpha-glucosidase inhibitory action.

Study design

The study was a prospective, 90 Days, randomized, double blind, placebo controlled, clinical safety and efficacy trial conducted.

The efficacy of OXY-280 was assessed by measuring changes in body anthropometric measurements (weight, body fat, waist and hip circumferences). Safety was assessed by self-reported symptoms and reasons for withdrawal from the study.

Randomization of equal numbers of subjects to placebo or treatment groups was achieved using a random number table. Sealed copies of these codes were provided to the investigators for emergency identification.

Statistical analyses were designed on an “intention to treat” basis to achieve a statistical power of 0.90 and a 0.05 type I error for a two sided test.

Subjects

Subjects were admitted into the study if they qualified to the following inclusion criteria:

- Male or female, 25-55 years old in overall good health.
- Body mass index (BMI) between ≥ 25 and ≤ 30 kg/m.
- Elevated LDL-cholesterol.

Subjects were excluded if any:

- Presence of any systemic, infectious diseases or chronic conditions, including leukemia and/or cancers.
- Having recently lost weight or participated in other diet or drug studies.
- Currently taking medications for infection, systemic or chronic conditions.

- Any history or evidence on examination, of clinically significant autoimmune, rheumatological, gastrointestinal, renal, hepatic, endocrine, respiratory, cardiovascular, dermatological or hematological disease, which in the opinion of the investigator may affect the interpretation of the efficacy and safety data.
- Current smoker or history of smoking for at least 6 months prior to enrolment into the study and active alcohol/drug dependence or abuse.

Subjects who did not fall into any the exclusion categories after these baseline measures were randomized to either placebo or the OXY-280 preparation.

Early Withdrawal of Subjects

Patients may have been withdrawn from the study (i.e. from any further study product or study procedure but not from analyses) for the following reasons:

- At their own request: If, in the investigator's opinion, continuation in the study would be detrimental to the patient's well-being.
- If they are lost to follow-up: In any occurring cases, the reason for and date of withdrawal were recorded in the Exit Form and in the patient's medical records, and the sponsor's representative was notified within 5 days. The patients were followed up to establish whether the reason was an adverse event, and, if so, this was reported in accordance with the procedures detailed in Safety and Adverse Events.

The investigator made every effort to contact patient lost to follow-up. Attempts to contact such patients were documented in the patient's records (e.g., dates and times of attempted telephone contact).

Treatment

At randomization, subjects were counselled to eat normally, and maintain their usual activities. Active and placebo tablets were supplied in opaque white plastic bottles containing a known number of tablets. Subjects were directed to take one tablet, before lunch meal and diner meal (2 tablets per day) and to return unused tablets, which were counted to determine adherence.

The active preparation was a mix of plant extracts containing Olive, Rosemary, and Black Bean as the only active ingredients. Each tablet was specified to contain 250 mg of the mix of plant extracts. Each tablet was formulated with 100 mg of active ingredients and 150 mg of excipient. The placebo was an identical appearing tablet containing inert ingredients.

Certificates of analyses were validated by the investigators.

During the subsequent 90 days, subjects had a control visit at 30 days, 60 days and 90 days. The following parameters or variables were measured and analyzed:

- Body weight (at 30, 60 and 90 days)
- BMI (at 30, 60 and 90 days)
- Waist & hips circumference (at 30, 60 and 90 days)
- Body fat (at 30, 60 and 90 days)
- Mental and physical feeling (by questionnaire) (at 30, 60 and 90 days)
- Fasting HDL-,LDL-, and Total-cholesterol (at 90 days)
- Fasting triglycerides (at 90 days)

Normal laboratory values for selected parameters.

Test (variable)	Specimen	Method	Normal Range (Values Units)	
			Conventional	International
Triglyceride	Serum	Enzymatic	<200 mg/dL	<2.26 mmol/L
Total cholesterol	Serum	Enzymatic	<200 mg/dL *High: ≥240 mg/dL*	< 5.17 mmol/L *High:≥6.21 mmol/L*
HDL-cholesterol	Serum	Direct	≥35 mg/dL *"Negative" risk factor: ≥ 60 mg/dL*	≥0.9 mmol/L *"Negative" risk factor: ≥1.55 mmol/L*
LDL-cholesterol	Serum, calculated	Calculate from Total & HDL-c	Same as above	Same as above
Fasting Glucose	Plasma	Hexokinase	<110 mg/dL	<6.1 mmol/L

Source: The Merck Manual Seventeenth Edition and the US National Institute of Health, unless stated differently.

Safety Profile

The safety profile of the study product was determined by evaluating the incidence and severity of adverse events that occurred. The adverse events that were monitored included: abdominal pain, abdominal bloating, distended feeling/gas, diarrhea/constipation, flatulence, vomiting, regurgitation, and heartburn. Participants were instructed to contact the study coordinator by phone in the event that the participant experienced any of the aforementioned adverse events. The study coordinator then documented the adverse event's occurrence and severity.

Study Objectives

Primary Objectives: To evaluate the effects of OXY-280 as compared to placebo on weight loss, on BMI reduction, waist & hips OXY-280 as compared to placebo fat reduction

Secondary Objective: To evaluate whether OXY-280 has a positive effect on regulation of serum lipids through fasting cholesterol (HDL, LDL, VLDL) and triglycerides. To evaluate the safety profile of OXY-280

Measurements

Anthropometric Measurements: All measurements were taken between 8:00 and 10:00am. The lead investigator oversaw the anthropometric measurements. BMI is calculated as weight in kilograms (measured to the nearest 0.1 kg without coats and shoes) divided by height in meters squared (measured to the nearest cm without shoes), kg/m2. Waist- and hip-circumferences were measured in cm as the minimum value between iliac crest and the lateral costal margin and the maximum value over the buttocks respectively, with values taken twice.

Blood Samples and Biochemical Analysis

A form was provided to the nurses to record the value for each designated date. Blood samples were drawn on days 0, and 90 between 8 and 10 am after an overnight fasting (12-14 hours). The blood samples (10 mL) were collected into tubes, and centrifuged with in 30 minutes at 3000 rpm and 40C for 10 minutes to separate and collect the plasma and stored at -700C until the laboratory assays are performed for the different biochemical parameters.

The biochemical determinants [fasting TG, total-, HDL-, LDL-), were measured according to the following procedures:

Fasting plasma TG, HDL-, and total-cholesterol were measured enzymatically using an automated clinical analyzer (Bayer 650).

LDL cholesterol was calculated using the Friedewald equation (Friedewald et al., 1972): [LDL-cholesterol = total cholesterol –HDL-cholesterol-triglycerides/5])

General Medical History Questionnaire

A general/medical history questionnaire was completed at baseline. Only participants who have not been excluded from the study completed the questionnaire. The questionnaire included general medical history questions pertaining to history of disease, such as heart disease, diabetes, cancer, etc.

Physical and Mental Status Assessment

Mexican standards were applied to determine emotional parameters and a series of culturally appropriate questions regarding the level of mood and functionability for normally applied daily routines.

Statistical methods

Values are presented in the text and tables as means ± standard deviation (s.d.) and in the figures as means ± standard errors (s.e.). The tables show statistical comparisons between the groups by the "last observation carried forward" (LOCF) method for dealing with missing data. Values for subjects who dropped out after the acute phase were carried forward to each subsequent time point in the trial. Figures present analyses of only data that was actually available for subjects at each time point, with no values carried forward for subjects who dropped out.

Effect of treatment on weight, body fat, waist and hips circumferences, sitting blood pressure, heart rate and blood chemistries were assessed by using a repeated measures ANOVA test for group by time interaction, followed by pair-wise t-tests.

Results

Baseline characteristics: baseline characteristics of the participants. 130 participants (OXY-280, n=65 and placebo group, n=65) started the study. About 95% of the participants (OXY-280, n=63 and placebo group, n=62) completed the study.

Side effects and adverse events

During the course of the study, there were no reported adverse events in either OXY-280 group or the placebo group. There were also no issues of intolerance in the OXY-280 and the placebo group including GI complaints such as gas, bloating, diarrhea, or other related symptoms. There were no missed doses in either the OXY-280 or the placebo group during the course of the study.

Baseline physical characteristics of subjects

Subjects in two treatment groups (OXY-280 – Placebo) did not differ ($P>0.05$) initially in age (43.0±12.2 (mean±s.d.); 41.5±12.4y), body weight (82.1.1±11.8; 81.9±11.1kg), or BMI (26.9±2.8; 27.0±2.2kg/m^2). Distribution of gender is not significantly different between groups (OXY-280, 40% female; Placebo, 43% female).

Baseline characteristics of all randomized subjects

Characteristic	OXY-280 (n=63)	Placebo (n=62)
Male (n (%))	23 (36%)	19 (31%)
Female (n (%))	40 (64%)	43 (69%)
Age (y)	43.0±12.2	41.5±12.4
Weight (kg)	82.1±11.8	81.9±11.1
Body mass index (kg/m²)	26.9±2.8	27.0±2.2

Anthropometric Measurements

Weight

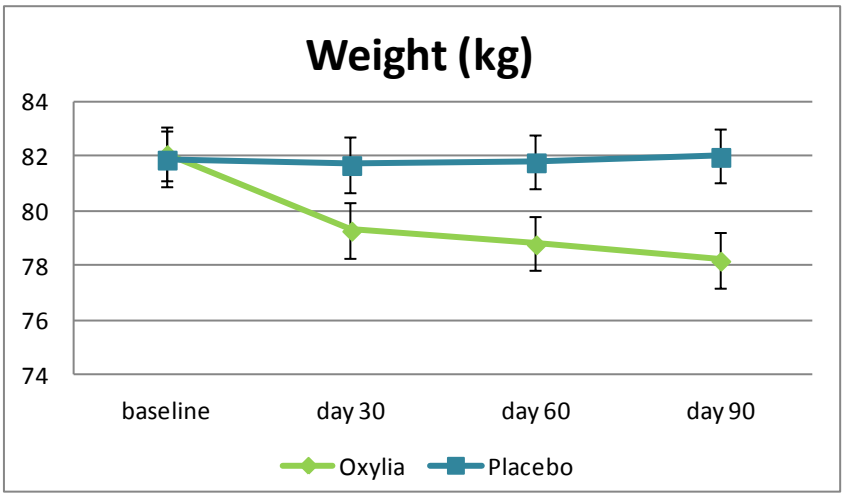
Overall, the participants in the OXY-280 group lost more weight than the participants in the placebo group. There is a significant decrease in weight noted throughout each of the 3 measurements taken. The placebo group remained fairly stable in weight during the course of the three measurements. The difference between the two groups was not statistically significant at the baseline. However, the data clearly show that the OXY-280 group showed the greatest weight reduction compared to the placebo group. This was shown with a significant weight difference between the OXY-280 group and the placebo group from Day 30 ($p=0.042$). In the OXY-280 group, all participants lost weight ranging from 2.6 to 5.3 kg (average 3.9 Kg) during the 90 days of trial, while only 15 participants from the placebo group lost weight ranging from 0.2 to 1.1 Kg (average 0.3 Kg).

Participants in the OXY-280 group had significant weight loss from Day 30 compared to the baseline measurement ($p < 0.001$). On average each subsequent weight measurement was also significantly lower than the previous weight for the OXY-280 group ($p<0.001$) over the course of the study.

In the OXY-280 group (n=63), participants who completed the study lost weight by an average of 3.9 kg ranging from 2.6 to 5.8 kg during the 90 days trial period. 100% (n=63) of the participants in OXY-280 group were able to lose weight at least 2.6 kg over the 90 days. Only 16 % (n=62) of participants in the placebo group did manage to lose weight by an average of 0.3 kg. There was only one person in the placebo group who lost 1.1 kg over the 90 days of trial.

Males and females participants in the OXY-280 group had both significant weight loss from Day 30 compared to the baseline measurement ($p < 0.001$). No significant mean weight loss difference was observed between the males and females. In the OXY-280 group, males participants (n=23) who completed the study lost weight

by an average of 3.93 kg during the 90 days trial period. In the OXY-280 group, females participants (n=40) who completed the study lost weight by an average of 3.88 kg during the 90 days trial period.



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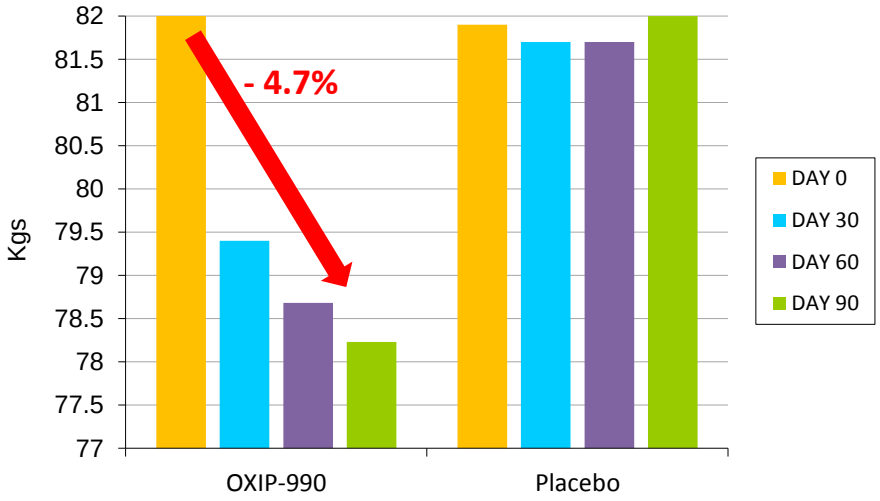
Weight loss measurements at Day 0, 30, 60 and 90



Waist Circumference

There was a significant difference in waist circumference measurements noted between the two groups (placebo and OXY-280 groups). The OXY-280 group on average lost about 3.9 cm ranging from 3.1 to 5.7 cm in waist circumference from baseline to 90 days. The placebo group has a relatively stable level of waist circumference values over the course of the study. A significant waist circumference drop was noted since Day 30 ($p< 0.05$) compared to the baseline (week 0). Therefore, these data suggest that OXY-280 has effect on waist circumference after about 30 Days of supplementation, with constant effect at Day 90.

Regarding individual waist circumference measurement, all participants in the OXY-280 group lost an average of 3.9 cm and none lost from the placebo group.

Hip Circumference

There was a significant difference in hip circumference measurements noted between the two groups (placebo and OXY-280 groups). The OXY-280 group on average lost about 5.6 cm ranging from 4.4 to 7.9 cm in hip circumference from baseline to 90 days. The placebo group has a stable level of hip circumference values over the course of the study. A significant hip circumference drop was noted since Day 30 ($p < 0.05$) compared to the baseline (week 0). Therefore, these data suggest that OXY-280 has effect on hip circumference after 30 Days of supplementation, with constant effect at Day 90.

Regarding individual hip circumference measurement, all participants in the OXY-280 group lost an average of 5.6 cm and none lost from the placebo group.

LOCF analysis of physical values

Measure	Study period	OXY-280 X±s.d. (P-*value*)	Placebo X±s.d. (P-*value*)	P
Body weight (kg)	Baseline	82.1±11.8	81.9±11.1	0.955
	Day 90	78.2±11.2	82.0±11.9	0.319
	Change	-3.9±5.0 (<0.001)	0.1±3.2 (<0.001)	<0.001
	ANOVA	Time x group interaction: P<0.001		
Body fat mass (kg)	Baseline	26.9±2.8	27.0±2.2	0.451
	Day 90	24.8±3	27.0±2.1	0.150
	Change	-2.1±3.1 (<0.001)	0.3±2.8 (<0.001)	0.020
	ANOVA	Time x group interaction: P<0.020		
Waist circumference (cm)	Baseline	85.7±11.4	85.9±11.6	0.699
	Day 90	81.8±11.5	85.9±11.5	0.135
	Change	-3.9±5 (<0.001)	0±6 (0.004)	0.005
	ANOVA	Time x group interaction: P<0.004		
Hip circumference (cm)	Baseline	108.2±10.6	108.5±10.1	0.270
	Day 90	102.6±10.1	108.5±10	0.033
	Change	-6±5 (<0.001)	0±4 (0.001)	0.018
	ANOVA	Time x group interaction: P<0.044		

Blood chemistries

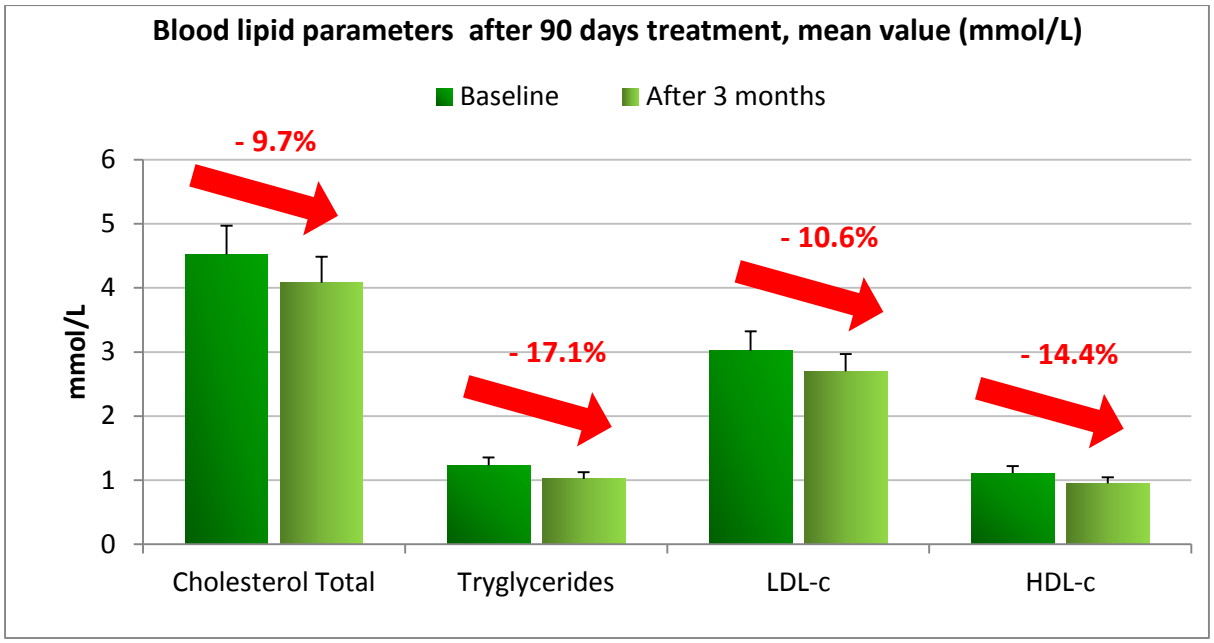
Baseline blood lipid parameters by gender:

		OXY-280		Placebo	
Variable		Females	Males	Females	Males
LDL-cholesterol (mmol/L)	mean	3.04	2.99	3.06	3.01
	SD	0.64	0.70	0.65	0.72
Total cholesterol (mmol/L)	mean	4.77	4.26	4.81	4.28
	SD	0.70	0.76	0.74	0.80
HDL-cholesterol (mmol/L)	mean	1.07	1.14	1.09	1.12
	SD	0.27	0.28	0.30	0.29
Triglyceride (mmol/L)	mean	1.216	1.261	1.219	1.266
	SD	0.410	0.453	0.418	0.456

Changes in the LDL-, HDL-, total cholesterol and triglyceride levels, after 90 days treatment by gender an overall (mm/L)

		Females	Males	Total
LDL-cholesterol (mmol/L)	mean	-0.27	-0.10	-0.22
	SD	0.56	0.47	0.54
	Median	-0.30	-0.10	-0.15
	Min	-1.20	-0.90	-1.20
	Max	1.00	0.80	1.00
	p-value[1]	0.011	0.547	0.012
Total cholesterol (mmol/L)	mean	-0.32	-0.17	-0.27
	SD	0.55	0.59	0.56
	Median	-0.30	-0.20	-0.25
	Min	-1.50	-1.10	-1.50
	Max	0.90	1.40	1.40
	p-value[1]	0.002	0.370	0.003
HDL-cholesterol (mmol/L)	mean	-0.08	-0.04	-0.06
	SD	0.21	0.21	0.21
	Median	-0.10	0.00	0.00
	Min	-0.40	-0.50	-0.50
	Max	0.50	0.20	0.50
	p-value[1]	0.085	0.671	0.090
Triglycerides (mmol/L)	mean	-0.16	-0.09	-0.11
	SD	0.39	0.35	0.37
	Median	0.04	0.03	0.04
	Min	-0.79	-0.69	-0.79
	Max	0.83	0.68	0.83
	p-value[1]	0.002	0.250	0.003

By LOCF analysis, there were statistically significant 90 Days improvements with OXY-280 treatment in serum levels of total cholesterol (-0.27±0.56 mmol/L, P=0.003); LDL-cholesterol (-0.22±0.54 mmol/L, P=0.012); triglycerides (-0.11±0.37 mmol/L, P=0.003); with no significant changes on HDL-cholesterol (-0.08±0.21, P=0.090).



Conclusion

In this study O|N|R® administered for a 90 Days period, showed significant reductions on body weight, fat and other anthropometric measurements in overweight subjects compared with placebo treated participants. Other beneficial effects that accompanied the greater weight loss of the OXY-280 treatment group included decreased serum LDL-cholesterol, Total-cholesterol and Triglycerides.

The numbers of subjects removed from the study for potential treatment related adverse events were similar in the OXY-280 group and placebo group. No side effects were reported in both groups.

Body composition related effects

The increased weight reduction with the OXY-280 group in the present study is consistent. As in the 90 Days study, the reductions in body fat, waist and hips circumferences and the favorable changes in serum LDL cholesterol, Total-cholesterol and Triglycerides levels are probable consequences of the greater reductions in body weight in the subjects treated with OXY-280.

The present study demonstrated significant beneficial effects on body weight, body fat and blood lipids of a mix of plant extracts Olive, Rosemary, Black Beans in overweight men and women who were otherwise healthy. Compared with placebo, the tested product produced no adverse events and no side effects. In total, these suggest that OXY-280, when used as directed by healthy overweight men and women may be beneficial for weight reduction without significantly increased risk of adverse events.

5.4. INSULIN RESISTANCE & GLYCEMIC RESPONSE

5.4.1. Effects of O|N|R® on weight management, glycemic response and LDL / HDL cholesterol

TITLE CLINICAL TRIAL

Effects of O|N|R® on Weight Management, Glycemic response and LDL /HDL cholesterol

TYPE OF STUDY

A clinical double-blind trial comparing O|N|R® vs Placebo

SPONSOR

SANKI MAYOR

LABORATORY

CERN (Centre d'Enseignement et de Recherche en Nutrition) CHBS – BP223356322 Lorient Cedex France. Managed by Dr Bernard Schmitt

PRODUCT INFORMATION

Product name: O|N|R®

Appearance: Powder

Colour: Brown

Taste: Characteristic

STUDY OBJECTIVES

This clinical trial was meant to assess the possible efficacy of O|N|R® on several biological and anthropometric functions: Diabetes, Lowering Glycemic Index, Weight Management, Balance of LDL / HDL, Antioxidants presence in blood serum.

VARIABLE VALUED

Anthropometric profile (Weight, BMI, Waist and Hips circumferences), Biological profile (Glycemia, Insulinemia, HOMA) as well as Lipid parameters (Triglycerides, Cholesterol, HDL-c, LDL-c) have been monitored. A crossover for the test meal with glycemic test was done only by half of the subjects.

TOTAL NUMBER OF PATIENTS

30 subjects (men and women), 15 subjects in each group. 15 subjects will do the glycemic test with the test meal in cross over.

DURATION OF TREATMENT AND DOSAGE

Ingestion of O|N|R® supplement and placebo during 30 days of treatment with a dosage of 500mg/day (each tablet contains 200 mg of active ingredients and 300 mg of excipient).

Introduction

This clinical trial was meant to assess the possible efficacy of a product on several biological and anthropometric functions:

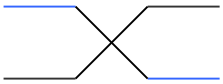
- Diabetes
- Lowering Glycemic index
- Weight management
- Balance of LDL/ HDL

Materials and method

Design of the study

- Test meal

Crossover for the test meal with glycemic test only which was done by 15 persons.



Breakfast with product 508

Breakfast with product 509

- One month protocol

Then parallel for a one month test with 30 persons.

Group A j0 30

Group B

Group A: Product 508

Group B: Product 509

Population

30 subjects for the one month period, 15 subjects in each group.

15 subjects will do the glycemic test with the test meal in cross over.

Group 508: 4 men and 11 women

Group 509: 5 men and 10 women

Table 1: Anthropometric profile of the study population

		Age	Height (m)	Weight	BMI	WC	HC	WC/HC
Group 508	Mean	**55**	**1,63**	**81,33**	**30,40**	**98,73**	**109,67**	**0,90**
	Std Error	7,8	0,07	13,02	3,37	10,25	6,49	0,08
Group 509	Mean	**55**	**1,63**	**82,41**	**30,82**	**100,77**	**109,00**	**0,93**
	Std Error	9,4	0,06	12,37	3,85	8,34	7,76	0,07

BMI: Body Mass Index=weight/Height² WC: waist circumference HC: Hips Circumference

The population of this study is overweight with a high waist circumference which is an indicator of diabetic and cardiovascular risk.

Table 2: Biological profile of the study population

		Glycemia (mmol/l)	Insulinemia µmol/L	HOMA	Triglycerides mmol/L	Cholesterol mmol/L	HDL-c mmol/L	LDL-c mmol/L
Group 508	Mean	**6,05**	**15,73**	**4,23**	**1,42**	**5,76**	**1,48**	**3,64**
	Std Error	0,89	9,56	3,10	0,49	0,91	0,24	0,88
Group 509	Mean	**5,78**	**12,86**	**3,30**	**1,97**	**6,03**	**1,42**	**3,73**
	Std Error	0,53	5,95	1,82	1,39	1,05	0,33	0,79

As the major aim of this study was to assess the effect on glucidic metabolism of the product, the population was chosen with a high post prandial glycemia (>1,15g/l =6,325mmol/l) which is a caracteristic of a beginning of intolerance to glucose which usely leads to type 2 diabetes. The fasting insulinemia shows also a beginning of insulin resistance with values higher than the limit (2,6-11,1 mcmol/l).

This is a population on which it is important to prevent the appearance of diabetes and cardiovascular disease with a good lifestyle including physical exercice and healthy food habits.

Dietary protocol

Test meal:

80g of white bread
15 g of butter
100g of "fromage blanc" (fresh cheese: like yogourt but with more protein and no bacteria)
250 ml coffee or tea

This test meal represents 357,1 kcal, with 13,6g protein (15,23%),12,3g lipids (31%), 48 g carbohydrates (53,77%).

Test meal was taken by 15 persons after an overnight fast two times with at least one week delay between the two mornings.

One time 2 pills of product 508 was taken at the beginning of the meal the other time with product 509 on a random basis.

This took place before the beginning of the one month protocol.

One month protocol:

All the 30 persons were advice to keep on with their usual diet and to take one pill with their breakfast and one pill in the evening with their dinner.

Measurements

Test meal

- OGTT (measures of the glycemia with autotest every 15 minutes after a test meal with the product or the placebo)

One month protocol

Frequency: Day 0 and day 30

Anthropometric measurements:

- Weight
- Body Mass Index
- Waist circumference
- Hip circumference
- Waist/hip ratio

Biological measurements:

- Insulinemia
- Glycemia
- HOMA index (Insulinemia*Glycemia/22,5= Insulin Resistance index)
- Cholesterol
- LDL-C
- HDL-C
- Triglycerides

Product satisfaction: The use of the product, the satisfaction, the side effects.

Products

508: placebo
509: O|N|R®

Results

Test Meal

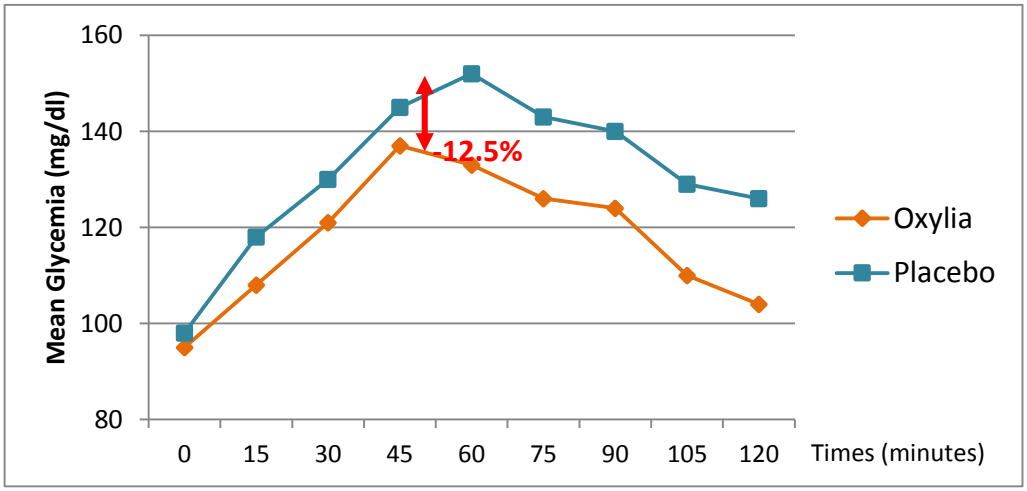
To evaluate the impact of O|N|R® on the glycemic response of the subject, we compare the value of the higher glycemia and the glycemia at the end of the test.

A positive result would be a smaller value for the test meal with O|N|R®.

The glycemia peak is bigger and earlier with placebo. The decrease of glycemia is also quicker with O|N|R® than with the placebo.

Scheme 1: Test meal mean glycemia

These results show statistically valid evidence of an efficacy of O|N|R® on glycemic response to a test meal.



One month protocol

Anthropometric measurements

Table 4: Weight and Body Mass Index

		Weight 0 (Kg)	Weight 30	W30-W0	BMI 0 (kg/m²)	BMI 30	BMI 30-BMI 0
508 (15 p)	Mean	**81,33**	**81,01**	**-0,31**	**30,40**	**30,29**	**-0,11**
	Std error	13,02	12,45	1,06	3,37	3,16	0,40
509 (15 p)	Mean	**82,41**	**80,15**	**-2,26**	**30,82**	**29,98**	**-0,84**
	Std error	12,37	11,83	1,57	3,85	3,62	0,58

The two groups have lost weight during one month but there is a statistically significative difference between the groups.

Table 5: Waist and Hip circumferences

		WC 0 (cm)	WC 30	WC 30-WC 0	HC 0 (cm)	HC 30	HC30-HC 0	WC/HC 0	WC/HC 30	WC/HC 30-WC/HC0
508	Mean	**98,73**	**99,28**	**0,55**	**109,67**	**110,04**	**0,37**	**0,90**	**0,90**	**0,00**
	Std error	10,25	9,51	2,07	6,49	5,73	1,71	0,08	0,08	0,02
509	Mean	**100,77**	**99,29**	**-1,48**	**109,00**	**107,21**	**-1,79**	**0,93**	**0,93**	**0,00**
	Std error	8,34	9,24	2,65	7,76	6,87	2,45	0,07	0,08	0,03

The group with the product 509 has lost hip circumference and waist circumference as the group 508 has gain a little.

These results provide good evidence for an efficacy of O|N|R® on weight and waist and hip circumference in this study where people kept their food habits.

Biological analysis

Table 6: Glycemic metabolism

		Glycemia 0 (mmol/L)	Glycemia 30	Gl 30-Gl 0	Insulinemia 0 (µmol/L)	Insulinemia 30	I 30-I0	HOMA 0	HOMA 30	HOMA 30-HOMA 0
	Mean	**6,055**	**6,043**	**-0,012**	**15,733**	**13,267**	**-2,467**	**4,23**	**3,56**	**-0,67**
508	Std error	0,892	0,939	0,444	9,560	9,531	2,944	3,10	0,93	2,703
	Mean	**5,78**	**5,12**	**-0,66**	**12,86**	**9,64**	**-3,22**	**3,30**	**2,19**	**-1,10**
509	Std error	0,53	0,40	0,42	5,95	6,14	3,84	1,82	0,65	1,21

In the two groups glycemia decreased a little after one month. Insulinemia decreases more in group 509 than in group 508. As a consequence of these changes in glycemia and insulinemia, the HOMA index of insulin resistance decreases more in group 509 than in group 508.

Table 7: Lipid metabolism

		TG 0 (mmol/l)	TG 30	TG 30-TG 0	TCh (mmol/l)	TCh 30	TCh30 - Tch0	HDL-c 0 (mmol/L)	HDL-c30	HDL-c 30-HDL-c 0	LDL-c 0 (mmol/l)	LDL-c 30	LDL-c 30 - LDL-c 0
	Mean	**1,423**	**1,352**	**-0,071**	**5,757**	**5,655**	**-0,101**	**1,478**	**1,478**	**0,000**	**3,639**	**3,563**	**-0,08**
508	Std er	0,493	0,481	0,345	0,913	0,976	0,616	0,240	0,244	0,141	0,882	0,905	0,519
	Mean	**1,97**	**1,76**	**-0,21**	**6,03**	**5,59**	**-0,44**	**1,42**	**1,21**	**-0,21**	**3,73**	**3,35**	**-0,38**
509	Std er	1,39	1,03	0,58	1,05	0,69	0,75	0,33	0,39	0,13	0,79	0,49	0,67

TG: triglycerides; TCH: total cholesterol

We can see a higher decrease in lipid parameters in group 509 than group 508.

Tolerance and impression

One person in each group complains at the end of the test about side effects but the events didn't lead to stop consuming the product. The product was reported easy to use.

GROUP 509

- 4 persons (n°2, 11, 15, 12) felt a lowering effect of the product on appetite
- 4 persons(n°2, 11, 15, 9) in this group felt a slimming effect
- 2 persons (n°11, 16) felt a positive effect on stress

GROUP 508

- 1 person (n°4) felt a slimming effect

There are more efficiency impression in group 509.

Discussion

This clinical trial shows evidences of an efficacy of O|N|R® on glycemic response to a test meal with a tendancy for a quicker decrease after the glycemia peak. It also shows evidences of efficacy of O|N|R® on insulin resistance and weight management after a one month treatment. 6 subjects of group 509 (15 p) found a positive impact of their product (lowering appetite, slimming effect and antistress) as only one person found a slimming effect in placebo group.

5.5. ANTI-INFLAMMATORY ACTION

5.5.1. Effect of daily use of OXI-280 on C-reactive protein levels

TITLE CLINICAL TRIAL

Study 1: Effect of daily use of OXI-288 on C-reactive protein levels.

TYPE OF STUDY

Double-blind, placebo-controlled study comparing OXI-288 vs. Placebo.

SPONSOR

Sanki Mayor

LABORATORY

MEDICA TOKYO Co.LTD

20-1, 3Chome Nishi-Shinjuku, Shinjuku-ku Tokyo JAPAN

Managed by Dr Taro Hirata - clinical@medica-tokyo.jp

PRODUCT INFORMATION

Product name: OXI-288

Appearance: Powder

Colour: Brown

Taste: Characteristic

STUDY OBJECTIVES

Elevated C-reactive protein levels are associated with the risk of cardiovascular disease and diabetes. We examined whether OXI-288 can reduce C-reactive protein levels.

VARIABLE VALUED

Subjects were asked to give two fasting blood draws to measure C-reactive protein levels: one prior to starting the study; and one at the end of the study (60 days).

TOTAL NUMBER OF PATIENTS

25 subjects (men and women aged 40 to 60 years old), 15 subjects in OXI-288 group and 10 subjects in placebo group.

DURATION OF TREATMENT AND DOSAGE

Ingestion of 500mg/day of OXI-288 supplement and placebo for a period of 60 days (each tablet contains 200mg of active ingredients and 300mg of excipient).

Introduction

This study investigates the effect of OXI-288 on the serum level of C-reactive protein (CRP). The study was a placebo-controlled, parallel study of 60 days duration.

C-reactive protein (CRP) is one of the acute phase proteins that increase during systemic inflammation. It has been suggested that testing CRP levels in the blood may be a new way to assess cardiovascular disease risk. Other research suggests that systemic or silent inflammation may be implicated in many life threatening diseases, such as cancer, heart disease, stroke, diabetes and Alzheimer's, among others.

Materials and methods

Subjects

Volunteers were admitted into the study if they qualified according to the following inclusion criteria:

(1) men and women 40 to 60 years older, excluding pregnant and lactating women;

(2) no diagnosis of cardiovascular disease, kidney disease, diabetes, or cancer;

(3) on stable doses of medication, if taking any;

(4) not participating in any other study that might conflict in some way with this one.

Twenty five participants were selected according to the above criteria and agreed to participate in the study and completed the study (Appendix 1).

The study protocol had been approved by an independent investigational review committee and was explained to each subject who then signed an informed consent.

All subjects were instructed to continue their current prescription medications, over-the-counter preparations and supplements; not to change their diet or lifestyle; and to notify investigators of any change they or their health care professionals may make in their medication(s) during the study period. They were also instructed to maintain their usual intake of coffee, tea, alcoholic beverages and soft drinks; their exercise routine; and not to make any special effort toward changing their weight.

Treatment

Subjects in the treatment group consumed 500mg of OXI-288 per day, 250mg with breakfast meal and 250mg with dinner for a period of 60 days. Subjects in the placebo group consumed 500mg of placebo per day, 250 mg with each meal for a period of 60 days.

Study Design

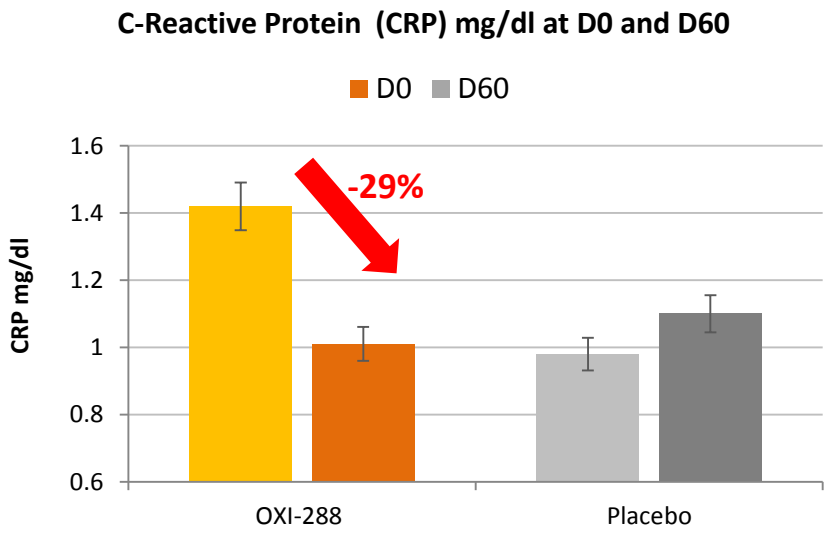
This double-blind, placebo-controlled study was conducted over a 60 days duration. Twenty-five subjects completed all aspects of the study (15 in treatment group, 10 in placebo group). Subjects were asked to give two fasting blood draws: one prior to starting the study; and one at the end of the study (60 days).

Results

Table 1 presents blood chemistry data for CRP for twenty-five subjects, 15 in the treatment group and 10 in the placebo group. Data for each subject is presented in Appendix 1.

Table 1. C-Reactive Protein Measurements

	C-Reactive Protein (CRP) mg/dl	
Group	D0	D60
OXI-288	1.42 ±1.62	1.01 ±1.35
Placebo	0.98 ±0.84	1.10 ±0.48



Discussion

The results of this study showed a significant decrease in the average CRP levels of the subjects who were in the treatment group. The mean of the pre-treatment CRP scores in the treatment group was 1.42, and the mean of the post-treatment scores was 1.01. These subjects, on the average, showed a 29% decrease in their CRP scores after taking the OXI-288 for 60 days. There was no such reduction in CRP scores in the placebo group, and in fact, the scores showed an average increase: the mean beginning score was 0.98 and the mean end score was 1.10, on the average the placebo group showed a 12% increase.

Conclusion

In conclusion, the study found that subjects who took OXI-288 for 60 days showed an average decrease in their measured CRP levels of over 29%. Subjects who took a placebo showed no decrease. Analysis of the data showed that there was a statistically significant difference between the two treatment conditions, suggesting that OXI-288 may reduce CRP levels. This finding was based on small sample sizes, and should be replicated in a larger study. However, considering recent literature on the importance of CRP as an indicator of cardiac health and other life threatening issues, the finding of an average reduction in CRP after a regimen of OXI-288 is a promising result that deserves further attention.

5.6. BLOOD PRESSURE

5.6.1. Effect of daily use of OXI-280 on blood pressure levels

TITLE CLINICAL TRIAL

Study: Effect of daily use of OXI-288 on blood pressure levels.

TYPE OF STUDY

Double-blind, randomized, placebo-controlled study comparing OXI-288 vs. Placebo.

