A. Obesity Definition

Overweight and obesity are defined as abnormal or excessive fat accumulation in the fatty tissue of humans and mammals that presents a risk to health (WHO Health Topics: Obesity and Overweight).

Obesity is the consequence of an energy imbalance between calories consumed and calories expended, it is not a single disorder, but a complex multifactorial disease involving environmental and genetic factors, and it develops from the interaction of individual biology and the environment.

There are nevertheless several factors that contribute to the appearing of this disease, like (Levy et al. 2012; Aller et al. 2011; Burkhalter & Hillman 2011):

- Bad nutritional habits, eating disorders, an increased intake of energy-dense foods that are high in fat, salt and sugars but low in vitamins, fibres, minerals and other micronutrients;
- Sedentary lifestyle with a decrease in physical activity;
- Hereditariety;
- Malfunctioning of some vital organs that would prevent the elimination of toxins, fat burning or complicate the organism’s metabolism;
- Stressful mentality. According to psychologists, emotional issues and unresolved and increased social pressure can influence a person to eat too much. Obese people have a tendency to emotional eating;
- Insufficient sleep;
- Smoking cessation;
- Hormonal changes that alter the normal functioning of the organism (depressions, etc.).

Changes in dietary and physical activity patterns are often the result of environmental and societal changes associated with development and lack of supportive policies in sectors such as health, agriculture, transport, urban planning, environment, food processing, distribution, marketing and education, all problems we will approach later on in this study (Ogunbode et al. 2011; Mushtaq et al. 2011; WHO – Obesity and Overweight).
Body mass index (BMI) is a simple ratio between weight and height, commonly used to classify overweight and obesity in adults (WHO Obesity Fact File). It is calculated by dividing the subject’s weight in Kg by the square of his height in meters.

\[
\text{BMI} = \frac{\text{weight (Kg)}}{\text{height}^2 \ (m^2)} \quad (1)
\]

The World Health Organisation’s (WHO) Classification of adult underweight, overweight and obesity according to BMI (Table 1.1):

<table>
<thead>
<tr>
<th>BMI (Kg / m²)</th>
<th>Classification</th>
<th>Sub-Classification</th>
<th>BMI (Kg / m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 18,50</td>
<td>Underweight</td>
<td>Severe Thinness</td>
<td>&lt; 16,00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate Thinness</td>
<td>16,00 ÷ 16,99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mild Thinness</td>
<td>17,00 ÷ 18,49</td>
</tr>
<tr>
<td>18,50 ÷ 24,99</td>
<td>Normal Range</td>
<td>Normal</td>
<td>18,5 ÷ 24,99</td>
</tr>
<tr>
<td>≥ 25</td>
<td>Overweight</td>
<td>Pre-Obese</td>
<td>≥ 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Obese</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Obese Class 1</td>
<td>30,00 ÷ 34,99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Obese Class 2</td>
<td>35,00 ÷ 39,99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Obese Class 3</td>
<td>≥ 40</td>
</tr>
</tbody>
</table>
Obesity represents a major health issue nowadays.

The global epidemic of overweight and obesity - "globesity" - is rapidly becoming a major public health problem in many parts of the world. Paradoxically coexisting with undernutrition in developing countries, the increasing prevalence of overweight and obesity is associated with many diet-related chronic diseases including diabetes mellitus, cardiovascular disease, stroke, hypertension and certain cancers, apart from serious psychological problems (WHO: Global Database on Body Mass Index).

Overweight and obesity are two major risk factors for a vast number of chronic diseases and other health issues (e.g.: congestive heart failure, asthma, cancer, etc.) (Shao et al. 2011; Peairs et al. 2011; World Health Organization Fact Sheet nº 311).

Obesity was once considered a problem only in the richest countries but it is now present and increasing in low- and middle-income countries, especially in urban settings, affecting children, men and women of all ages and races (Ogunbode et al. 2011).

I) A Growing concern

The most recent WHO projections indicate that at least 1 in 3 of the world's adult population is overweight and almost 1 in 10 is obese. Additionally there are over 20 million children under age five who are overweight.