SPONSOR

Sanki Mayor

LABORATORY

MEDICA TOKYO Co.LTD

20-1, 3Chome Nishi-Shinjuku, Shinjuku-ku Tokyo JAPAN

Managed by Dr Taro Hirata

PRODUCT INFORMATION

Product name: OXI-288

Appearance: Powder

Colour: Brown

Taste: Characteristic

STUDY OBJECTIVES

The objective of this study was to evaluate the action of OXI-288 for Blood Pressure reduction in humans.

VARIABLE VALUED

On days 0, 15 and 30 of the study, the subject's blood pressure was measured on their right hand in seated position.

TOTAL NUMBER OF PATIENTS

25 subjects (men and women aged 18-60 years old), with an average blood pressure between 120-160/80-100.

DURATION OF TREATMENT AND DOSAGE

Ingestion of 500mg/day of OXI-288 supplement and placebo for a period of 30 days (each tablet contains 200mg of active ingredients and 300mg of excipient).

Abstract

OXI-288 is a nutritional supplement which has been formulated from edible Mediterranean plants extracts (olive and rosemary) and kidney bean. OXI-288 has no known toxicity associated with its use.

Olive and rosemary polyphenols have been associated with several cardiovascular health benefits. This study aims to examine the influence of OXI-288 on blood pressure (BP in healthy humans with high-normal blood pressure or stage 1 essential hypertension.

A controlled clinical trial was conducted on twenty-five healthy adult subjects to evaluate the efficacy of OXI-288 for Blood Pressure reduction in humans.

Introduction

High blood pressure (BP) frequently coexists with diabetes, occurring twice as frequently in diabetic as in non-diabetic persons. It accounts for up to 75% of added cardiovascular disease risk in people with diabetes, contributing significantly to the overall morbidity and mortality in this high-risk population. It is one of the most important treatable risk factors for cardiovascular diseases because of its high prevalence and lethal outcomes.

Different studies have reported the global prevalence of hypertension in adults as 3.4–72.5%. It is estimated that in 2025, there will be 333 million patients in developed countries and 639 million patients in developing countries suffering from hypertension. Although it has been most dominant in industrialized countries in the past decades, now it is a challenging issue and its prevalence is rapidly increasing in many developing countries.

This chart reflects blood pressure categories defined by the American Heart Association.

Blood Pressure Category	**Systolic mm Hg (upper #)**		**Diastolic mm Hg (lower #)**
Normal	less than 120	and	less than 80
Prehypertension	120 – 139	or	80 – 89
High Blood Pressure (Hypertension) Stage 1	140 – 159	or	90 – 99
High Blood Pressure (Hypertension) Stage 2	160 or higher	or	100 or higher
Hypertensive Crisis (Emergency care needed)	Higher than 180	or	Higher than 110

Materials and methods

This sequential randomized controlled clinical trial was conducted on 25 subjects with a Blood Pressure between 120-160/80-100 mmHg. After obtaining informed consent from all subjects, they were randomly assigned to one of these two groups: OXI-288 group or placebo group. Assignment to the two groups was made by using a sequential list prepared on the basis of randomized numbers table. Subjects in the OXI-288 were given OXI-288 pills and those in the placebo group were given pills that were similar in shape and weight. The subjects were instructed to use take 250mg at breakfast and 250mg at night for 30 days.

Measurements

On days 0, 15 and 30 of the study, the subject's blood pressure was measured on their right hand in seated position. Blood pressure was measured twice at 5–10min intervals and the average was recorded. The Pulse Pressure (PP) was calculated as the difference between SBP and DBP.

The trial included 25 healthy male and female volunteers, ages 18-60 years, with a body mass index (BMI) ≤ 35 and an average blood pressure between 120-160/80-100.

Patients with diabetes mellitus, nephropathy, peripheral arterial disease, retinopathy, history of stroke, or heart disease (including left ventricular hypertrophy, prior myocardial infarction, angina pectoris, a prior revascularization procedure, or heart failure) were excluded from the study.

Subjects using medications that could produce weight loss, or who were on unstable doses (stable dose=same dose for previous three months) of medicines that influence blood pressure, were also excluded. Subjects were required to refrain from smoking and caffeine consumption for four hours before blood pressure measurements. Subjects did not change their baseline diet or physical activity during the study.

At the completion of the study, subjects were instructed in the appropriate dietary and lifestyle recommendations for hypertension, and were advised to follow-up with their treating physicians.

The outcome variable was the difference in blood pressure between the OXI-288 and placebo groups from baseline to 30 days.

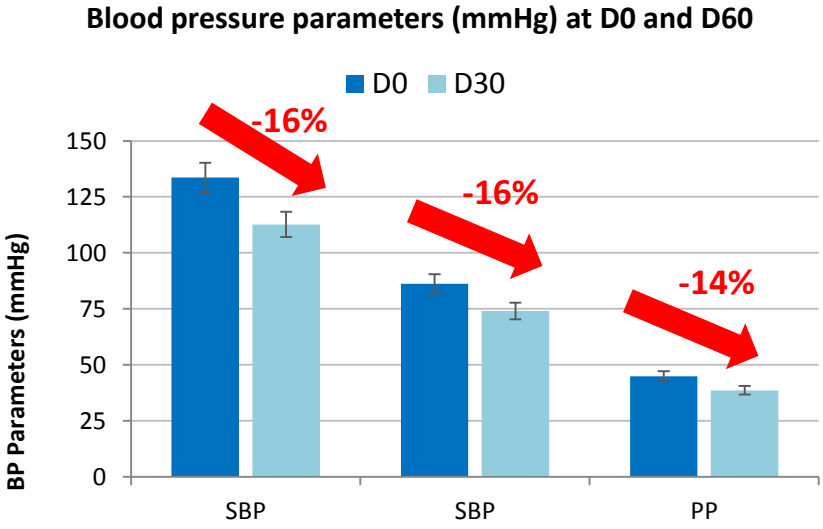
Results

Twenty five subjects completed the trial. Two subjects, one in the OXI-288 group and one in the placebo group, encored moderate headache, which were resolved during the study.

Table1: Means of SBP, DBP, and PP in different stages of the study

Variable	Group	Day 0	Day 15	Day 30
Systolic blood pressure (mm Hg)	Placebo	134.4	132.8	134.3
	OXI-288	133.6	120.7	112.7
Diastolic Blood Pressure (mm Hg)	Placebo	86.2	87.3	85.5
	OXI-288	88.7	79.9	74.1
Pulse Pressure (mm Hg)	Placebo	48.2	45.5	48.8
	OXI-288	44.9	40.8	38.6

Comparisons between the means of quantitative variables at the beginning of the study are shown in Table 1. The means of DBP, SBP and PP were significantly different for the OXI-288 group.



In the OXI-288 group, the means of SBP and PP showed statistically significant difference throughout the study (in the base-line, on days 15 and 30 of the intervention), as SBP decreased from 133.6 mmHg at the beginning (Day0) to 112.7 mmHg (Day30) at the end of the study. In the same period of time, the mean of DPB decreased from 88.7 mmHg (Day0) to 74.1 mmHg (Day30).

The OXI-288 group have a lower pulse rate at the end of the study: the mean of PP decreased from 44.9 mmHg (Day0) to 38.6 mmHg (Day30).

In the placebo group, the mean of DPB, SBP and PP did not show any statistical difference during the intervention. At the end of the study, the means of SBP and PP in the Placebo group were significantly higher than those in the OXI-288 group.

Discussion and conclusion

At the beginning of the study, the distribution of participants based on sex, treatment method, DBP and SBP classification in both groups was not significantly different. The main objective of this study was to evaluate the short-term therapeutic effects of OXI-280 on the blood pressure of subjects.

Our findings showed that the mean of SBP in OXI-288 group decreased from decreased from 133.6 mmHg at the beginning (Day0) to 112.7 mmHg (Day30), which is statistically significant. SBP decreased from 88.7 mmHg to 74.1 mmHg and the mean of PP decreased from 44.9 mmHg to 38.6 mmHg. In this study, positive therapeutic effectiveness was defined as decreasing 20mmHg or more in DBP and 10 mmHg or more in SBP.

Thus, OXI-288 might be employed as a “dietary measure” to maintain a healthy blood pressure in individuals with prehypertension or in mildly hypertensive range.

6. CONCLUSION

O|N|R® is a complex of active compounds functioning synergistically to maintain healthly sugar metabolism balance in human body. This unique complex has been formulated with bioactives issued from mediterranean ingredients (Olive, Rosemary and Black Beans), well-know for reducing the risks linked to metabolic syndrome (cardiovascular diseases, diabetes, strokes etc.)

Metabolic discipline is essential for successful weight management, but also impact directly cells and body general health. By supporting sugar metabolism, O|N|R® helps increasing cell's insulin uptake, promotes energy production and reduces inflammation.

O|N|R® targets 3 zones of impact on the human body:

- The first mechanism targets the stomach and intestine level to reduce oxidation and sugar absorption on the gastrointestinal zone and balance high GI food.
- The next stage is the blood circulation, an important level where by reducing glycation and improving blood micro circulation, we can improve sugar metabolism.
- Our research focus on the cellular level, by enhancing mitochondrial function, reducing inflammation, we can reduce fat accumulations in adipocytes cells, and reduce insulin resistance.

The results of O|N|R® clinical trials showed consequent amelioration of population health regarding:

- Improve antioxidant capacity after 30 days (ORAC test +300%)
- Reduction of weight (-4.6%), waist circumference (-4.6%) and hip circumference (-5.2%) after 3 months
- Reduction of cardiovascular risks after 3 months: TCh (-9.7%), LDL-c (-10.6%), HDL-c (-14.4%) and Tg (-17.1%)
- Lower glycemia peak during test meal (-12.5%)
- Reduce blood pressure levels after 1 month DBP (-16%), SBP (-16%) and PP (-14%)
- Reduction of inflammatory maker TNF-a after 1 month (-29%)

1kg fat silicon model -real size and weight-, can be sent to you for demonstration

By balancing sugar metabolism, O|N|R® improves the general health of subjects, benefits that can be felt in their daily life.

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**Full-text available*
***Abstract only (free publication restricted)*

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Rosemary Compounds as Nutraceutical Health Products

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1. Introduction

The oxidation of lipids in food, cosmetic and pharmaceutical products, together with the growth of undesirable microorganisms results in the development of spoilage, off-flavor, rancidity and deterioration of such products turning them unacceptable for human consumption. In consequence, the addition of exogenous antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole is frequently required to improve the stability of these products. As synthetic butylated derivatives may have toxic effects there is an increasing interest in the use of natural antioxidants, such as phenols isolated from plants to avoid undesired food borne diseases (Shylaja & Peter, 2004).

In recent years, a greater emphasis has been placed on the link between the prevention of chronic diseases and the human diet. The present popularity of natural antioxidants, as the dietary polyphenols, on human health has promoted the surge of in vitro studies examining the effects of these physiological active components (Williams et al., 2004).

During the past decade, consumers began to view food in a new way. The antioxidant properties of polyphenols have been widely studied, but it has become clear that the mechanisms of action of these compounds go beyond the modulation of oxidative stress (Scalbert et al., 2005). These compounds have great potential in the emerging nutritional industry, because they are often considered as food and medicines as well, therefore they may be used in the prevention and curative treatments (Sies, 2010). This issue has been of interest from ancient times, Hippocrates, 400 B.C. said, "Let food be your medicine and medicine your food".

Different foods have been identified as containing health-promoting properties beyond their basic nutritional value, and stimulating innovation in the field of nutrition and health is the search for nutraceuticals. A nutraceutical is defined as a food or part of a food that provides medicinal benefits or health, including prevention and/or treatment of diseases.

In order to use nutraceuticals in the prevention and treatment of human pathologies, many questions still remain unanswered: Which natural source is to be used? Which is a good candidate to be used as nutraceutical?.

Rosemary (Rosmarinus officinalis L., Lamiaceae) is considered one of the most important sources for the extraction of phenolic compounds with strong antioxidant activity. This specie grows worldwide and has been cultivated since long ago, in ancient Egypt, Mesopotamia, China and India (Bradley, 2006). Rosemary extracts, enriched in phenolic compounds are effective antioxidants due to their phenolic hydroxyl groups but they also possess plenty of other beneficial effects like antimicrobial, antiviral, anti-inflammatory,

anticarcinogenic activities and is also known to be an effective chemopreventive agent (al-Sereiti et al., 1999; Aherne et al., 2007).

Therefore, this specie contains bioactive ingredients which provide a complementary value other than the nutritional one, to be applied in the food industry. However, particular bioactives of rosemary responsible for some biological activities, as antimicrobial, have not been deeply characterized. A lesser amount of information exists about their mechanism of action. The present chapter focuses on the most significant rosemary biological properties, reviewing the free radical scavenging and antibacterial actions of non-volatile constituents and essential oils, the antibacterial activity of main rosemary bioactives in combination with antibiotics, as well as possible antibacterial mechanism of action is proposed, among other topics. In addition, a toxicity assay using the nematode Caenorhabditis elegans is covered.

2. Rosemary the best natural antioxidant

2.1 Antioxidant action of different fraction of rosemary

Rosemary plants have many phytochemicals which constitute potential sources of natural compounds as phenolic diterpenes, flavonoids phenolic acids and essential oils. About 90% of the antioxidant activity is attributed mainly to a high content of non-volatile components as carnosic acid and carnosol (phenolic diterpenes) and rosmarinic acid (Bradley, 2006). It is clear that R. officinalis constituents have antioxidant activity according to traditional use and scientific evidence, although little information is available on the relationship between chemical composition and antioxidant activity of the essential oils and non-volatile extracts.

We investigated the antioxidant activity of volatile and non-volatile fractions isolated from two leaf phenotypes of rosemary plants growing in the same farm in Argentina. Plants showing a wide (W) and a narrow (N) leaves phenotypes were collected from Jardín Botánico Arturo E. Ragonese from National Institute of Agricultural Technology-INTA Castelar, Argentina, January 2008. The essential oils were obtained by hydrodistillation of dried leaves using a Clevenger-type apparatus and samples were analyzed by high resolution gas chromatography coupled with mass spectrometry. Ethanol extracts were prepared according to the method previously reported (Moreno et al., 2006) and stored at - 20 °C. To determine the dry weight of each extract, 1 ml of the sample was dried in an oven to constant weight. The extracts were centrifuged using a 5804 Eppendorf centrifuge at 5000 rpm for 15 min at room temperature before HPLC analysis. Quantification of phenolic compounds and identification was performed on an HPLC (LKB Bromma) equipped with a diode array detector, using a 250 mm × 4 mm C18 Luna analytical column (Phenomenex, USA), as previously described (Moreno et al., 2006).

The antioxidant activity was tested using a stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Brand-Williams et al., 1995. The percentage of DPPH radical was calculated measuring the change in absorbance at 517 nm and EC50 (extract concentration necessary to decolorate DPPH radical in a 50%) was determined for each fraction.

Results showed that different amounts of key bioactive compounds were present in the essential oils of both phenotypes. The main constituents of W phenotype essential oil were α-pinene and 1,8-cineole, while the N phenotype contained a comparable amount of 1,8 cineole and high contents of myrcene (Table 1). Therefore, the first is referred as an α-pinene chemotype and the other one as a myrcene chemotype. The essential oil of the myrcene chemotype exhibited approximately a double fold antioxidant activity than the α-pinene

phenotype. Recently, other authors reported that pure myrcene showed antioxidant activity and eliminated oxidative stress in rats in a time-dependent manner (Ciftci et al., 2011).

Essential oil	EC_{50} (µl/ml)	Main constituents	(%)
W Phenotype	25.0 ± 1.0	α-Pinene	31.2 ± 2.5
		1,8 -Cineole	21.6 ± 1.6
N Phenotype	10.0 ± 0.4	Myrcene	31.1 ± 2.3
		1,8-Cineole	18.7 ± 1.4

Table 1. Antioxidant activity and main constituents of essential oils isolated from two phenotypes of rosemary plants. Values ± SD

Chemical analysis of ethanol rosemary extracts indicated that W phenotype contained about 36.8% of diterpenes (carnosic acid plus carnosol) and 8.4% of rosmarinic acid, while the other phenotype contained a higher amount of diterpenes and lesser amounts of rosmarinic acid (Table 2). The ethanol extract isolated from the N phenotype, containing a high amount of diterpenes, exhibited approximately double fold antioxidant activity than plants of W phenotype.

Our results showed that the volatile and non-volatile fractions isolated from the same phenotype had similar antioxidant activity.

Ethanol extract	EC_{50} (µg/ml)	Main constituents	(%)
W Phenotype	20.0 ± 0.8	Carnosic acid + carnosol	36.8 ± 2.7
		Rosmarinic acid	8.4 ± 0.5
N Phenotype	10.8 ± 0.4	Carnosic acid + carnosol	50.0 ± 4.5
		Rosmarinic acid	2.9 ± 2.5

Table 2. Antioxidant activity and main constituents of ethanol extracts isolated from two phenotypes of rosemary plants. Values ± SD

2.2 Rosemary as protective agent against oxidative protein damage

A large number of reports have shown rosemary constituents to be an efficient antioxidant against lipid peroxidation and DNA damage induced by radical oxygen species in rat liver mitochondria and microsomes at concentrations of 3 - 30 µM, demonstrating their ability to protect tissues and cells against oxidative stresses (Bradley, 2006). On the other hand, it is well-known that several antioxidants exhibited pro-oxidant effect producing protein damage under certain conditions as in the presence of transition metals such as Fe and Cu. In order to study the protection of rosemary compounds against protein damage in comparison with ascorbate and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), hydroxyl radical-mediated oxidation experiments, were carried out using a metal-catalyzed reaction. Bovine serum albumin (4 µg) was incubated with or without Cu+ (100 µM) and H2O2 (1 mM) in the absence or presence of ascorbate, Trolox or the methanol rosemary extract (obtained as described in Moreno et al., 2006). Reactions were performed in opened tubes at 37°C, mixed with loading buffer and loaded in 12.5% dodecyl sulfate-polyacrylamide gel electrophoresis as reported by Mayo et al., 2003. Figure 1 shows that 20 µg of the plant extract used containing a concentration of 18 µM diterpenes, reduced significantly protein damage compared with 20 µM of ascorbate (compare the intensity of the protein's monomer in lane 8 vs. lane 6). Figure 1 also shows that Trolox and ascorbate only protected protein modifications when they were present at lower concentrations,

and no protection of the protein was observed at higher concentrations. Ascorbate reveals a higher pro-oxidant action than Trolox.

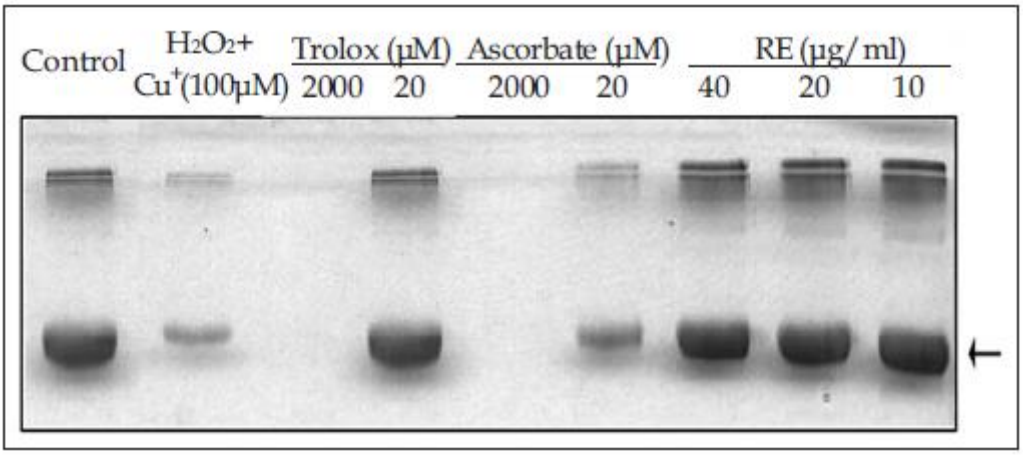

Fig. 1. Gel electrophoresis of bovine serum albumin protein after treatment with H2O2 + CuSO4 in the presence of Trolox, ascorbate and methanol rosemary extract (RE) at the concentrations indicated. Control was performed with the addition of ethanol 0.2%. Arrow indicates the bovine serum albumin monomer

3. Antibiotics and antioxidants of rosemary working together

Due to the recent trend in green consumers, there is an increasing interest in the antimicrobial properties of rosemary compounds. Essential oils and organic or aqueous extracts of isolated from this specie not only have antioxidant activity but also present antibiotic effects, therefore they have gained acceptance in industry to replace existing synthetic preservatives in foods (Davidson, Sofos, & Branen, 2005).

In the area of health, the near-term interest of plant products as antimicrobial agents is related to the development of new strategies/therapies for infections caused by bacterial species (Cowan, 1999; Lewis and Ausubel, 2006). Recently, it was reported that natural plant products can potentiate the activity of antibiotics in combination (Coutinho et al., 2009). Moreover, the use of bacterial resistance modifiers derived from natural sources, mainly from plants, such as efflux pump inhibitors, was suggested to be useful to suppress the emergence of multidrug resistant strains (Stavri et al., 2007).

To determine the validity of rosemary compounds as nutraceuticals, a rigorous analysis of the biological activities of their bioactives is required as well as the further study of their antibacterial mechanism of action.

3.1 Antibiotic action of different fractions of rosemary

We previously reported the effective antimicrobial action of non-volatile rosemary extracts containing 33 – 46% of diterpenes (carnosic acid plus carnosol) against common food pathogenic Gram positive bacteria as Staphylococcus aureus and Enterococcus faecalis as well as the Gram negative bacteria Escherichia coli (Moreno et al., 2006). These microorganisms cause severe problems in human health (NNIS, 2004). According to a 2011 research study, S. aureus was found in ~50% of beef, pork, and poultry products throughout the United States, 96% of these isolates were resistant to at least 1 antibacterial agent relevant in human medicine and 52% were resistant to three or more types (Waters et al., 2011).

Here, we show the antibacterial performance of a methanol rosemary extract (obtained as described in Moreno et al., 2006) in comparison with common food preservatives. The results are expressed in percent of inhibition of bacterial growth (see Equation 1)

$$\% \text{ Inhibition of bacterial growth} = \frac{(A_{595}\text{Control} - A_{595}\text{Sample})}{A_{595}\text{Sample}} \times 100 \qquad (1)$$

Where, A595 Control is the absorbance of the bacterial culture without compounds.

Rosemary extracts presented a higher antimicrobial efficacy than benzoic acid, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) to inhibit S. aureus growth (Fig. 2A), while a similar action than BHT and benzoic acid and a minor antimicrobial activity in relation to BHA was seen against E. coli (Fig. 2B).

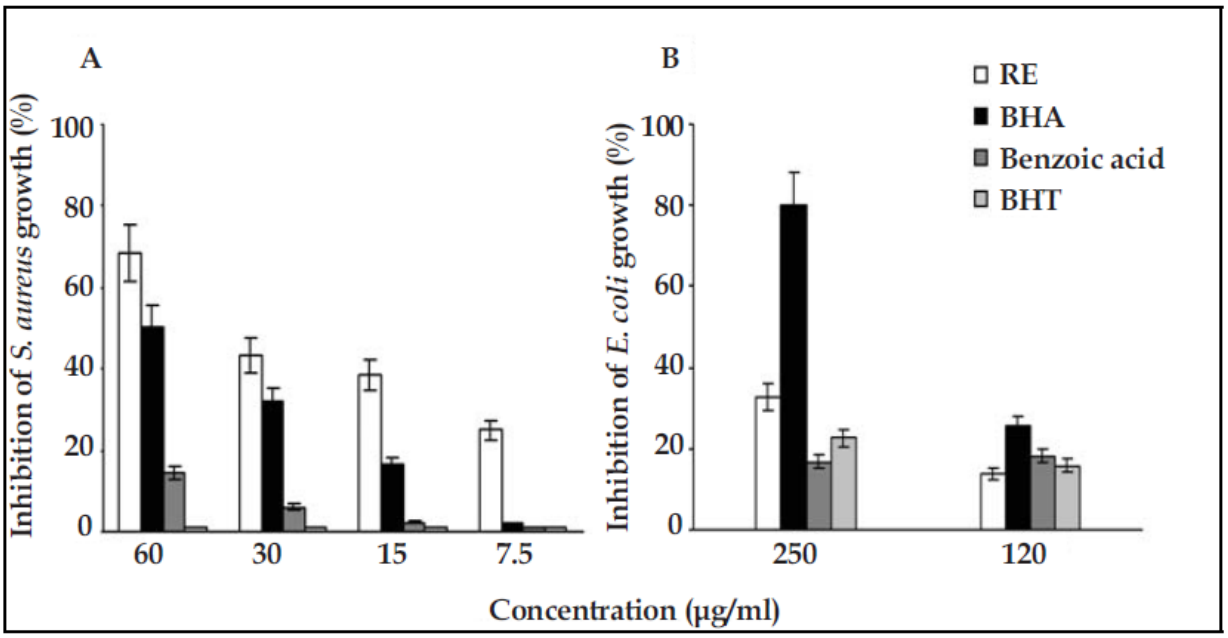

Fig. 2. Effect of the methanol rosemary extract (RE) on the S. aureus growth (A) or on the growth of E. coli (B), in comparison with BHA, BHT and benzoic acid. Values are shown as the mean of three independent experiments ± SD

The performance of rosemary oils and ethanol extracts isolated from the W phenotype and N phenotype plants against the human pathogen E. faecalis, was also compared (Table 3). Results showed that the essential oil from the α-pinene chemotype (W phenotype) exhibited a higher antibacterial activity than the essential oil isolated from N phenotype. By contrast, the ethanol extract isolated from the N phenotype containing higher amounts of carnosic acid and carnosol exhibited the highest antimicrobial action (Table 4).

Phenotype	Essential oil	Inhibition of *E. faecalis* growth (%)
W	26 µl/ml	100
N	26 µl/ml	45.5 ± 3

Table 3. Antibiotic activity of essentials oils isolated from two phenotypes of rosemary plants. Values are shown as the mean of three independent experiments ± SD

Phenotype	Ethanol extract	Inhibition of *E. faecalis* growth (%)
W	0.25 mg/ml	50.0 ± 6
N	0.25 mg/ml	100

Table 4. Antibiotic activity of ethanol extracts isolated from two phenotypes of rosemary plants. Values are shown as the mean of three independent experiments ± SD

All findings suggested that carnosic acid and α-pinene are key ingredients responsible that confer the antibacterial properties in the non-volatile and volatile fractions isolated from rosemary plants, respectively.

The essential oils isolated from N phenotype exhibited minor antibacterial activity than the other one, although it presented a higher performance as antioxidant.

We are investigating the antibacterial action against several Gram positive and negative microorganisms. Others authors demonstrated antibacterial activity of volatile compounds against Listeria monocytogenes, Salmonella typhimurium, Escherichia coli, Shigella dysenteria, Bacillus cereus (For review see Burt, 2004).

3.2 Antibiotic potentiation by rosemary bioactives

The use of combined antioxidants has gained acceptance in industry and has been applied to different aspects of food preservation (Davidson, Sofos & Branen, 2005). Although, up to date, a rational basis for the use of phytochemicals enhancing and/or broadening the biological antioxidant and antimicrobial activities against food-borne pathogens, is still poorly explored (Wei & Shibamoto, 2007). In the health's area, natural compounds are usefull strategies for the development of therapies against infections caused by bacterial species, and they are used in combination with common antibiotics potentiating their activity (Coutinho et al., 2009).

We previously reported a synergistic antioxidant effect between the methanol rosemary extract and BHT and a synergistic interaction with BHA to inhibit E. coli and S. aureus growth (Romano et al., 2009). Here, we reported the in vitro antibiotic interaction of rosemary extracts with common antibiotics using the broth microdilution method against S. aureus and E. faecalis.

Figure 3A shows the dose-response curve, in which the addition of 6.25 µg/ml of the plant extract clearly displaced the curve to the left, meaning that an increment in the antimicrobial action against S. aureus took place in the binary mixture at all gentamicin concentrations tested. It can be extrapolated from the curve that 0.035 µg/ml of pure gentamicin is needed to achieve 50% of inhibition, while the same effect can be achieved with 0.015 µg/ml of the aminoglycoside in the presence of rosemary extract.

Then, to study the type of interactions binary mixtures with different concentrations of rosemary extract and pure carnosic acid with gentamicin were analyzed by isobolograms (Fig. 3B).

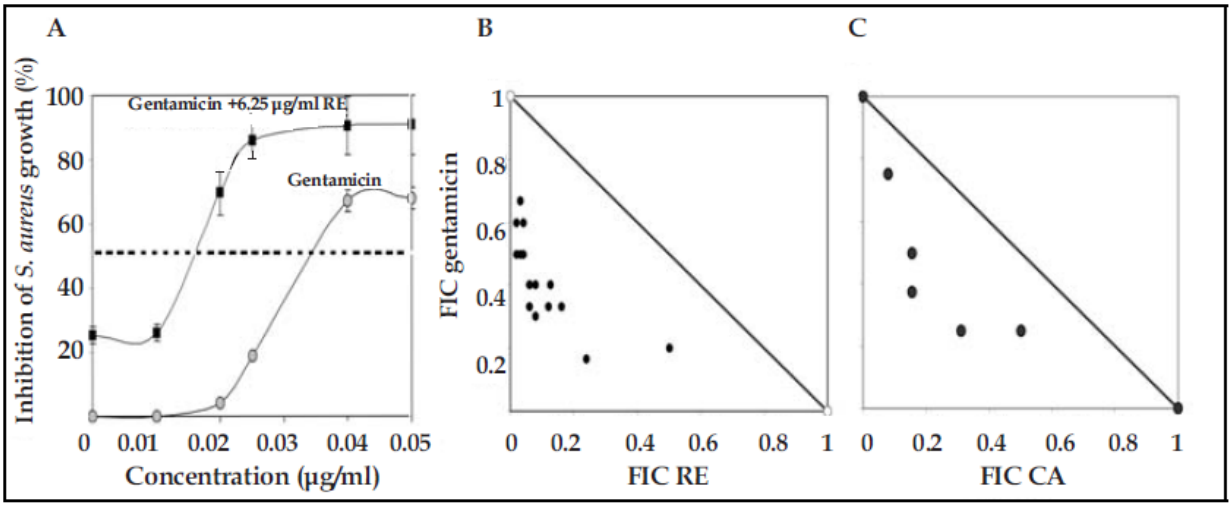

Fig. 3. Rosemary extract (RE) and carnosic acid (CA) acts synergistically with gentamicin against S. aureus. Dose-response curve (A), isobologram of gentamicin in combination with RE (B) or pure carnosic acid (C). Inhibition of S. aureus 25923 growth was determined after 24 h of incubation. FIC, fractional inhibitory concentration, normalized to the unit of RE, gentamicin or pure carnosic acid, corresponds to the MIC50 = 12 µg/ml, 0.035 µg/ml and 8 µg/ml, respectively. Values are shown as the mean of three independent experiments ± SD

Compounds concentrations lower than their MIC50 values were prepared for every combination tested as described previously (Romano et al., 2009) and isobolograms were performed. When compounds in combination are more effective than what might be expected from their dose-response curves (synergy), smaller amounts will be needed to produce the effect under consideration, and a concave-up isobole results (Tallarida, 2001).

The Fractional inhibitory concentration (FIC index) was determined (see Equation 2).

$$\text{FIC index} = \text{FICA} + \text{FICB} = [A]/\text{MICA} + [B]/\text{MICB} \quad (2)$$

FICA, FICB: Fractional inhibitory concentration of drug A & B respectively. MICA, MICB: Minimum inhibitory concentration of drug A & B respectively. [A], [B]: Concentration of drug A & B respectively.

FIC index obtained by checkerboard method is interpreted as follows: ≤ 0.5- synergy; > 0.5 and ≤ 4- additivity; and > 4– antagonism.

Figure 3B shows that different combinations of rosemary extracts corresponding to FIC 0.01 to 0.25 with gentamicin (FIC of 0.2 to 0.4) resulted in fixed-ratio points below the additive line and exhibited values of FIC index ≤ 0.5, verifying synergistic antimicrobial effects of the plant extract with gentamicin.

As carnosic acid was shown to be the main antimicrobial compound of non-volatile rosemary extracts against S. aureus (Moreno et al., 2006), this compound was assayed in combination with gentamicin at concentrations equivalent to those found in the extract (1 to 10 µg/ml). The minimal inhibition concentration (MIC) of carnosic acid against the bacteria was 16 µg/ml and was referred as a FIC of 1. Values of FIC index ≤ 0.5 (FIC 0.3 of carnosic acid in combination with FIC 0.2 to 0.3 of gentamicin) were observed (Fig. 3C).

On the other hand, rosmarinic acid exhibited a minor antibacterial effectiveness than carnosic acid and exhibited additive antibacterial actions with gentamicin (Data not shown). Therefore, data showed that the addition of both the extract and carnosic acid allows a reduction of the gentamicin amount in approximately

3 - 4 folds to obtain the same antibiotic action. The bactericidal action of rosemary extracts and pure carnosic acid were confirmed by time-kill curves.

Table 5 shows that carnosic acid plus tetracycline, tobramycin, kanamycin, ciprofloxacin and gentamicin exhibits significant synergistic antibiotic activity against S. aureus.

Antibiotics	MIC values of antibiotic in combination with MIC of carnosic acid[a] (µg/ml)				FIC Index
	0	1/8	1/4	1/2	
Tetracycline	0.50	0.06	0.06	0.06	0.25
Tobramycin	0.50	0.12	0.12	0.06	0.37
Kanamycin	2.00	1.00	0.50	0.25	0.50
Vancomycin	2.00	1.00	1.00	0.50	0.62
Ciprofloxacin	0.50	0.06	0.12	0.12	0.25
Penicillin	0.06	-	0.03	0.03	0.75
Gentamicin	0.50	0.06	0.06	0.06	0.25

Table 5. Antibiotic activity of carnosic acid in combination with several antibiotics against S. aureus ATCC 25923. aUnderlined values: combinations of minimum FIC used to calculate the FIC index. Values are the mean of three independent experiments

In order to confirm the role of carnosic acid as the main bioactive compound of rosemary extracts involved in the synergistic effect with gentamicin, additional experiments were carried out, examining carnosic acid in combination with gentamicin against E. faecalis (Fig. 4). Results showed that 30 µg/ml of the diterpene in combination with 0.25 µg/ml of gentamicin resulted in an strong antibiotic synergistic effect as the expected value for the sum corresponding to individual effects was 42% of inhibition, whereas experimental data showed a100 % of inhibition on bacteria growth for the combination mixture.

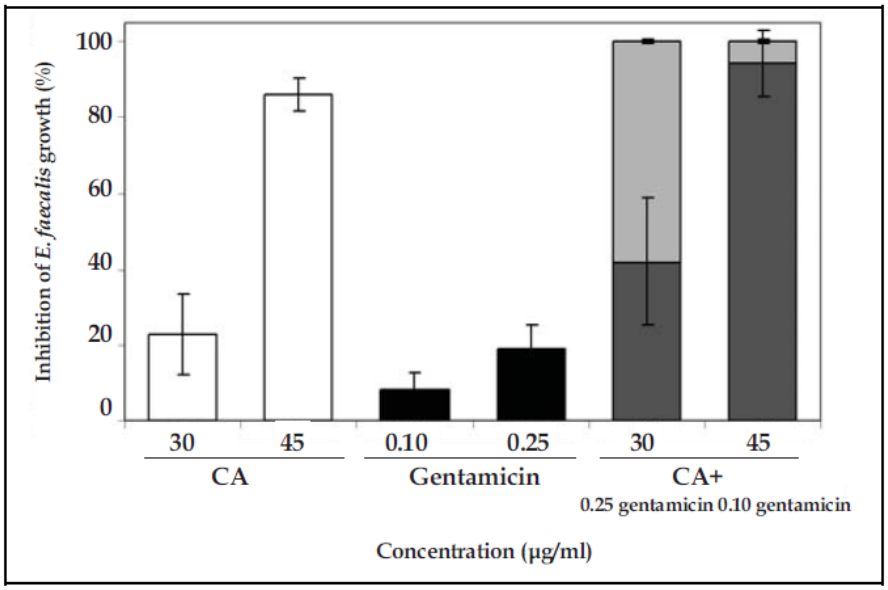

Fig. 4. Antibiotic activity of carnosic acid in combination with gentamicin against E. faecalis ATCC 29212. Dark grey bars represent the expected results if an additive interaction took place and light grey bars show the experimental data

3.3 Mechanism of antibacterial action of rosemary bioactives

R. officinalis plants exhibited antibiotic activity although less is known regarding the mechanisms of action of the particular bioactives. Polyphenols still constitute a promising source of new drugs and there is a high interest in understanding their mechanisms of action (Scalbert, 2005). We carried out studies to determine the antibacterial effect of the carnosic acid. This liphophilic diterpene has been found associated with chloroplasts membranes (Perez-Fons et al., 2006). Herein the cell membrane permeabilization effect by carnosic acid using the membrane potential sensitive cyanine dye DiS-C3-(5). This fluorescent probe is a caged cation, which distributes between cells and medium depending on the cytoplasmic membrane potential. Once it is inside the cells, it becomes concentrated and self-quenches its own fluorescence. The fluorescence monitored with an excitation wavelength of 610 nm and an emission wavelength of 670 nm. A blank with only cells and the dye was used to subtract the background and compounds as the proton motive force inhibitor carbonyl cyanide-m-chlorophenylhydrazone (CCCP) and Polimycyn B were used as controls for their ability to decreased or increase the membrane potential, respectively. S aureus suspension was incubated with 1.6 µM DiS-C3-(5) until a stable reduction in fluorescence and 100 mM KCl was added to equilibrate the cytoplasmic and external K+ concentration. Figure 5 shows time decreases of the fluorescence intensities upon addition of 32 µg/ml of carnosic acid to S. aureus cells in a similar way than the inhibitor CCCP, while the cationic antimicrobial peptides Polimyxyn B produced an increment on the fluorescence. CCCP is a small amphipathic molecule which dissolves in phospholipid bilayers, providing a polar environment for the ion and an hydrophobic face to the outside world, it is an uncoupling agent that specifically increases the proton permeability, and disconnects the electron transport chain from the formation of ATP, discharging the pH gradient, and destroying the membrane potential.

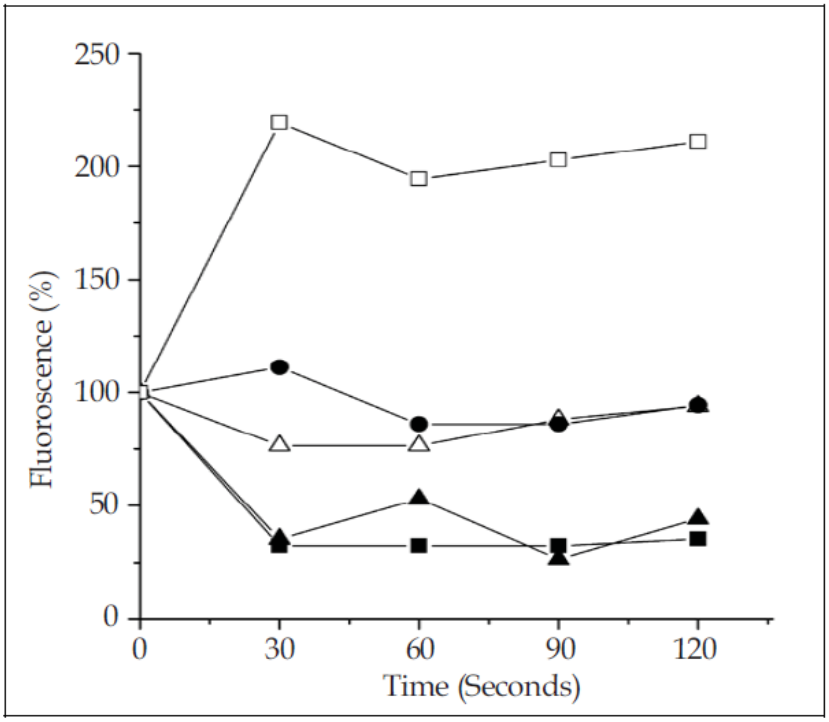

Fig. 5. Time changes of the fluorescence intensities of DiS-C_3-(5) upon addition of carnosic acid to *S. aureus* cells: 32 µg/ml (▲), 16 µg/ml (△), cells treated with CCCP (■), cells treated with Polimyxyn B (□) and untreated cells (●). Representative records from two similar independent experiments are shown

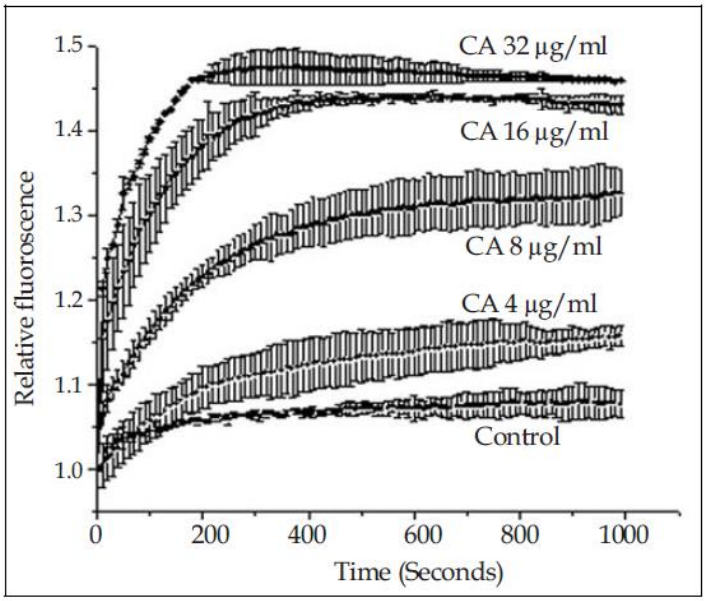

Fig. 6. Effect of carnosic acid on EtBr efflux in S. aureus cells. Control corresponds to the initial intracellular fluorescence of EtBr at time 0. Representative efflux records from two similar independent experiments are shown

As it can be seen above, carnosic acid at 1/4 - 1/8 MIC value (2 to 4 µg/ml) showed a selective synergistic interaction with tetracycline and aminoglycosides (Table 5) that may be explained by selective modulation of the antibiotics accumulation possibly by inhibition of their efflux in S. aureus. To assess whether this compound may have an efflux pump inhibitor activity in S. aureus, the efflux substrate ethidium bromide (EtBr) was used. EtBr shows a characteristic fluorescence when it enters the cell and binds to DNA and its uptake was determined by monitoring the fluorescence using a multiwell plate reader (DTX 880 Multimode detector, Beckman Coulter) at 29ºC every 10 sec intervals for 3 min with excitation and emission wavelengths of 535 nm and 485 nm, respectively. Figure 6 shows that carnosic acid rapidly increased EtBr uptake and this effect was dose dependent indicating a positive effect on the intracellular accumulation of the fluorescent molecule.