Here is some information provided by the WHO as to how this disease has been escalating, just how serious it already is and how it tends to get even worse.

(a) Facts and Numbers (WHO Website)

- Worldwide
  - Worldwide obesity has more than doubled since 1980.
  - 65% of the world's population lives in countries where overweight and obesity kills more people than underweight.
  - Overweight and obesity are the fifth leading risk for global deaths. At least 2.8 million adults die each year as a result of being overweight or obese, over 5% of annual worldwide deaths (these numbers account for deaths that are indubitably due to obesity, several more can be caused by this condition without there being registry of it as cause of death).
In 2008, more than 1.4 billion adults, 20 and older, were overweight, amongst these, approximately 10% and nearly 300 million women (about 14%) were obese, accounting approximately 500 million obese adults worldwide. When Asian-specific cut-off points for the definition of obesity (body mass index >28 kg/m²) are taken into account, this number rises to over 600 million.

- Globally, about 200 million children under the age of 5 are either overweight or obese, of those, 40-50 million are classified as obese.
- 44% of the diabetes burden, 23% of the ischaemic heart disease burden and between 7% and 41% of certain cancer burdens are attributable to overweight and obesity.
- Overweight and obesity are important risk factors for cardiovascular disease, which is the number one cause of death worldwide and accounts for over 17 million deaths every year.
- The World Health Organization predicts there will be 2.3 billion overweight adults in the world by 2015 (about 1/3) and more than 700 million of them will be obese (approximately 1/10).

**Europe**

- In 2008, over 50% of both men and women in the WHO European Region were overweight, and roughly 23% of women and 20% of men were obese.
- In Europe alone, nearly 15 million children under 5 are overweight, and more than 25% of these obese.
- The latest estimates (2010) in European Union countries point to between 30 to 70% adults affected by overweight, and 10 to 30% by obesity.
- Over 60% of children who are overweight before puberty will be overweight in early adulthood.
- About 60% of adults and over 20% of school-age children are overweight or obese, around 260 million adults & over 12 million children.
- Obesity accounts for 2%–8% of Europe’s health costs and 10%–13% of deaths in different parts of the Region.
Obesity in Portugal has been steadily growing. In 2004, 14.2% of Portuguese adults were obese. The latest numbers (2010) show that 21.6% of the Portuguese population are obese, and 55.3% overweight.

According to the WHO's COSI study (Childhood Obesity Surveillance Initiative):

Table 1.2. Childhood Overweight and Obesity in Portugal (Padez et al. 2005)

<table>
<thead>
<tr>
<th></th>
<th>Boys</th>
<th>Girls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overweight</td>
<td>34.0%</td>
<td>30.3%</td>
</tr>
<tr>
<td>Pre-obesity</td>
<td>18.4%</td>
<td>16.8%</td>
</tr>
<tr>
<td>Obesity</td>
<td>15.6%</td>
<td>13.5%</td>
</tr>
</tbody>
</table>

Table 1.3. Overweight and Obesity in Portuguese adults (do Carmo I. et al.2005).

<table>
<thead>
<tr>
<th></th>
<th>Women</th>
<th>Men</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Low (&lt;18.5)</td>
<td>126</td>
<td>3.4</td>
<td>27</td>
</tr>
<tr>
<td>Normal (18.5-24.9)</td>
<td>1830</td>
<td>49.4</td>
<td>1069</td>
</tr>
<tr>
<td>Overweight (25.0-29.9)</td>
<td>1256</td>
<td>33.9</td>
<td>1216</td>
</tr>
<tr>
<td>Obesity I (30.0-34.9)</td>
<td>371</td>
<td>10.0</td>
<td>341</td>
</tr>
<tr>
<td>Obesity II (35.0-39.9)</td>
<td>89</td>
<td>2.4</td>
<td>49</td>
</tr>
<tr>
<td>Obesity III (≥40.0)</td>
<td>33</td>
<td>0.9</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>3705</td>
<td>100.0</td>
<td>2706</td>
</tr>
</tbody>
</table>

Along the last few decades, people have adopted an increasingly sedentary lifestyle. Adults and children tend to increasingly reduce their physical activity (Mushtaq et al. 2011; Williams 2011).
Simultaneously, dietary habits of the world’s population tend to deteriorate with the growth and expansion of fast food chains. They are more and more exposed to high-fat, high-sugar, high-salt, energy-dense, micronutrient-poor foods, which tend to be lower in cost. Overweight is also regarded by many less informed people as a sign of good health, as many parents are happy to see their sons being overweight, for it means that they are not enduring lack of nourishment as some of them might have had to (Burke & Heiland, 2007).

C. Anti-obesity strategies

Prevention will always be a better strategy than treatment. The simple fact that, in avoiding a disease rather than curing, it you are steering clear of any side effects, is big enough reason to support this affirmation (TP Gill, 1997).

At the individual level, in order to prevent obesity, people can (WHO Redefining obesity and its treatment; WHO Obesity: preventing and managing the global epidemic):

- limit energy intake;
- shift the fat consumption away from saturated to unsaturated fats and towards the elimination of trans-fatty acids;
- increase consumption of fruit and vegetables, whole grains and nuts;
- limit the intake of sugars and fats;
- engage in regular physical activity.

When diet and exercise simply aren’t enough to bring a person back to a healthy weight, there are several methods nowadays for treating an obese person, depending on the person’s level of obesity, their associated noncommunicable diseases (NCDs) and individual needs, each with its own effectiveness and side effects (Fabricatore & Wadden, 2003). The most common ones are behavioural therapy, surgery and medication (Orzano & Scott, 2004).

I) Behavioural therapy

Behavioural therapy is primarily based on principles of classical conditioning which set that eating is preceded by certain events that become strongly linked to food intake. Behavioural treatment helps patients identify the cues that lead to unhealthy eating, and learning new behaviours that will become linked to those cues (Foster et al. 2005).
In spite of it being a very healthy option, this method might prove to be very expensive if the patient chooses private. Also, not all people respond well to this kind of treatment. It can also be a very slow process (Foster et al., 2005).

II) Surgery

Next on the list is Bariatric (Obesity) surgery, which works primarily by helping to reduce the number of calories that are available in your body. Bariatric surgery offers the best chance of losing a large amount of weight in a moderate period of time (Buchwald & Williams, 2003).

Several types of surgery are available nowadays, such as Laparoscopic adjustable gastric banding (lagb) (or lap-band®), Vertical banded gastroplasty (also known as stomach stapling), etc.

However, there are certain criteria that qualify a patient for these types of intervention (Hollywood et al. 2012):

- BMI ≥ 40,
- BMI between 35 and 39.9, and you also have developed, in consequence of overweight, a serious NCD, such as diabetes, hypertension, etc.;
- You’re committed to making the lifestyle changes that are necessary for surgery to be effective on improving your condition.

Nevertheless, a surgical procedure, which, ultimately, can even result in the patient’s death, naturally has side effects, apart from the severe discomfort of being submitted to such an invasive procedure, it can pose serious risks and several limitations and it does not guarantee that you’ll lose all of your excess weight or that you won’t gain it back on the long term. The surgery may also result in inadequate weight loss, requiring additional procedure to reach the adequate weight. Weight-loss success after surgery demands a very high degree of compliance from the patient to dietary and exercise changes (Buchwald et al. 2004).

III) Medication

Medication is another option for obese people.

Just like bariatric surgery, in order to be effective, medication is to be used along with dietary, exercise and behavioural changes (Haddock et al. 2002; Greenway et al. 2008).

Mainly, doctors will resort to weight-loss medication on two situations:

- No other method has worked;
There are several weight-loss drugs that work in three different ways (Haddock et al. 2002; Greenway et al. 2008):

- Stimulant-like drugs that stimulate the central nervous system and reduce appetite, medication that increases levels of serotonin and norepinephrine, helping the patient feel full, and drugs that prevent fat absorption.