Whether carnosic acid is a general disruptor of the transmembrane electrochemical potential, it should display a synergistic effect with chloramphenicol which is effluxed out of the cell by secondary transport relying in the membrane potential. Additionally, an antagonist effect with ampicillin and penicillin G, which enter inside cells by a proton symport mechanism, should be displayed however, none of these effects were observed (Table 5).

Other studies from our laboratory on the antibiotic action of carnosic acid against E. faecalis showed similar results. In time-kill studied, carnosic acid displayed a bacteriostatic effect at its MIC value (64 µg/ml), whereas a bactericidal effect was achieved at 2 x MIC (128 µg/ml) increasing the permeability of cell membrane effects. At sub MIC values, the diterpene inhibited the drug efflux pumps of secondary transporters (Repetto, 2009). Other authors reported the antibacterial and resistance modifying activity of R. officinalis and suggested that the carnosic acid has the capacity to modify the resistance pattern of strains of S. aureus expressing multidrug efflux pumps (Oluwatuyi et al., 2004). Regarding this feature, we decided to investigate several bioactive compounds of R. officinalis with antimicrobial activity per se, or as modulators of bacterial resistance, against multidrug-resistant clinical strains of S. aureus isolated from pediatric patients (Ojeda Sana et al., 2011).

Our findings, together with results from other authors, suggest that this diterpene is a potential antibacterial agent to be used in combinational therapy (at least with aminoglycosides, tetracyclin and fluoroquinolones) against sensitive as well as multidrug resistant, vancomicyn and/or methicillin resistant Gram-positive cocci. Moreover, as an antibacterial compound, carnosic acid can not only target membrane permeability and enhance drug uptake, but also this compound, at sub MIC values inhibited the drug efflux transport probable by altering the cell membrane potential. We proposed a model for the antibacterial action of carnosic acid (Fig. 7).

3.4 Rosemary bioactives kill inthaphagocytic S. aureus cells

We examined the intracellular antibacterial activity of carnosic acid against S. aureus. A model of S. aureus infected RAW 654.7 mouse macrophages has been monitored for long-term (24 h) experiments. This bacterium adheres to phagocytes and easily invades them and tends to restrict the phagolysosomal compartment, where it largely escapes destruction and survives in a semiquiescent state for prolonged periods (Maurin et al., 2001). These intraphagocytic forms are considered responsible for the well-known recurrent character of staphylococcal infections as well as for the many failures of apparently appropriate antibiotic treatments.

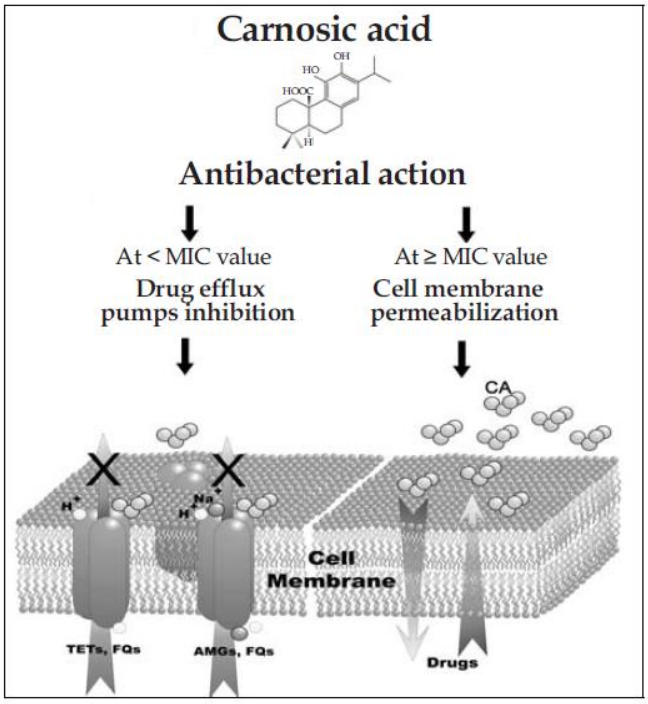

Fig. 7. Proposed model for the antibacterial action of carnosic acid. NMGs, aminoglycosides; TETs, Tetracyclins and FGs, fluoroquinolones

Confocal microscopy was used to ascertain the localization of the bacteria inside RAW 264.7 macrophages observed 24 h after phagocytosis of opsonized S. aureus. As shown in Fig. 8 A and B S. aureus clearly appeared intracellular. Pericellular membrane, located mostly on the inner face was labeled with FITC-phalloidin (green signal), and bacteria were labeled with DAPI (Blue signal). Later, macrophages were incubated for 24 h with carnosic acid from the MIC (16 µg/ml, determined in broth at pH 7.3). Carnosic acid caused a marked reduction in CFU, diminishing 3-4 log the bacterial growth at a concentration of 1 ½ MIC (24 µg/ml) (Fig. 8C). This effect was comparable to the control cells incubated with 0.5 µg/ml ciprofloxacin, a fluoroquinolone antibiotic that has excellent antibacterial activity against gram positive bacteria and intracellular penetration.

Earlier we demonstrated the in vivo antibacterial efficacy of a rosemary extract containing high amounts of carnosic acid against S. aureus in two skin infection models in mice (Barni et al, 2009).

Clinically effective antimicrobial agents exhibit selective toxicity towards the bacterium rather than the host. It is this characteristic that distinguishes antibiotics from disinfectants. The basis for selectivity will vary depending on the particular antibiotic. Carnosic acid at the concentrations that kill S. aureus has a high selectivity to bacteria and is not toxic to macrophages.

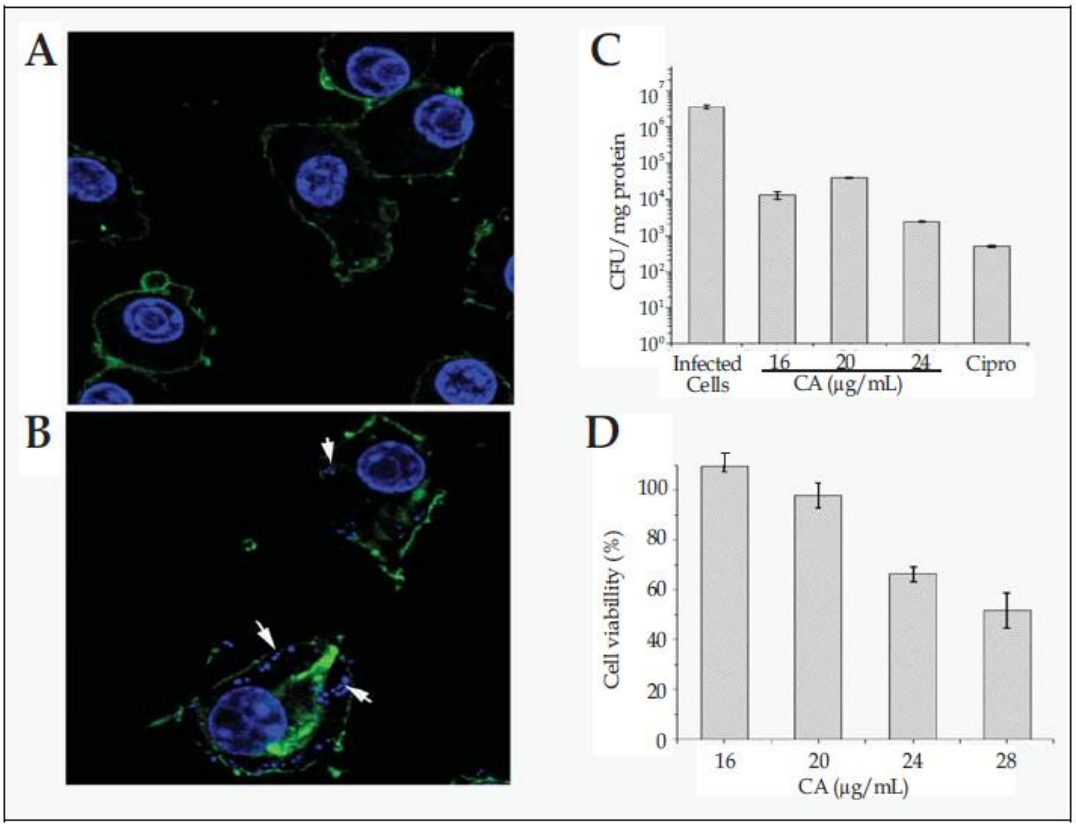

Fig. 8. Intraphagocitic killing of S. aureus by carnosic acid. Confocal microscopy of uninfected RAW 264.7 macrophages (A) or after phagocytosis of S. aureus (B). Arrowheads indicate intraphagocytic bacteria. (C) variations in the number of CFU per mg of protein (± SD; n= 2) after 24 h of incubation with carnosic acid or with 0.5 µg/ml of ciprofloxacin (Cipro). (D) Effect of carnosic acid following a 24-h exposure using a standard MTS cell viability assay

4. Safety of rosemary: Toxicity evaluation

Due to the potential use in human nutrition and health in foods of carnosic acid, it is important to determine its toxicological effects in a living organism.

In this study, we used Caenorhabditis elegans as a model system to examine the toxic effects of rosemary bioactives. This nematode, present in soil and found in temperate regions of the world, has emerged as an important animal model in various fields including toxicological research and rapid toxicity assessment for new chemicals (Moy T et al., 2006). C. elegans is easily cultured on agar medium plates with Escherichia coli OP50 as a food source. The ease of laboratory cultivation, its small size, large brood size, short development time (the entire complete life cycle from egg to egg producing hermaphrodite occurs over 3 days at 20°C under normal laboratory conditions) and the well-studied biology, makes the C.elegans an ideal model organism for biological studies. The transparency of this nematode allows for high quality microscopic images to be taken. Toxicity assays will be used in this study to test how carnosic acid affects C. elegans

survival by exposing them to various concentrations of this compound. The C. elegans life cycle is comprised of an embryonic stage, four larval stages designated L1-L4 and adulthood. As it had been previously described that some nematode killing observed in this assays was a consequence of eggs being retained in the uterus and hatching internally, we substituted wild type worms by the glp-4 temperature-sensitive sterile mutants to prevent internal hatching of progeny. glp-4 does not make a germline at the restrictive temperature and survive without a bacterial food source, whereas WT C. elegans die by internal hatching of progeny when transferred to bacteria-free media. Worms were synchronized by isolating eggs from gravid adults, hatching the eggs overnight in M9 buffer, and plating L1-stage worms onto lawns of OP50 E. coli on nematode growth medium (NGM) agar media. Worms were grown to sterile, young adults by incubation at 25°C for 48 – 52 h, washed off the plates with M9 buffer. Approximately 40 worms were transferred to 24 well plates containing 500 microliters of M9 medium and the compounds to be tested. Each compound was tested in individual wells, and the screen was performed by using triplicate 24-well plates. To score for survival, the plates were shaken by hand, worms were considered to be dead if they did not move or exhibit muscle tone when viewed using a stereo microscope at 20X magnification. Worms were observed every two days and scored for survival analysis at day 8 after transfer. As carnosic acid was dissolved in ethanol, we first tested if the 1.6% final ethanol concentration found in the vehicle of this assay had any effect over the survival of worms. We didn't find significative differences between the survival of ethanol treated and untreated worms. Our results demonstrated that up to 250 µg/ml of carnosic acid tested the compound does not adversely affect the normal physiology of the nematodes, while the highest concentration tested (500 µg/ml) of carnosic acid had moderate worm mortality (30%) (Fig. 9).

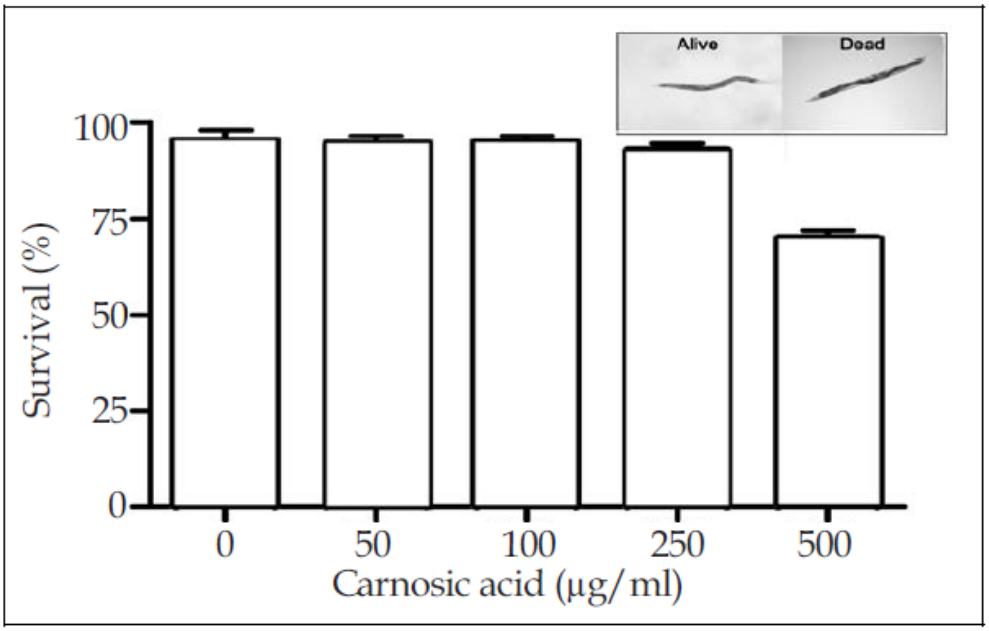

Fig. 9. Effect of carnosic acid over C.elegans survival. (Inset) typical morphology of living nematodes maintaining a sinusoidal shape (left) and dead ones appearing as straight, rigid rods (right) (at ×20 magnification). Values are shown as the mean of three independent experiments ± SD

5. Conclusions

The present chapter summarizes the most significant rosemary biological properties, reviewing its free radical scavenging, antibacterial actions of essential oils and non-volatile pure constituents and in combination with antibiotics.

Both volatile and non-volatile secondary metabolites isolated from rosemary plants growing in the same place exhibited comparable antioxidant activities. Non-volatile constituents exhibited at least a minor pro-oxidant property than ascorbic acid and Trolox, at the concentration assayed. The non-volatile fractions containing high content of carnosic acid as well as the volatile oils rich in α-pinene exhibited the highest antibiotic activity.

Rosmarinus officinalis extract as well pure carnosic acid, showed synergistic effect in combination with aminoglycosides and ciprofloxacin against S. aureus. We proposed a model for the antibacterial action of carnosic acid which may be useful for future applications.

Unexpectedly, carnosic acid showed a clear antistaphylococcal action towards extracellular and intraphagocytic forms of Staphylococcus aureus, pointing out a potential use of this compound in the treatment of S. aureus infections, without significant effect on the macrophage viability. Moreover, carnosic acid at bactericide concentrations did not show adverse effects on the viability of a living organism as the nematode C. elegans.

6. Rosemary in human nutrition and health: Future and prospect

All together, the available evidence indicates that rosemary compounds might be of therapeutic benefit in bacterial infections and be an ideal candidate for nutraceutical health products. Non-volatile extracts of rosemary containing approximately an amount of 20 μg/ml (18 μM) of carnosic acid as the key compound, killed several bacteria and represent a therapeutic alternative against extracellular-intracellular S. aureus infections. This compound did not show pro-oxidant effects and its use is safe at least until a concentration of 250 μg/ml (750 μM). The in vivo antibacterial efficacy of an ethanol extract of Rosmarinus officinalis L. containing high amounts of carnosic acid against the pathogenic bacteria S. aureus has been demonstrated previously in mouse (Barni et al, 2009). Even though, prospective controlled clinical studies are still lacking.

Rosemary is the only spice commercially available for use as an antioxidant in Europe and the United States. This specie has the advantage to contain different antioxidant molecules (lipophilic monoterpenes and diterpenes, as well as hydrophilic derivatives of caffeic acid as rosmarinic acid) that could be effective in both, aqueous fluids as well as in lipophilic parts of the body as a very effective antioxidant to scavenge free radicals. In addition, non-volatile extracts of rosemary can also be used to decrease 4.4 - to 17-fold the amounts of the synthetic butyl derivatives used as food or cosmetic preservatives (Romano et al., 2009).

Although not discussed in this chapter, due to the associated bioactivities of carnosic acid (anti-inflammatory-and anticarcinogenic effects), rosemary polyphenols can be considered as a potential source of promising new nutraceuticals formulations (Mengoni et al., 2011).

Improvements in the processes of regulation of rosemary bioactives are needed, and the general tendency is to perpetuate the German Commission E experience, which combines scientific studies and traditional knowledge (monographs).

There is still work to be done regarding how to use rosemary derivatives inside the human diet. Particular attention needs to be given to stability studies and interactions of rosemary constituents with other food constituents. Further investigations will be directed towards the application of phenolic compounds in various food matrices. The use of functional foods enriched with rosemary compounds need technologies for incorporating health-promoting ingredients into food without reducing their bioavailability or functionality. In this sense, we are also working on the addition of rosemary bioactives into edible films (Proyect CYTED, 309AC0382 action: Getting additive materials from plant by-products of the region and its

application in the development of biodegradable packaging food processing and nutraceutical use). Finally, the production and biotechnological studies and genetic improvement of rosemary plants will offer great advantages, since it will be possible to obtain uniform and high quality raw materials which will ensure the efficacy and safety of rosemary products.

7. Acknowledgment

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Carnosic acidrich rosemary (*Rosmarinus officinalis* L.) leaf extract limits weight gain and improves cholesterol levels and glycaemia in mice on a highfat diet

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Abstract

Rosemary (Rosmarinus officinalis L.) extracts (RE) are natural antioxidants that are used in food, food supplements and cosmetic applications; exert anti-inflammatory and anti-hyperglycaemic effects; and promote weight loss, which can be exploited to develop new preventive strategies against metabolic disorders. Therefore, the aim of the present study was to evaluate the preventive effects of rosemary leaf extract that was standardised to 20% carnosic acid (RE) on weight gain, glucose levels and lipid homeostasis in mice that had begun a high-fat diet (HFD) as juveniles. The animals were given a low-fat diet, a HFD or a HFD that was supplemented with 500mg RE/kg body weight per d (mpk). Physiological and biochemical parameters were monitored for 16 weeks. Body and epididymal fat weight in animals on the HFD that was supplemented with RE increased 69 and 79% less than those in the HFD group. Treatment with RE was associated with increased faecal fat excretion but not with decreased food intake. The extract also reduced fasting glycaemia and plasma cholesterol levels. In addition, we evaluated the inhibitory effects of RE in vitro on pancreatic lipase and PPAR-g agonist activity; the in vitro findings correlated with our observations in the animal experiments. Thus, the present results suggest that RE that is rich in carnosic acid can be used as a preventive treatment against metabolic disorders, which merits further examination at physiological doses in randomised controlled trials.

Key words: Rosmarinus officinalis L.: Carnosic acid: Pancreatic lipase: PPAR-*g*

The prevalence of metabolic disorders, such as obesity, hyperlipidaemia and hyperglycaemia, is rising dramatically in developing and industrialised nations. Obesity is reaching epidemic proportions worldwide(1) and is an established risk factor for various comorbidities, such as type 2 diabetes mellitus and CVD(2 – 4). The development of obesity induces systemic oxidative stress(5) and affects the inflammatory state(6). The constant increase in fat intake that is linked with sedentary lifestyles is the chief cause of this phenomenon (2).

Developing preventive and therapeutic solutions that impede the rise in metabolic disorders has become a primary goal in the past decade. In addition to pharmaceutical approaches, the use of natural products in physiological doses has been recognised as an effective regimen to improve several health conditions(7 – 9). Plant-based treatments have been validated as strategies in the prevention of obesity and type 2 diabetes mellitus(10). Rosemary (Rosmarinus officinalis L.) extracts are natural antioxidants that are used in food, food supplements and cosmetic applications(11 – 15). Recently, rosemary extracts that have been standardised for carnosic acid and carnosol attained antioxidant status, garnering an additive E classification from the European Food Safety Authority, confirming its importance as a natural preservative in foods and beverages(13).

Carnosic acid-rich rosemary extract has been reported to have antioxidant activity in vitro by oxygen radical absorbance capacity and ferric-reducing/antioxidant power assays, and it inhibits the oxidation of Cu2t-induced LDL ex vivo (15). These antioxidant effects have been recapitulated in vivo. Consequently, carnosic acid-rich

rosemary extract reduces oxidative stress in aged rats(16). Carnosic acid has anti-inflammatory effects in cellular(17) and animal(18) models.
Furthermore, carnosic acid has promising anti-obesity and anti-glycaemic effects. In in vitro trials, carnosic acid inhibits pancreatic lipase(19), activates PPAR-g(20) and prevents the differentiation of mouse pre-adipocytes into adipocytes(21) – all of which are important mechanisms in glucose and lipid homeostasis. The capacity of rosemary to regulate weight gain(22,23) and glycaemia(22 – 24) has been observed in vivo. Nevertheless, no randomised clinical trials have been reported using rosemary extracts to control obesity and hyperglycaemia, but this evidence encourages further study of carnosic acid-rich rosemary extracts to prevent the development of metabolic disorders. In the present study, we aimed to determine the preventive effects of a rosemary extract that was standardised to contain 20% carnosic acid (RE) on weight gain, glycaemia levels and lipid homeostasis in mice that were started on a high-fat diet (HFD) as juveniles. The animals were given a low-fat diet (LFD), a HFD or a HFD with 500 mg RE/kg body weight per d (mpk) (HFD-RE). Physiological and biochemical parameters were measured throughout the 16 weeks of treatment, and the effects on pancreatic lipase and PPAR-g agonist activity in vitro were examined.

Experimental methods

Rosemary leaf extract
RE was prepared as described by Ibarra et al.(15).

Animals and diet
Male C57BL/6J mice, aged 4 weeks, were purchased from Elevage Janvier (CERJ, Le Genest Saint Isle, France). All mice were housed in a cage on a 12 h light–12 h dark cycle in a temperature-controlled environment during a 2-week acclimatisation, with ad libitum access to water and a control standard diet – an energy-balanced diet. After acclimatisation, the mice were randomised by body weight into three groups of eight animals. Each group was fed an experimental diet (Research Diets, Inc., New Brunswick, NJ, USA) for 16 weeks, as described in Table 1 (LFD, HFD and HFD-RE). Body weightwas measured twice per week, and food intake was recorded once per week. All procedures were performed as per French guidelines for the care and use of experimental animals.

Blood biochemistry
Blood was collected from the retro-orbital sinus into EDTAcoated tubes under isoflurane anaesthesia after overnight fasting. Samples were collected at the beginning of the study (day 0) and after 16 weeks on the experimental diets. Blood samples were centrifuged at 4000 rpm for 15 min at 48C to recover the plasma.
Biochemical levels were measured using commercial kits.Total cholesterol, TAG, glucose (kits CH3810, TR3823 and GL3815; Randox Laboratories Limited, Newbury, Berkshire, UK) and NEFA (kit 434-91717; Wako Pure Chemical Industries Limited, Osaka, Japan) were measured by spectroscopy. Insulin (kit INSKR020; Crystal Chem, Inc., Downers Grove, IL, USA) was measured by ELISA.

Faecal lipid measurements
Faeces were collected at weeks 0, 8 and 16, frozen at 2808C and pulverised. For each condition, faeces from eight mice, harvested during a 24 h period, were pooled. Total lipids were extracted from 100 mg of dried faeces as described(25). Total lipid levels from several independent extractions were estimated by traditional gravimetric analysis: 500ml of total lipids in chloroform were dried by evaporation and weighed.
The amount of faecal fat energy that was excreted, expressed in kJ/animal per d, was calculated in the lyophilised total fat that was excreted and collected throughout the experiment, assuming that 1 g lipid equals 37·7 kJ.

Pancreatic lipase activity assay

Human pancreatic lipase was purchased from Lee Biosolutions, Inc. (St Louis, MO, USA). Orlistat tetrahydrolipstatin, a pancreatic lipase inhibitor) was purchased from Sigma Chemical Company (St Louis, MO, USA). Other chemicals were of reagent grade. The pancreatic lipase was diluted in dimethyl sulfoxide to obtain a final activity of 0·1 £ 106 U/l. Orlistat was tested at two concentrations in dimethyl sulfoxide.

Lipase activity was measured using the ENZYLINETM Lipase Colour Assay kit (Biome´rieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Briefly, pancreatic lipase, substrate and the test sample were mixed gently, and incubated for 5 min at 378C. Activator reagent was added, and the mixtures
were incubated again for 6 min at 378C. The recorded rate of increase in absorbance at 550 nm, due to the formation of quinone diimine dye, reflected pancreatic lipase activity.

PPAR-g assay

PPAR-g activation was measured in a cell-based luciferase assay. COS-7 cells (African Green Monkey SV40-transformed kidney fibroblast cell line), cultured in Dulbecco's modified Eagle's medium that was supplemented with 10% fetal calf serum, were transiently transfected with a fusion protein GAL4/PPARg
and a DNA construct that harboured the gene reporter.

The plasmid pGal5-TK-pGL3 was obtained by inserting five copies of the Gal4 (a yeast transcription factor) DNA-binding site upstream of the thymidine kinase promoter in pTK-pGL3. The plasmid pGal4-human PPAR-g was constructed by PCR amplification of the human PPAR-g DEF domain (nuclear receptor Hinge region (D) t ligand binding domain (E) + C-terminal domain (F)) (aa 318–505). The resulting amplicons were cloned into pBD-Gal4 (Stratagene, La Jolla, CA, USA), and the chimera was subsequently subcloned into pCDNA3.

After transfection, the COS-7 cells were incubated for 24 h with RE to assess its capacity to activate PPAR-g. Dimethyl sulfoxide was used as the reference control, and rosiglitazone was used as a positive control. The activation of PPAR-g by RE induced the expression of luciferase and a consequent increase in luminescence.

Table 1. Composition of the experimental low-fat diet (LFD), high-fat diet (HFD) or a HFD plus rosemary extract (RE) and macronutrient content (%)

	HFD				LFD				HFD-RE 0·5 % (500 mpk)			
	g	kJ	g/kg	kJ/kg	g	kJ	g/kg	kJ/kg	g	kJ	g/kg	kJ/kg
Compound									4·31	0	5·00	0
Casein 80 mesh	200	3349·4	233	3902·1	200	3349·4	190	3173·6	200	3349·4	231·89	3902·1
L-Cysteine	3	50·2	3	58·6	3	50·2	3	46·1	3	50·2	3·48	58·6
Maize starch	72·8	1219·2	85	1419·3	315	5275·4	299	4999·0	72·8	1219·2	84·41	1419·3
Maltodextrin 10	100	1674·7	117	1951·0	35	586·2	33	556·8	100	1674·7	115·95	1951·0
Sucrose	172·8	2893·9	201	3370·4	350	5861·5	332	5555·9	172·8	2893·9	200·36	3370·4
Cellulose BW200	50	0	58	0	50	0	47	0	50	0	57·97	0
Soyabean oil	25	942·0	29	1096·9	25	942·0	24	891·8	25	942·0	28·99	1096·9
Lard	177·5	6688·4	207	7795·8	20	753·6	19	715·9	177·5	6688·4	205·81	7795·8
Mineral mix	10	0	12	0	10	0	9	0	10	0	11·59	0
Calcium phosphate	13	0	15	0	13	0	12	0	13	0	15·07	0
Calcium carbonate	5·5	0	6	0	5·5	0	5	0	5·5	0	6·38	0
Potassium citrate	16·5	0	19	0	16·5	0	16	0	16·5	0	19·13	0
Vitamin mix	10	167·5	12	196·8	10	167·5	9	159·1	10	167·5	11·59	196·8
Choline bitartrate	2	0	2	0	2	0	2	0	2	0	2·32	0
Dye	0·05	0	0	0	0·05	0	0	0	0·05	0	0·06	0
Total	858·2	16985·4	1000·0	19791·0	1055·1	16985·8	1000·0	16098·2	862·5	16985·4	1000·0	19791·0
Protein (%)	23·66	20·02			19·24	20·01			23·66	20·02		
Carbohydrate (%)	40·27	34·08			66·35	69·02			40·27	34·08		
Fat (%)	23·60	44·92			4·27	9·98			23·60	44·92		

mpk, mg rosemary extract/kg body weight per d.

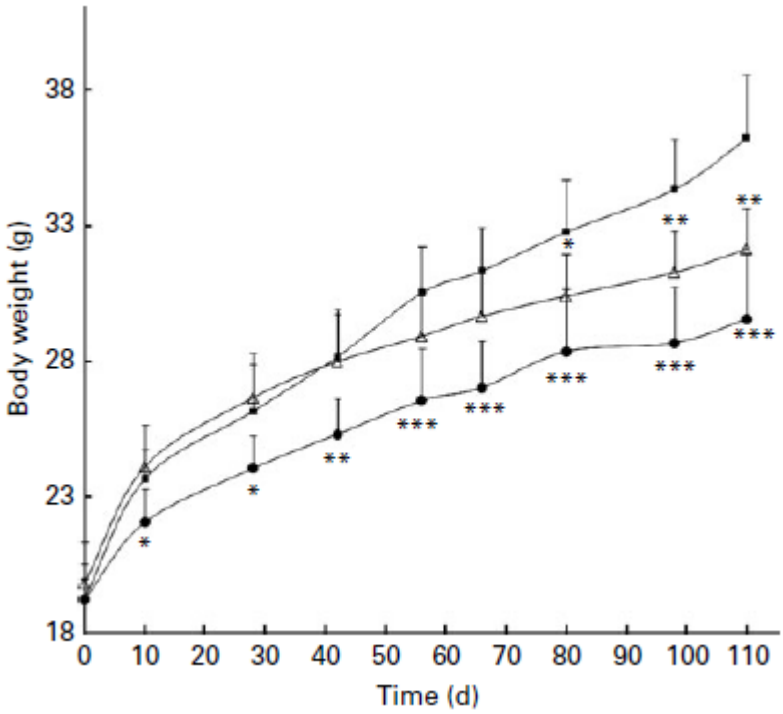

*Fig. 1. Effects of rosemary extract (RE) standardised to 20% carnosic acidon body weight after 16 weeks. Low-fat diet ( ), high-fat diet (HFD,) and HFD supplemented with RE (HFD-RE,) at a concentration equivalent to 500mg RE/kg body weight per d. Values are means, with standard deviations represented by vertical bars. Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): *P,0·05, ** P,0·01, *** P,0·001.*

After 24 h, the cells were collected, and the luciferase assay was performed according to the manufacturer's instructions (SteadyGlow; Promega, Charbonnie`res, France). Luminescence was measured on a Tecan Ultra spectrophotometer (Tecan, Ma¨nnedorf, Switzerland). All experiments were performed in quadruplicate. Relative luciferase activity of a sample was calculated as the ratio of mean luciferase activity in the test cells to that in the control cells, and PPAR-g ligand-binding activity was expressed as the ratio of relative luciferase activity to that of the reference control.

Analysis of results

The animals were randomised based on total body weight by principal component analysis (GENFIT, Loos, France), resulting in groups of animals between which no statistical difference was observed for any parameter.

The data from the in vivo and in vitro studies are expressed as means and standard deviations. One-way ANOVA (one-way Bonferroni) and Student's t test were performed to compare groups using Sigma Plot 11.0 (2008) (Systat Software, Inc., Chicago, IL, USA). Statistical significance was considered at P,0·05.

In the in vivo study, gains in the HFD-RE group are expressed as a percentage compared with those in the HFD and LFD control groups, which is calculated as: Parameter d%T . ddHFD 2 HFD_RET=dHFD 2 LFDTT x 100:

In the in vitro studies, changes are expressed relative to their respective controls.

Results

Effect of rosemary extract on body and organ weight and food intake in mice fed a high-fat diet

Body weight between the HFD and LFD groups began to differ significantly after the first week of treatment. In HFDRE animals, body weight differed significantly after day 80 (Fig. 1) compared with that in HFD mice and weight gain peaked at 69% (P,0·01) at the end of the study (Table 2). This effect was associated with a 79% (P,0·001) less of an increase in epididymal fat mass; liver weight was unaffected by the treatment. No significant changes in food or energy intake were observed between the groups (Table 2).

Effects of rosemary extract on serum biochemical parameters

At 16 weeks of treatment, total fasting glycaemia, total cholesterol and NEFA levels rose significantly in the HFD group compared with the LFD animals. HFD-RE mice experienced 72% (P,0·01) less increase in plasma glucose levels and 68% (P,0·001) less an a rise in total cholesterol compared with HFD mice. No significant effects were observed in NEFA or TAG levels in HFD-RE mice compared with the HFD group (Table 3).

Fasting insulinaemia was also monitored; insulin levels remained low during the entire experiment, and no significant differences were observed between the groups (data not shown).

	Food intake (g/animal per d)		Energy intake (kJ/animal per d)		Weight gain (g)		Liver weight (g)		Epididymal fat weight (g)	
Animal group	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LFD	3·40	0·28	61·7	5·3	10·33***	2·18	0·99	0·10	0·45***	0·26
HFD	3·09	0·19	67·5	2·2	17·01	1·94	1·12	0·13	1·52	0·48
HFD-RE	3·01	0·10	62·8	1·3	12·37**	1·59	1·14	0·15	0·67***	0·15

Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): ** $P<0·01$ and *** $P<0·001$.

Table 2. Effects of chronic administration of rosemary extract (RE) standardised to 20% carnosic acid on nutritional and weight parameters in mice fed a low-fat diet (LFD), a high-fat diet (HFD) or a HFD plus RE after 16 weeks (Mean values and standard deviations)

	Total cholesterol (mg/l)		TAG (mg/l)		NEFA (mmol/l)		Glucose (mg/l)	
Animal group	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LFD	1076·6***	48	891·4*	125·9	1·46	2·2	1286***	182·5
HFD	1475·8	136·3	1297·3	374·6	1·45	1·1	1978·1	492·6
HFD-RE	1205***	154·6	1021·1	256·8	1·47	2·8	1480**	113·3

Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): * $P<0·05$, ** $P<0·01$ and *** $P<0·001$.

Table 3. Effects of chronic administration of rosemary extract (RE) standardised to 20% carnosic acid on plasma lipid and glucose levels in mice fed a low-fat diet (LFD), a high-fat diet (HFD) or a HFD plus RE after 16 weeks (Mean values and standard deviations)

Effect of rosemary extract on faecal fat excretion

HFD animals had higher faecal fat excretion values (7·63 (SD 1·27) mg/100 mg) compared with LFD mice (2·74 (SD 0·10) mg/100 mg, P,0·001) after 16 weeks. Moreover, Retreated mice experienced a significant 1·2-fold increase P,0·01) in total faecal content compared with HFD animals (Fig. 2).

Throughout the experiment, there was no significant difference in fat energy intake between the HFD and HFD-RE groups. However, faecal fat energy excretion rose 1·3-fold (P,0·05) in RE-treated mice (1·13 (SD 0·16) kJ/animal

per d) v. the HFD group (0·87 (SD 0·15) kJ/animal per d). Faecal fat energy excretion differed between the LFD group (0·32 (SD 0·01) kJ/animal per d, P,0·001) and HFD mice (Fig. 3).

In vitro analysis of the mechanism of rosemary extract

As shown in Fig. 4, 100 mg/ml RE (P,0·001) inhibited pancreatic lipase activity by 70% compared with Orlistat. RE also activated PPAR-g by 1·66-fold (P,0·001) in a dosedependent manner compared with the blank control at 30mg/ml (Fig. 5), whereas activation in the positive control, rosiglitazone, was 4·35-fold (P,0·001) that in the blank control at 10 nM. Therefore, 30mg/ml RE are able to activate PPAR-g by 19·70% compared with the positive control.

Discussion

The production of RE, estimated to exceed 100 tonnes annually, has risen considerably in recent years due to its widespread use in food, beverage, flavour, food supplements and cosmetic applications(11 – 15). Consequently, technologies to develop standardised extracts from rosemary have evolved tremendously with regard to quality and reproducibility. Recently, we have demonstrated the importance of standardisation in determining the biological activity of plant extracts.

Furthermore, depending on the content, we have observed that the antioxidant activities of various preparations of RE vary(15).

Rosemary exerts various biological activities with which preventive nutritional strategies against metabolic disorders, such as obesity, dyslipidaemia and diabetes, can be developed (19 – 23). Nevertheless, individual studies have examined specific extracts, the chemical description of which is not always available. Thus, it can be difficult to extrapolate the health benefits of a unique RE to another solely on the basis of published data.

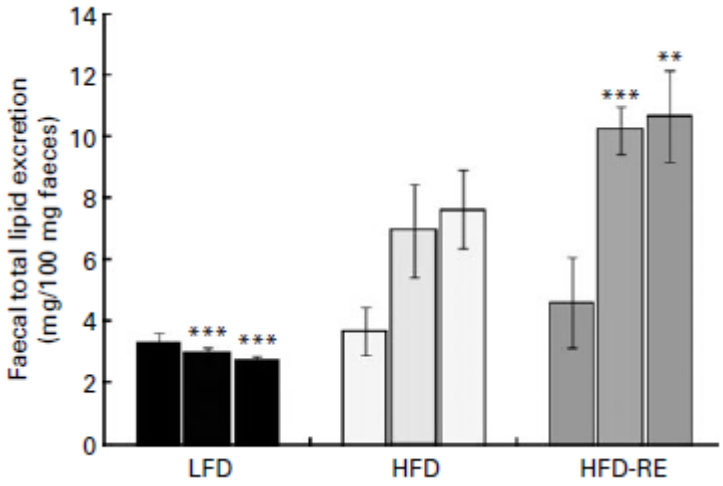

*Fig. 2. Effects of rosemary extract (RE) standardised to 20% carnosic acid on total faecal lipid content at weeks 0, 8 and 16. Low-fat diet (LFD), high-fat diet (HFD) and HFD supplemented with RE (HFD-RE) at a concentration equivalent to 500mg RE/kg body weight per d. Values are means, with standard deviations represented by vertical bars of pooled data (n 6; except for LFD, n 3). Mean values were significantly different from HFD control (ANOVA one-way Bonferroni): **P,0·01, *** P,0·001.*

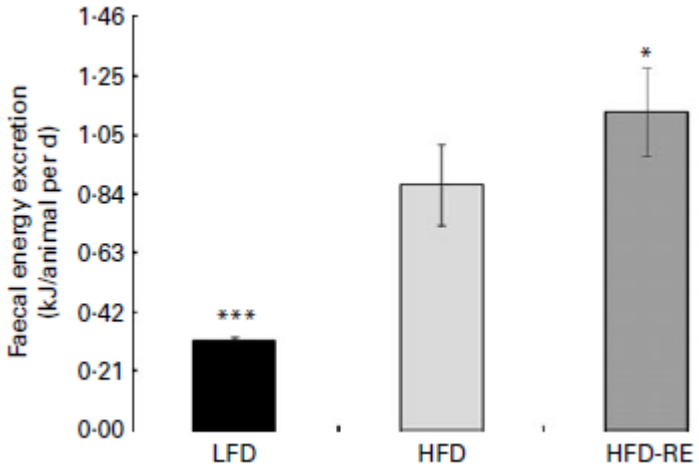

*Fig. 3. Effects of rosemary extract (RE) standardised to 20% carnosic acid on total faecal energy excretion over 16 weeks. Low-fat diet (LFD), high-fat diet (HFD) and HFD supplemented with RE (HFD-RE) at a concentration equivalent to 500mg RE/kg body weight per d. Values are means, with standard deviations represented by vertical bars of pooled data (n 6; except for LFD, n 3). Mean values were significantly different from HFD control (Student's t test): * P,0·05, *** P,0·001.*

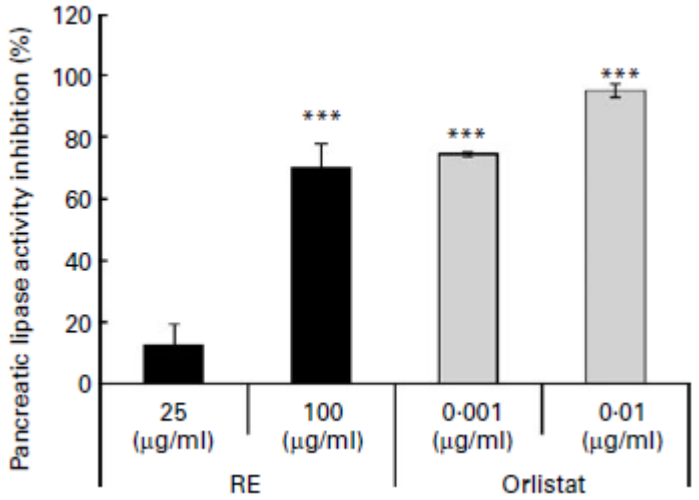

*Fig. 4. Effects of rosemary extract (RE) standardised to 20% carnosic acid on pancreatic lipase inhibition in vitro. Values are means, with standard deviations represented by vertical bars expressed as a percentage of inhibition of three independent experiments. Mean values were significantly different from the blank control (Student's t test): *** P,0·001.*

In a previous study, we identified RE as the most potent antioxidative extract in an ex vivo LDL oxidation model(15), prompting us to determine whether it affects other metabolic parameters in a HFD mouse model. In the present study, the administration of a 44·92% fat diet to 6-week-old C57BL/6J mice for 16 weeks resulted in significant increases in body weight, epididymal fat mass, glycaemia and cholesterol, compared with a LFD, confirming previous reports(26,27).

Treatment of mice with 500 mpk of RE reduced the gains in weight that were induced by the HFD without affecting food intake or fat energy intake. It also lowered epididymal fat tissue weight significantly compared with HFD mice. In addition, total faecal lipid content increased in HFD-RE mice compared with the HFD group, which correlates with the amount of total faecal fat energy that was excreted. Based on the present study and other reports(19,22), limiting lipid absorption in the intestine is a potential mechanism by which RE prevents weight gain.

This hypothesis is strongly supported by evidence of the in vitro inhibitory effect of RE on pancreatic lipase activity, a key enzyme in the digestion and absorption of fat. Moreover, similar effects have recently been reported with an ethanolic extract of rosemary that contains rosmarinic acid, carnosol and carnosic acid, wherein the treatment of 15-week-old diet-induced obesity mice with 200 mpk of extract limited the weight gain that was induced by a 50 d HFD and increased the lipid faecal content by 2·2-fold. Ninomiya et al.(19) observed that after 2 weeks of treatment with 20 mpk of carnosic acid alone, the weight of ddY (Deutschland, Derker, Yoker) mice fell by 7·6% compared with the control group. Thus, in the present study, the effects of RE on faecal fat excretion and, consequently, faecal fat energy excretion partially explain the observed reductions in body weight.

In addition to its effects on physiological measures, RE significantly reduced elevated cholesterol levels that were induced by the HFD. Although these effects were not observed by Harach et al.(22) or Ninomiya et al.(19), they were observed in human subjects with Orlistat(28 – 30) and in animal models with other plant-based pancreatic lipase inhibitors (31,32). Dietary cholesterol absorption has been proposed to be associated with fat digestion; Young & Hui(33) have shown that minimal TAG hydrolysis is sufficient to increase cholesterol transport significantly from lipid emulsions to intestinal cells. Consequently, pancreatic lipase inhibition has been proposed to be a target against which lipid malabsorption can be triggered to control TAG and cholesterol levels(33).

In the present study, fasting glycaemia was reduced in animals in the HFD-RE group compared with the HFD control group. Few studies have evaluated the effect of rosemary on diabetes. Anti-hyperglycaemic effects can be induced by an ethanolic RE in the alloxan diabetic rat model(34) and by a water RE in a mouse model(24), whereas no such effect has been observed in the diet-induced obesity mouse model with an extract that contains rosmarinic acid, carnosol and carnosic acid(22). Based on these data, it appears that the anti-hyperglycaemic activity and preventive effects of an extract against type 2 diabetes mellitus depend on its composition and the animal model in which it is tested.

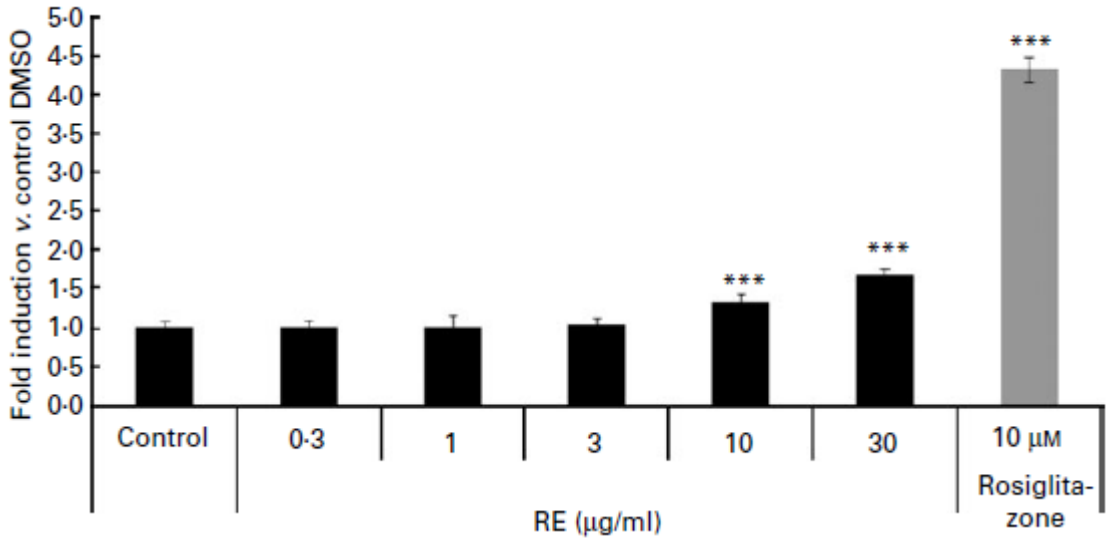

*Fig. 5. Effects of rosemary extract (RE) standardised to 20% carnosic acid on PPAR-g activation in vitro. Values are means, with standard deviations represented by vertical bars expressed as a percentage of inhibition of three independent experiments. Mean values were significantly different from the blank control (Student's t test): *** P,0·001. DMSO, dimethyl sulfoxide.*

Recently, it has been reported that the glucose-lowering effect of rosemary is attributed to PPAR-g activation, in which carnosic acid and carnosol were proposed be the active compounds(20). Therefore, we examined the in

vitro effects of RE on PPAR-g activation, hypothesising that the glucose-lowering effects are mediated through this mechanism.

In the present study on C57BL/6J mice, we used an effective dose of RE – 500 mpk – that contains 100 mpk of carnosic acid. The European Food Safety Authority Panel on Food Additives has estimated the dietary exposure for adults and pre-school children (aged 1·5–4·5 years) to carnosol plus carnosic acid to be 0·04 and 0·11 mpk, respectively(13). Thus, considering the normal dietary exposure of carnosic acid, we used a pharmacological dose of RE. In addition, the Panel also notes that the margin between the not observable adverse effect level of carnosol plus carnosic acid, as calculated in 90 d rat studies, is equivalent to 20–60 mpk, and the mean intake of carnosic acid-rich rosemary extracts is estimated to be 500–1500 mg/d in adults and 182–546 mg/d in pre-school children(13). Therefore, future randomised clinical trials that aim to confirm the efficacy of RE in humans should consider these values to establish an effective and safe dose.

In conclusion, we have demonstrated that a carnosicstandardised RE limits weight gain and improves plasma lipid and glucose levels in a HFD mouse model. These data confirm its potential for use in preventive strategies against metabolic disorders and encourage the initiation of further studies to recapitulate the physiological activity of RE in human subjects.

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Faixova Z, Faix S

Biological effects of rosemary (Rosmarinus Officialis L.)

Many herbs and plant extracts are added to the diet not only for their aromatic properties but they have been identified as a source of various phytochemicals, many of which possess an important biological activity. Results of many experiments showed that rosemary essential oil had antimicrobial, antioxidant, anti-carcinogenic, cognition-improving and certain glucose level lowering properties which makes it useful as a natural animal feed additive. This review describes the most important biological activities of rosemary (Rosmarinus officinalis L.) essential oil in animals and humans. In vitro and in vivo effects of rosemarv essential oil are discussed.

European Journal of Clinical Nutrition (2009), 1–7

A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain

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Background/Objectives: The aim of this study was to analyze the influence of a Mediterranean dietary pattern on plasma total antioxidant capacity (TAC) after 3 years of intervention and the associations with adiposity indexes in a randomized dietary trial (PREDIMED trial) with high cardiovascular risk patients.
Subjects/Methods: 187 subjects were randomly selected from the PREDIMED-UNAV center after they completed 3-year intervention program. Participants were following a Mediterranean-style diet with high intake of virgin olive oil or high intake of nuts, or a conventional low-fat diet. Adiposity indexes were measured at baseline and at year 3. Plasma TAC was evaluated using a commercially available colorimetric assay kit.
Results: Plasma TAC in the control, olive oil and nuts groups was 2.01±0.15, 3.51±0.14 and 3.02±0.14mM Trolox, respectively after adjusting for age and sex. The differences between the Mediterranean diet and control groups were statistically significant (Po0.001). Moreover higher levels of TAC were significantly associated with a reduction in body weight after 3 years of intervention among subjects allocated to the virgin olive oil group (B¼_1.306; 95% CI¼_2.439 to _0.173; P¼0.025, after adjusting for age, sex and baseline body mass index).
Conclusions: Mediterranean diet, especially rich in virgin olive oil, is associated with higher levels of plasma antioxidant capacity. Plasma TAC is related to a reduction in body weight after 3 years of intervention in a high cardiovascular risk population with a Mediterranean-style diet rich in virgin olive oil.

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Keywords: Mediterranean diet; plasma antioxidant capacity; olive oil; body weight; PREDIMED

Introduction

An imbalance between tissue-free radicals, reactive oxygen species (ROS) and antioxidants causing oxidative damage might be a major mechanism underlying obesity-related comorbidities (Higdon and Frei, 2003). Numerous studies have found elevated oxidative stress biomarkers in obesity (Keaney et al., 2003), and have suggested that oxidative stress may be the linking mechanism in the pathway leading from obesity to obesity-related diseases (Higdon and Frei, 2003; Morrow, 2003). In this sense, a number of pathways capable of generating injury-inducing ROS are known to be present in obesity, including lipoprotein oxidation, increased production of cytokines, upregulation of nicotinamide adenine dinucleotide phosphate oxidase(s) and other

oxidative enzymes present in vascular tissue. In addition, if obesity is a condition of increased oxidative stress, obese individuals may benefit from a better antioxidant status (Morrow, 2003). Therefore an antioxidant treatment should delay or prevent obesity phenotypes and obesity-related diseases.

The Mediterranean dietary pattern has been associated with a lower risk of obesity or weight gain (Schroder et al., 2004; Mendez et al., 2006; Sanchez-Villegas et al., 2006) and also with lower cardiovascular morbidity and mortality (Trichopoulou et al., 2003; Knoops et al., 2004; Martinez-Gonzalez and Sanchez-Villegas, 2004; Sanchez-Tainta et al., 2008; Sofi et al., 2008). Current studies have suggested that this protective effect may be related to a decrease in oxidative stress mediated by the antioxidant capacity of the diet (Visioli and Galli, 2001; Pitsavos et al., 2005; Fito et al., 2007; Dai et al., 2008). Some authors have underlined the idea that is preferable to analyze the whole dietary pattern rather than single components thought to be antioxidants (Martinez-Gonzalez and Sanchez-Villegas, 2004). In this context, there are studies that analyzed the adherence to a Mediterranean diet and its effects on plasma antioxidant capacity, however, to the best of our knowledge, there are no randomized controlled intervention studies assessing the effect of this dietary pattern on plasma total antioxidant capacity (TAC) and adiposity indexes. The assessment of TAC considers the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated approach rather than the simple sum of measurable antioxidants. With this approach the capacity of both known and unknown antioxidants and their synergistic interaction is, therefore, included, and provides a better insight into the delicate in vivo balance between cellular oxidants and antioxidants (Serafini and Del Rio, 2004).
In the frame of a randomized dietary trial assessing the effect of a Mediterranean-style diet for primary cardiovascular prevention among high cardiovascular risk patients (PREDIMED), this substudy was aimed to analyze the effect of this dietary pattern on plasma TAC after 3 years of intervention and the association between the dietary pattern and its antioxidant capacity with adiposity indexes.

Subjects and methods

Study design

The PREDIMED study is a large, parallel-group, multicenter, randomized controlled, 4-year clinical trial aimed to assess the effects of the traditional Mediterranean diet (TMD) on the primary prevention of cardiovascular disease. The methods of this trial have been described in detail elsewhere (Estruch et al., 2006; Zazpe et al., 2008). The inclusion criteria were either diabetes mellitus type II or at least three of the following risk factors: current smoking, hypertension, hyperlipidemia, high-density lipoprotein cholesterolo1.034 mmol/l, overweight/obesity or family history of premature coronary heart disease. 1055 subjects, at high cardiovascular risk, were recruited in the AP-UNAV center of the PREDIMED trial, and were randomly assigned to three intervention groups: TMDþfree provision of extra virgin olive oil (VOO); TMDþfree provision of nuts; and a low-fat diet. All participants provided informed consent and the protocol was approved by the institutional review boards according to the Declaration of Helsinki Principles.

Participants

This study assesses the effects of the intervention after 3 years of recruitment. The population sample consisted on 187 subjects (59 control, 65 TMDþVOO and 63 TMDþNuts subjects) randomly selected within those who had been 3 years in the intervention program.

Dietary assessment

The dietary habits of the participants, both at baseline and after follow-up for 36 months, were assessed using a semiquantitative 137-item Food Frequency Questionnaire previously validated in Spain (Martin-Moreno et al.,

1993). After the screening visit, and based on a baseline short (14-item) questionnaire specifically targeted to assess adherence to the Mediterranean diet (Martinez-Gonzalez et al., 2004; Zazpe et al., 2008; Razquin et al., 2009), each participant was given personalized dietary advice by the dietician during a 30-min session. Participants allocated to a low-fat diet were advised to reduce all types of fat and were given written recommendations according to American Heart Association guidelines (Krauss et al., 2000). The TMD participants received instructions directed to upscale the TMD 14-item score, including (1) the use of olive oil for cooking and dressing; (2) increased consumption of vegetables, nuts, and fish products; (3) consumption of white meat instead of red or processed meat; (4) preparation of home-made sauce by simmering tomato, garlic, onion and aromatic herbs with olive oil to dress vegetables, pasta, rice and other dishes; and (5) for alcohol drinkers, to follow a moderate pattern of red wine consumption.

No energy restrictions were suggested for the TMD groups. Participants in the TMD groups were given free VOO (15 liter for 3 months) or sachets of walnuts, hazelnuts and almonds (1350 g of walnuts (15 g per day), 675 g of hazelnuts (7.5 g per day) and 675 g of almonds (7.5 g per day), for 3 months). To improve compliance and account for family needs, participants in the corresponding TMD groups were given excess VOO or additional packs of nuts. One week after a participant's inclusion, 1-h group session (up to 20 participants) for each TMD group was held by the dietician. Each session consisted of an informative talk and written material with elaborated descriptions of typical Mediterranean foods, seasonal shopping lists, meal plans and recipes. All participants had free and continuous access to their dietician throughout the study (Fito et al., 2007; Zazpe et al., 2008; Corella et al., 2009).

The samples were obtained from overnight fasting peripheral blood. Plasma TAC was measured with a colorimetric test (Cayman Chemical Corporation, Ann Arbor, MI, USA) on plasma samples. It is based on the determination of antioxidant capacity (measured as the ability of inhibiting the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) by metmyoglobin) of both aqueous and lipid-soluble antioxidants by comparison with that of Trolox, a water-soluble tocopherol analogue (TEAC).

Statistical analysis

The Kolmogorov–Smirnov test was used to determine variable distribution. Descriptive analyses of variables between the three interventional groups were performed using parametric tests (Student's t-tests, analysis of variance followed by Bonferroni's post hoc tests). Assuming two-tailed a error of 0.05 and a minimum difference between group of 0.8mM Trolox, with two equally sized groups (n¼50) the statistically power would be 1.0 (100%). Means of TAC were also compared among the three randomized groups using general linear models adjusting for age and sex. Multiple linear regression models were used to analyze the effects of diet on plasma TAC levels and the effects of these levels on changes on adiposity indexes after 3 years of nutritional intervention.

Results

Characteristics of participants according to the nutritional group at baseline and after 3 years of intervention are presented in Table 1. Although the baseline body weight and waist circumference were significantly different in these subsamples between control and VOO groups (P¼0.015 and 0.005, respectively), when they were adjusted for height (body mass index (BMI) and waist to height), no statistically significant differences were found.

The measurement of plasma TAC after 36 months of intervention showed that both Mediterranean diet groups presented significantly higher levels of this antioxidant capacity parameter compared to control subjects (Po0.001). Moreover participants in the TMDþVOO tended to exhibit a slightly higher TAC than those in the TMDþ Nuts group (P¼0.066). When these values were adjusted for age and sex in a general linear model, the differences between control and Mediterranean diet groups remained statistically significant, and the TMDþVOO presented significantly higher levels of TAC when compared to TMDþNuts group (P¼0.048). To analyze the effects of the diet on TAC levels, we investigated the effectiveness of the nutritional intervention. For this purpose the

macronutrient distribution and the intake of some specific food items of the TMD (VOO and nuts) were analyzed and were compared with the control group (advised to follow a low-fat diet) (Table 2). We observed that the distribution of macronutrient intake was significantly different among the three groups (Table 2). The control group had the highest protein and carbohydrate intake, whereas TMD subjects had the highest intake of mono- and polyunsaturated fat but not of saturated

fat. Moreover, we confirmed that the highest intake of VOO was present in the TMDþVOO group (Po0.001), and that the Nuts group had also significantly higher intake of VOO than the control group (P¼0.008). Likewise, the highest intake of nuts was observed in the TMDþNuts group (Po0.001), having the TMDþVOO group significantly higher intake of nuts compared to the control group (P¼0.001).

Table 1 Characteristics of the population according to the nutritional intervention group

	Control (n = 59)	*Virgen olive oil (n = 65)*	*Nuts (n = 63)*
Baseline			
Age	69.00 ± 5.94	67.48 ± 5.82	68.40 ± 5.82
Sex (% females)	54	52	46
Weight (kg)	71.98 ± 11.59	78.46 ± 12.11[a]	74.10 ± 9.80
BMI (kg/cm^2)	28.55 ± 3.36	29.96 ± 2.96	28.95 ± 2.93
Waist circumference (cm)	93.79 ± 9.78	98.83 ± 10.14[b]	96.67 ± 9.30
Waist to height	0.59 ± 0.05	0.61 ± 0.05	0.60 ± 0.05
3 years			
3-year weight change (kg)	0.36 ± 3.49	0.10 ± 5.11	−0.02 ± 3.18
3-year waist change (cm)	0.11 ± 4.56	−0.63 ± 4.76	−0.23 ± 3.60
Plasma TAC (mM Trolox)[c]	2.05 ± 0.97[d]	3.49 ± 1.08[e]	3.03 ± 0.90
Plasma TAC (mM Trolox)[f]	2.01 ± 0.15[d]	3.51 ± 0.14[g]	3.02 ± 0.14

Abbreviations: BMI, body mass index; TAC, total antioxidant capacity.
The data are presented as mean ± standard deviation, except when indicated.
[a]The differences between control and virgin olive oil groups were statistically significant ($P = 0.015$).
[b]($P = 0.005$).
[c]Unadjusted means.
[d]The differences between control and virgin olive oil or nuts group were statistically significant ($P < 0.001$).
[e]The differences between virgin olive oil and nuts groups tended to be statistically significant ($P = 0.066$).
[f]Means adjusted for age and sex.
[g]The differences between virgin olive oil and nuts groups were statistically significant ($P = 0.048$).

Table 2 Distribution of macronutrients and Mediterranean diet specific nutrients after 3 years of nutritional intervention according to the nutritional group

	Control (n = 59)[a]	*TMD + VOO (n = 65)*[a]	*TMD + Tree nuts (n = 63)*[a]	*P-values for the between-group differences*		
				TMD + VOO vs control	*TMD + Nuts vs control*	*TMD + Nuts vs TMD + VOO*
Total energy intake (kcal/day)	2266.4 ± 657.1	2565.6 ± 562.2	2607.3 ± 648.1	**0.025**	**0.009**	1.000
Carbohydrates (% total energy intake)	43.69 ± 7.0	40.26 ± 5.8	37.82 ± 5.4	**0.006**	**<0.001**	0.075
Proteins (% total energy intake)	16.51 ± 2.8	15.30 ± 2.3	15.36 ± 2.2	**0.019**	**0.030**	1.000
Total fat (% total energy intake)	38.19 ± 5.9	41.84 ± 5.1	43.67 ± 5.1	**0.001**	**<0.001**	0.169
Saturated fat (% total energy intake)	9.20 ± 2.5	9.19 ± 1.7	9.64 ± 1.7	1.000	0.692	0.616
MUFA (% total energy intake)	19.60 ± 3.9	22.71 ± 3.2	23.23 ± 3.5	**<0.001**	**<0.001**	1.000
PUFA (% total energy intake)	5.77 ± 1.8	6.52 ± 1.6	7.48 ± 1.09	**0.023**	**<0.001**	**0.002**
VOO (10 g) (servings/day)	3.32 ± 2.3	6.39 ± 1.2	4.54 ± 2.8	**<0.001**	**0.008**	**<0.001**
Nuts (25 g) (servings/day)	0.15 ± 0.3	0.33 ± 0.3	0.53 ± 0.2	**0.001**	**<0.001**	**<0.001**

Abbreviations: MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; TMD, traditional Mediterranean diet; VOO, virgin olive oil.
[a]Mean ± standard deviation.
P-values in bold indicate that they are statistically significant.

Table 3 Multiple regression model to predict the plasma TAC according to age, sex and the nutritional intervention

		B (95% CI)[a]	*P-value*
Age	(×1 additional year)	0.016 (−0.838 to 3.158)	0.253
Sex	Males	0 (ref.)	
	Females	−0.160 (−0.490 to 0.171)	0.341
Nutritional intervention	Control	0 (ref.)	
	Virgin olive oil	1.497 (1.095–1.900)	**<0.001**
	Nuts	1.011 (0.605–1.416)	**<0.001**

Abbreviation: B, coefficient of the multiple linear regression model.
[a]Dependent variable: total plasma antioxidant capacity (mM Trolox).
P-values in bold indicate that they are statistically significant.

Table 4 Multiple regression model assessing the association between plasma TAC and body weight changes after 3 years of nutritional intervention with a Mediterranean diet rich in VOO ($n = 65$)

		B *(95% CI)*[a]	P-*value*
Age	(× 1 additional year)	0.119 (−0.097 to 0.335)	0.274
Sex	Males	0 (ref.)	
	Females	0.735 (−1.725 to 3.195)	0.551
BMI		−0.768 (−1.170 to −0.366)	**<0.001**
TAC (mM Trolox)		−1.306 (−2.439 to −0.173)	**0.025**

Abbreviations: B, coefficient of the multiple linear regression model; BMI, body mass index; TAC, total antioxidant capacity.
[a]Dependent variable: body weight changes after 3 years of nutritional intervention (body weight at year 3-baseline body weight).
P-values in bold indicate that they are statistically significant.

Taking into account these data, we performed a multiple regression model to predict the plasma TAC levels at year 3 according to the nutritional group and adjusting for age and sex (Table 3). Both TMDþVOO (B¼1.497) and TMDþNuts (B¼1.011) interventions predicted significantly higher levels of plasma TAC (Po0.001) independently of sex and age.

Accordingly to our objective, we investigated whether the plasma TAC levels modified body weight or adiposity (waist circumference) after 3 years of intervention. First of all, changes in body weight were analyzed. Therefore, a multiple regression model, adjusted for sex, age and baseline BMI, for each intervention group was fitted. In Table 4, we observed that, within the TMDþVOO group, the 3-year plasma TAC was significantly associated with a reduction in body weight change (B¼_1.306; P¼0.025). Moreover this model showed that the highest baseline BMI, the highest body weight change reduction (B¼_0.768; Po0.001). No statistically significant association was observed within the control or TMDþNuts group (data not shown). When waist circumference change at year 3 was analyzed, the same decreasing tendency within VOO group was observed, although the results were not statistically significant (data not shown).

To clarify the potential explanation for the significantly higher levels of plasma TAC found in the TMDþVOO group that seemed to be leading to a reduction in weight change, we analyzed the association between VOO intake and plasma TAC levels. A statistically significant partial correlation, adjusted for age and sex, was observed between the two parameters (r¼ þ0.302; Po0.001), showing that the higher the VOO intake, the higher plasma TAC levels.

Discussion

We have found a robust association between a nutritional intervention with a Mediterranean dietary pattern and the plasma TAC in subjects at high cardiovascular risk. This relationship was higher in subjects with higher intake of VOO. Interestingly, plasma TAC levels were associated with a reduction in weight changes after 3 years of intervention. To the best of our knowledge, this is the first study analyzing the effects of a Mediterranean diet on plasma TAC in the context of a randomized nutritional intervention. There are short-term interventional studies analyzing the effects of the Mediterranean diet on circulating oxidative stress biomarkers, but not measuring the TAC (Hagfors et al., 2003; Ambring et al., 2004; Zulet et al., 2008; Puchau et al., 2009). Moreover, this study goes further and analyzes the influence of this antioxidant capacity of the three nutritional interventions on adiposity indexes.

The Mediterranean diet has been related to lower rates of obesity (Schroder et al., 2004) and cardiovascular disease (Trichopoulou et al., 2003; Knoops et al., 2004; Martinez-Gonzalez and Sanchez-Villegas, 2004; Sanchez-Tainta et al., 2008; Sofi et al., 2008). Our dietary pattern is not restrictive on the quantity of fat as other hypocaloric dietary programs directed to decrease body weight. The main characteristic of the TMD is the change in the distribution of fat (high intake of mono- and polyunsaturated fat) being the main source of fat, olive oil and especially VOO. Thus, the mechanisms that link the Mediterranean diet with lowering the risk of obesity need to be further clarified. There are studies showing that obese subjects present high levels of oxidative stress biomarkers (Keaney et al., 2003), suggesting that potential therapies may act in two ways: decreasing body weight

that would be accompanied by lower oxidative stress or decreasing oxidative stress what may result in lower body weight. Thus, an antioxidant treatment could be a satisfactory therapy for obesity.

There are some cross-sectional studies analyzing the antioxidant capacity and the adherence to a Mediterranean diet and reporting an association between this dietary pattern and high levels of antioxidant capacity (Lapointe et al., 2005; Pitsavos et al., 2005; Dai et al., 2008). There are also short-term interventional studies analyzing the Mediterranean diet and its potential association with other oxidative stress biomarkers (Hagfors et al., 2003; Ambring et al., 2004), but there are not long-term interventional studies about this issue. Our study is a 3-year randomized trial that supports the idea that a greater adherence to Mediterranean diet is associated with higher plasma antioxidant capacity (Pitsavos et al., 2005; Dai et al., 2008). It shows that both Mediterranean diet interventions, with high intake of VOO as well as high intake of nuts, presented significantly higher levels of plasma TAC compared to a control group following a low-fat diet. Another important finding is the significant correlation found between the intake of VOO and plasma TAC in our population. Related to this result, some authors highlighted the idea that VOO is important in the antioxidant capacity of Mediterranean diet (Perez-Jimenez et al., 2005; Mataix et al., 2006), explained by the fact that VOO is rich in monounsaturated fatty acids and polyphenols that have high antioxidant activity. On the other hand, we observed that the TMDþNuts group presented higher plasma TAC compared to control group levels as reported in previous studies (Torabian et al., 2009). However, the supplementation with nuts is not equally effective to increase antioxidant capacity as compared to VOO. It seems that a high intake of VOO in a Mediterranean-style diet is essential to obtain higher dietary antioxidant contribution. Moreover, a potential connection between plasma antioxidant capacity and lower body weight is observed. In the TMDþVOO group, higher plasma TAC predicted higher reduction in body weight change. This result agrees with the potential link between oxidative stress and obesity and also with the hypothesis that decreasing oxidative stress could improve obese phenotypes. It has been suggested that inadequacy of antioxidant defenses observed in obese subjects probably begins with a low dietary intake of antioxidants and phytochemicals that possess antioxidant capacity. In fact, several studies showed that obese individuals have a lower intake of phytochemical-rich foods (fruits, vegetables, whole grains, legumes, wine, olive oil, seeds and nuts) compared with nonobese persons. In addition, phytochemical intake is inversely correlated with waist circumference, BMI and plasma lipid peroxidation in several populations (Wallstrom et al., 2001; Reitman et al., 2002; Vincent et al., 2007).Therefore, the intake of a Mediterranean dietary pattern rich in VOO appeared to be a good strategy to increase antioxidant capacity of the organism and thus allowing to decrease oxidative stress and adiposity.

This study has some limitations because the TAC was measured rather than a specific antioxidant biomarker that could be more precise. Despite this, TAC was measured because it considers the single antioxidant activity as well as the synergistic interactions of the redox molecules present in complex matrixes, giving an integrated insight into the assessment of the nonenzymatic antioxidant network (Serafini et al., 2006). On the other hand, we did not measure baseline TAC but we could assume that there were little differences between groups due to the randomization. This study used 187 subjects at high cardiovascular risk who were a random subsample of the 1055 subjects enrolled in the PREDIMED trial in our center (AP-UNAV). Although further studies with larger sample size are necessary to corroborate these findings, we have enough power to detect statistically significant differences between groups. Participants who died during this follow-up period (n¼40) or failed to comply with the 3-year measurement protocol in our center (n¼99) were not eligible for this substudy, and, thus, the randomization advantage was not completely preserved in the subset of participants here included. However, the multivariable adjustment of estimates that we have used is very likely to correct the small potential between-group imbalances. On the other hand, this study has several strengths. First of all, its design allows us to find results in real-life conditions such as with home-prepared foods (Fito et al., 2007). Moreover, the period of

intervention is longer than any previous study carried out with the Mediterranean diet. Thus, the results obtained may be more accurate and valid.

In conclusion, Mediterranean diet, especially rich in VOO, is associated with higher levels of plasma antioxidant capacity. Interestingly, the antioxidant capacity is related to a reduction in body weight in a high cardiovascular risk population after 3 years of intervention with a Mediterranean-style diet rich in VOO.

Conflict of interest

The authors declare no conflict of interest.

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Benavente-Garcia O, Castillo J, Lorente J, Ortuno A, Del Rio JA
Antioxidant activity of phenolics extracted from Olea europaea L. leaves

The purpose of this study was to identify the main phenolic compounds present in an olive leaf extract (OL) in order to delineate the differential antioxidant activities of these compounds through the extent of their abilities to scavenge the $ABTS^{\bullet+}$ radical cation and to clarify the structural elements conferring antioxidant capacity in aqueous systems. The results show that the relative abilities of the flavonoids from olive leaf to scavenge the $ABTS^{\bullet+}$ radical cation are influenced by the presence of functional groups in their structure, mainly the B-ring catechol, the 3-hydroxyl group and the 2,3-double bond conjugated with the 4-oxo function. For the other phenolic compounds present in OL, their relative abilities to scavenge the $ABTS^{\bullet+}$ radical cation are mainly influenced by the number and position of free hydroxyl groups in their structure. Also, both groups of compounds show synergic behaviour when mixed, as occurs in the OL.

Reference [6] Trends in Food Science & Technology 2000 **11**(9-10), 357-363

Saija A, Uccella N
Olive biophenols: functional effects on human wellbeing

With increasing interest in novel descriptors of hedonic-sensory (HS) and functional (F) quality, scientific documentation of the dietary habits associated with the Mediterranean Aliment Culture (MAC) lifestyle, shows low risk for many chronic diseases. This has been interpreted as the F effect of widespread plant antioxidant intake. The antioxidant and antimicrobial activity of some of the most typical biophenols (BPs) contained in table olives (TOs) and olive oil, such as extra virgin olive oil (EVOO), was revealed through biomimetic experiments on the scavenging effects of chain-propagating lipid peroxyl radicals within membranes, and for human skin protection. Dietary intake of TO and EVOO BPs might lower the risk of degenerative diseases and microbial infections for consumers, *Homo consumans* (*Hc*). MAC foodstuffs, also referred to as life-stage foods, could emerge as F products, engineered to tackle the specific dietary requirements of the aged population.

Reference [7] Lancet 2005 366(9491), 1059-62

The metabolic syndrome—a new worldwide definition

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The metabolic syndrome (visceral obesity, dyslipidaemia, hyperglycaemia, and hypertension), has become one of the major public-health challenges worldwide.[1] There has been growing interest in this constellation of closely related cardiovascular risk factors. Although the association of several of these risk factors has been known for more than 80 years,[2] the clustering received scant attention until 1988 when Reaven described syndrome X: insulin resistance, hyperglycaemia, hypertension, low HDL-cholesterol, and raised VLDL-triglycerides.[3] Surprisingly, he omitted obesity, now seen by many as an essential component, especially visceral obesity.[1] Various names were subsequently proposed, the most popular being metabolic syndrome.[1]

The cause of the syndrome remains obscure. Reaven proposed that insulin resistance played a causative role,[3] but this remains uncertain. Lemieux et al suggested visceral obesity and the hypertriglyceridaemic waist phenotype as a central component,[4] but this too has been contested. Several different factors are probably involved, many related to changes in lifestyle.[1] The ultimate importance of metabolic syndrome is that it helps identify individuals at high risk of both type 2 diabetes and cardiovascular disease (CVD). Several expert groups have therefore attempted to produce diagnostic criteria. The first attempt was by a WHO diabetes group in 1999, which proposed a definition that could be modified as more information became available.[5] The criteria had insulin resistance or its surrogates, impaired glucose tolerance or diabetes, as essential components, together with at least two of: raised blood pressure, hypertriglyceridaemia and/or low HDL-cholesterol, obesity (as measured by waist/hip ratio or body-mass index), and microalbuminuria. The European Group for the Study of Insulin Resistance[6] then produced a modification of the WHO criteria excluding people with diabetes and requiring hyperinsulinaemia to be present. Waist circumference was the measure of obesity, with different cutoffs for the other variables.

A fresh approach came from the US National Cholesterol Education Program: Adult Treatment Panel III in 2001, with a focus on cardiovascular disease risk.[7] The specific remit was to facilitate clinical diagnosis of highrisk individuals. It was less glucocentric than the definition from WHO and the European Group for the Study of Insulin Resistance, requiring the presence of any three of five components: central obesity, raised blood pressure, raised triglycerides, low HDL-cholesterol, and fasting hyperglycaemia.

The different definitions inevitably led to substantial confusion and absence of comparability between studies. One difficulty has been that the conceptual framework used to underpin the metabolic syndrome (and hence drive definitions) has not been agreed on. Opinions have varied as to whether the metabolic syndrome should be defined to mainly indicate insulin resistance, the metabolic consequences of obesity, risk for CVD, or simply a collection of statistically related factors. Prevalence figures for the syndrome have been similar in any given population regardless of which definition is used, but different individuals are identified.[8] What matters, of course, is which produces the best prediction of subsequent diabetes and CVD. Thus Adult Treatment Panel III was superior to WHO in the San Antonio Study, but WHO gave better prediction of CVD in Finnish men.[9,10] Another problem with the WHO and the Adult Treatment Panel definitions has been their applicability to different ethnic groups, especially as relates to obesity cutoffs.[11] For example, the risk of type 2 diabetes is apparent at much lower levels of adiposity in Asian populations than in European populations.[12] With current metabolic syndrome definitions, particularly Adult Treatment Panel III, suspiciously low prevalence figures in Asian populations resulted,[12] suggesting the need for ethnic-specific cutoffs, at least for obesity.

The International Diabetes Federation (IDF) felt there was a strong need for one practical definition that would be useful in any country for the identification of people at high risk of CVD, but also diabetes. This definition would also allow comparative long-term studies, which could then be used, if necessary, to refine the definition on the basis of solid endpoints. As a result, an IDF consensus group met in 2004, with representatives from the organisations that had generated the previous definitions and members from all IDF regions. Their recommendations are now available[13] There was consensus that the components identified by Adult Treatment Panel III were a sensible starting point. It was also agreed that diabetes and insulin resistance had been overemphasised as core measurements in the earlier definitions. Measurement of insulin resistance was deemed impractical, although it is clear that several metabolic syndrome components, especially waist circumference and triglycerides, are highly correlated with insulin sensitivity.[4]

Panel: **International Diabetes Federation: metabolic syndrome definition**

Central obesity
Waist circumference*—ethnicity specific (see table 1)
Plus any two:
Raised triglycerides
>150 mg/dL (1·7 mmol/L)
Specific treatment for this lipid abnormality
Reduced HDL-cholesterol
<40 mg/dL (1·03 mmol/L) in men
<50 mg/dL (1·29 mmol/L) in women
Specific treatment for this lipid abnormality
Raised blood pressure
Systolic ⩾130 mm Hg
Diastolic ⩾85 mm Hg
Treatment of previously diagnosed hypertension
Raised fasting plasma glucose†
Fasting plasma glucose ⩾100 mg/dL (5·6 mmol/L)
Previously diagnosed type 2 diabetes
If above 5·6 mmol/L or 100 mg/dL, oral glucose tolerance test is strongly recommended, but is not necessary to define presence of syndrome

*If body-mass index is over 30 kg/m², central obesity can be assumed and waist circumference does not need to be measured. †In clinical practice, impaired glucose tolerance is also acceptable, but all reports of prevalence of metabolic syndrome should use only fasting plasma glucose and presence of previously diagnosed diabetes to define hyperglycaemia. Prevalences also incorporating 2-h glucose results can be added as supplementary findings.

Ethnic group	Waist circumference (as measure of central obesity)
Europids*	
Men	⩾94 cm
Women	⩾80 cm
South Asians	
Men	⩾90 cm
Women	⩾80 cm
Chinese	
Men	⩾90 cm
Women	⩾80 cm
Japanese	
Men	⩾85 cm
Women	⩾90 cm
Ethnic south and central Americans	Use south Asian recommendations until more specific data are available
Sub-Saharan Africans	Use European data until more specific data are available
Eastern Mediterranean and middle east (Arab) populations	Use European data until more specific data are available

Data are pragmatic cutoffs and better data are required to link them to risk. Ethnicity should be basis for classification, not country of residence. *In USA, Adult Treatment Panel III values[7] (102 cm male, 88 cm female) are likely to continue to be used for clinical purposes. In future epidemiological studies of populations of Europid origin (white people of European origin, regardless of where they live in the world), prevalence should be given, with both European and North American cutoffs to allow better comparisons.

Table: **Ethnic-specific values for waist circumference**

Central obesity, as assessed by waist circumference, was agreed as essential (panel), because of the strength of the evidence linking waist circumference with cardiovascular disease and the other metabolic syndrome components, and the likelihood that central obesity is an early step in the aetiological cascade leading to full metabolic syndrome. The waist circumference cutoff selected was the same as that used by European Group for the Study of Insulin Resistance, and lower than the main Adult Treatment Panel III recommendations, because most available data suggest an increase in other cardiovascular disease risk factors in Europids (white people of European origin, regardless of where they live in the world) when the waist circumference rises above 94 cm in men and 80 cm in women.[1] Ethnic-specific waist circumference cutoffs have been incorporated into the definition (table), and have been based on available data linking waist circumference to other components of the metabolic syndrome in different populations.[12,14,15] The levels of the other variables were as described by Adult Treatment Panel III, except that the most recent diagnostic level from the American Diabetes Association for impaired fasting glucose (5·6 mmol/L [100 mg/dL]) was used.[16] Although this new definition will still miss substantial numbers of people with impaired glucose tolerance (because an oral glucose-tolerance test is not required), it retains the simplicity of the instrument.