Orlistat (Xenical) is one of the latter, a Fat Absorption Inhibitor, and it is the only one that has been approved by the USA’s Food and Drug Administration (FDA) for long-term weight loss. Orlistat irreversibly blocks gastrointestinal lipase, thus inhibiting fat hydrolysis and reducing cholecystokinin (CCK) secretion. Orlistat lipase inhibition mechanism acts through a covalent bond to the lipase active site serine (Hadváry et al., 1988, 1991; Tsujita et al., 2006). By covalently blocking the lipase, orlistat inhibits the hydrolysis of dietary triglycerides (TGs) to generate free fatty acids; the inhibition of fat hydrolysis reduces the subsequent intestinal absorption of the lipolysis products monoglycerides (MGs) and free fatty acids. Unabsorbed fat is eliminated in the stool. Average weight loss with orlistat is only about 2.5 to 3.2 kilograms, more than you can get from diet and exercise after one or two years of taking the medication (Haddock et al. 2002; Greenway et al. 2008).

The FDA has approved orlistat for use in adults, children and adolescents. The FDA also has approved a reduced-strength version of Orlistat (Alli) to be sold over-the-counter, without a prescription.

Apart from lipase inhibition, there are also drugs that alter hormone levels, hence increasing satiety/suppressing appetite. Sibutramine is the first new drug for treating obesity via appetite suppression to be approved by the FDA within the past 30 years (Tziomalos et al., 2009). Its main mechanism causes an increase in the feeling of satiety by controlling noradrenalin, serotonin, 5-hydroxytryptamine, and dopamine (Lean, 2001; Poston and Foreyt, 2004).

As with surgery, medication also presents serious risks and side-effects. Patients need close medical monitoring while taking a prescription weight-loss medication. Physicians may avoid using certain prescription diet pills in patients with health issues such as hypertension, cardiac disease, hyperthyroidism or glaucoma for example. Weight-loss medication also may not work for everyone and, if the medication does work, its effects tend to level off after six months of use, so a patient may need to take this medication indefinitely to avoid regaining much or all of the lost weight (Haddock et al. 2002; Greenway et al. 2008).
All of these problematic methods make novel therapeutics to reduce food intake and body weight with minimal adverse reactions highly desirable. Thus, natural products appear as a safe and effective solution for obesity prevention and therapy.

It is a well-known fact that Nutrition is a fundamental pillar of life, health and development throughout a person’s entire life. Good nutrition is essential for physical growth, mental development, performance, health and well-being and, ultimately, survival.
Functional foods are foods that have a potentially positive effect on health beyond basic nutrition (European Commission 2010).

An everyday example is wholegrain cereals, considered as functional food because they naturally contain soluble fibre that helps lower cholesterol levels (Bell et al. 1990).

Apart from natural functional foods, some foods are modified to have extra health benefits. For example, Activia Yogurt, from Groupe Danone, is enriched with a strain of bifidobacteria that reduces intestinal transit time, thus improving bowel regularity (Kendall Powell, 2007).

Naturally, all foods can be considered functional because they do provide nutrients and energy to sustain growth, support vital processes and assure a person’s health and well-being (Linda Orh, 2004). Despite no legal definition existing for functional foods, they are generally considered to offer additional benefits that may reduce the risk of disease or promote optimal health (JA Milner 2002).

**Difference between functional foods and nutraceuticals**

There is actually no regulatory definition for a nutraceutical. Dr Stephen DeFelice coined the term "Nutraceutical" from "Nutrition" and "Pharmaceutical" in 1989.

A proposed definition to help form distinction between the two terms is that, when a functional food aids in the prevention and/or treatment of disease(s) and/or disorder(s) (except anaemia), it is called a nutraceutical. This definition may still bears further confusion, as a functional food for one person, can therefore act as a nutraceutical to another one (Ekta K. Kalra, 2003).

Another suggested definition for functional foods and nutraceuticals is, according to Health Canada, the "Federal department responsible for helping Canadians maintain and improve their health, while respecting individual choices and circumstances."

A functional food is similar in appearance to, or may be, a conventional food that is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions, i.e. they contain bioactive compound.