The consensus group also recommended additional criteria that should be part of further research into metabolic syndrome, including: tomographic assessment of visceral adiposity and liver fat, biomarkers of adipose tissue (adiponectin, leptin), apolipoprotein B, LDL particle size, formal measurement of insulin resistance and an oral glucose-tolerance test, endothelial dysfunction, urinary albumin, inflammatory markers (C-reactive protein, tumour necrosis factor _, interleukin 6), and thrombotic markers (plasminogen activator inhibitor type 1, fibrinogen). These factors should be combined with assessment of CVD outcome and development of diabetes so better predictors can be developed. Researchers and clinicians should use the new criteria for the identification of high-risk individuals and for research studies. Preventive measures are obviously needed in the people identified. Mounting evidence suggests that lifestyle modification with weight loss and increased physical activity will be beneficial, although specific studies in metabolic syndrome are needed. There are suggestions from the Finnish Diabetes Prevention Study that individuals with metabolic syndrome show less development of diabetes with lifestyle advice.[17] In many people, however, pharmacological intervention will be needed. There is no specific treatment for the metabolic syndrome so individual abnormalities will have to be attended to. Again, long-term studies will help establish whether existing or newer agents, such as agonists for the peroxisome-proliferator-activated _/_receptors or cannabinoid-1 receptor blockers,[18] could be of specific benefit.

Recently, the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) have published a provocative discussion paper on the syndrome.[19] They raise several interesting questions, based on a critique of the earlier WHO and Adult Treatment Panel III criteria: 1) is it indeed a syndrome, particularly as the precise cause is unknown, 2) does it serve a useful purpose, and 3) is it labelling (and medicalising) people unnecessarily? Additionally, it has been suggested in an editorial that recognition of the metabolic syndrome has been largely driven by industry to create new markets.[20] A major part of the ADA/EASD19 stance is based on pure semantics, but the IDF (and the cardiovascular community) feel strongly that this clustering of closely related risk factors for CVD and type 2 diabetes is indeed a very good basis for calling this a syndrome. Many examples exist of conditions being given a name even when the precise underlying cause or causes, are unknown (eg, type 2 diabetes). The IDF feels that it serves a useful purpose to focus on people, in both the community and clinical settings, who are at high risk of developing CVD and type 2 diabetes, particularly using the new IDF criteria proposed above. Indeed, the ADA has just reinvented and redefined the condition of "prediabetes" for people who only have a 50% chance of developing diabetes.[20] We also emphasise most strongly in our longer article[13] that treatment must be focused on lifestyle change—and on the individual components if the former fails. This is a far cry from a condition claimed to be invented by industry.[21] Themetabolic syndrome concept has been around for over 80 years.[1] The burgeoning epidemic of type 2 diabetes and CVD worldwide, particularly in the developing world seem adequate reasons for identifying and treating people with the syndrome. We would stress that the new IDF criteria are not the final word, but hopefully will help identify people at increased risk, and through further research will lead to more accurate predictive indices.

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Guize L, Pannier B, Thomas F, Bean K, Jégo B, Benetos A
Recent advances in metabolic syndrome and cardiovascular disease

Metabolic syndrome is defined as an association of central obesity and several other cardiometabolic risk factors. Dysfunctional visceral adipose tissue and inflammatory status appear to be involved in its genesis. New definitions have decreased the threshold for glycaemia and one has lowered the threshold for waist circumference, leading to an increase in the prevalence of metabolic syndrome. However, the impact on mortality with these new definitions is lower than with the National Cholesterol Education Program–Adult Treatment Panel III 2001 definition. An increase in waist circumference, along with increased glycaemia, triglycerides and/or blood pressure is more highly associated with an increased risk of mortality than are other associations, while a decrease in high density lipoprotein cholesterol increases risk of coronary heart disease. The risk of sudden death and stroke is particularly notable with metabolic syndrome. Metabolic syndrome is associated with an increase in heart rate, pulse pressure, arterial stiffness and left ventricular hypertrophy, impairment of diastolic function, enlargement of the left atrium and atrial fibrillation. In the 2007 European recommendations for the management of high blood pressure, metabolic syndrome is now taken into consideration for both risk stratification and in selecting the optimal therapeutic strategy for arterial hypertension.

Reference [9] International journal of obesity 1997 **21**(9), 715-737

Glenny A-M, O'Meara S, Melville A, Sheldon TA, Wilson C
Review: The treatment and prevention of obesity: a systematic review of the literature

OBJECTIVE: To determine the effectiveness of interventions designed to prevent and treat obesity, and maintain weight loss.
DESIGN: A systematic review of randomised controlled trials.
SUBJECTS: Overweight and obese adults and children.
MEASUREMENTS: Post-intervention changes in weight, fat content and fat distribution, measured relative to baseline.
RESULTS: For obese children, family therapy and lifestyle modification appear to be effective in prevention and treatment, respectively. The effectiveness of interventions to prevent and treat obesity in adults remains unclear, although behavioural therapy and multicomponent strategies may be useful. Continued therapist contact appears to be useful for maintaining weight loss. Pharmacological interventions appear to be effective for up to 9 months, after which regain occurs. Surgery appears to be effective for the morbidly obese and gastric bypass is more effective than gastroplasty. In general, the methodological quality of studies was poor.
CONCLUSION: Due to problems with methodological quality, it is recommended that research findings indicative of promising interventions are replicated.

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Screening for antidiabetic activity and phytochemical constituents of common bean (Phaseolus vulgaris L.) seeds

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Seeds of Phaseolus vulgaris were given individually at different doses to different batches of rats (both normal and hyperglicemic rats) after an overnight fast. Seeds contain the bioactive components – alkaloids, flavonoids, fiber, proteins, tannins, terpenoids, saponins, quercetin, anthocyanin and catechin. The blood glucose levels were measured at 0, 1, 2, 3, 4, 5 and 6 h after the treatment. Most active doses were further studied to dose-dependent (300, 200 and 100 g/kg bw) antihyperglycemic effects alone and in combination with glibenclamide (0.20, 0.10 and 0.05 g/kg bw). Seeds of P. vulgaris at a dosage of 300 g/kg bw is showing maximal blood glucose lowering effect in diabetic rats after third hour. The antihyperglycemic activity of P. vulgaris seeds was compared with the treatment of glibenclamide, an oral hypoglycemic agent. The combination of seeds of most dose (300 mg/kg bw) and higher dose of glibenclamide (0.20 g/kg bw) showed safer and potent hypoglycemic as well as antihyperglycemic activities without creating severe hypoglycemia in normal rats.

Key words: Phaseolus vulgaris, seeds, diabetes mellitus, antihyperglycemic activity, glucose-infused diabetes, glibenclamide.

INTRODUCTION

The origin and domestication of common bean (Phaseolus vulgaris) has been established in America (Papa et al., 2005). Dry common bean is a legume widely consumed throughout the world and it is recognized as the major source of dietary protein in many Latin-American and African countries (Guzman- Maldonado and Paredes-Lopez, 1998). Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia. These metabolic disorders include alterations in the carbohydrate, fat and protein metabolisms associated with absolute or relative deficiencies in insulin secretion and/or insulin action. The characteristic symptoms of diabetes are polyuria, polydipsia, polyphagia, pruritus and unexpected weight loss, etc. There is an increasing demand by patients to use the natural products with antidiabetic activity, due to the side effects associated with the use of insulin and oral hypoglycemic agents (Botero and Wolfsdorf, 2005). In 2010, according to World Health Organisation (WHO), 221 million people in the world were diabetic. In Africa, diabetes has a rapidly and was currently regarded as a public health problem (N'guessan, 2008; Konkon et al., 2010). World Health Organization estimated that there are 14 million people with diabetes in Africa in 2000, which is projected to rise to 30 million by the year 2025. Globally, the number of people that has been diagnosed with diabetes has exploded in the past two decades. With a long course and serious complications often resulting in high death rate, the treatment of diabetes spent vast amounts of resources including medicines, diets, physical training, and so on in all countries. Therefore, it is very important to search new therapeutic strategies which might be cheaper, safe, and convenient for treatment of diabetes.

In Africa, use of plant medicines is very common practice from ancient time, and it is considered as much safer

and less expensive therapeutic strategies for treatment of various diseases (Ambady and Chamukuttan, 2008). The use of plant medicines for treatment of diabetes has been reported long ago. There is plethora of literature that is available for antidiabetic plants (Modak et al., 2007; Carai et al., 2009; Mishra et al., 2010; Konkon et al., 2010. Pravin (2006) reported that about 70% of the human population is dependent (wholly or partially) on plant-based medicines and the World Health Organisation estimates that 80% of the world population presently uses herbal medicine for some aspect of primary health care (WHO, 2008). The potential of medicinal plants research results in health care is no longer in doubt, having gained recognition in several nations of the world.

The available literature shows that there are more than 400 plant species showing hypoglycemic activity (Ivorra et al., 1989; Konkon et al., 2010). Though some of the plants are reputed in the indigenous systems of medicine for their activities, it remains to be scientifically established. To date, however, only a few of these medicinal plants have received scientific scrutiny, despite the fact that the World Health Organization has recommended that medical and scientific examinations of such plants should be undertaken (WHO, 1980). P. vulgaris have a notable place in the folklore throughout the world and in the traditions of many cultures such as antidiabetic activity (Tormo et al., 2004; Carai et al., 2009; Mishra et al., 2010).

Preclinical investigations have unanimously reported how the acute, repeated administration of extracts of P. vulgaris, as well as some of their isolated ingredient reduced food intake, body weight and lipid accumulation in lean and obese laboratory animals have been carried out on this plant. Although, a surfeit of literature is available for the antidiabetic potential of P. vulgaris, the selected seeds (which are well-known as a good dietary source) are an interesting pharmacological activity according to several authors (Hangen and Bennink, 2002; Helmstädter, 2010). Currently, the data on antidiabetic properties and bioactive constituent of P. vulgaris seeds are limited in literature. Hence, the present study was undertaken to determine the chemical constituent and investigate the antihyperglicmic effects of P. vulgaris seeds.

MATERIALS AND METHODS

Plant material

Vegetable material is constituted of common bean (P. vulgaris) seeds, which were purchased from Abidjan (Côte d'Ivoire) markets.

Phytochemical screening

Screening of phytochemical constituents of the plant was done using standard procedures described by several authors (Harbone, 1978; Sofowora, 1984; Trease and Evans, 1996; Adetuyi and Popoola, 2001; Eogona et al., 2005; N'Guessan, 2008; Konkon et al., 2010; Yadav et al., 2010). The extracts obtained with the powder coming to freeze-dried seeds were used to identify and characterize some chemical groups.

Animals and experimental schedule

Male Wistar rats of 4 - 5 weeks old were housed in polypropylene cages at 22 ± 2°C ambient temperature and 55 ± 5% humidity in 12/12 light and dark cycle. Rats were fed until a weight of approximately 180 g.

animals and put out in individual cage for the study. A total of 42 male rats were used for the study. This study was completed in two phases. Phase 1: seeds of bean were selected and administered in 12-h-fasted normal animals at 300 g/kg bw. To study antihyperglycemic activity, 200 g/kg bw dose of glucose was administered to 12-h-fasted animals (for induction of hyperglycemia) at the same time of glucose ingestion. Phase 2: The

antihyperglycemic effect of bean seeds was studied for dose-dependent effects and its drug interactions with standard antidiabetic medicine, that is, glibenclamide. Three different doses of common bean seeds (300, 200 and 100 g/kg bw) and glibenclamide (0.20, 0.10, and 0.05 g/kg bw) were tested for antihyperglycemic potential.

Estimation of blood glucose

After administration of seeds and/or standard drug, the blood samples were collected from tail tip, and glucose was monitored using glucometer strips (Roche Diagnostics, Indiana, USA) at 1 h interval for 6 h.

Statistical analysis

Data were analyzed using Statistical software (release 7.5). Differences in mean values were tested by analysis of variance, and significance levels were obtained with Duncan's test. A significance level of $p < 0.05$ was used. Data are the means of three replicates.

RESULTS AND DISCUSSION

Basic phytochemical screening comprising chemical tests to detect the presence of alkaloids, anthraquinones, glycosides, polyphenols, saponins, steroids, trepenoid and tannins (Table 1). All compounds analyzed in the seeds were significantly differents. Hyperglycemia is a chronic state of diabetic condition; in fact, chronic hyperglycemia is the defining characteristic of the disease. The pathophysiology of hyperglycemia in diabetic state is very complicated and affects by many daily activities such as food intake, exercise, etc. Long-term hyperglycemia causes several microvascular and macrovascular complications of diabetes (Brindisi et al., 2006; Calcutt et al., 2009). Therefore, the control of hyperglycemia needs special attention in diabetic conditions. Although, there are various oral hypoglycemic regimens are available in market, but conventional therapies for diabetes have many shortcomings like side effects and high rate of secondary failure.

Table 1. Phytochemical analysis of *Phaseolus vulgaris* seeds

Active principle	Extract
Alkaloids	+
Anthraquinone	++
Catechic tannins	+++
Flavonoids	++++
Gallic tannins	+
Glycosides	++++
Polyphenols	+++
Saponins	+++
Steroids	+
Terpenoids	+

(+) Present; (-) Absent.

On the other hand, plant extracts are expected to have similar efficacy without side effects as that of conventional drugs. Hence, in present study, we evaluated the antihyperglycemic potential of P. vulgaris seeds. After a thorough reviewing the literature on antidiabetic effects of P. vulgaris, it has been found that various studies reported the effect of different part of this plant in different diabetic models (Carai et al., 2009 ; Tormo et al., 2004; Mishra et

al., 2010; Yadav et al., 2010).

According some authors, P. vulgaris is gaining increasing attention as a functional or nutraceutical food, due to its rich variety of phytochemicals which have a potential benefits on health (N'guessan, 2008; Mishra et al., 2010). Important biological activities have been described from common beans like enhancement of the bifidogenic effect (Queiroz-Monici et al., 2005); anti- oxidant (Heimler et al., 2005); anticarcinogenic (Hangen and Bennink, 2002) effects. Interestingly, we found that all doses of P. vulgaris seeds were active for antihyperglycemic potential. The reason for this active antihyperglycemic activity of P. vulgaris seeds might be due to their availability phytoconstituents, that is, alkaloids, flavonoids, tannins, terpenoids and saponins (Henningson et al., 2001; Sharma et al., 2003). It has also been reported earlier that terpenoids (Murakami et al., 2001), saponins (Kambouche et al., 2009), are precise bioactive components responsible for the antidiabetic activity of P. vulgaris seeds. In addition, we also studied the combination of most bioactive dose of P. vulgaris seeds with standard drug; glibenclamide was also investigated to find out how much dose of its can be reduced by combining most active dose (Table 2). This part of study shows very interesting results that the combination of most active dose of P. vulgaris seeds significantly reduced the dose of glibenclamide (from 0.20 to 0.05 g/kg bw) in hyperglycemic animals, and it was safer in normal rats also, while combination with higher dose (0.20 g/kg bw) was chronic to produce hypoglycemia in normal rats. The antihyperglycemic effects of P. vulgaris seeds might be either stimulating pancreatic B cells to secrete more insulin (insulin secretor) or increased insulin sensitivity in peripheral tissues, that is., adipose tissue, muscle, and live to clear blood glucose at faster rate. The exact mechanism of action of P. vulgaris seeds is not well-known, but some authors give tracks of responses. Literature suggests the involvement of two possible mechanisms of action in the reducing effect of P. vulgaris extracts on glycemia. Both these mechanisms focus on the role of phytohemoagglutinin and α-amylase inhibitors. Pancreatic α- amylase is an enzyme that catalyzes hydrolysis of α- (1,4)-glycosidic bonds of starch polymers (Santimone et al., 2004). Thus, inhibition of α-amylase results in the suppression of starch metabolism and, in turn, a decrease in glycemia (Ishimoto et al., 1995). It has also been reported that -amylase inhibitors delay gastric emptying, producing feelings of satiety (Jain et al., 1991), thus resulting in reduced food intake (Tormo et al., 2006).

Phytohemoagglutinin is known to bind to the stomach epithelial cells and to the brush border membrane of small intestine, cecum, and colon (Herzig et al., 1997). This binding results in the stimulation of the release of cholecystokinin and glucagonlike peptides (King et al., 1986; Radberg et al., 2001), two hormones playing a relevant role in digestive processes. In close agreement with the latter hypothesis, recent data indicate that treatment with the cholecystokinin receptor type A phytohemoagglutinin is the stimulation of pancreatic secretion of α-amylase in rats (Baintner et al., 2003); this should result in an accelerated metabolism of ingested starch and, in turn increase in glycemia. Nevertheless, based on these results, it may be speculated that most active P. vulgaris seeds had similar activity as glibenclamide as an insulin secretor in hyperglycaemic rats. Together, these data suggest that extracts of P. vulgaris seeds may constitute potentially interesting, novel remedies for the treatment of metabolic syndrome such as diabetes. Physiological impact of P. vulgaris seeds on post-ingestif glucose could be integrated in strategies of treatment and the prevention. In the preceding strategies, the consumption of glucides is often limited, but deliberated selection of foodstuffs with low glycemic index is a more modern approach with the problem.

Conclusion

From this study, it may be concluded that the P. vulgaris seeds have antihyperglycemic potential and may use as complementary medicine to treat the diabetic population by significantly reducing dose of standard drugs. They may suggest that the combination of most active dose of P. vulgaris seeds with glibenclamide may play an important role to reduce the blood glucose levels in chronic diabetic conditions. Moreover, further study is

required, pharmacological and biochemical investigations are underway to elucidate the mechanism of the antidiabetic effect of P. vulgaris seeds. In the same way, it could be interesting to carry out isolation, purification and characterization of bioactive active components from seeds, which might pave a good independent and/or complementary regiment for the treatment of diabetes mellitus, seem to be necessary.

Table 2. Effect of *Phaseolus vulgaris* seeds and glibenclamide on fasting blood glucose levels (mg/dl) of normal and diabetic rats.

Groups	Blood glucose at different hours after the treatment						
	0 h	1 h	2 h	3 h	4 h	5 h	6 h
Normal rats							
Seeds							
300 g/kg bw	80 [a]	110 [c]	130 [f]	100 [b]	85 [a]	74 [n]	64 [g]
200 g/kg bw	81 [a]	100 [b]	125 [df]	87 [a]	82 [a]	71 [n]	65 [g]
100 g/kg bw	84 [a]	115 [c]	120 [d]	89 [a]	81 [a]	78 [j]	68 [g]
Glibenclamide							
0.20 g /kg bw	87 [ab]	108 [c]	124 [de]	85 [a]	81 [a]	74 [n]	63 [g]
0.10 g/kg bw	85 [a]	105 [bc]	127 [f]	85 [a]	80 [a]	75 [n]	66 [g]
0.05 g/kg bw	86 [a]	112 [c]	125 [df]	89 [a]	85 [a]	80 [n]	69 [g]
Seeds + glibenclamide							
300 + 0.20 g/kg bw	85 [a]	70 [e]	60 [g]	48 [k]	35 [m]	25 [b]	08 [c]
300 + 0.10 g/kg bw	80 [a]	77 [ae]	68 [h]	40 [l]	33 [lm]	22 [b]	12 [c]
300 + 0.05 g/kg bw	82 [a]	79 [a]	65 [h]	4l [f]	37 [n]	40 [f]	45 [f]
Hyperglycemic rats							
Seeds							
300 g/kg bw	94 [b]	120 [d]	138 [e]	130 [il]	128 [l]	123 [d]	121 [d]
200 g/kg bw	90 [b]	117 [d]	135 [ei]	133 [ei]	131 [l]	127 [l]	123 [d]
100 g/kg bw	89 [b]	118 [d]	133 [ei]	130 [ei]	127 [gl]	125 [dl]	120 [d]
Glibenclamide							
0.20 g /kg bw	90 [b]	130 [f]	145 [j]	140 [j]	135 [f]	130 [f]	125 [f]
0.10 g/kg bw	93 [b]	132 [f]	137 [ij]	144 [i]	139 [f]	133 [f]	130 [f]
0.05 g/kg bw	92 [b]	127 [fg]	136 [j]	146 [j]	143 [j]	139 [fj]	135 [f]
Seeds + glibenclamide							
300 + 0.20 g/kg bw	93 [b]	125 [d]	112 [c]	115 [c]	123 [d]	120 [d]	122 [d]
300 + 0.10 g/kg bw	94 [b]	120 [d]	118 [c]	110 [c]	124 d	126 [l]	121 [d]
300 + 0.05 g/kg bw	90 [b]	124 [d]	111 [c]	113 [c]	121 [d]	123 [d]	120 [d]

Values are means six animals in each group. Values with different superscripts (lowercase letters) in a row are significantly different at the level of $p<0.05$.

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The nutraceutical role of the Phaseolus vulgaris a-amylase inhibitor

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The present review assesses the potential of the Phaseolus vulgaris a-amylase inhibitor isoform 1 (a-AI1) starch blockers as a widely used remedy against obesity and diabetes. Consumption of the a-amylase inhibitor causes marginal intraluminal a-amylase activity facilitated by the inhibitor's appropriate structural, physico-chemical and functional properties. As a result there is decreased postprandial plasma hyperglycaemia and insulin levels, increased resistance of starch to digestion and increased activity of colorectal bacteria. The efficacy and safety of the amylase inhibitor extracts, however, depend on the processing and extraction techniques used. The extracts are potential ingredients in foods for increased carbo- hydrate tolerance in diabetics, decreased energy intake for reducing obesity and for increased resistant starch. Research developments in the dis- tribution and biosynthesis of the a-amylase inhibitor, relevant physico-chemical properties, the molecular starch-blocking mechanism, anti-obesity and anti-diabetes effects, safety of extracts and the need for research into their potential anti-colorectal cancer effect are discussed.

a-Amylase inhibitor: Common beans (Phaseolus vulgaris): Diabetes: Hyperglycaemia: Obesity: Toxicity

Common beans (Phaseolus vulgaris L.) are among the world's grain legumes most used for direct human consump- tion(1). The common bean a-amylase inhibitor isoform 1 (a-AI1), one of their non-nutritive bioactive factors(2), dis- covered in 1945 by Bowman(3), has been extracted and used in several commercial anti-obesity and anti-diabetes products referred to as starch blockers. A starch blocker is a substance that interferes with the breakdown of complex carbohydrate leading to a reduced digestibility or prolonged digestion such that energy derived from the carbohydrate is reduced or the rate of body absorption of the energy in form of glucose is reduced(4).

In the 1980s, use of the starch blockers from common beans to control obesity and diabetes was a research issue, but it has presently re-emerged with efforts being taken for its consider- ation as 'generally regarded as safe'(5). Detailed investigations revealed that many of the commercially available amylase inhibitor extracts (starch blockers) failed to influence starch digestion due to low a-amylase inhibition activity in humans(6,7). Recent developments, however, with improved extraction methods such as supercritical carbon dioxide extrac- tion, fractionation and heat treatment(8) have led to demon- strable efficacy of the starch blockers in humans. Despite some contrary reports, the starch blockers from common beans have been demonstrated to at least cause subtle weight loss, which has been shown to have advantages relative to dras- tic weight loss(9). On the other hand, extensive research has shown that obesity is on the increase worldwide and predis- poses individuals directly or indirectly to diabetes mellitus and various forms of cancer(10-13).

The common bean a-amylase inhibitor extracts are legally more acceptable based on the de minis concept(14) than new synthetic pharmaceutical products and recently some patents have been documented on their effective extraction(8). Safety and efficacy of such dietary supplements, however, are of critical importance since regulatory authorities such as the United States Food and Drug Administration consider them as conventional foods and manufacturers do not need to register and get product approval(15). Although there have been advancements in

the several aspects of the a-amylase inhibitor from common beans, few attempts have been made to sum-marise and integrate them from a nutritional point of view. In response, the present paper assesses the potential of the P. vulgaris a-amylase inhibitor as an extensive remedy against obesity and diabetes based on research developments in its distribution, relevant physico chemical properties, starch-blocking mechanism, evidence of beneficial effects and its safety.

Distribution and biosynthesis of the Phaseolus vulgaris a-amylase inhibitor

Natural a-amylase inhibitors have been extracted from various sources. The P. vulgaris a-amylase inhibitor, however, has relatively wide potential as an extensive anti-obesity and anti-diabetes remedy because common beans are grown widely in the world(16); the pure form has not been associated with deleterious effects such as asthma and dermatitis which have been associated with some cereal amylase inhibitors(17-19), and it has unifunctionality relative to other potential inhibitors which are bifunctional(20).

Although common beans have three isoforms of a-amylase inhibitor (isoform 1 (a-AI1); isoform 2 (a-AI2); a-amylase inhibitor like (a-AIL)), the a-AI1 isoform with anti- amylase activity in humans is the most widely distributed of the isoforms and is found in most of the common bean accessions grown worldwide(21-24). This makes efforts of extraction from any part of the world possible and, in addition, common beans are adapted to different ecological environments(1).

In the bean plant, a-AI1 is only found in the seeds and is concentrated in the axis(25). It is three times more concentrated in the axis than in the cotyledon. Apparently this is because there is more efficient glycosylation in the axis relative to the cotyledon. There is no a-AI1 in other organs of the plant(25). According to Moreno & Chrispeels(26), a-AI1 accumulates in seeds to make up about 9 – 11 % of the total seed protein. This percentage can provide a substantial yield of the inhibitor from a given amount of common beans although the extraction method may limit the yield.

Synthesis of a-AI1 occurs at the same time as that of phaseolin and phytohaemagglutinin (PHA) and also it accumulates in the protein storage vacuoles(25). The a-AI1 is a typical bean lectin, which is synthesised in the rough endoplasmic reticulum, modified in the Golgi body through removal of a signal peptide and N-glycosylation, and trans- ported to the protein storage vacuoles where it is proteolytically processed. SDS-PAGE, used for microsomal fractions, shows that Mr 30 000 – 35 000 fractions are associated with endoplasmic reticulum, while 14 and 19kDa are associated with Golgi body and storage vacuoles(25,27). The a-AI1 is detectable 17d after pollination in the cotyledons and axis of the plant seed. The amounts increase to a constant maxi- mum after 28 d until maturity, although the amount on a dry basis decreases slightly during drying(25). The a-amylase inhibitor is therefore suitably obtained from non-dried common beans. However, there is need for research to access maturity indexes for optimum inhibitor levels in beans to be used for extraction of the inhibitor for maximum economy. The distribution and biosynthesis show that the common bean a-amylase inhibitor is a suitable candidate as a widely used remedy against diabetes, obesity and for other related beneficial effects.

Favourable physico-chemical properties of the Phaseolus vulgaris a-amylase inhibitor

The inhibition efficiency, specificity, absence of deleterious carbohydrate-binding action associated with PHA and the action of the a-amylase inhibitor relative to similar agents such as acarbose or cyclodextrins have been shown to be based on its structure and molecular weight. In addition, to enable improvements in the use and application of the inhibitor, an understanding of the starch-blocking activity in terms of functional and biochemical factors is necessary.

Structural properties of the Phaseolus vulgaris a-amylase inhibitor

The three common bean lectin compounds PHA, arcelins and a-AI (a-AI1, a-AI2, a-AIL) have an amino acid sequence homology of about 50 – 90 %(28). In a study on genes that encode for a-AI1 in white and black beans, Lee et al.(29) found similarities of 40 and 43 %, 52 and 53 %, and 93 and 95% with PHA, arcelins and previously determined a-AI1 sequences respectively. These observations corresponded to major differences in the number of surface loops in the three-dimensional structures of the lectins. PHA has three loops, arcelin has two of the loops, a-AIL has one shortened loop, while the loops are completely absent in a-AI1 and a-AI2(30). The inhibitor has no carbohydrate-binding activity due to lack of carbohydrate-binding loops that are present in PHA(27,31,32). The inhibitor, therefore, if extracted efficiently, is bound not to possess the deleterious effects associated with PHA. Several researchers using various methods have shown the deletions in the sequences to be an indication of evolutionary relationship between the lectins(26,29,30,33,34). Le Berre-Anton et al.(35), using graphical docking methods, concluded that the extra loops, presence of extra glycan moieties and lack of proteolytic processing in PHA, arcelins and a-AIL were responsible for their lack of inhibitory activity relative to a-AI1. The extra loops caused steric hindrance that prevented them from entering the active site of mamma- lian amylases to enable binding(35).

The a-amylase inhibitors a-AI1 and a-AI2 exist in their native form as typical lectin tetramer structures (a2b2)(35). The a and b chains are formed through a two-step proteolytic processing in the protein storage vacuoles which leads to formation of the active form of the inhibitor from a precursor(27,30,36,37). The process involves removal of a short-chain carboxy terminus and proteolytic cleavage at the carboxyl side of Asn77 by action of a carboxypeptidase or an asparagyl-specific endopeptidase leading to the formation of the two chains(26,27,30,36). When compared with the precursor, a-AIL and with a transgenically produced inhibitor in tobacco which all have the proteolytic processing site, the proteolytic processing is responsible for the removal of a structural constraint in the inhibitor which enables it to acquire the inhibitory activity(27,34,37). Based on structural models resulting from nucleotide sequences of a-AI1, Lee et al.(29) showed this structural constraint to consist of a bend in the region next to Asn 77.

Between a-AI1 and a-AI2, only the former shows inhibitory activity against mammalian amylases. This has been explained in terms of inhibitor structural properties. There is a 78 % homology in amino acid sequence between them and both undergo post-translational cleavage, yet a-AI2 has no inhibitory effect on mammalian amylases(34). The differences in the sequence between the two therefore have a significant effect on the inhibitory activity(34). Le Berre-Anton et al.(35) explained the difference in specificity between a-AI1 and a-AI2 to result from lower stability of binding interactions with mammalian amylases by a-AI2. They explained that two hairpin loops were responsible for the stability of an a-AI1– porcine pancreatic amylase (PPA) complex, by the formation of fifteen hydrogen bonds with PPA in the active site cleft. With a-AI2, however, there were only eight of the hydrogen bonds formed due to deletions and replacements of residues in the loops of a-AI2 relative to a-AI1. The deletions and replacements included two residues (Tyr34 and Asn35) pre- sent in loop L1 of a-AI1, which were deleted, and residues Tyr186, Tyr37 and Tyr190, which were replaced by His175, Val35 and Phe179 in a-AI2. These replacements could not interact with any residue from the PPA active site by hydrogen bonding(35).

According to Santimone et al.(38) the inhibitor protomers are bound together non-covalently mainly through hydro- phobic interactions. Higaki & Yamaguchi(39) suggested that glycan moieties played a role in holding the protomers together. The N-glycosylation according to Sawada et al.(40) does not have an effect on the activity of the inhibitor since it occurs in positions that do not interact with mammalian amylases during binding. Removal of the glycan moieties by Gibbs & Alli(41) did not also affect the activity of a purified a-amylase inhibitor from white kidney beans. Bompard- Gilles et al.(42), however, noted that although it did not take part directly in

amylase binding, the glycan moiety at Asn 12, during inhibitor – enzyme complex formation, lay in a sol- vent channel that linked the dimers to the enzyme with the two glycan moiety branches forming an extended conformation that was parallel to the surface of the dimer through water- mediated hydrogen bonding that stabilised the dimers. They concluded, however, that the glycan moiety did not take part in the binding action of the inhibitor. Sawada et al.(40) showed that there is limited variation in glycosylation at this point (Asn 12) between a-AI1 from different accessions. The role of glycan moieties in the inhibitor binding of the a-amylase therefore is of limited significance and does not affect relative inhibitory activity between accessions.

There are differences in the primary structures of the a-AI1 from different accessions that have been determined and deposited in the Expasy database(40,43). These differences, however, do not affect the specific activity of the a-amylase inhibitors from different accessions(42). There is a difference in activity of the a-amylase inhibitor extracts from different accessions, however, due to the existence of varying amounts of particular isoforms and isoinhibitors between accessions(22,23). An accession to be used to obtain starch blockers therefore should be accessed in terms of its average amylase content in order to get higher extraction and activity yields.

According to Le Berre-Anton et al.(35) and Kasahara et al.(44), the tetrameric (a2b2) nature of the inhibitor explains why there are observations that the a-AI1 inhibitor inhibits two PPA molecules per molecule. This makes it divalent in its mode of inhibitory action and has thus been reported in various studies to have a stoichiometric ratio of 2:1 relative to the 1:1 ratio of acarbose and cyclodextrins(38,44-46). According to Koukiekolo et al.(46) a-AI1 is a much stronger inhibitor of PPA than acarbose based on molar concentration. There is 74% inhibition of amylose digestion by a-AI1 compared with 71 % by acarbose, and a 57 % inhibition by a-AI1 com- pared with 49% by acarbose for maltopentaose hydrolysis. However, based on weight, due to lower molecular weight, acarbose is a stronger inhibitor(46). Lee & Whitaker(47) showed that the molecular weight of the inhibitor is actually 56·7 kDa, and values in the range 14 – 20 kDa resulted from chemical modification due to the SDS-PAGE method. The rate of reaction of acarbose with the amylase is, however, faster, since there is no requirement for conformational change during binding(46).

Factors that affect the Phaseolus vulgaris a-amylase inhibitor activity

Various researchers have shown the dependence of the amylase inhibitor activity on pH, temperature, incubation time and presence of particular ions.

The optimum pH for the inhibitory action has been reported as 4·5(48,49), 5·5(32,49,50) and 5·0(51), rather than 6·9 – the optimum for mammalian amylase (PPA). The different pH optima reported were probably due to the different incubation temperatures used in the studies. Lajolo & Finardi Filho(49) noted different pH optima for salivary and pancreatic a-amy- lase of 4·5 and 5·5 respectively. Le Berre-Anton et al.(48) demonstrated that there is a narrow range around the optimum in which high activity is observed beyond which activity drops drastically. Kluh et al.(43) illustrated that for maximum activity, the inhibitor requires pre-incubation at low pH (pH 4) relative to the optimum.

Temperature has been reported to have an effect on the activity of the inhibitor. The effect of temperature, however, is less felt at pH 4·5 which is the optimum pH for inhibitor activity than at pH 6·9, the optimum pH for PPA(43,50). According to Le Berre-Anton et al.(48), the a-amylase inhibitor shows no activity at 08C, then activity increases to a maxi- mum between 22 and 378C with little change within this range(51). Although Marshall & Lauda(32) also reported no activity at 08C, they showed a 10-fold increase in activity within this range (22 and 378C). Le Berre-Anton et al.(48) attributed this discrepancy to different incubation pH used, with the increase occurring when incubated at pH 6·9, the optimum pH of the enzyme. The inhibitor is completely inactivated at

1008C by boiling for 10 min(32,52). Collins et al.(53) showed that the inhibitor transgenically expressed in peas was only inactivated after heating at over 908C for 5min. There is need to characterise the temperature-inactivation pro- file of the inhibitor further since many potential products in which it can be incorporated would require heat treatment during processing.

The incubation time required for optimum activity has been reported as 10 min by Le Berre-Anton et al.(48), 40 min by Marshall & Lauda(32) and 120 min by Powers & Whitaker(51). These differences were suggested to be a result of the different pH conditions used in the experiments, with the latter two being obtained when the optimum for a-amylase activity (6·9) was used and the first when the optimum for the inhibitor (4·5) was used(48). The longer incubation times at pH 6·9 imply that it would require the inhibitor to be taken before or at least with meals in order to achieve substantial in vivo inhibitory activity.

Various ions have also been shown to affect the activity of the inhibitor. Lajolo et al.(49) reported increases in the activity of the inhibitor against salivary amylase mediated by ions in the order nitrate . chloride . bromide . iodide . thiocyanate. Gibbs & Alli(41) reported that chloride ions are important for maximum activity while Ca ions increase the rate of initial binding of the inhibitor to the amylase. They also reported that K, Mg, sulfate and Na ions did not have any effects on the amylase inhibitor activity and so did increased ionic strength(41).

Generally, there is need to further characterise the effect of various functional and biochemical factors on the activity of the inhibitor in order to enable improvements in the use and application of the inhibitor.The starch-blocking mechanism of the Phaseolus vulgaris a-amylase inhibitor

Research into the mechanism of the P. vulgaris a-amylase inhibitor action shows that the inhibitor is effective in preventing starch digestion by completely blocking access to the active site of the enzyme. The molecular-level binding of the action of the amylase inhibitor on human pancreatic amy- lase and PPA was reviewed in detail by Payan(54). During inhibition, several components of the inhibitor molecule, amylase molecule and the whole system have been reported to play important roles in the mechanism. The main components that participate in the mechanism include two loops of the inhibitor (L1 and L2) made up of residues 29 – 46 and 171 – 189 respectively(35,38,42), the amylase domains A and B plus the active site surface loop (residues 303 – 312)(32,40,41), the active site non-loop residues (Cl binding site and Asp197, Glu233; Asp300 and Arg74 in human pancreatic amylase only(42,55)), the active site lining and gate aromatic residues(42), the chlorine ion of the amylase(56) and system aspects such as the inhibitor: enzyme ratio(38) and pH(55). Based on the effects of chemical modifications on activity of the inhibitor, Ho & Whitaker(57) proposed that His, Trp, Tyr and Arg residues were important in the mechanism of the inhibitor. Mirkov et al.(58) suggested the active site of a-AI1 to be made up of Arg in the a-subunit, and Trp and Tyr in the b-subunit, which are located in a TrpSerTyr motif. Takahashi et al.(59) who, however, postulated that the arginine residues were not essential in the mechanism, supported these results. Bompard-Gilles et al.(42) attributed these observations to the participation of the residues in hydrophobic interactions. On the other hand, Da Silva et al.(60) showed that no particular structure in the amylase inhibitor – amylase complex was solely responsible for the inhibitory action.

In the course of the binding action, the inhibitor approaches the enzyme active site cleft by way of the loops, which leads to the formation of an extensive network of bonds between the loop residues and parts of the active site(42). The network of bonds involves mainly hydrogen bonds which may be direct or water mediated, hydrophobic bonds and protein – protein bonds, especially in areas outside the active site(42). The bond network formation is accompanied by conformational changes in parts of the amylase in adjustment to docking of the inhibitor, which occurs in the active site surface loop (residues 303 – 312)(41,42,55,61,62), the domains of the amylase (domains A and B) and in the areas near the surface loop in the active site(42). Although several

researchers have elucidated the inhibitor binding reactions, there is need for more work to establish and confirm the actual sequence of events during the inhibitory mechanism. This would provide more insight into the binding reactions and provide more knowledge that would help in developing similar synthetic inhibitors. It is, however, clear from the research in its mechanism that the inhibitor is effective in preventing starch digestion by completely blocking access to the active site of the enzyme(42).

Efficiency of a-amylase inhibitor isoform 1 extracts in reducing activity of amylases in man

An effective reduction in activity of intraluminal amylases is the underlying source of all the beneficial effects obtained from the inhibitor. Several researchers have shown a decrease of intraluminal amylase activity in vivo, in all parts of the gastrointestinal tract, hence reducing the rate of evolution and absorption of glucose in the lumen (Table 1). In human subjects Layer et al.(6) reported a decrease in duodenal amylase activity and length of inhibition time, which were dependent on the dose of application of the inhibitor. In another human study, decreased duodenal, ileal and jejunal amylase activity, with no apparent effect on trypsin levels, was observed(7). Brugge & Rosenfeld(63) showed a 96 % decrease in duodenal amylase activity in human subjects after taking starch-containing meals with an incorporated laboratory-purified amylase inhibitor.

Studies have shown marginal middle and proximal gastro- intestinal tract amylase activity a few hours after feeding with meals containing the inhibitor and a complete abolition of activity after 4h of feeding(6). Inhibition results in malabsorption of starch and passage into distal parts of the ileum(6,7). Various levels of the resultant malabsorption have been reported. Layer et al.(6) reported a malabsorption level of 20% of ingested starch, while other workers have reported lower levels. Brugge & Rossenfeld(63) reported a level of 7·0 (SD 1·4) % and Boivin et al.(64) documented a concentration-dependent level of up to 18 % with 2·9 mg of inhibitor. The different levels reported could have been due to differences in activity and amounts of a-AI1 used. Some changes occur in response to the presence of excess starch in the duodenum and the passage of excess starch into the distal parts of the ileum in order to increase the rate of digestion(65). They include reduced rate of gastric emptying(6) and increased secretion of amylase by the pancreas, in addition to general changes in pancreaticobiliary secretions(65,66). The onset of reduced gastric emptying occurs after the first 2 postprandial hours(65,66). The mechanism that initiates these changes was postulated to involve carbohydrate-mediated hormonal and non-vagal neural responses, since changes in plasma hormonal levels (peptide YY, neurotensin and gastric inhibitory peptide) were associated with changes in gastric emptying(66). These changes, however, were associated with subtle increase in glycaemia relative to controls without the inhibitor(66). The anti-amylase activity of the inhibitor in vivo is also decreased by the amount and type of starch in the duodenum, with liquid starch being more potent than solid starch in the reduction(6).

According to Brugge & Rosenfeld(63), the form in which the inhibitor is applied, whether powder or tablet form, has no effect on the inhibitory activity when incorporated in meals. This implies that various forms of extract products can be developed depending on a particular targeted functionality and still have the desirable inhibitory activity.

Table 1. Human studies on the efficacy of *Phaseolus vulgaris* α-amylase inhibitor isoform 1 extracts on starch digestion and resultant effects

Dose/duration	Main results*	Reference
Acute, two commercial starch-blocker tablets, 16 666 units of activity with six healthy subjects	No difference in postprandial plasma glucose, insulin and breath hydrogen	Carlson *et al.* [68]
Acute, 500 mg commercial starch blocker with two healthy subjects	Starch blocker ineffective in reducing energy intake	Bo-Linn *et al.* [69]
Acute, 500 mg commercial starch blocker with eight healthy subjects	Commercial starch blocker ineffective *in vitro* and *in vivo*	Hollenbeck[67]
Acute, three healthy subjects; 2–5 mg/ml for 90 min	Purified extract effective but commercial blocker ineffective	Layer *et al.* [6]
Acute, 5 and 10 g with four healthy subjects	Purified inhibitor orally taken blocks starch digestion; no abdominal problems; decreased postprandial hyperglycaemia and insulin levels	Layer *et al.* [7]
Acute, 3·8 g with thirteen healthy subjects	Physical form has no effects on inhibitory activity of orally taken inhibitor	Brugge & Rosenfeld[63]
Acute, perfused over 7 h at 9·9 mg/min with eighteen healthy subjects	Orally taken inhibitor starch digestion blocking effect; changes in GIT motility and GIT-related hormones	Jain *et al.* [65]
Acute, perfused at 3·3 mg in 570 ml, with eighteen healthy subjects	Orally taken starch-blocking effect; GIT motility accompanied; changes in pancreaticobiliary secretions	Jain *et al.* [66]
Acute, dose 2·0 and 2·9 g with eight healthy subjects	Starch digestion blocked; no abdominal problems; changes in plasma insulin and glucose	Boivin *et al.* [64]
445 mg Phase 2® tablet before meals with thirty slightly obese subjects	Highly significant combined change in anthropometric parameters ($P<0·001$)	Celleno *et al.* [4]
1500 mg Phase 2® before each meal with sixty healthy subjects	Subtle weight loss with body-fat loss and no observed deleterious effects	Meiss & Ballerini[85]
1500 mg Phase 2®, twice daily before meals for 8 weeks, with twenty-seven healthy subjects	Subtle loss of body weight and three-fold decrease in plasma TAG levels relative to controls	Udani *et al.* [86]
Two and eight tablets of commercial starch blocker for 3 and 4–5 months respectively, with twenty-two obese subjects	Starch blocker has no synergistic effect on weight reduction under reduced energy conditions	Diaz *et al.* [87]

GIT, gastrointestinal tract.
*Statistical significance at $P<0·05$ unless mentioned.

The inefficiency of the amylase inhibitor reported by researchers in the early 1980s was mainly due to low activity and purity of the commercial starch blockers(67-69). The manufacturers employed methods based on extraction of a-AI1 by Marshall & Lauda(32). A simple partial extraction of the inhibitor by Layer et al. led to a 30–40-fold increase in inhibitor concentration by dry weight(6). The resultant in vivo inhibitory activity and length of inhibitory time were dose dependent compared with commercial inhibitor and crude extracts that were only effective at high doses. This showed that low activity was the cause of apparent inefficiency and hence the highest possible a-amylase activity should be a target for extraction processes.

Impurities were also reported in the starch blockers which were found ineffective(70,71). The trypsin inhibitor, one of the potential inhibitor extract impurities(70), would lead to increased trypsin secretion which has been associated with decreased a-AI1 activity due to non-specific secretion of excess amylase by the pancreas(66,72), while the pure amylase inhibitor is not associated with changes in chymotrypsin activity in rats(73). According to Yoshikawa et al.(74), chymotrypsin reduces inhibitor activity in vitro rapidly within 2h, pepsin slightly and the inhibitor is highly resistant to trypsin digestion. The amylase inhibitor had been earlier hypothesised ineffective in reduction in energy intake due to proteolysis by gastric enzyme, high amylase activity and unfavourable pH conditions in the duodenum(68). Gibbs & Alli(41), on the other hand, showed that the inhibitor was resistant to proteolysis in vitro by physiological amounts of chymotrypsin and pronase. It has also been shown that the amylase inhibitor is stable in gastric and duodenal juices(65,75) and reduces in vivo amylase activity(63,65,72). The activity, however, is slightly reduced (15 %) by the unfavourable pH in the duode-num(6,64).

In summary, despite several factors that may reduce the amylase inhibitor activity in vivo, the activity has been shown to be sufficient and hence the P. vulgaris inhibitor is applicable as an intraluminal a-amylases inhibitor.

The beneficial effects of the Phaseolus vulgaris a-amylase inhibitor

Decreased obesity due to Phaseolus vulgaris a-amylase extracts

Currently there is a shift from synthetic anti-obesity prescribed medications to natural ones, due to undesirable long-term side effects of synthetic prescribed medications(76,77). Though acarbose and voglibose, which are

approved by the Food and Drug Administration, reduce blood glucose levels, they also induce abnormalities in hepatic enzyme levels, yet natural anti-glyco- sidase extracts do not exhibit such effects(78). The P. vulgaris a-amylase inhibitor extracts have an anti-obesity effect as shown by the various researches although there are some uncertainties (Table 1). The effect is derived from the mobilisation of body fat reserves due to energy restriction as a result of the a-amylase inhibitory action.

In studies by Pusztai et al.(79), there was a reduction in body fat in rats due to the consumption of raw kidney beans. They, however, attributed the effect to the presence of PHA through some unknown mechanism. The effect could also have arisen due to the presence of amylase inhibitors in the common beans since the lean body content of the obese rats was not affected. Hangen & Bennink(80) showed that rats fed diets containing black and navy beans were able to achieve a reduction in body weight and the fat percentage directly associated with anorexia and starch escape of digestion in the ileum. In their studies the amount of starch that escaped digestion was higher than the amount of resistant starch originally in the diet.

Incorporation of the inhibitor in diets leads to a reduced integrated postprandial plasma glucose area by 85% and a lower than fasting level of late postprandial plasma glucose according to Layer et al. (7). The total energy in form of glucose obtained from the diet is therefore reduced leading to mobilisation of fat in the body.

Several reports have shown increases in breath hydrogen on ingestion of food with an active amylase inhibitor. This is as a result of action of distal ileum enterocytes on undigested starch that passes digestion sites(63 65,81,82). Although action of the enterocytes releases energy to the body, 50 to 20 % of the total energy in the by-passed starch is not released(4). The total energy therefore is still bound to be reduced resulting in mobilisation of fat reserves.

The amylase inhibitor was found to induce reduced growth in weaned young male rats by Maranesi et al.(83), which they attributed to reduced energy intake due to the inhibitor. The reduced energy intake was accompanied by increase in levels of plasma NEFA. There have been several positive results indicating reduced obesity by researchers using a commercial a-AI1 extract referred to as Phase 2w (Pharma- chem Laboratories, Inc., Kearny, NJ, USA). According to Chokshi(84), Phase 2w is prepared using thermoprocessing conditions to substantially inactivate haemagglutinating activity and trypsin inhibitory activity while preserving sub- stantial a-amylase inhibition activity. The product is also tested for the presence of other antinutritional factors or potentially toxic substances with standard levels of .3400 haemagglutinating units/g and .40 trypsin inhibitor units/g(84). Celleno et al.(4) reported a highly significant difference (P, 0·001) in combined obesity anthropometric measures between subjects taking a dietary supplement containing 445 mg Phase 2w in a 30 d study with controls on microcrystalline cellulose – maltodextrin. In their study, changes relative to controls were observed in body weight, adipose tissue thickness, waist circumference, hip circumference, right thigh circumference and fat mass. Although these were accompanied by a just significant lean mass loss, the total weight loss was more due to fat mass loss than lean mass loss(4). It was shown in a double-blind placebo-con- trolled clinical trial by Meiss & Ballerini(85) that feeding Phase 2w for 30 d resulted in a 4 % decrease in body weight, accompanied by a 10·45% reduction in body fat, and a skin echography revealed an 11·63 % reduction in adipose membrane. This study also showed that Phase 2w caused a change in hip, thigh and waistline circumferences. In a similar study, Udani et al.(86) also reported an average weight loss of 95 g (0·21 lb)/week and an average of 263 mg/l reduction in TAG for individuals taking Phase 2w. These results were, however, not statistically significant due to the low sample size used.

On the other hand, Bo-Linn et al.(69), in a study of commercial starch blockers, found no changes in faecal energy output when the inhibitor was taken compared with inhibitor-less controls. In a controlled double-blind placebo study, a commercial starch blocker was found to be ineffective relative to controls in reducing the weight of obese

women on a BMR-equivalent diet(87). More recently in toxicity studies of amylase inhibitor in rats, no effects of plasma lipoproteins(77) and weight gain have been observed(5).

Given the exhibited starch-blocking ability of the amylase inhibitor by Phase 2w relative to earlier forms of commercial extracts(4,85,86), the amylase inhibitor has anti-obesity effects, although the effect of the extracts that results from reduced energy intake depends on a given manufacturer's methods of manufacture and extraction as regards the maintenance of high anti-amylase activity and purity.

The anorexigenic effect of Phaseolus vulgaris a-amylase inhibitor isoform 1 extracts

Some works have suggested an anorexigenic effect as an underlying cause of obesity reduction. The mechanism of the anorexigenic effect of the amylase inhibitor is, however, not clearly understood(88). It has been reported that the amylase inhibitor fed chronically to rats reduces feed intake(82). The inhibitor in further studies also reduced water intake in diabetic rats in addition to reduced food intake(81). However, the a-amylase inhibitor in a study on the toxicity of a commercial starch blocker was found to have no anorexigenic effect after 28d(5). A similar study showed that the anorexigenic effect in Sprague – Dawley rats was felt only after 77 d of feeding(77). The anorexigenic effect may therefore be only achieved with prolonged exposure to the inhibitor. More research is, however, needed in human subjects to assess the anorexigenic effect of the inhibitor further.

Reduced postprandial plasma hyperglycaemia and insulin due to a-amylase inhibitor isoform 1 extracts

Changes in postprandial plasma glucose levels have been reported when the amylase inhibitor is taken with a starch- containing meal or before the meal (Fig. 1). Earlier reports, using commercial starch blockers with low activity, could not show changes in postprandial plasma glucose(67,68). Kotaru et al.(73) and Menezes & Lajolo(72) showed smoothed and retarded hyperglycaemia in rats fed rations containing the purified a-amylase inhibitor. A reduction of 85 % in postprandial plasma glucose integrated area accompanied by lower than fasting late post-prandial plasma glucose were shown on acute consumption with meals of the inhibitor in human subjects(7). Boivin et al.(64) also reported decreased integrated area and lower peak plasma postprandial glucose in human subjects on acute application. According to Tormo et al.(82), a reduction of hyperglycaemia due to the inhibitor in rats starts 50min after the consumption of a starch-containing meal. Chronic consumption of the amylase in meals in rats led to reduced mean glycaemia over the period of application. There was variation of significance of the reduced mean hyperglycaemia from day to day, ranging from P,0·01 to P, 0·05(81,82).

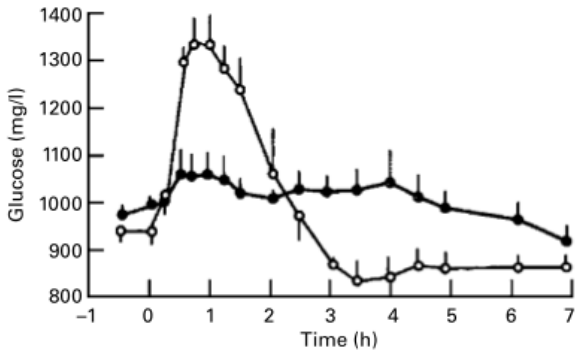

Fig. 1. Effect of α-amylase inhibition by *Phaseolus vulgaris* α-amylase inhibitor isoform 1 on postprandial plasma concentration of glucose in response to a starch meal. (○), Placebo (*n* 4); (●), 5 or 10 g inhibitor (*n* 4). Values are means, with standard deviations represented by vertical bars. (Adapted from Layer *et al.* [7].)

The ingestion of the amylase inhibitor with meals has also been shown to alter postprandial plasma insulin levels.

Boivin et al. (64) reported in human subjects a decrease in the integrated areas of plasma insulin secretion-related hormones of gastric inhibitor peptide and C-peptide over baseline values when the inhibitor was part of a composite meal. An abolition of postprandial plasma insulin, C-peptide and gastric inhibitory peptide in human subjects was also documented by Layer et al.(7) (see Fig. 2). Lowering of plasma insulin levels was shown to occur 30–40min after the consumption of a composite ration containing a purified cranberry bean (P. vulgaris L.) amylase inhibitor in rats. In another study in rats Menezes & Lajolo(72) showed decreased serum insulin levels in both diabetic and normal rats fed meals containing the amylase inhibitor.

Earlier reports on tests using commercial starch blockers that were found to lack in vivo amylase inhibitory activity found the inhibitor ineffective in reducing plasma insulin levels(67,68). It was also found that plasma insulin levels in Wistar rats are not affected by both chronic and acute administration of a-AI1(82). The levels were lower than in the fasting state but not statistically significant.

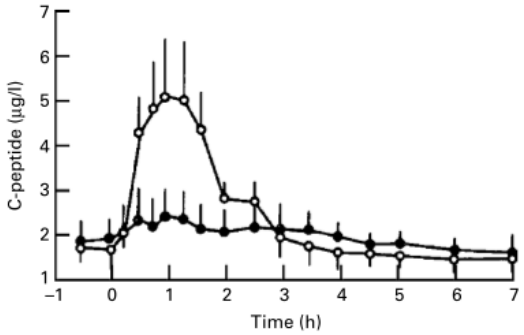

Fig. 2. Effect of α-amylase inhibition by *Phaseolus vulgaris* α-amylase inhibitor isoform 1 on postprandial plasma concentration of C-peptide in response to a starch meal. (○), Placebo (*n* 4); (●), 5 or 10 g inhibitor (*n* 4). Values are means, with standard deviations represented by vertical bars. (Adapted from Layer *et al.* [7].)

Despite these findings, the reduction in plasma insulin and related hormonal levels can increase the carbohydrate tolerance of diabetics. This has been shown to occur on consumption of the a-amylase inhibitor. There is a need therefore for more research to confirm the effect of the inhibitor on postprandial insulin levels in man and its incorporation in starch-containing foods.

A few studies have been reported on the application of the a-amylase inhibitor in food products. Udani(89,90) reported successful incorporation of the amylase inhibitor in the form of a proprietary fractionated white bean extract powder (FWBEw) ($3000 a-amylase inhibitor units/mg) into six commercial baked products at levels deemed sufficient for inhibitory activity (750 mg/serving) without significant changes in the acceptability of the products. The main factors that influenced the incorporation were the order of ingredient incorporation and the time – temperature requirements for dough development and baking. Combinations of these factors through trials and iterations were obtained that did not affect consumer acceptability of products with the required amounts of extracts per serving. These results, however, did not report the effect of the incorporation on the glycaemic index of the products. In a similar study (J Udani, unpublished results), using an open label six-arm cross-over design with thirteen randomised subjects, the glycaemic index of white bread was reported to have been significantly reduced (P1⁄40·0228) by the addition of 3000 mg StarchLitew powder – a commercial a-amylase bean extract. There is a need for more research into the application of the amylase inhibitor in these and other products to enable wide application.

Safety and toxicity of the Phaseolus vulgaris a-amylase inhibitor extracts

Toxic effects associated with common beans

Haemagglutinin poisoning due to the consumption of raw common beans by animals and humans has been documented in several reports(91-98). In man, acute consumption in all documented cases led to severe symptoms requiring hospitalisation(97,98). In addition, slimming pills consisting of extracts from common beans were found by Kilpatrick et al.(71) to cause a skin rash after ingestion. The rash was linked to haemagglutinating activity in the pills at levels of up to 150 mg protein and agglutinated human A, B, or O erythrocytes; the specific lectin activity was 2000 lectin units/mg protein(71). The haemagglutinating activity of common beans varies between accessions in terms of amount and specificity of activity(99-102). Varieties low in PHA such as pinto beans(99) are therefore more suitable candidates as raw material for a-AI1 extracts. Some acute and subchronic studies have been conducted on the toxicity of a-AI1 extracts in man and rats.

Acute toxicity studies

Acute toxicity is a toxicity response that often occurs immediately after ingestion and is induced by a single exposure. It is measured by the lethal dose 50 (LD50) value, which is the amount of a given substance under test that causes death of 50 % of the test animals after consuming the substance only once(14). There were no significant signs of acute toxicity or mortality when 3 g/kg of Blockalw (a dietary supplement containing Phase2w at a rate of 1668 mg/kg body weight) was fed to rats(5). The symptoms observed at the acute experimental levels of feeding (1668mg/kg body weight of Phase 2w) were not similar to those caused by PHA, indicating that the Phase 2w component used did not contain adequate PHA to cause deleterious effects(5). Variations from normal were not observed in liver function markers, kidney function markers, plasma levels of electrolytes, cholesterol and TAG. The acute toxicity level was established at . 5 g Phase 2w/kg body weight in another acute oral administration study in adult male and female Wistar rats(77) and there was no observed toxicity based on clinical evaluation, biochemical and histopathological analyses at this level of single-dose feeding(77).

Chronic toxicity studies

Chronic measurement requires a longer time of study, usually about 20 – 24 months of continuous feeding to rodents. The maximum tolerance dose is the level at which a substance can be fed to an animal without inducing any obvious sign of toxicity(14). In chronic studies, the maximum tolerance dose is typically used with two or more lower levels below(14). Studies have been done on the effect of chronic feeding of the amylase inhibitor. In a subchronic study on the oral toxicity of a standardised white kidney bean extract Phase 2w in rats, it was found that there were no mortalities and clinical signs considered of toxicological significance on rats fed doses up to 2500mg/kg (7d/week) for a period of 31d (males) or 32d (females)(84). No gross abnormalities were observed apart from some isolated cases, which were considered unrelated to the treatments. The microscopic findings in body organs observed which apparently deviated from normal were similar to those commonly observed in the studied rat strain(84). In addition, on the basis of lack of correlation of these findings to microscopic and clinical pathological data, they were considered to have no toxicological relevance(84). The no observed adverse effect level was found to be at least 2500mg/kg per d for rats, which corresponds to 175 g Phase 2w/d in a 70 kg person. It was proposed that the upper limit level of aggregate intake of Phase 2w/d from dietary supplement and qualified food use be 6g/kg per d for a 70kg person based on the fact that a 30-fold safety factor was used in the experiment(84).

In another study a lower no observed adverse effect level of at least 1112 mg Phase 2w/kg body weight was observed in a 4-week toxicity study involving feeding Blockalw at 2g/kg body weight to rats(5). Variations were observed in different parameters during the study but were also considered irrelevant because they were not

associated with any histo- pathological changes, did not vary with sex and were within the range of the historical results obtained in the laboratory. These variations occurred in weight, micro and macro appearance of organs, and some haematological, clinical and urine analyses(5).

Subchronic feeding testing has also been carried out in adult human subjects. In one randomised double-blind placebo-con- trolled study, tablets of a commercial blocker were given to individuals before carbohydrate-rich meals. An 800 mg tablet containing 445mg Phase 2w was given once per d in an 8370 – 9200 kJ (2000 – 2200 kcal) diet with a microcrystalline cellulose and maltodextrin placebo as the control for 30 d. There were no significant deleterious effects reported(4). The average weight of individuals in the study was 74·1 (SD 2·1)kg, hence the level corresponded to a rate of 6 mg Phase 2w/kg body weight per d. Udani(86), in a randomised double-blind placebo-controlled subchronic human subjects, showed that there were no deleterious effects on safety markers of kidney function. The level of Phase 2w used in this test was 1500 mg/ d with the average weight of the individuals being 87·6 (SD 12·22)kg(86). When subchronically applied to rats at two to twenty times the human subchronic levels recommended by Udani(86) the commercial extract Phase 2w did not produce signs of toxicity(77). It was concluded that feeding Phase 2w to rats at the rate of over 350 g/kg for a 70 kg individual did not produce any adverse effects(77). It was, however, noted in a study on the efficacy of the amylase inhibitor by Tormo et al. [82] and Pusztai et al. [88] that chronic administration of the a-amylase inhibitor in rats leads to changes in organ weights. There is need therefore for more research to completely ensure safety of the amylase inhibitor extracts. However, the use of a starch blocker with at least 3000 a-amylase inhibitor units/ g, , 3400 haemagglutinating units/g and , 40 trypsin inhibitor units/g at the subchronic level of 6·0 g/kg body weight per d for a 70 kg individual has so far been shown to be safe by studies using Phase 2w.

Future research on beneficial effects: the potential of a-amylase inhibitor isoform 1 extracts against colorectal cancer

Several studies have pointed to increased microbial activity in the hindgut on consumption of a-AI extracts although there are no reports on its effect on butyrate production, which is necessary for anti-colorectal cancer functionality. Based on the definition of resistant starch as the sum of starch and pro- ducts of starch degradation not absorbed in the small intestine of healthy individuals(103,104), the presence of the amylase inhibitor in the gut causes an action similar to that of resistant starch or rather increases the amount of resistant starch. Resistant starch has been shown by many workers to have a prebiotic effect and several reviews have been written documenting the effect(103 – 107). Human and animal studies have shown that butyrate leads to a reduced incidence of colon cancer. Le Leu et al. [108,109] found that butyrate had an apoptotic response to DNA damage by genotoxic carcinogens in the distal colon of rats, leading to the removal of mutated clones that would progress to malignancy. Distinct patterns of SCFA production are associated with particular polysaccharides and substantial butyrate formation was found to be associated mainly with starch(110).

The amylase inhibitor has been shown to increase the amount of breath hydrogen after the consumption of starch- containing meals as a result of passage of starch into the proximal parts of the colon that is accompanied by microbial activity(7,63,64,67,68,87). This was reported in studies with in vivo active inhibitor extracts while studies with extracts that showed no activity did not show increases in breath hydrogen. Collins et al. [53], in a study on transgenic pea a- AI1 in pigs, showed a significant difference in energy content between terminal ileum and faecal matter which they attributed to energy recovery by hindgut micro-organisms from ileum by-passed starch. No reports on butyrate production were given from these studies. On the other hand, several reports have shown that acarbose, a synthetic pharmaceutical starch blocker that functions in a similar manner to the common

bean a-amylase inhibitor (a-AI1), leads to alteration of colon microbe pathways. The alterations lead to an increase in the overall SCFA production with an increase in the butyrate:total SCFA ratio(111-114). The total faecal SCFA and butyrate output on prolonged acarbose use correlates inversely with proliferation in the rectal upper crypt – a biomarker of risk for colonic neoplasia(114). Future research on the beneficial effects of the a-amylase inhibitor therefore should also be focused on checking its potential in colorectal cancer prevention as a result of increased butyrate production due to starch in the colon after consumption of reasonable amounts of the inhibitor.

Conclusion

Although obesity and diabetes are on the increase worldwide, based on the research developments discussed, the common bean (P. vulgaris) a-amylase inhibitor (a-AI1) has potential to serve as a widely used remedy against these conditions while there is need for research on its probable anti-colorectal cancer effect. The potential lies in the fact that the amylase inhibitor is present in most P. vulgaris accessions which are widely grown in the world, it has a significant in vivo inhibitory capacity based on appropriate structural, physico-chemical and functional properties, and has mediating effects on these conditions although there are some uncertainties. In studies carried out more recently the a-amylase inhibitor has been found to be safe. There are several aspects of the inhibitor that require further research. These include wider clinical trials over longer times to confirm the efficacy and safety of the inhibitor, ingredient functionality of the inhibitor in various food systems and further elucidation of molecular-level binding interactions to enable synthetic blockers based on the inhibitor to be designed.

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Barrett ML, Udani JK

A proprietary alpha-amylase inhibitor from white bean (Phaseolus vulgaris): A review of clinical studies on weight loss and glycemic control

Obesity, and resultant health hazards which include diabetes, cardiovascular disease and metabolic syndrome, are worldwide medical problems. Control of diet and exercise are cornerstones of the management of excess weight. Foods with a low glycemic index may reduce the risk of diabetes and heart disease as well as their complications. As an alternative to a low glycemic index diet, there is a growing body of research into products that slow the absorption of carbohydrates through the inhibition of enzymes responsible for their digestion. These products include alpha-amylase and glucosidase inhibitors. The common white bean (Phaseolus vulgaris) produces an alpha-amylase inhibitor, which has been characterized and tested in numerous clinical studies. A specific and proprietary product named Phase 2® Carb Controller (Pharmachem Laboratories, Kearny, NJ) has demonstrated the ability to cause weight loss with doses of 500 to 3000 mg per day, in either a single dose or in divided doses. Clinical studies also show that Phase 2 has the ability to reduce the post-prandial spike in blood glucose levels. Experiments conducted incorporating Phase 2 into food and beverage products have found that it can be integrated into various products without losing activity or altering the appearance, texture or taste of the food. There have been no serious side effects reported following consumption of Phase 2. Gastro-intestinal side effects are rare and diminish upon extended use of the product. In summary, Phase 2 has the potential to induce weight loss and reduce spikes in blood sugar caused by carbohydrates through its alpha-amylase inhibiting activity.

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Yamamoto T, Sakashita T, Suhara T

Safety and Weight Reduction Effect of foods Containing Phaseolus vulgaris

Phaseolus vulgaris which is component of white kidney bean is deemed to be effective to reduce obesity and weight reduction of people whose Japanese style food habit are rich of carbohydrate. Starchiness and sugariness such as glycogen taken into living body by foods are digested in small intestine by carbohydrate dissolving enzyme and amylase into maltose and further disaccharide are dissolved by carbohydrate dissolving enzyme (.ALPHA.-glucosidase) into glucose and absorbed by living body. We already know that Phaseolus vulgaris keep down activity of amylase and mitigate absorption of glucose from digestive tract. We verified effect of weight decrease and safety by giving health food, combination of Phaseolus vulgaris, Fenugreek, which has action of keeping down blood pressure and fat, Ginger, Fructo-oligosaccaride, Fennel, Chrome, Cardamon, Gymnema Sylvestre for 3 months. In conclusion, we can confirm statistically significant difference of weight decrease and safety of foods containing Phaseolus vulgaris

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Onakpoya I, Aldaas S, Terry R, Ernst E

The efficacy of Phaseolus vulgaris as a weight-loss supplement: a systematic review and meta-analysis of randomised clinical trials

A variety of dietary supplements are presently available as slimming aids, but their efficacy has not been proven. One such slimming aid is the bean extract, *Phaseolus vulgaris*. The aim of the present systematic review is to evaluate the evidence for or against the efficacy of *P. vulgaris*. Electronic and non-electronic searches were conducted to identify relevant human randomised clinical trials (RCT). Hand searches of bibliographies were also conducted. No age, time or language restrictions were imposed. The eligibility of studies was determined by two reviewers independently, and the methodological quality of the included studies was assessed. We identified eleven eligible trials, and six were included. All the included RCT had serious methodological flaws. A meta-analysis revealed a statistically non-significant difference in weight loss between *P. vulgaris* and placebo groups (mean difference (MD) − 1·77 kg, 95 % CI − 3·33, 0·33). A further meta-analysis revealed a statistically significant reduction in body fat favouring *P. vulgaris* over placebo (MD − 1·86 kg, 95 % CI − 3·39, − 0·32). Heterogeneity was evident in both analyses. The poor quality of the included RCT prevents us from drawing any firm conclusions about the effects of *P. vulgaris* supplementation on body weight. Larger and more rigorous trials are needed to objectively assess the effects of this herbal supplement.

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Preuss HG

Bean amylase inhibitor and other carbohydrate absorption blockers: effects on diabesity and general health

Many believe that excessive intake of refined carbohydrates (CHO) plays a major role in the development of obesity/overweight, type 2 diabetes mellitus and insulin resistance, a collection of events commonly referred to as "diabesity," and have sought natural means to overcome these linked perturbations. As a first approach, planned diets with low portions of refined CHO have become popular. However, these diets do not satisfy everyone; and many are concerned over replacing CHO with more fats. As a second option, addition of soluble fiber to the diet can slow absorption of refined CHO, i.e., lower the glycemic index of foods and overcome or at least ameliorate many of the adverse reactions resulting from increased refined CHO ingestion. Unfortunately, the general public does not favor diets high in fiber content, and various fibers can lead to gastrointestinal problems such as gas and diarrhea. A third choice to favorably influence CHO absorption is to use natural dietary supplements that block or slow CHO absorption in the gastrointestinal tract via inhibiting enzymes necessary for CHO absorption -amylase and alpha-glucosidases. Although a number of natural supplements with anti-amylase activity have been recognized, the most studied and favored one is white kidney bean extract. Animal and human studies clearly show that this agent works in vivo and has clinical utility. This paper reviews many aspects of diabesity and the use of "carb blockers" to prevent and ameliorate the situation. In many respects, carb blockers mimic the beneficial effects of fibers.

Impact of Dietary Polyphenols on Carbohydrate Metabolism

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1. Introduction

Polyphenols are a large and heterogeneous group of phytochemicals of plant-based foods, such as tea, coffee, wine, cereal grains, vegetables, legumes, fruits and berries. The structural diversity of polyphenols extends from simple one-phenol hydroxybenzoic and hydroxycinnamic acids to large polymeric macromolecules like proanthocyanidins and ellagitannins. An essential group of phenolic compounds are flavonoids encompassing structural classes like flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavones. The estimated intake of dietary polyphenols is approximately 1 g/day [1]. Consumption of plant foods is associated with lowered risk of major chronic diseases including diabetes, cardiovascular diseases and cancer [2–5]. *In vitro* and *in vivo* studies on polyphenols show that polyphenols possess anti-inflammatory, antioxidative, chemopreventive and neuroprotective activities, suggesting that they could contribute to the health-protective properties of plant foods. Growing evidence indicates that dietary polyphenols also influence glucose and lipid metabolism

The majority of dietary polyphenols are metabolised by colonic microbiota before absorption, only smaller amount being absorbed directly from upper gastrointestinal tract [6]. Gut bacteria modulate polyphenols by various mechanisms including hydrolysis, ring-cleavage, reduction, decarboxylation and demethylation. The microbial metabolism is a pre-requisite for absorption, and it also modulates the biological activity of the compounds. The systemic effects of dietary polyphenols depend largely on the synergistic action that polyphenols may exert after entering circulation, and are affected by other constituents present in the diet as well as endogenous factors [7,8].

Starch and sucrose are the most important dietary carbohydrates. Their digestion, absorption and metabolism may be influenced by dietary polyphenols and their metabolites. Most dietary carbohydrate is digested in the upper gastrointestinal tract to monosaccharides which are then absorbed to the circulation. The elevated glucose concentration in blood promotes secretion of insulin from the β-cells of the islets of Langerhans in the pancreas, and insulin mediates the uptake of glucose in peripheral tissues including muscle, adipose tissue and kidney, promotes storage of glucose in liver as glycogen, and inhibits lipolysis in adipose tissue. Another essential hormone in maintaining the glucose homeostasis is glucagon that is secreted from the pancreatic α-cells once the blood glucose level begins to fall below normal. Glucagon promotes liver glucose production by inducing glycogenolysis and gluconeogenesis to ensure adequate circulating glucose to fuel the body functions.

Maintenance of glucose homeostasis is of utmost importance to human physiology, being under strict hormonal control. Failure of this control can result in the metabolic syndrome, a multi-symptom disorder of energy homeostasis encompassing obesity, hyperglycemia, impaired glucose tolerance, hypertension and dyslipidemia [9]. The most characteristic abnormality in the metabolic syndrome is insulin resistance, which results from interactions between genetic and environmental factors, including diet and sedentary lifestyle [10,11]. Metabolic

syndrome is the major predisposing factor to type 2 diabetes, where defects in both insulin action and insulin secretion are present, but their relative contribution varies individually. The disturbance of glucose metabolism is often related to the increase of fat mass, especially in the abdominal area and ectopically, to the tissues where fat is not stored in normal energy homeostasis [12]. This in turn results in inflammation and exacerbated oxidative stress at the whole body level, and malfunction in several organs including pancreas, liver, muscle and adipose tissue [13].

The prevalence of type 2 diabetes is rising exponentially, estimated to reach over 300 million cases by year 2030 [14]. Presently, the treatment of metabolic syndrome and prevention of type 2 diabetes involves lifestyle modifications like increased physical activity and weight control by reduced caloric intake [15,16]. Increasingly, the dietary recommendations for individuals at risk of type 2 diabetes emphasise the intake of plant food products, such as whole grains, berries, fruits and vegetables, all known to be excellent sources of dietary fibre, but also good sources of variable polyphenolic compounds. These compounds may influence glucose metabolism by several mechanisms, such as inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β–cells, modulation of glucose release from liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Figure 1).

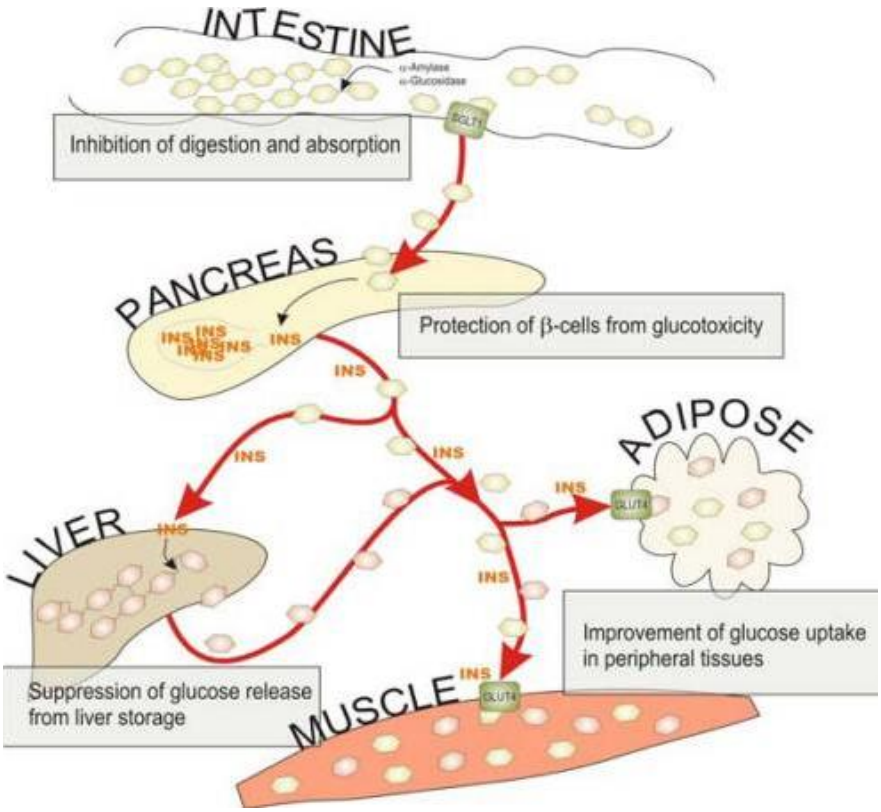

Figure 1: *Potential sites of action of dietary polyphenols on carbohydrate metabolism and glucose homeostasis.*

Dietary polyphenols are found in distinctive combinations of metabolites from different chemical classes. The biochemical properties and resulting health-beneficial bioactivities in different plant groups or even different species are thus discrete, having different impact on different health conditions [3]. In terms of metabolic syndrome and type 2 diabetes, the up-to-date most extensively studied plants and metabolites include soy, that is one of the few edible plants having high concentrations of isoflavonoids [17]; tea, mainly for condensed tannins, in particular epigallocatecin gallate [18]; coffee, for phenolic acid content [19]; grape especially for the presence of resveratrol [20]; apple for rich flavonoid content [21] and several herbs often possessing highly distinct

phytochemical profiles, e.g., high content of terpenoids [22]. Also different berry species like cranberry, strawberry and blueberry have been addressed to possess capacity to protect from diabetes, and the studies have most often focused on the anthocyanin metabolite class [23]. Similarly, the whole grain products are intensively studied not only for the high fibre content but also for the rich phenolic compound repertoire that may have beneficial effect on glucose homeostasis [24]. Whilst the results from dietary human interventions are still scarce, there is a wealthy of data published with different diabetic animal models. The most common ones are rat and mice models with diet-induced diabetes, thereby resembling the type 2 diabetes in humans, and the models with destruction of pancreas by allozan or streptotozin treatment resulting in insulin deficiency. Various *in vitro* studies have been performed by different cell lines of adipose, hepatic, pancreatic and myotube origin.

This review will demonstrate the potential of dietary phenolic phytochemicals in maintenance of glucose and energy homeostasis and in suppression of metabolic syndrome and type 2 diabetes as evidenced by rapidly expanding literature. However, the antioxidant role of these compounds in metabolic syndrome, extensively reviewed recently [25,26], is not discussed herein.

2. Influence of Polyphenols on Carbohydrate Digestion and Glucose Absorption in the Intestine

Food and beverages high in available carbohydrates such as starch or sucrose induce postprandial hyperglycemia, hyperinsulinemia and other hormonal and metabolic disturbances. The rapid absorption of glucose challenges the regulatory mechanisms of glucose homeostasis, and habitual consumption of high-glycemic diets may therefore increase the risk for obesity, type 2 diabetes and cardiovascular disease [27]. Carbohydrate digestion and glucose absorption are obvious targets for better glycemia control after high-carbohydrate meals. α-Amylase and α-glucosidase are the key enzymes responsible for digestion of dietary carbohydrates to glucose. The liberated glucose is absorbed across the intestinal enterocytes *via* specific transporters. Inhibition of the digestive enzymes or glucose transporters would reduce the rate of glucose release and absorption in the small intestine and consequently suppress postprandial hyperglycemia.

2.1. Carbohydrate Digestion

Starch is composed of amylose, which is a linear α-1,4-linked glucose polymer, and highly branched amylopectin consisting of linear α-1,4-linked glucose chains with α-1,6-linked branch chains. Salivary and pancreatic α-amylases catalyze the endo-hydrolysis of α-1,4-glucosidic linkages releasing mainly maltose, maltotriose and related α-1,6-oligomers. Further digestion takes place in the small intestinal brush border by α-glucosidases, which hydrolyze the terminal α-1,4-linked glucose residues as the final step in the digestion of dietary carbohydrates to release glucose. The α-glucosidase activities, first described as maltases, are associated with maltase-glucoamylase and sucrase-isomaltase [28–30]. In addition to α-1,4-glucosidic activity, sucrase-isomaltase displays specific activities against the α-1,2 linkages of sucrose and α-1,6 linkages of isomaltose.

A variety of polyphenols have been shown to inhibit α-amylase and α-glucosidase activities *in vitro*. The inhibitory polyphenols include flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanidins and ellagitannins). In addition, *in vitro* inhibitory activities have been reported for polyphenolic extracts of foods, including berries (strawberries, raspberries, blueberries and blackcurrants), vegetables (pumpkin, beans, maize and eggplant), colored grains such as black rice, green and black tea, and red wine. In the studies, maltose, sucrose or *p*-nitrophenyl-α-d-glucopyranoside have been used as substrate for α-glucosidase activity.

2.2. Glucose Absorption

Intestinal absorption of glucose is mediated by active transport *via* the sodium-dependent glucose transporter SGLT1 and by facilitated sodium-independent transport *via* the glucose transporter GLUT2 [31,32]. On the luminal side of the intestinal brush border membrane, two Na^+ ions bind to SGLT1 and produce a conformational change

that permits glucose binding, followed by another conformational change to allow glucose and Na^+ to enter the enterocyte. Glucose is released from the enterocyte *via* GLUT2, a high capacity facilitative transporter in the basolateral membrane, to enter the circulation.

The influence of polyphenols on glucose transporters has been studied *in vitro* by using intestinal brush border membrane vesicles or everted sacs and Caco-2 cells. Several flavonoids and phenolic acids have been shown to inhibit glucose transport (Table 2). The Na^+-dependent SGLT1-mediated glucose transport was inhibited by chlorogenic, ferulic, caffeic and tannic acids [33], quercetin monoglucosides [34], tea catechins [35–37] and naringenin [38]. The glucose transport by GLUT2 was inhibited by quercetin, myricetin, apigenin and tea catechins [37,39].