A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with foods. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease.
The use of nutraceuticals, as an attempt to accomplish desirable therapeutic outcomes with reduced side effects, as compared with other therapeutic agents, has met with great monetary success, hence making the discovery and production of nutraceuticals over pharmaceuticals well seen in pharmaceutical and biotechnology companies (Jadhav et al. 2005).

**FUNCTIONAL FOOD COMPONENTS**

Natural products may represent a precious help in fighting obesity, as they provide a vast pool of components that act as pancreatic lipase inhibitors which, as will be explained later on in this work, are the key element in a very effective strategy for weight reduction and satiety promotion (Chapter 3).

Several food components can be considered functional. They can be macro- or micronutrients or a component not even considered as a nutrient. These are divided into several classes, such as dietary fibre, fatty acids, probiotics, phytochemicals, etc. On this work, we will focus on the latter.

**Phytochemicals**

Phytochemicals are complex chemicals found in plants, notably in fruits and vegetables (BJ Xu et al. 2007; Sun et al. 2002)

The term "phyto" originated from a Greek word meaning plant. Phytonutrients are certain organic components of plants, and these components are thought to promote human health. Fruits, vegetables, grains, legumes, nuts and teas are rich sources of phytonutrients (Pascal & Segal 2006).

The classification of phytochemicals can be rather complex but phytochemicals can be classified into three major classes:

<table>
<thead>
<tr>
<th>Phytochemical class</th>
<th>Phytochemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpenoids</td>
<td>Monoterpenoids, iridoids, sesquiterpenoids, sesquiterpene lactones, diterpenoids, triterpenoid saponins, steroid saponins, carotenoids, bufadienolides, phytosterols, cucurbitacins, nortriterpenoids, triperpenoids, carotenoids, limonoids</td>
</tr>
<tr>
<td>Phenolic metabolites</td>
<td>Anthocyanins, anthochlors, benzofurans, chromones, coumarins, flavonoids, flavonones, flavonols, isoflavonoids, lignans, phenols, phenolic acid, phenolic ketones, phenyl-propanoids, quinonoids, stilbenoids, tannins, xanthones</td>
</tr>
<tr>
<td>Alkaloids and other nitrogen-containing constituents</td>
<td>Amaryllidacea, betalain, diterpenoid, indole, isoquinoline, lycopodium, monoterpene, sesquiterpene, peptide, pyrrolidine, piperidine, pyrrolizidine, quinoline, quinolizidine, steroidal, tropane compounds,-non-protein aminoacids, amines, cyanogenic glycosides, glucosinilates, purines, pyrimines</td>
</tr>
</tbody>
</table>
Heneman et al. (2008) presented a few of the benefits associated with some phytochemicals:

**Table 2.2. Potential health benefits from some phytochemical compounds**

(Heneman et al., 2008)

<table>
<thead>
<tr>
<th>Food</th>
<th>Phytochemical</th>
<th>Possible benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy beans, soy milk and tofu</td>
<td>Isoflavones</td>
<td>A reduction in blood pressure and increased vessel dilation</td>
</tr>
<tr>
<td></td>
<td>(Genistein and Daidzein)</td>
<td></td>
</tr>
<tr>
<td>Strawberries, red wine, blueberries</td>
<td>Anthocyanins</td>
<td>Improvement of vision, inhibition of nitric oxide production, induction of apoptosis</td>
</tr>
<tr>
<td>Red Wine, grape juice, grape extracts, cocoa</td>
<td>Proanthocyanidins and flavan-3-ols</td>
<td>Inhibition of low-density lipoprotein (LDL) oxidation, inhibition of cellular oxygenases and inhibition of proinflammatory responses in the arterial wall</td>
</tr>
<tr>
<td>Garlic, onions, leeks, olives, scallions</td>
<td>Sulphides, thiols</td>
<td>Decrease in LDL cholesterol</td>
</tr>
<tr>
<td>Carrots, tomatoes and tomato products, various types of fruits and vegetables</td>
<td>Carotenoids such as lycopene, beta-carotenes</td>
<td>Neutralization of free radicals that cause cell damage</td>
</tr>
<tr>
<td>Broccoli and other cruciferous vegetables such as kale, horseradish</td>
<td>Isothiocyanates</td>
<td>Neutralization of free radicals that cause cell damage and protection against some cancers</td>
</tr>
</tbody>
</table>

The following table presents the organization of and relationships between all classes and subclasses of phytochemicals:
Phytochemicals are divided into several sub-classes, as shown in Figure 2.1.