2.3. Postprandial Glycemia

Effects of polyphenols, polyphenolic food fractions, and foods and beverages rich in polyphenols on postprandial blood glucose responses have been investigated in animal models and in human studies. Either glucose, maltose, sucrose, starch or various meals have been used as the carbohydrate challenge.

Animal studies. Diacylated anthocyanin as well as an anthocyanin extract from purple sweet potato reduced the blood glucose and insulin responses to maltose administration in rats [40]. The lack of effect after sucrose or glucose administration indicates that the anti-hyperglycemic effect was achieved by maltase inhibition, and not by inhibition of intestinal sucrase activity or glucose transport. Also a tea polyphenol, theaflavin 3-O-gallate, was effective in suppressing the postprandial glucose response to maltose [41].

A crude Acerola polyphenol fraction (containing anthocyanins) significantly reduced the plasma glucose level after administration of maltose or glucose in mice, suggesting inhibition of α-glucosidase and intestinal glucose transport [42]. A leaf extract of *Nerium indicum*, a plant used as a folk remedy for type 2 diabetes in Pakistan, was found to reduce the postprandial rise in blood glucose in maltose-or sucrose-loaded rats [43]. A similar response was obtained with chlorogenic acid, which was identified as the major α-glucosidase inhibitor in the leaf extract.

Gingko biloba extracts and their flavonoid fraction reduced the elevation of rat plasma glucose level after oral administration of starch, maltose, sucrose or glucose [44]. Also in diabetic rats, the flavonoid fraction attenuated the glucose response to sucrose and glucose administration. When diabetic rats were administered glucose with quercetin, hyperglycemia was significantly decreased compared to administration of glucose alone [39].

Human studies. Apple juice contains polyphenols such as chlorogenic acid and phloridzin, with higher levels in cloudy juice compared to clear juice. When nine healthy subjects consumed a 25 g glucose load in 400 mL of commercial apple juices, the mean plasma glucose concentrations were significantly lower at 15 and 30 min after ingestion of clear apple juice, and significantly lower at 15 min but significantly higher at 45 and 60 min after ingestion of cloudy apple juice compared to control drink [45]. The effects of apple juices on plasma glucose, insulin, GIP and GLP-1 concentrations were consistent with delayed absorption of glucose.

Berries are rich sources of polyphenols, especially anthocyanins, flavonols, proanthocyanidins and phenolic acids. In twelve healthy subjects, ingestion of sucrose (35 g) with berries (150 g of purée made of bilberries, blackcurrants, cranberries and strawberries providing nearly 800 mg polyphenols) produced a different postprandial glycemic response compared to the control without berries but with comparable profile of available carbohydrates [46]. The shape of the plasma glucose curve with reduced concentrations in the early phase and a slightly elevated concentration in the later phase indicates delayed response due to berry consumption. Berries also significantly decreased the peak glucose increment. Reduced rates of sucrose digestion and/or absorption from the gastrointestinal tract are the most probable mechanisms underlying the delayed and attenuated glycemic response. In another study, consumption of cranberry juice sweetened with high-fructose corn syrup resulted in different (but not statistically significant) pattern of postprandial glycemia compared to the similar amount of the sweetener in water [47].

In ten type 2 diabetic patients, red wine (200 mL) taken during a midday meal induced a smaller increase in blood glucose *versus* the same meal accompanied by an equivalent amount of water, with no effect of plasma insulin levels [48]. Comparable results were obtained with tannic acid, a polyphenolic component of red wine. Ethanol had no effect. In ten healthy young adults, sugar cane bioflavonoid extract reduced the postprandial glycemic response to a high-glycemic starchy meal composed of wheat biscuits and milk [49]. Ingestion of cinnamon (6 g) with rice pudding significantly lowered blood glucose response in the postprandial phase (15, 30 and 45 min) in 14 healthy subjects [50,51]. However, in another study of the same group [51], cinnamon (3 g) reduced postprandial serum insulin and increased GLP-1 concentrations without significantly affecting blood glucose response. Cinnamon has high content of proanthocyanidins.

Gastrointestinal hormone (GIP and GLP-1) profiles after consumption of 25 g glucose with coffee (400 mL containing 350 mg chlorogenic acid) indicated delayed intestinal absorption of glucose in nine healthy subjects [52]. The authors concluded that chlorogenic acid, the major polyphenol of coffee, might attenuate the intestinal glucose absorption rates and shift the site of glucose absorption to more distal parts of the intestine. In overweight men, chlorogenic acid (1 g) reduced early glucose and insulin responses during an oral glucose tolerance test [53]. Attenuated glycemic response has also been observed when sucrose (25 g) was consumed in chlorogenic acid enriched instant coffee [54].

When either 250 mL of coffee or tea was consumed with test meals, they increased the overall mean peak blood glucose concentration, but did not significantly affect the incremental area under the glucose response curve of the meals [55]. Coffee and tea contain caffeine, which increases postprandial glycemia and impairs glucose tolerance [52,56,57]. Caffeinated coffee ingested with either a high or low glycemic meal significantly impaired acute blood glucose management and insulin sensitivity compared with ingestion of decaffeinated coffee [57]. Instant black tea consumed with glucose reduced the late phase plasma glucose response with a corresponding increase in insulin [58]. The attenuation of late postprandial glycemia may be explained by an elevated insulin response following stimulation of pancreatic β-cells rather than by retarded absorption of glucose.

In conclusion, the scientific evidence on the potential of polyphenolic compounds to retard carbohydrate digestion and absorption and to suppress hyperglycemia in the postprandial state is promising. However, it is mostly based on simple *in vitro* and animal studies. Current evidence from human studies suggests that beverages such as apple juice, red wine and decaffeinated coffee as well as berries and cinnamon may improve short-term glycemic control. For substantiation of the benefits of polyphenols in the control of postprandial glucose homeostasis, more clinical studies involving subjects with normal and impaired glucose metabolism are needed. These studies should be focused on the effects of dietary polyphenols on glycemic responses induced by starch and sucrose, the main high-glycemic carbohydrates in our diet.

3. Influence of Polyphenols on Pancreatic β-cell Function

Insulin secretion by the pancreas involves numerous reactions which are potential targets for the action of polyphenols. Upon high blood glucose concentrations pancreatic β-cells respond to the increased demand of insulin by various mechanisms including increased insulin secretion, hypertrophy, proliferation of existing β-cells and formation of new ones from progenitor cells. The insulin release from β-cells is a cascade starting from the uptake of glucose by the GLUT2 transporters. Glucose enters a cycle of enzymatic reactions involving phosphorylation, leading to increased ATP content in the cells, and causing inactivation of ATP-sensitive potassium channels on the cell membrane. The membrane depolarizes and leads to calcium channel opening and subsequent flow of Ca^{2+} into cell. The rise in Ca^{2+} concentration promotes release of insulin by exocytosis from existing storage granules [59,60].

Prolonged hyperglycemia and hyperlipidemia, typically within development of metabolic syndrome, leads to the dysfunction of the pancreatic β-cells, reflected in autocrine insulin resistance, impaired insulin secretion, decreased expression of genes involved in insulin production and finally decrease in β-cell mass caused by

apoptosis. Therefore the insulin deficiency related to metabolic syndrome in pancreas is due to both the cellular damage and the impaired efficiency in the synthesis of insulin [61].

The most extensively studied sources of dietary polyphenols in terms of pancreatic function and insulin secretion is soy, and especially its isoflavonoids, genistein and daidzein. The most commonly applied approaches in determining the effect of polyphenols on pancreatic insulin metabolism are measurement of insulin secretion or/and content in cultured pancreatic cell lines, either with or without glucose stimulation, and examination of perfused pancreas either after feeding trial/intraperitoneal injection or by directly applying the compound of interest on the isolated islets. Many of these studies, reviewed below and summarized in Tables 1 and and2,2, examine also the molecular mechanisms behind the observed effects of polyphenols.

3.1. *In Vivo* Studies with Animal Models

There are few recent studies where soy isoflavonoids at physically achievable concentrations have shown positive impact on β-cell function. Choi *et al.* [62] used genistein and daidzedin in order to study factors related to glucose and insulin metabolism using a non-obese diabetic mouse model which spontaneously develops autoimmune insulin dependent diabetes mellitus. Both isoflavonoids (0.2 g/kg genistein or daidzein for nine weeks) preserved the insulin production by the β-cells, whereas mice fed the control diet had no insulin production [62]. Another *in vivo* study performed in non-obese mice (streptozotocin (STZ) induced diabetic model) fed with fermented soybean, a Korean food 'chungkukjang' (5 g/100 g of diet for 6 weeks), similarly showed that the insulin concentration in pancreas was higher in the soybean- fed mice than in the non-treated control mice. In addition to enhancing the insulin production in pancreas the treatment also seemed to contribute to improved insulin sensitivity in peripheral tissues, thus necessitating smaller amounts of insulin and preventing pancreatic exhaustion [63]. The same line in results was obtained also by Lu *et al* on high-isoflavone soy protein fed STZ-diabetic rats [64].

3.2. Effects Observed in Cell Culture Analyses

Epigallocatechin gallate (EGCG) and rutin were examined for their ability to attenuate the glucotoxicity in rat insulinoma pancreatic β-cells (RIN m5F) [65]. The treatment increased glucose dependent insulin secretion, and was able to promote effective secretion of insulin also under chronic high glucose incubation when insulin secretion is suppressed by glucotoxicity (33 mM, 48 h), suggesting that both EGCG and rutin might preserve the glucose- sensing ability during hyperglycemia. EGCG and rutin elevated the intracellular ATP, suggesting that the increase in insulin secretion is mediated by enhancing the normal, glucose induced insulin secretion that is dependent on ATP concentrations. Interestingly, epicatechin, the precursor of EGCG, was found to inhibit insulin secretion when tested on INS-1 cells [66].

A very detailed study on the effects of dietary phenolic acids on pancreas function was carried out with cinnamic acid derivatives in INS-1 cell culture and perfused rat pancreas [67]. Among the differentially substituted cinnamic acid derivatives, the most prominent insulin releasing agents were the ones containing m-hydroxy and p-methoxy residues on the phenol ring structure, whereas cinnamic acid (no substituents in the phenol ring) was inactive. The structure promoting insulin secretion most effectively was the one of ferulic acid, containing p-hydroxy and m-methoxy structure, as it enhanced insulin secretion in a dose-dependent manner (1–100 μM), being significant already at 1 μM concentrations. Notably, the assays were performed in absence of glucose, whereas the majority of other reports have focused on glucose dependent insulin release. The results were verified also with treatment of perfused rat pancreas and intravenous administration in normal rats, where the increase in plasma insulin was detected in fasting state. Interestingly, isoferulic acid, the stereoisomer of ferulic acid did not have any effect on insulin releasing properties. This finding corroborated earlier results showing that plasma glucose lowering properties of isoferulic acid are due to increase in glucose uptake and retarding of hepatic gluconeogenesis, without any effect on pancreatic insulin output [68]

In one of the most recent studies isoflavonoids were shown to improve glucose stimulated insulin secretion in INS-1E pancreatic cell line but this effect was not due to modulation of insulin synthesis, since there was no difference on the insulin concentration in the genistein treated and non-treated cells. However, the insulin secretion upon glucose stimulation was significantly increased after 48h pre-treatment with genistein (1–5 μM). It was suggested that the effect of genistein on promoting glucose dependent insulin secretion was not mediated by the same mechanism as glucose stimulation alone, since several cellular factors related to glucose-induced insulin secretion, e.g., cellular ATP concentration, were not changed. The finding that cellular Ca^{2+} levels were elevated by the genistein treatment suggests that the improvement in insulin secretory function may be attributable to modulation of Ca^{2+} signaling and cAMP/protein kinase A (AMPK) function, but the mechanism is not yet clear [69]. The effect of genistein on insulin secretion was observable also in mouse and human pancreatic islets showing non-species-specific and biologically relevant effect.

Also numerous other publications report on the insulin secretagogic activities of dietary phenolics e.g., anthocyanidin and anthocyanin compounds in INS-1 cell line [70], aspalathin, component from rooibos tea *Aspalathus linearis*, in RIN-5F cells [71], and compounds isolated from *Eriobotrya japonica* in INS-1 Cells [66].

There are also indications for the function of polyphenols on β-cells by other mechanisms besides affecting insulin secretion. Ethanol extracts from the root, stem, leaf and fruit of the Canadian lowbush blueberry *Vaccinium angustifolium*, a very rich source of flavonoids, were analyzed for insulin secretagogue and proliferative effects [72]. The insulin secretion was measured from growth arrested (tetracycline-treated) β-cells in order to distinguish the insulinotropic effect from the cell proliferative effect. Only slight enhancement was observed in the glucose stimulated insulin secretion with the treatment by leaf and stem extracts, but the effect on the cell proliferation rate was found to be significantly increased by the treatment with the fruit extract when compared to vehicle-only control, suggesting a potential capability to restrain β-cell damage in metabolic syndrome.

Another study showing β-cell protective effect of flavonoids was performed by mixtures of flavonoids quercetin, luteolin and apigenin in RINmF5 cells [73]. Flavonoids showed anti-inflammatory action in a treatment with interleukin 1β (IL-1β) and interferon γ (IFN- γ), and the effect was verified at transcriptional analysis of inflammation-related genes, suggesting a role for flavonoids in the restoration of insulin secretion capacity by preventing the cytokine-induced β-cell damage.

3.3. Effects Observed in Isolated/Perfused Pancreas

Oral administration of rutin (100 mg/kg, 45 days) was shown to promote β-cell viability in STZ induced diabetic rats [74]. It was suggested that the β-cell restoring effect of rutin was due to enhanced ability to scavenge free radicals and mediate antioxidant enzyme activity in the pancreas. Similarily, quercetin, the aglycon molecule of rutin, showed β-cell restoration when used as dietary supplement (0.5% of diet for 14 days) in STZ induced diabetic mice [75]. Gene expression analysis showed that quercetin restored the cell proliferation capacity inhibited by STZ treatment, and resulted in higher plasma insulin levels. In addition oxidative stress markers were reduced in pancreas, further ameliorating the oxidative damage associated with diabetes. Quercetin has been studied also in STZ-diabetic rats by intraperitoneal injection, and the preservation of islet cells and restoration of insulin production has been observed in two studies [76,77].

Intraperitoneal injection of (−)epicatechin in alloxan treated mice demonstrated β-cell- regenerative capacity [78]. Similarily, (−)epicatechin or quercetin promoted increased release of insulin when isolated rat islets were exposed to them, whereas naringenin and chrysin inhibited it [79]. Additional observations with dietary sources of polyphenols include the protection of non-obese diabetic mice pancreatic islets from infiltration of immune cells and induction of insulitis by feeding grape powder and high vitamin A supplement [80]. An interesting approach was taken to study olive mill waste which is a rich source of phenolic compounds, especially phenylethanol compound hydroxytyrosol. Fractions of olive mill waste were studied for a range of hypoglycemic and antioxidative effects, including the effect in insulin secretion in alloxan- induced diabetic rats administered by

intraperitonial injection. Mainly the purified hydroxytyrosol fraction showed protective action on alloxan-damaged β-cells [81].

Phytochemical- rich extracts from other than dietary plants have also been studied for their impact on pancreatic insulin production and release. Studies have focused especially on medicinal plants known for their anti-diabetic effects. Seed extracts of *Eugenia jambolana* enhanced insulin secretion from isolated islets of STZ-induced diabetic rats in the presence of 10 mM glucose [82]; eupatilin, a flavone from *Artemisia princes*, elevated pancreatic insulin concentration in type 2 diabetic mouse model (db/db) [83]; and aqueous extract from *Abutilon indicum*, a plant used as traditional medicine in Thailand, stimulated insulin secretion from isolated rat islets and INS-1E cells [84]. A fraction containing apigenin and rutin from *Teucrium polium*, a medicinal plant from Iran, mediated insulin secretion increase in the presence of STZ on isolated rat pancreatic islets [85].

In conclusion, it is obvious that the pancreas is one of the targets of dietary polyphenol bioactivity, as several of the studied plant extracts and purified compounds exhibit beneficial effects on β-cell function and insulin release in different diabetic models. However, no single mechanism has been identified to be responsible for the response. For instance, in INS-1E cells genistein did not increase the level of intracellular ATP upon the glucose stimulation, whereas treatment of the RIN-m5F cells with EGCG and rutin elevated the ATP level [65]. This suggests that the latter treatment enhanced the signaling route mediated normally by glucose, whereas the genistein treatment had effect on alternative mechanism of insulin secretion. A range of different compounds and plant food extracts studied show various activities relevant for insulin secretion, and the activities are different on normoglycaemic controls and the subjects with symptoms of metabolic syndrome. The different effects of various molecules were highlighted in a study showing that even small changes (e.g., hydroxylation) on the molecular backbone result in different insulin- releasing capacity [67]. The studies have been made mainly using cell cultures and animal models, and motivate to proceed to human controlled trials.

4. Influence of Polyphenols on Tissue Uptake of Glucose

Dietary polyphenols may also influence glucose metabolism by stimulating peripheral glucose uptake in insulin-sensitive and non-insulin sensitive tissues. Glucose transport pathways can be classified either as insulin or non-insulin mediated pathways. Non-insulin mediated glucose uptake takes place in all tissues and is responsible for the basic glucose transport into the cells in post-absorptive state. In contrast the insulin mediated glucose uptake takes place only in insulin sensitive tissues. Insulin stimulates the glucose uptake in skeletal muscle, which is the largest site for disposal of dietary glucose, and in adipose tissue, whereas in the liver it decreases the hepatic glucose output rate by increasing the storage of glucose as glycogen.

Glucose uptake is mediated by the action of glucose transporters (GLUTs) on the cell surface [86]. It is important to point out that among the 13 GLUTs identified so far [87], only GLUT4 is an insulin sensitive glucose transporter. Based on sequence comparison, the GLUT isoforms can be grouped into three classes. Class I comprises GLUT1–4; class II, GLUT6, 8, 10, and 12 and class III, GLUT5, 7, 9, 11 and H^+-myo-inositol cotransporter (HMIT) [88]. Tissue- and cell-specific expression of the well-characterized GLUT isoforms underlies their specific role in the control of whole-body glucose homeostasis. Numerous studies with transgenic or knockout mice support an important role for these transporters in the control of glucose utilization, glucose storage and glucose sensing, but more studies are needed to elucidate the mechanisms behind.

Glucose transporters from class I are actively involved in glucose mobilization and uptake. GLUT1 and GLUT3 are responsible for maintaining the basal glucose uptake, and contrary to GLUT4 are abundant in several tissues [89]. GLUT1 is widely distributed in fetal tissues and it is expressed at high levels in erythrocytes and endothelial cells of barrier tissues in adults, while GLUT 3 is mostly expressed in neurons and placenta. Glucose is transported into and out of liver cells by the concentration-driven GLUT2 [90], which is also expressed by renal tubular cells, small intestinal epithelial cells that transport glucose and pancreatic beta cells. GLUT4 is expressed by muscle, adipose

and kidney cells and remains stored in insulin-responsive compartments within the cells until insulin mediates its localization on the cell surface.

The most studied insulin signalling pathway leading to increased muscle glucose uptake involves binding of insulin to GLUT4, phosphorylation of downstream insulin receptor substrates (IRS) and activation of several signalling enzymes such as phosphatidylinositol-3 kinase (PI3K) and Akt-serine/threonine kinase. The cascade promotes GLUT4 glucose transporter translocation from an intracellular pool to the plasma membrane [91,92]. In addition to PI3K activity, there are also other signalling routes involved in the cellular response to insulin stimulation and a detailed overview of the basic insulin signalling and regulation of glucose metabolism was reviewed some years ago by Saltiel and Kahn [93]. In this sense, a molecular mathematical model of glucose mobilization and glucose uptake has been recently developed considering the kinetics of GLUT2, GLUT3 and GLUT4, the process of glucose mobilization by glycogen phosphorylase and glycogen synthase in liver, as well as the dynamics of the insulin signalling pathway [90].

Among the potential compounds stimulating glucose uptake, several foods and plant extracts rich in polyphenols have been the object of extensive research during the last years (Tables 1 and and2).2). The methods most commonly used to study the effects of phenolic compounds on peripheral glucose uptake are cell culture assays in rat skeletal muscle (rat L6 myotubes) and adipose (3T3-L1) cell lines. Most studies reported in the literature so far base their glucose uptake mechanisms in insulin mediated pathways, mainly cAMP/protein kinase A (AMPK) and PI3K activation. The insulin-stimulated glucose uptake shows to be dose-dependent in most cases.

4.1. Effects of Pure Compounds on Glucose Uptake

Chlorogenic acid and ferulic acid caused a modest, but significant increase in 2-deoxy-d-glucose transport into L6 myotubes, showing comparable performance to metformin and 2,4-thiazolodinedione, two common commercial oral hypoglycemic drugs [94]. Purified aspalathin from green roiboos extract increased dose-dependently and significantly glucose uptake by L6 myotubes at concentrations 1–100 μM, irrespective of insulin absence [71]. As aspalathin is capable of scavenging intracellular reactive oxygen species (ROS), its antioxidative function may be involved in the stimulation of glucose uptake and insulin secretion, and hence glucose homeostasis. An inhibitory effect of EGCG was observed in L6 skeletal muscle cells on insulin resistance induced by dexamethasone, a glucocorticoid [95]. A 24 h- treatment with EGCG attenuated the effect of dexamethasone on glucose uptake and improved insulin-stimulated glucose uptake in a dose-dependent manner by increasing GLUT4 translocation to plasma membrane [95]. EGCG was able to increase the phosphorylation of AMPK, suggesting that the AMPK signalling pathway is likely responsible for the EGCG-stimulated GLUT4 translocation.

Resveratrol increased glucose uptake in C2C12 skeletal muscle cells by activating AMPK [96]. In the absence of insulin, the effect of resveratrol on glucose uptake was primarily dependent on AMPK activation, without involving PI3K. In the presence of insulin, resveratrol also potentiated the effect of insulin on glucose uptake *via* AMPK activation, but leading to activation of the PI3K-Akt signal pathway [96]. Resveratrol treatment during 15 weeks increased both insulin-stimulated whole-body and steady-state glucose uptake of both soleus muscle and liver in high cholesterol-fructose-fed rats [97]. It enhanced membrane trafficking activity of GLUT4 and increased phosphorylation of IR in insulin-resistant soleus muscles. Interestingly the activation of estrogen receptor seems to be crucial for resveratrol-stimulating muscular glucose uptake *via* both insulin-dependent and –independent pathways [97]. Additional putative function for resveratrol was found in a study reporting that Akt/protein kinases B (PKB) and GLUT4 or GLUT1 translocation is not involved in resveratrol activation. The mechanism seems to involve sirtuin-dependent AMPK activation that may lead to stimulation of the intrinsic activity of GLUT4 [98]. Sirtuins are a family of histone/protein deacetylases, among which, SIRT1 has been suggested to play a role in regulating glucose homeostasis and may be involved in the insulin signalling cascade [99,100].

Kaempferol and quercetin isolated from the traditional Chinese medicine *Euonymus alatus* improved glucose uptake of insulin stimulated 3T3-L1 mature adipocytes and had no effects on GU without insulin [101]. The results

indicated that both flavonoids could ameliorate insulin resistance peripherally, similar to a PPARγ agonist such as rosiglitazone. Kaempferol 3-neohesperidoside, a flavonoid glycoside isolated from Cyathea phalerata, stimulated glucose uptake in rat soleus muscle mainly *via* the PI3K pathway [102]. Another kaempferol derivative, kaempferitrin (3,7-dirhamnoside), has recently been shown to inhibit GLUT4 mediated glucose uptake in differentiated 3T3-L1 cells by interfering with insulin signaling pathway and also by directly interacting with membrane GLUT4 [103]. Contradictory, at the same time other authors have found opposite results for kaempferitrin treatment of the same cell line, demonstrating increase in the glucose uptake [104]. The latter results agreed with the glucose uptake stimulation by kaempferitrin found in rat soleus muscle [105]. This suggests that the effect of kaempferitrin on insulin mediated glucose uptake might be a cell type specific function. Inhibitory effect on glucose uptake has been observed in adypocyte cells also by the isoflavone genistein with concentrations 20–50 μM [106].

4.2. Effects of Polyphenol Containing Foods and Plant Extracts on Glucose Uptake

Several plant based foods and extracts have been reported to enhance glucose uptake *in vitro.* A green tea polyphenolic extract was reported to regulate the expression of genes involved in glucose uptake and insulin signalling pathways in the muscle tissue from rats with metabolic syndrome induced by a high fructose diet [107]. The tea extract significantly increased the mRNA levels of GLUT4 in the muscle. A procyanidin extract from grape seed has been reported as an insulinomimetic agent since it stimulates glucose uptake in 3T3-L1 adipocytes and L6E9 muscle cells *via* PI3K – pathway [108]. A more detailed study with same approach showed recently that the grape seed extract interacts with the insulin receptor inducing its phosphorylation and consequently leading to increased glucose uptake *via* pathway requiring Akt. However, the treatment leads to differential phosphorylation of the insulin signalling pathway proteins than insulin does [109].

Fruit juice extract of *Momordica charantia* (bitter melon) was shown to stimulate glucose and amino acid uptakes into L6 muscle cells in a similar manner to insulin [110]. Pharmacological concentrations had inhibitory effects, while physiological concentrations had insulin-like stimulating effects, a finding that points out the importance of the concentration of the bioactive compounds in stimulating glucose uptake into muscle cells. Water-soluble components in bitter melon also enhanced the glucose uptake at sub-optimal concentrations of insulin in 3T3-L1 adipocytes, which was accompanied by an increase in adiponectin secretion [111]. Charantin, steroid, glycosides, flavonoids and their derivatives may in part be responsible for the observed up-regulatory activities of glucose uptake and mRNA expression of GLUT4, PI3K and PPARγ in bitter melon extracts but more research is needed to confirm this statement [112]. Another study on the effect of fruit juices on the glucose uptake was performed with blueberry juice. The biotransformation of the juice with a novel strain of bacteria isolated from the blueberry flora (*Serratia vaccinii)* increased its phenolic content and antioxidant activity [113] and modified its biological activity [72]. The juice extract increased AMPK phosphorylation and glucose uptake in both muscle cells and adipocytes, but it also inhibited adipogenesis [114].

Common spices, such as cinnamon, cloves, turmeric and bay leaves also show insulin-like activity *in vitro* [115]. For instance, cinnamon polyphenols with doubly linked procyanidin type-A polymers appear to be unique for their insulin- like activity [115]. A water-soluble cinnamon extract showed to increase the activity of autophosphorylation of the IR and decrease the activity of tyrosine phosphatase *in vitro* [116]. The mechanism of cinnamon's insulin-like activity may be in part due to increases in the amounts of IRβ and GLUT4 [117]. *In vivo* insulin-regulated glucose utilization was also enhanced by cinnamon extracts by increasing glucose uptake in rats with insulin resistance induced by a high-fructose diet [118,119].

Several plant extracts from plants used in traditional medicine have been as well reported to promote insulinotropic / insulinomimetic activities. Four isoflavonoids (genistein-derivatives), recently identified from a branch extract fraction of the Vietnamese traditional herb *Tetracera scandens*, exhibited significant glucose uptake activity both in basal and insulin-stimulated skeletal muscle cells in a dose-dependent manner. AMPK

activation and GLUT4 and GLUT1 expressions appear to be involved in the glucose uptake stimulation mechanism [120]. A recent review has also reported that penta-galloyl-glucose (PGG), a polyphenolic compound highly enriched in a number of medicinal herbals, exhibits multiple biological activities relevant in diabetes prevention [95]. Both β-PGG and its anomer α-PGG have showed insulin-mimicking activity in the absence of insulin, and α-PGG was more potent than β-PGG [121]. α-PGG itself stimulated glucose uptake in 3T3-L1 adipocytes. However, α-PGG weakened the activity of insulin if treated together. α-PGG induced phosphorylation of the IR, PI3K and Akt, and stimulated membrane translocation of GLUT4. Plant root extracts can also exert glucose uptake enhancement properties. For example, the aqueous extract of *Canna indica* root (Cannaceae), rich in flavonoid compounds, caused a dose- and time- dependent induction of glucose uptake activity in L8 muscle cells [122]. The authors suggested that GLUT1 protein synthesis and the activation of PI3K are critical for the increase in glucose transporter activity at the plasma membrane.

In conclusion, insulin stimulates glucose uptake in skeletal muscle and adipose tissue primarily by eliciting the translocation of GLUT4 from an intracellular pool to the plasma membrane [123]. Current data suggest that polyphenols mainly affect glucose transport and insulin-receptor function, both of which play essential roles in diseases related to carbohydrate metabolism [124]. To date glucose uptake data from polyphenols mainly derives from animal cell culture studies. The most likely mechanism implies the PI3K activity signaling route. Recent studies use amounts of phenolic compounds closer to physiological range. However, doses of relevance to human health are still unknown, and deserve further research.

5. Influence of Polyphenols on Liver Function to Maintain Glucose Homeostasis

Liver plays a major role in the regulation of blood glucose levels in tight cooperation with peripheral tissues. As estimated, liver is responsible of taking up one third of the postprandial glucose [125], and stores effectively glucose as glycogen *via* glycogenesis. In fasted state, liver is the main regulator of maintaining stable blood glucose levels and produces glucose by two different routes either by breaking down glycogen (glycogenolysis) or by synthesising glucose from other metabolites such as pyruvate, lactate, glycerol and amino acids (gluconeogenesis). The key enzymes responsible for the regulation of glycogenesis are glucokinase (GK) and glycogen synthase (GS). Pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase, and glucose-6-phosphatase are the major enzymes responsible of the regulation of gluconeogenesis [126].

Several factors influence hepatic glucose homeostatic control. At hormonal level insulin and glucagon directly regulate hepatic glucose metabolism. For instance, in fed state insulin suppresses liver glucose production and output *via* insulin receptor pathway [127]. Furthermore, the central nervous system mediates part of the effects of insulin and of other signals such as long chain fatty acids (LCFAs) to exert higher control on hepatic glucose metabolism [128,129]. In type 2 diabetes and insulin resistant state the control of hepatic glucose metabolism and hepatic glucose output are disturbed, and the inability of the liver to respond to insulin results in severe defects in the regulation of glucose homeostasis such as increased hepatic glucose output and hyperglycemia. Non-alcoholic hepatic steatosis, the accumulation of triglycerides in the liver that might lead to fibrosis, is clearly associated with hepatic insulin resistance. However, it is not clear whether insulin resistance causes the excessive accumulation of triglycerides (TG) in liver, or whether the increase in TG itself plays a causal role in the development of hepatic or systemic insulin resistance [130]. In mice, a high-fat diet has been shown to first deteriorate hepatic insulin sensitivity in association with hepatic accumulation of short to medium chain fatty acylcarnitines, prior to affecting peripheral insulin sensitivity [131]. Several studies indicate improved liver glucose and lipid metabolism in normal, obese and diabetic mouse or rat models after treatment with different polyphenol-rich diets. The following section discusses the potential mechanisms of effects of polyphenols on glucose metabolism in liver.

5.1. Effects of Green Tea and Epigallocatechin Gallate (EGCG)

Tea catechins and their effects on liver glucose metabolism have been effectively studied in animal and cell culture models. Green tea extracts and green tea catechins such as epigallocatechin alone have been shown to decrease blood glucose levels and concomitantly also liver triglyceride contents. In streptozotocin-induced diabetic rats oral administration of EGCG (25 mg/kg b.w./day) for eight weeks significantly alleviated the increase in serum glucose levels and serum TG levels [132]. However, the study did not include any tissue specific analyses. Supplementation of the diet with 0.5% and 1.0% green tea for six weeks reduced liver TG concentrations 27–30% in fructose-fed ovariextomized rats as compared to fructose and starch fed control diets [133]. Several other studies have also shown reduced blood glucose levels and liver TG contents after feeding with green tea or EGCG. For instance, supplementation of high-fat diet (60% energy as fat) fed mice with dietary EGCG (3.2 g/kg diet) for 16 weeks resulted in decreased blood glucose levels and decreased liver TG contents [134].

The potential mechanisms explaining how liver could contribute to the reduced blood glucose levels in green tea and EGCG treated animal models have been studied as well. Wolfram *et al*. [135] assessed glucose and insulin tolerance in db/db mice and investigated the effect of 5–7 weeks EGCG supplementation on gene expression in liver tissue using real-time quantitative PCR (RT-PCR). EGCG supplementation (2.5–10.0 g/kg) resulted in decreased blood glucose levels in a dose dependent manner as tested by OGTT. In the fed state plasma glucose, free fatty acid and TG levels were lower and insulin levels higher in EGCG-treated db/db mice than in control mice. EGCG treatment increased the expression of liver glucokinase (glycogenic enzyme), carnitine palmitoyl transferase-1β and decreased the expression of gluconeogenetic enzymes phosphoenolpyruvate carboxykinase (PEPCK). The authors suggested that the potential mechanisms to explain the T2DM amelioration by the dietary supplementation of EGCG could be the reduced endogenous liver glucose production and increase in glucose-induced insulin secretion [135]. Furthermore, DNA microarray analysis of H4IIE rat hepatoma cells exposed to EGCG (50μM), showed that genes involved in the synthesis of fatty acids, triacylglycerol, and cholesterol were strongly downregulated, also genes involved in gluconeogenesis were downregulated whereas genes involved in glycogenesis were upregulated.

These findings are in line with cell culture studies that have shown reduced hepatic gluconeogenesis and glucose output after exposure to EGCG or green tea extract [136,137]. For instance, Collins *et al*. [136,137] studied the role of EGCG in hepatic gluconeogenesis using isolated hepatocytes exposed to physiologically relevant concentrations of EGCG (≤1 μM). EGCG decreased glucose production by inhibiting expression of the gluconeogenetic enzymes (PEPCK and glucose-6-phosphatase) in a similar manner to insulin. However, EGCG was not found to activate the insulin signalling pathway. Further tests showed that EGCG activated AMPK, which was shown to be necessary for the observed inhibition of gluconeogenetic enzyme expression. AMPK activation was mediated by the calmodulin-dependent protein kinase kinase CaMKK [136]. Furthermore, ROS production induced by EGCG was shown to be required for the activation of AMPK and inhibition of gluconeogenesis. The study by Collins *et al*. showed that EGCG exerts toxic effects on primary hepatocytes already at concentration of 10 μM. Other studies have found EGCG to have similar effects on hepatic glucose metabolism, though with concentrations exceeding 10 μM [137,138].

5.2. Effect of Soy Isoflavones, Genistein and Daidzein

Similarly to green tea also soy and soy isoflavones genistein and daidzein supplementation (0.2 g/kg) have been found to decrease blood glucose levels and to reduce liver TG concentrations in db/db mice model [139] and in non-obese diabetic mice [62]. Both studies found reduced glucose-6-phosphatase and PEPCK liver activities and increased glucokinase activities suggesting that genistein and daidzein suppresses liver glucose output. Cederroth *et al*. [140] studied the mechanisms behind the effects of soy supplementation rich in equol, daidzein and genistein, in normal CD-1 mice. Phytoestrogen- rich supplementation (198 ppm daidzein and 286 ppm genistein equivalents in the high phytoestrogen diet) from conception to adulthood was found to activate AMPK in liver but also in white adipose tissue and muscle. The authors hypothesised that high-phytoestrogen-fed mice would have

altered mitochondrial metabolism and found that the expression of peroxisome proliferator-activated receptor α (PPARα) and its coactivator peroxisome proliferator-activated receptor γ coactivator (PGC-1α) were upregulated in liver, white adipose tissue and muscle suggesting improved fatty acid β-oxidation [140]. Potentially, in normal (non-obese) mice activation of PPARα could lead to change from glucose utilization to fatty acid oxidation to produce fuels, instead of creating new TGs [141]. Increased fatty acid β-oxidation might protect against non-alcoholic hepatic steatosis and therefore could also improve insulin sensitivity and glucose metabolism in liver. Furthermore, decrease in hepatic TG pools has been shown to correlate with improved insulin sensitivity [130]. However, the role of TGs in the development of insulin resistance is not yet clear. Chungkukjang (a fermented soybean food) supplementation also resulted in significantly higher hepatic GK activity and decreased activity of gluconeogenic enzymes G6Pase and PEPCK in db/db mice when compared to control group [142]. However, also insulin secretion was improved after Chungkukjang supplementation.

5.3. Effect of Citrus Flavonoids, Grape Polyphenols and Phenolic Acids

The citrus flavonoids, hesperidin and naringin (0.2 g/kg) were shown to lower blood glucose levels as compared to the control diet fed to db/db mice [143]. Similarly to green tea and soy, hesperidin and naringin also significantly reduced plasma free fatty acid, TG and total cholesterol levels in plasma as well as hepatic TG content. These physiological changes were postulated to be due to increase in hepatic glucokinase mRNA, decrease in expression of the gluconeogenetic enzymes PEPCK and G6Pase, and improvement in lipid metabolism caused by altered activities of hepatic lipid metabolizing enzymes [143,144]. Furthermore, naringenin (25–100 μM), the aglycone form of naringin, was shown to suppress hepatic glucose production from hepatoma cells in a dose dependent manner even though naringenin did not have any impact on gluconeogenetic gene expression [145]. However, naringenin exposure led to decrease in cellular ATP levels.

Grape seed-derived polyphenols such as procyanidins have been also shown to alleviate insulin resistance in mice fed with high-fat diet. Simultaneous supplementation of grape-seed derived procyanidin-rich extract and *G. pentaphyllum* extract (altogether 80 mg/kg) improved glucose tolerance and HOMA-IR index, as well as lowered the high-fat-diet induced serum glucose levels and also increased the activity of hepatic glucokinase [95].

Unlike polyphenols discussed above, resveratrol was shown to have opposite effects and increase the expression and activity of gluconeogenetic enzymes. As Ganjam *et al*. [146] showed, rats treated with resveratrol (5–10 mg/kg/day) by intraperitoneal injections for 2 days lead to decreased GK mRNA levels in liver in a dose-dependent manner. The decreased GK mRNA expression was accompanied by a reduction in GK protein levels. In primary rat hepatocyte cultures resveratrol (10–50 μM) also suppressed GK expression and conversely enhanced PEPCK expression. The suppression of GK by resveratrol was found to be mediated, at least partly, by the deacetylation of FoxO1-transcription factor and further binding to HNF-4 (hepatocyte nuclear factor) that can restrain it from its binding site in the proximal GK promoter [146]. Similar effects of resveratrol on hepatic glucose metabolism have been shown with H4IIE rat hepatoma cells [147]. However, there are controversial findings showing that activation of SIRT1 was repressing forkhead transcription factors including Foxo1 in different cell models [148]. On the other hand, the results are supported by the fact that knockdown of SIRT1 in liver leads to decrease in gluconeogenesis [149]. The roles of Foxos and sirtuins in the regulation of hepatic glucose metabolism clearly need further clarification.

Administration of phenolic acid fraction of rice bran containing considerable amounts of *trans*-cinnamic acid derivatives (ferulic acid, and *p*-coumaric acid) and ferulic acid alone for 17 days was shown to exert hypoglycemic effects and to elevate liver glycogen synthesis and glucokinase activity in db/db mice compared with the control group [150]. Insulin secretion was also improved, and it was postulated that the rice bran fraction and ferulic acid could have increased insulin action and the utilization of dietary glucose in the liver.

In conclusion, several different polyphenol classes have been shown to reduce hepatic glucose output by suppressing gluconeogenetic enzyme expression and increasing the activity of glucokinase to improve

glycogenesis and glucose utilization. EGCG has been shown to exhibit these effects by activating AMPK. Furthermore, theaflavins have been shown to activate AMPK in HepG2 cells and to attenuate hepatic lipid accumulation [151]. Activation of AMPK by dietary polyphenols leads to suppression of hepatic gluconeogenesis and induction of fatty acid β-oxidation that both improve hepatic glucose utilization and insulin sensitivity [152]. Resveratrol seems to function in opposite way by activating FoxO1 and inducing hepatic gluconeogenesis. In contrast, resveratrol has been also shown to activate hepatic AMPK [153,154]. Therefore the role of resveratrol in hepatic glucose metabolism needs further clarification. Recent findings suggest that FoxO1 integrates insulin signalling with hepatic mitochondrial function and inhibition of Foxo1 can improve hepatic metabolism during insulin resistance and the metabolic syndrome [155]. In addition to the changes in hepatic glucose utilization and output, most of the *in vivo* studies report changes in hepatic TG contents as well as in blood TG contents. As the hepatic lipid accumulation is connected to insulin resistance it is therefore possible that phytochemicals could exert their effects indirectly on hepatic glucose output by influencing lipid metabolism. *In vivo* studies have also reported increased insulin secretion or changes in blood insulin levels after polyphenol rich diet and therefore the hepatic effects could be also due to insulin signalling.