Here are a few facts on the main ones:

**Polyphenolic Compounds**

Polyphenolic compounds are a group of chemical substances found in plants, characterized by the presence of more than one phenol group per molecule. They are natural components of a wide variety of plants also being secondary plant metabolites (Manach et al. 2004).

Polyphenols are responsible for the colouring of some plants, for example, the colour of leaves in the autumn.

Food sources rich in polyphenols include apple, tea, red wine, red grapes, grape juice, strawberries, raspberries, blueberries, cranberries, black currants, yerba mate, peanuts, green tea, white tea, red wine, olive oil and olive derivatives, dark chocolate, and pomegranates, and other fruits and vegetables and certain nuts. High levels of polyphenols can generally be found in the fruit skins with some of the highest percentages in grape skin, apple skin and orange skin.

The flavonoids quercetin and catechins are the most extensively studied polyphenols relative to absorption and metabolism.
Research indicates that polyphenols have antioxidant characteristics with potential health benefits. These polyphenol antioxidants may reduce the risk of cardiovascular disease, present anti-inflammatory properties, anti-carcinogenicity properties and most have anti-microbial activity.

More recently reported is that these substances reduce the onset of Alzheimer's.

Polyphenols can be classified as non-flavonoids and flavonoids:

**Flavonoids** are a subclass of polyphenols (Hollman & Katan, 1999), diverse in chemical structure and characteristics than can be found ubiquitously in plants (Cook & Samman 1995). Their many functions include producing yellow or red/blue pigmentation in flowers and providing protection from attack by microbes and insects.

Flavonoids are most commonly known for their powerful antioxidant activity protecting against oxidative and free radical damage (PG Pietta 2000).

The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet.

Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity.

There are over 4,000 different flavonoids, divided into classes (Cook & Samman 1995) which include flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones.

Flavonoids are potent antioxidants, free radical scavengers, and metal chelators and inhibit lipid peroxidation.

Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancers and cardiovascular disease. The beneficial effects of fruit, vegetables, and tea or even red wine have been attributed to flavonoid compounds rather than to known nutrients and vitamins.

**Nonflavonoids** (also called Phenolic acids) account for about one third of the total intake of polyphenols in our diet, and flavonoids account for the remaining two thirds.

Phenolic acids are simple molecules such as caffeic acid, vanillin, and coumaric acid.
Phenolic acid compounds seem to be universally distributed in plants. They have been the subject of a great number of chemical, biological, agricultural, and medical studies.

**Terpenes**

The term "terpenes" originates from turpentine (lat. balsamum terebinthinae), the so-called "resin of pine trees", is the viscous pleasantly smelling balsam which flows upon cutting or carving the bark and the new wood of several pine tree species (Pinaceae) (Breitmaier, 2006).

Terpenes have been long associated with the term Essential Oils comprising resins, steroids and rubber. In fact, they are hydrocarbons that usually contain one or more C=C double bonds, while the terpenoids are oxygen-containing analogues of the terpenes. They are thoroughly distributed in the plant kingdom, especially in those plants that have abundant chlorophyll (Dewick, 2002).

There are several sub-classes of terpenes (Dewick, 2002):

- Hemiterpenes C$_5$H$_8$
- Monoterpenes C$_{10}$H$_{16}$
- Sesquiterpenes C$_{15}$H$_{24}$
- Diterpenes C$_{20}$H$_{32}$
- Sesterterpenes C$_{25}$H$_{40}$
- Triterpenes C$_{30}$H$_{48}$
- Tetramerpenes C$_{40}$H$_{64}$
- Polyterpenes (C$_5$H$_8$)$_n$

Hemiterpenes do not appear to accumulate in plant tissues, but are instead found to be associated with other compounds like alkaloids, coumarins and flavonoids.