6. Impact of Polyphenols on Maintenance of Glucose Homeostasis

The majority of the studies on the effects of dietary polyphenols on carbohydrate homeostasis are performed by specific assays focusing on certain parts of the regulatory system. There is, however, increasing data from long-term dietary studies on polyphenol supplementation in animal models and in humans. In such trials, the most common outcome parameters are the blood glucose and insulin levels, the measurements of body fat composition and circulating levels of triglycerides, free fatty acids or other lipid metabolism related biomarkers such as cholesterol, measurement of inflammatory markers, and factors related to the redox status of the organs. The most relevant mechanisms underlying the beneficial health effects are, however, difficult to postulate as the molecular mechanisms have not been comprehensively studied.

6.1. Evidence from Epidemiological Studies

In epidemiological studies, very few of the individual polyphenolic compounds alone have been so far demonstrated to have a beneficial effect on prevention of type 2 diabetes. A prospective study of flavonoid intake from the Finnish diet concluded that quercetin and myricetin are associated with reduced risk of type 2 diabetes [5]. On contrary, intakes of quercetin, kaempferol, myricetin, apigenin, and luteolin were not associated with reduced risk of type 2 diabetes in The Women's Health Study [156]. However, the inverse association with diabetes risk in epidemiological studies has been shown with whole polyphenol-rich diets/food items, which suggests that the effects of polyphenols on disease risk cannot be attributed to single compounds. This is an important issue for consideration for the mechanistic studies using *in vitro* models. Whole grain rich diets have been linked with decreased risk of obesity and type 2 diabetes in epidemiological studies [157,158], and high coffee consumption has been associated with lower prevalence of metabolic syndrome [19,159]. Apples and tea consumption have also been linked to lowered incidence of type 2 diabetes in middle-aged women [156], and apples and berries were the most important contributors lowering the risk in Finnish men and women [5]. In a meta-analysis including nine cohort studies with follow-up ranging from 5 to 18 years, tea consumption was associated with prevention of development of type 2 diabetes [160]. Over four cups of tea per day was required to produce the beneficial effect, although also smaller intake has been shown to be effective in lowering the risk of obesity and blood glucose levels [161]. These beneficial effects by both coffee and tea intakes have been demonstrated also in a recent cohort study where the effect of single compounds magnesium, potassium, and caffeine alone was excluded, and it also was concluded that the effect was not mediated by blood pressure lowering effect [162].

6.2. Evidence from Clinical Trials

There are only a few controlled interventions studying the effects of specific polyphenols or food products in amelioration of the symptoms of the metabolic syndrome. One of the polyphenols studied most frequently *in vitro* is EGCG and/or its source green tea extract. In spite of promising results from animal and *in vitro* testing, EGCG treatment has not been shown to improve insulin resistance in humans, although some beneficial health impacts have been observed [163,164]. One study on type 2 diabetes patients showed increased levels of insulin after 12 weeks of diet supplemented with catechin-rich (582.8 mg) green tea [165], and another study revealed correlation between high intake of tea polyphenols and improved insulin levels in type 2 diabetes patients [164]. In contrast to tea, interesting results have been produced in human trials by dark chocolate consumption. Dark chocolate (100 g dark chocolate bar containing approximately 500 mg of polyphenols for 15 days) improved insulin sensitivity along with reducing blood pressure in healthy subjects [166] and similar results were reported with the same treatment on hypertensive subjects [167]. However, consumption of a flavanol-rich cocoa drink (150 mL twice a day, approximately 900 mg flavanols) for 15 days did not improve insulin resistance or blood pressure in individuals with essential hypertension [168]. Grape seed extract given to type 2 diabetic patients for 4 weeks, had positive effect on several inflammatory markers and glycaemia, but did not result in statistically significant changes in HOMA-IR [169]. In regard of whole grain consumption the beneficial health effects may also be, at least partly, due to the polyphenol content of whole grain products, as a polyphenol-rich wheat bread had higher glucose lowering and antioxidative effect than a control wheat bread during a 9-day study period [170]. Other promising plant food candidates with diabetes preventive potential include cinnamon, bitter melon and fenugreek [171].

6.3. Evidence from Animal Experiments

On the other hand, considerable evidence is available on the effects of several polyphenols and polyphenol-rich food items in ameliorating insulin resistance and improving insulin sensitivity in experimental animals. In mice fed high-fat diet indications towards beneficial effect in glucose/insulin signaling have been obtained by catechin [172], EGCG [134], grape seed procyanidins [173], and blueberry [174]. Similarly in rats fed high-fat diet isoflavones [175], quercetin [176], and blueberry [177] have alleviated the markers of metabolic syndrome. Another type of high-calorie diet, fructose-rich diet, has been applied in rat experiments, producing promising results in balancing the glucose/insulin metabolism with myricetin [178], fenugreek seed extract or quercetin [179], longan flower extract [180], green tea [181] and cinnamon [119,179]. Moreover, insulin sensitivity was improved in the CD-1 mice that have genetic susceptibility to obesity and type 2 diabetes by feeding a diet containing soy [140].

In conclusion, the evidence from epidemiological studies on the protective role of polyphenol-rich foods against development of type 2 diabetes is suggestive, but in spite of the large array of studies *in vitro* and the positive results in animal models, only a handful of controlled human interventions confirm these results. The discrepancy between the results from animal and human studies may be due to species specific differences, but also other factors such as genetic variability and general study set-up (dosage of supplementation, number of study objects, length of intervention) most likely have an impact on the outcome. It is clear that more tightly controlled human studies should be conducted in order to draw conclusions about the role of polyphenols in insulin resistance.

7. Conclusions and Future Prospects

Foods or meals high in available carbohydrate such as starch or sucrose induce hyperglycemia and hyperinsulinemia. Regular consumption of diets with high glycemic impact may increase the risk of obesity, type 2 diabetes and cardiovascular disease by promoting excessive food intake, pancreatic β-cell dysfunction, dyslipidemia, and endothelial dysfunction [27]. The potential of polyphenols in controlling glycemia is a very intensively studied area, encompassing a large piece of scientific literature; studies listed in PubMed in this field

in 2009 alone gave over 70 hits. Indications for positive effects of a large number of polyphenols on glucose homeostasis have been obtained *in vitro* and in animal studies, but definitive conclusions, especially from controlled human studies and at the molecular mechanistic level have not been obtained. There is a shortage of human studies with clinically relevant end-points indicating effects during postprandial handling of dietary carbohydrates, pancreatic insulin secretion and its functions on glucose homeostasis in peripheral tissues.

The field is broad because carbohydrate metabolism constitutes one of the most important physiological functions in the human body involving numerous different organs, tissues and cell types. On the other hand, the amount of dietary constituents potentially contributing to glucose homeostasis is vast, and especially for bioactive non-nutrients, such as polyphenols, mostly unidentified. One important issue in research on dietary phytochemicals is the lack of knowledge on their absorption, metabolite composition and tissue distribution. Plants contain thousands of metabolites in different quantitative and qualitative combinations, and the identification of combinations of active molecules in a given metabolic pathway is an extremely challenging task. The studies performed in cell cultures with single plant phenolic compounds at concentrations exceeding pharmacological doses do not have much predictive value of the effects these compounds would produce when fed in diet and harnessing their target tissues after the metabolism of gut microbiota and the human organs. It is therefore understandable that data from controlled human interventions is missing or contradictory in spite of the positive epidemiological evidence with e.g., whole grains, apples, tea and coffee, and studies with pure compounds and extracts showing effects in various steps of glucose metabolism in cell and animal models. However, in comparison to the studies reported a decade ago, the current *in vitro* studies tend to use amounts of phenolic compounds closer to the range of physiological levels than pharmacological doses.

It is obvious that more human trials with well defined diets and controlled study set-ups should be made to test the hypotheses created by the mechanistic studies, and early biomarkers are needed to reveal the effects of subtle dietary changes in intervention studies. Dose response studies and pharmacokinetic profiling of the hypothetic active metabolites should also be made. More focus should be laid on the studies analysing the effect of whole plant/food extracts in order to follow the synergistic bioactivity of the different phytochemical compounds present in the food concomitantly. Also the interplay between the phenolic compounds and other food constituents such as fibre, is an interesting topic that undoubtedly deserves attention in the case of food products that are rich in both polyphenols and fibre, including whole grain products and fruits like apple.

The research on health effects of plant-based foods will benefit from taking holistic approaches with the aim to resolve an array of effects mediated by an array of bioactive metabolites on the whole body level. One of the key factors will be the combination of the different omics-profiling techniques in the concept of systems biology, or nutrigenomics as termed in the context of nutrition related sciences. Whilst transcriptomics and proteomics characterization are already available on relatively routine laboratory analyses, metabolomics analyses are also rapidly developing, and are expected to be an even more useful tool in making the link between food constituents and subsequent clinical outcome, also in diabetes related research [182]. Especially the non-targeted profiling assays where the metabolite pools of control group and test group (e.g., after dietary challenge) are compared and the metabolite signals significantly differing are resolved with statistical analysis methods. In the elucidation of the effects of dietary phytochemicals on human health, such analyses will likely play a key role in pointing out the factors from bioavailability, absorption, microbial metabolism, whole body distribution, tissue localization and mechanisms of action that would not be achievable by targeted single compound assays.

Main conclusions

There are indications for positive effects on glucose homeostasis with polyphenols and polyphenol-rich plant extracts from *in vitro* & animal studies.

Epidemiological evidence supports beneficial effects of polyphenol- rich diets.

Clinical studies so far have not undoubtedly succeeded in pointing out any specific polyphenols or food products in reducing the risk of insulin resistance.

It is evident that in clinical studies whole diets instead of single compounds or food components should be addressed.

Combination of specific clinical measurements determining glucose tolerance and insulin sensitivity together with systems biology profiling technologies is needed to get a holistic view on the health effects of diets and foods rich in polyphenols.

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Khalil OA, Ramadan KS, Danial EN, Alnahdi HS, Ayaz NO

Antidiabetic activity of Rosmarinus officinalis and its relationship with the antioxidant property

Oxidative stress plays an important role in diabetic pathogenesis. Rosmarinus officinalis L. was first used as an antioxidant agent for inhibition of diabetic nephropathy. Oxidative stress induced by Streptozotocin (STZ) has been shown to damage pancreatic beta cell and produce hyperglycemia in rats. In the present study, an attempt was made to examine the action of R. officinalis against experimental diabetes as well as the antioxidant potential of the leaf extract. Water extract of R. officinalis (200 mg/kg body weight for 21 days) was found to be significantly reducing the blood sugar level. The oxidative stress produced by Streptozotocin was found to be significantly lowered when compared to control rats. This was evident from a significant decrease in blood sugar level and oxidative stress makers including serum TBARS and nitric oxide (NO). Serum enzymatic (glutathione transferase (GST), catalase (CAT), glutathione peroxidase (GPx) and non enzymatic antioxidants (vitamin C and reduced glutathione) were found to be increased by the administration of R. officinalis. These results indicate that R. officinalis extract effectively reduced the oxidative stress induced by Streptozotocin and potential reduction in blood sugar level.

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Posadas SJ, Caz V, Largo C, De la Gandara B, Matallanas B, Reglero G, De Miguel E

Protective effect of supercritical fluid rosemary extract, Rosmarinus officinalis, on antioxidants of major organs of aged rats

Rosemary leaves, "Rosmarinus officinalis", possess a variety of antioxidant, anti-tumoral and anti-inflammatory bioactivities. We hypothesized that rosemary extract could enhance antioxidant defenses and improve antioxidant status in aged rats. This work evaluates whether supplementing their diet with supercritical fluid (SFE) rosemary extract containing 20% antioxidant carnosic acid (CA) reduces oxidative stress in aged rats. Aged Wistar rats (20 months old) were included in the study. Rats were fed for 12 weeks with a standard kibble (80%) supplemented with turkey breast (20%) containing none or one of two different SFE rosemary concentrations (0.2% and 0.02%). After sacrifice, tissue samples were collected from heart and brain (cortex and hippocampus). Enzyme activities of catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD) and nitric oxide synthase (NOS) were quantitatively analyzed. Lipid peroxidation and levels of reactive oxygen species (ROS) were also determined. Rosemary decreased lipid peroxidation in both brain tissues. The levels of catalase activities in heart and cortex were decreased in the rosemary-treated groups. The SFE rosemary-treated rats presented lower NOS levels in heart and lower ROS levels in hippocampus than the control rats. Supplementing the diet of aged rats with SFE rosemary extract produced a decrease in antioxidant enzyme activity, lipid peroxidation and ROS levels that was significant for catalase activity in heart and brain, NOS in heart, and LPO and ROS levels in different brain tissues. These observations suggest that the rosemary supplement improved the oxidative stress status in old rats.

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Pan MH, Lai CS, Ho CT

Anti-inflammatory activity of natural dietary flavonoids

Over the past few decades, inflammation has been recognized as a major risk factor for various human diseases. Acute inflammation is short-term, self-limiting and it's easy for host defenses to return the body to homeostasis. Chronic inflammatory responses are predispose to a pathological progression of chronic illnesses characterized by infiltration of inflammatory cells, excessive production of cytokines, dysregulation of cellular signaling and loss of barrier function. Targeting reduction of chronic inflammation is a beneficial strategy to combat several human diseases. Flavonoids are widely present in the average diet in such foods as fruits and vegetables, and have been demonstrated to exhibit a broad spectrum of biological activities for human health including an anti-inflammatory property. Numerous studies have proposed that flavonoids act through a variety mechanisms to prevent and attenuate inflammatory responses and serve as possible cardioprotective, neuroprotective and chemopreventive agents. In this review, we summarize current knowledge and underlying mechanisms on anti-inflammatory activities of flavonoids and their implicated effects in the development of various chronic inflammatory diseases.

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The Mediterranean diet pattern and its main components are associated with lower plasma concentrations of tumor necrosis factor receptor 60 in patients at high risk for cardiovascular disease

Adherence to a Mediterranean diet (MD) is associated with a reduced risk of coronary heart disease. However, the molecular mechanisms involved are not fully understood. The aim of this study was to compare the effects of 2 MD with those of a low-fat-diet (LFD) on circulating inflammatory biomarkers related to atherogenesis. A total of 516 participants included in the Prevention with Mediterranean Diet Study were randomized into 3 intervention groups [MD supplemented with virgin olive oil (MD-VOO); MD supplemented with mixed nuts (MD-Nuts); and LFD]. At baseline and after 1 y, participants completed FFQ and adherence to MD questionnaires, and plasma concentrations of inflammatory markers including intercellular adhesion molecule-1(ICAM-1), IL-6, and 2 TNF receptors (TNFR60 and TNFR80) were measured by ELISA. At 1 y, the MD groups had lower plasma concentrations of IL-6, TNFR60, and TNFR80 ($P < 0.05$), whereas ICAM-1, TNFR60, and TNFR80 concentrations increased in the LFD group ($P < 0.002$). Due to between-group differences, participants in the 2 MD groups had lower plasma concentrations of ICAM-1, IL-6, TNFR60, and TNFR80 compared to those in the LFD group ($P \leq 0.028$). When participants were categorized in tertiles of 1-y changes in the consumption of selected foods, those in the highest tertile of virgin olive oil (VOO) and vegetable consumption had a lower plasma TNFR60 concentration compared with those in tertile 1 ($P < 0.02$). Moreover, the only changes in consumption that were associated with 1-y changes in the geometric mean TNFR60 concentrations were those of VOO and vegetables ($P = 0.01$). This study suggests that a MD reduces TNFR concentrations in patients at high cardiovascular risk.

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Adipocyte metabolism and obesity

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Abstract

Adipose tissue metabolism exerts a profound impact on whole-body metabolism. We review how fuel partitioning between adipocytes and other tissues affects insulin signaling pathways. We discuss the role of adipose tissue inflammation in adipocyte metabolism and whole-body insulin sensitivity. Finally, we mention the role of adipokines in autocrine and paracrine signaling.

Keywords: adiponectin, leptin, inflammation

Obesity is associated with changes in adipocyte gene expression spanning many pathways. These changes alter fuel partitioning between adipose tissue and other tissues. In addition, they alter the hormonal milieu by changing the relative expression of adipose hormones, adipokines. Finally, many metabolites act as signaling molecules; thus, their redistribution alters signaling pathways in adipose and other tissues.

The role of inflammation in obesity has gained attention, beginning with the discovery that the macrophage content of adipose tissue in obese humans and rodents increases dramatically [reviewed in (1)]. Thus, in evaluating gene expression from adipose tissue, one has to consider the change in tissue composition and the role of macrophage-derived molecules in the alteration of adipocyte gene expression.

In the context of overnutrition, most of the attention has been directed to dietary fat and carbohydrates. However, high-calorie diets are often associated with increased protein intake. Of particular interest in relation to extrahepatic tissues are the branched chain amino acids (BCAAs). BCAA levels are increased in the bloodstream of obese humans and in animal models of obesity [reviewed in (2)]. Owing to the absence of the mitochondrial branched chain amino transferase in the liver, BCAAs bypass the liver and are selectively metabolized in extraphepatic tissues. BCAAs modulate food intake through hypothalamic signaling and regulate leptin production in adipose tissue. The increase in BCAAs in obesity appears to be due in part to reduced expression of mitochondrial branched chain amino transferase in adipose tissue (2).

Amino acid excess affects insulin signaling and glucose metabolism. This results in a direct stimulation of gluconeogenesis (3) and decreased glucose transport and glycogen synthesis in muscle (4). Through their activation of the mTor/S6 kinase pathway, amino acids stimulate serine phosphorylation of IRS1 and blunt insulin signaling (5).

Fuel partitioning

Although animals go through feeding/fasting cycles, the liver maintains a relatively constant fatty acid flux into triglyceride biosynthesis [reviewed in (6)]. During fasting, the free fatty acid is derived from lipolysis of adipose tissue triglyceride, is transported to the liver, and is re-esterified to form hepatic triglyceride. With a lipogenic diet,

the fatty acids are synthesized de novo in liver and adipose tissue. In a healthy animal, hepatocytes are able to maintain a rate of VLDL secretion sufficient to prevent the accumulation of excess triglyceride, a condition termed hepatic steatosis. Hepatic steatosis occurs in >20% of the US population, with some ethnic groups as high as 45% (7). It is especially common in obese individuals. The heritability of hepatic steatosis is quite high, and a candidate susceptibility gene, *PNPLA3*, a gene expressed in liver and adipose tissue, has recently been identified in a genome-wide association study (8).

Early microarray experiments indicated that genes involved in lipogenesis are downregulated in adipose tissue of obese leptin-deficient mice (9, 10). This also includes the master regulator of these genes, *Srebp1c*. Conversely, these same genes are upregulated in the liver. Interestingly, a comparison hepatic gene expression in diabetes-resistant (C57BL/6) versus diabetes-susceptible (BTBR) leptin-deficient mice showed that only the nondiabetic strain exhibited the induction of lipogenic genes in the liver (9). In the livers of obese mice from a diabetes-susceptible strain, *Srebp1c* and its target genes were not upregulated. The same trend is seen for *Pparg*, a gene normally not expressed at a high level in the liver, but one that is induced in the liver of obese and lipodystrophic animals (9, 11). The hepatic steatosis phenotype is rescued by ablation of the hepatic *Pparg* gene (11). This creates an interesting dichotomy between hepatic steatosis and diabetes susceptibility; the diabetes-resistant mouse strain is more susceptible to hepatic steatosis. Similar results have been observed in mice with lipodystrophy when the lipodystrophy mutation is studied in C57BL/6 versus a diabetes-susceptible strain, FVB (11). In short, the relative expression level of at least two key transcription factors, *Srepb1c* and *Pparg*, in adipose tissue and liver, plays a role in fuel partitioning between the two tissues. There is reason to believe that several factors in adipose tissue locally have an impact on peroxisome proliferator-activated receptor γ (PPARγ) activity. Such factors include the cytoplasmic lipid binding protein aP2, whose absence leads to increased PPARγ activity (12), as well as the adipokine adiponectin, whose overexpression in adipose tissue causes a net increase in the transcription of PPARγ targets (13).

The partitioning of lipids between adipose tissue and other tissues plays an important role in insulin signaling and cellular viability. Adipose tissue is specialized for triglyceride storage and has a very high capacity to accumulate triglycerides. Thus, the enhanced lipolysis and consequent free fatty acid flux from adipose tissue in obesity exposes other tissues to a substantial fatty acid burden (14). These other tissues can accumulate triglycerides, and this is associated with cell pathology and insulin resistance. However, it is important to point out that current evidence argues against triglyceride itself being the culprit in these fatty-acid-mediated actions. Rather, strong evidence supports a role for diacylglycerol in the blunting of insulin signaling. Increased expression of diacylglycerol acyl transferase improves insulin sensitivity (15), whereas inhibition of diacylglycerol kinase suppresses insulin signaling (16). A likely target for diacylglycerol action is protein kinase C-Θ (17), which upon stimulation by diacylglycerol, phosphorylates serine residues on insulin receptor substrates, blunting the insulin signal (18).

In addition to diacylglycerol, there is also strong evidence implicating ceramide in the modulation of insulin sensitivity. Ceramide is structurally analogous to diacylglycerol by having two fatty acid moieties attached to a backbone. Instead of glycerol, the backbone is serine, and instead of giving rise to glycerolipids, ceramide is an intermediate in sphingolipid synthesis. Like diacylglycerols, ceramides are signaling molecules. They activate protein phosphatase 2A, which dephosphorylates and thus inactivates Akt/PKB, a key arm of the insulin signaling pathway (19). In addition, ceramides inhibit the translocation of Akt/PKB to the plasma membrane (20).

Leptin and adiponectin

Leptin also plays a profound role in fuel partitioning. Diet-induced obesity simultaneously leads to increased leptin secretion and a blunting of the autocrine leptin signal in adipocytes. The leptin signal tends to be anti-adipogenic, a conclusion that emerged from the protection from obesity afforded to transgenic mice overexpressing the leptin

b receptor in adipocytes (21). This illustrates the point that adipose tissue expansion during a positive energy balance (i.e., overeating) is an integral component of the maintenance of energy homeostasis. These mice are deficient in both adipocyte hypertrophy and hyperplasia, resulting in an inability to expand adipose tissue mass under these conditions. Functionally, the net result is comparable to states of partial or complete lipodystrophy; for example, excess triglycerides accumulate ectopically in tissues such as liver, muscle, and β-cells, where they contribute significantly toward the lipotoxic effects of lipids, as discussed above.

On the other end of the spectrum, we find a recently described mouse obesity model that overexpresses the adipocyte-derived circulating factor adiponectin (13). In the context of a challenge with the *Leptin*$^{ob/ob}$ mutation, these mice retain adiponectin levels at concentrations found in a lean mouse. Under these conditions, the mice further expand their adipose tissue mass quite dramatically, far beyond the excess adiposity conventionally seen in the *Leptin*$^{ob/ob}$ model. Surprisingly, despite the *Leptin*$^{ob/ob}$ mutation and the huge excess of adipose tissue, these mice have a fairly good metabolic profile and retain near normal insulin sensitivity, a normalized lipid profile, and hallmarks of a significant improvement in adipose tissue histology. An increased number of smaller fat cells is apparent, with a reduced infiltration of macrophages. Hepatic steatosis, which is conventionally seen at high levels in the *Leptin*$^{ob/ob}$ mouse, is reduced. This is an example in which the ectopic accumulation of lipids is reduced, presumably because excess lipids are neutralized by storage in subcutaneous fat pads. As a result, insulin sensitivity is preserved.

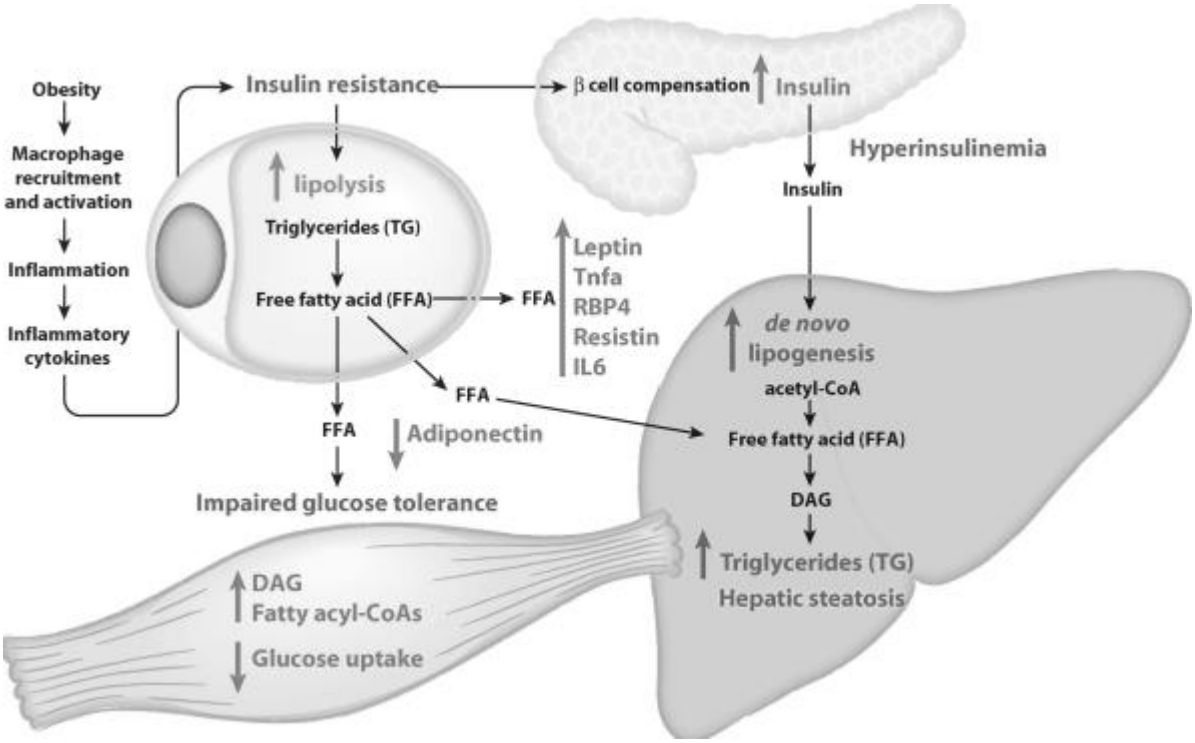
These results suggest that adipose tissue can play a protective and a detrimental role in maintaining a favorable metabolic profile. This was quite elegantly demonstrated in a fat transplantation study that followed a 2 × 2 experimental design in which subcutaneous and visceral fat was transplanted into either a subcutaneous or visceral adipose site (22). Transplantation of subcutaneous fat into a visceral site produced significant improvement in insulin sensitivity and a drop in plasma glucose and insulin levels. Surprisingly, the animals also experienced a drop in leptin and adiponectin, suggesting that other fat-derived factors play a role in the improved metabolic profile. Since transplantation into the subcutaneous site did not alter the metabolic profile, there appear to be both donor and recipient site-specific factors required for these metabolic changes.

This raises the important question as to what the critical initial events are that prevent adipose tissue from assuming its appropriate physiological role as a triglyceride storage compartment, leading to the forced accumulation of ectopic lipids in alternative tissues. From an evolutionary point of view, the “thrifty gene hypothesis” suggests that we are all geared toward maximizing our ability to store fat during times of plenty so these reserves can be tapped during times of food shortage. On the other hand, “too much of a good thing” (i.e., excess adipose tissue accumulation) may significantly impair the ability to effectively escape a predator. Leptin is clearly one leg of a feedback loop designed to reduce food intake and increase energy expenditure during times of excessive adipose tissue growth. However, the system is clearly not very effective, as clear signs of “leptin resistance” occur during early stages of obesity. On the other hand, the well-established obesity-associated downregulation of adiponectin may be a second leg on which this feedback loop critically depends. If adiponectin is indeed an anabolic hormone that potently drives free fatty acids into adipocytes for esterification, its downregulation during adipose tissue growth may prevent excessive expansion of fat mass. However, this comes at the price of ectopic accumulation of a fraction of these excess lipids in other tissues.

Adipose tissue inflammation

The obesity-associated increased infiltration of immune cells, especially macrophages, is well established at this stage (23). The infiltration of macrophages per se can trigger increased local and systemic inflammation, which is associated with decreased insulin sensitivity (**Fig. 1**). For instance, transgenic overexpression of monocyte chemotactic protein-1 in adipose tissue (24) is necessary and sufficient to trigger increased infiltration of

macrophages. However, it is not clear whether under normal physiological conditions increased macrophage infiltration and subsequent inflammation is the result of a sophisticated chemokine-based signaling mechanism or simply the result of an increased incidence of necrotic adipocytes, frequently seen during rapid tissue expansion. Independent of its origin, a reduction of local inflammation in adipose tissue, either through pharmacological intervention or through genetic manipulation of pro-inflammatory pathways, is invariably associated with improvements in local and systemic insulin sensitivity. While inflammatory pathways are the ultimate mediators of insulin resistance, other events in adipose tissue may precede the initiation of inflammation-induced adipose tissue dysfunction. In addition, since the macrophage-induced inflammation is preceded by the activation of the classical inflammatory transcription program in the macrophages [the "M1" activation pathway (25)], there may be critical factors in adipose tissue that elicit this program in adipose tissue macrophages.



Three links between adipocyte biology and metabolic syndrome. Obesity leads to the recruitment by adipocytes of macrophages. These macrophages are activated to produce inflammatory cytokines, which blunt insulin signaling. In adipocytes, insulin resistance leads to an impaired ability of insulin to suppress lipolysis, leading to an increased flux of free fatty acids from adipocytes to other tissues. In muscle, increased fatty acid flux leads to impaired glucose uptake, leading to whole-body impaired glucose tolerance. In the liver, the increased flux of free fatty acid contributes to increased triglyceride synthesis and hepatic steatosis. Insulin resistance causes pancreatic β-cells to compensate with increased insulin production, leading to hyperinsulinemia. This in turn stimulates de novo lipogenesis in the liver, contributing to the pool of free fatty acids available for triglyceride production. Obesity also alters the balance of adipokines produced by adipocytes, with an increase in leptin, TNFα, RBP4, resistin, and IL6, and a decrease in adiponectin. This altered balance contributes to impaired glucose tolerance and insulin resistance.

An attractive model that is supported by limited data is that adipocyte growth (both hypertrophy and hyperplasia) places demands for increased vascularization and tissue remodeling. If these two processes lag behind the expansion of adipose mass, hypoxic conditions emerge, which then stimulate a specific program of gene expression and may also lead to the recruitment of macrophages to the adipose tissue. Gene expression changes

consistent with this model have been observed in a comparison of responsive versus nonresponsive (in terms of weight gain) mice of a single strain fed a high-fat diet (26).
Hypoxia has been directly detected by immunohistochemistry and by measuring the perfusion of adipose tissue with radiolabeled microspheres in genetically obese [KKAy (27) or leptin-deficient (28)] mice. These changes are associated with reduced adiponectin expression and an increase in the expression of genes associated with hypoxia, *Hif-1α*, *Glut1*, and *Pdk1*, and inflammation, *TNFα*, *IL-1*, *IL-6*, and *TGF-β* (28). The latter genes were induced in both adipocytes and macrophages.
In many other tissues, hypoxia is associated with an increased level of fibrosis through an upregulation of many extracellular matrix proteins [reviewed in (29)]. As such, it is likely that the prevailing hypoxia in expanding adipose tissue may also be associated with an increased degree of extracellular matrix deposition in adipose tissue. It is not clear whether this increased density of extracellular support structures in adipose tissue contributes directly toward an increased rate of cell death observed in expanding adipose tissue. Genetic and pharmacological studies will be required to see if the upregulation of these extracellular matrix components in adipose tissue is a simple epiphenomenon associated with expanding adipose tissue or if it presents a worthwhile area to interfere with in the context of an increased fibrotic content in adipose tissue.

The adipocyte: do endoplasmiv reticulum stress and the unfolded protein response play a role?

Endoplasmic reticulum stress and the associated unfolded protein response (UPR) have been associated with cellular dysfunction and cell death in a number of different cell types relevant for metabolic homeostasis. This is particularly relevant for cell types with a very active secretory pathway. The pancreatic β-cell, with its high-level production of insulin, is highly prone to stress in the endoplasmic reticulum. The rate of protein secretion from adipocytes is frequently underestimated. Whereas the role of the adipocyte as an endocrine cell is widely appreciated, many of the adipokines that the fat cell produces have relatively short half-lives but circulate at rather high levels (e.g., adiponectin, several complement factors, and acute phase reactants). This imposes major challenges for the proper folding and assembly of some of these factors, which in some instances need to form highly complex quaternary structures. Thus, the UPR may play an important role in the cellular homeostasis of the adipocyte in the lean as well as in the obese state.

All three “classical” pathways of the UPR (such as the PERK, IRE-1, and the ATF-6 pathways) are present and can be activated in the adipocyte [reviewed in (30)]. Even in human adipose tissue, these pathways are highly relevant, and the degree of activation correlates positively with overall adiposity within an individual (31). However, in this study, the activation state of the pathway did not correlate with systemic insulin resistance. This is surprising in light of the fact that endoplasmic reticulum stress can trigger activation of the jun kinase pathway and NF-κB, while at the same time, local inflammation in adipose tissue can trigger the UPR. The connection between the UPR and inflammation is a reflection of crosstalk at multiple levels, including the increased production of reactive oxygen species that are generated as a result of the activation of the UPR. These questions will need to be further studied, particularly because a recent article implicated Xbp1, an important downstream mediator of the UPR, as a master regulator of lipogenesis in the liver; deletion of *Xbp1* in the liver caused hypocholesterolemia and reduced triglyceride accumulation as a result of decreased lipogenesis (32). Whether Xbp1 exerts similar functions on lipogenesis and/or lipid storage in adipocytes may indicate that the differential activation of the UPR in liver and adipose tissue plays a role in the fuel partitioning of lipids between these two tissues.

Mitochondrial dysfunction: also important for the white adipocyte?

Mitochondrial function is key for proper maintenance of energy homeostasis. This also holds true for white adipocytes, where proper mitochondrial function is likely to be key for systemic insulin sensitivity. Insulin-sensitizing drugs, such as the PPARγ agonists, induce a host of mitochondrial proteins and improve mitochondrial function in adipocytes (33). Impaired mitochondrial respiratory function triggers a reduction in translocation of Glut4 to the plasma membrane, but surprisingly, enhances Akt signaling (34). Even modest changes at the level of mitochondrial function have a dramatic effect on production and release of adiponectin (34). Particularly in the hyperglycemic state, excess intracellular glucose availability causes a dramatic increase in mitochondrial ROS production and hence increased local inflammation (35). It is therefore very likely that proper mitochondrial function in white adipocytes is key for appropriate energy balance between different tissues, particularly during times of excess energy intake.

Conclusions and outlook

Adipose tissue and the liver constitute an interesting organ pair that is in constant communication with each other via adipokines, lipid factors, and lipoprotein particles. The adipohepatic axis affects lipid and carbohydrate usage and flux. Dysregulation in either of the two tissues is detrimental to the other and ultimately for the entire system. One of the first organs to be affected when adipose tissue becomes dysfunctional and inflamed is the liver. Secondary to that, changes in free fatty acid concentration and flux affect other cell types, such as muscle cells and pancreatic β-cells, which are susceptible to fatty-acid-induced lipotoxicity. The associated insulin resistance imposes increased demands on the secretory capacity of β-cells, which under these conditions, is vulnerable to UPR-induced cell death.

Many new avenues of research are currently opening up that will allow for further study of the complex relationship between proper adipocyte hypertrophy and hyperplasia and downstream events such as inflammation, mitochondrial dysfunction, and lipid accumulation in secondary organs such as the liver, muscle, and β-cells.

Abbreviations

BCAA, branched chain amino acid
PPARγ, peroxisome proliferator-activated receptor γ
UPR, unfolded protein response

Notes

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Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3 T3-L1 cells

AIMS: Oleuropein and hydroxytyrosol, which are antioxidant molecules found in olive leaves and oil, have been reported to exert several biochemical and pharmacological effects. These polyphenols are able to prevent low-density lipoprotein oxidation and protect cells against several diseases. Here, we studied the effect of these compounds on adipocyte differentiation in 3 T3-L1.

MAIN METHODS: To perform this study, 3 T3-L1 preadipocytes viability was analysed via Trypan blue and MTT assays, and triglycerides were stained with Oil Red O. Adipogenesis related genes expression were checked by RT-PCR and qRT-PCR. Also, cells counting and flow cytometry were used to analyse the mitotic cell cycle during the adipogenesis clonal expansion phase.

RESULTS: Oleuropein and hydroxytyrosol dose-dependently suppressed intracellular triglyceride accumulation during adipocyte differentiation without effect on cell viability. PPARγ, C/EBPα and SREBP-1c transcription factors and their downstream targets genes (GLUT4, CD36 and FASN) were down-regulated after treatment by oleuropein and hydroxytyrosol. At 200 and 300 μmol/L oleuropein or 100 and 150 μmol/L hydroxytyrosol, the greatest effect on the adipogenesis process was observed during the early stages of differentiation. Flow cytometry revealed both polyphenols to inhibit the division of 3T3-L1 preadipocytes during mitotic clonal expansion and cause cell cycle delay. Furthermore, oleuropein and its derivate hydroxytyrosol decreased the transcriptional activity of SREBP-1c in a stable transfected 3T3-L1 cell line.

SIGNIFICANCE: These findings indicate that both compounds are able to prevent 3T3-L1 differentiation by inhibition of the mitotic clonal expansion and downregulation of the adipogenesis related genes.

Reference [23] Biochemistry Research International 2011, article ID 285618, doi:10.1155/2011/285618

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Effect of Plant Polyphenols on Adipokine Secretion from Human SGBS Adipocytes

Introduction. Adipose tissue contributes to atherosclerosis with mechanisms related to adipokine secretion. Polyphenols may exhibit antiatherogenic properties. The aim of the study was to investigate the effects of three polyphenols, namely, quercetin, epigallocatechin gallate (EGCG), and resveratrol on adipokine secretion from cultured human adipocytes. Methods. Human SGBS adipocytes were treated with quercetin, EGCG, and resveratrol for 24 and 48 hours. Visfatin, leptin, and adiponectin were measured in the supernatant. Results. Visfatin secretion was inhibited by quercetin 10 μM by 16% and 24% at 24 and 48 hours respectively. The corresponding changes for quercetin 25 μM were 47% and 48%. Resveratrol 25 μM reduced visfatin by 28% and 38% at 24 and 48 hours. EGCG did not have an effect on visfatin. None of tested polyphenols influenced leptin and adiponectin secretion. Conclusion. Quercetin and resveratrol significantly decreased visfatin secretion from SGBS adipocytes. This effect may contribute to their overall antiatherogenic properties.

8. CERTIFICATES & SAFETY TESTS

8.1. Heavy metals

Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan http://www.jfrl.or.jp/

No. 12085482001-01 1/1
September 05, 2012

CERTIFICATE OF ANALYSIS

Client: Mayor Invivo
Genopole, 5 rue Henri Desbrueres 91030 Evry cedex-France

Sample name: OXYLIA

Received date: August 27, 2012

This is to certify that the following result(s) have been obtained from our analysis on the above-mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Arsenic (as As_2O_3)	Not detected	0.1 ppm		1
Lead	Not detected	0.05 ppm		1
Cadmium	0.01 ppm	.....		1
Mercury	Not detected	0.01 ppm		2

QL: Quantitation limit N: Notes M: Method
Method
1:Atomic absorption spectrometry 2:Cold vapor atomic absorption spectrometry

JAPAN FOOD RESEARCH LABORATORIES

T. Arai
Takeko Arai
Principal Investigator

Sep. 05, 2012
Date

日本食品分析センター

RCA0217-03

8.2. Microbial tests

Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan http://www.jfrl.or.jp/

No. 12085482001-02 1/1
September 06, 2012

CERTIFICATE OF ANALYSIS

Client: Mayor Invivo
Genopole, 5 rue Henri Desbrueres 91030 Evry cedex-France

Sample name: OXYLIA

Received date: August 27, 2012

This is to certify that the following result(s) have been obtained from our analysis on the above-mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Aerobic plate count	Not more than 300/g	----		1
Coliform bacteria	Negative/2.22g	----		2
Viable molds count	Negative/0.1g	----		3
Viable yeasts count	Negative/0.1g	----		3
Salmonella	Negative/25g	----		4

QL: Quantitation limit N: Notes M: Method

Method

1:Standard Agar plating method
2:BGLB broth inoculating method
3:Potato Dextrose (10 %) Agar plating method
4:Enrichment culture method

JAPAN FOOD RESEARCH LABORATORIES

T. Arai
Takeko Arai
Principal Investigator

Sep. 06, 2012
Date