Monoterpenes are naturally occurring compounds, the majority being unsaturated hydrocarbons (C10). But, some of their oxygenated derivatives such as alcohols, ketones, and carboxylic acids are known as monoterpenoids.

Monoterpenes together with sesquiterpenes and diterpenes form the majority of essential oils.

Like other phytochemicals we have described, these also present pharmacological properties: Antiseptics, Expectorants and diuretics, Spasmolytic and sedative, anti-inflammatory; cicatrising, etc.
Diterpenes occur in all plant families and consist of compounds having a C\textsubscript{20} skeleton. There are about 2500 known diterpenes that belong to 20 major structural types (Dewick, 2002).

In a similar manner to monoterpenes, diterpenes arise from metabolism of geranyl geranyl pyrophosphate (GGPP). These C\textsubscript{30} constituents are abundant in nature, particularly in resins and may occur as either esters or glycosides (often called saponins - molecules made up of sugars linked to steroids or triterpenes - due to their ability to make aqueous solutions appear foamy) (Dewick, 2002).

Steroids are modified triterpenes. They are most familiar from their role as hormones, i.e., androgens such as testosterone and estrogens such as progesterone. Steroids, such as cortisol, are most often used as anti-inflammatory agents, but many have other uses such as in birth control pills. Triterpenes belong to a large group of compounds arranged in a four or five ring configuration of 30 carbons with several oxygens attached. Cholesterol is one example of a triterpene (Dewick, 2002).

Probably the best known Tetraterpenes are the C\textsubscript{40} yellow or orange-red carotenoid pigments, of which about 180 have been reported (Dewick, 2002).

Polyterpenes are composed of many isoprene units. Common examples, both having macromolecules of molecular weight over 100 000, are found in India rubber and gutta-percha. Doubtless, the rubber-like substances of many other plants have similar composition (Dewick, 2002).

Polyisoprenes have yet to show any biological function (Dewick, 2002), none has yet been referred in the literature.

Saponins

Saponins are a group of phytochemicals that occur in a wide range of plant species (Hostettmann and Marston, 1995) which play important roles in plant defense against pathogens and animals (Price et al. 1987; Morrissey and Osbourn, 1999).

These compounds also are nowadays processed as drug, foaming agents, sweeteners, taste modifiers and cosmetics (Hostettmann and Marston, 1995), attributing them a considerable commercial value.

Saponins can be divided into three major groups:

Triterpenoid, steroid, or steroidal glycoalkoloid saponins (steroidal amines) (Bruneton J, 1995).

As explained earlier, saponins are closely related to triterpenes. Triterpenoid saponins are triterpenes which belong to the group of saponin compounds. They are found primarily in dicotyledonous plants but also in some monocots, steroid saponins occur mainly in monocots, such as the Liliaceae, Droscomaceae and Agavaceae and in certain dicots, such as foxglove.
Oats (Avena spp.) are unusual because they contain both triterpenoid and steroid saponins (Price et al., 1987). Steroidal glycoalkaloids are found primarily in members of the family Solanaceae, which includes potato and tomato. The steroidal glycoalkaloid ß-Tomatine is the major saponin present in tomato. However, the highest concentration of this saponin occurs in the leaves, flowers and green fruits of tomato (Roddick, 1974).

Many studies have reported pharmacological activities of saponins, such as anti-inflammatory (Takagi et al., 1980), molluscicidal (Huang et al., 2003), antimicrobial (Tamura et al., 2001), antispasmodic (Corea et al., 2005), antidiabetic and anticancer (Yuan et al., 2010), hypocholesterolemic (Seth & Sarin, 2010), antioxidant (Lu et al., 2005), anticonvulsant and analgesic (Pal et al., 2009), antitussive and cytotoxic activities (Sparg et al., 2004).

Some saponins are known to reduce the feed intake and growth rate of non-ruminant animals while others are not very harmful. For example, the saponins found in oats and spinach increase and accelerate the body's ability to absorb calcium and silicon, thus assisting in digestion. Saponins are also diverse in ginseng. Many of the known effects of this plant are due to these compounds. The anti-inflammatory activity of English ivy, Hedera helix, also is due to compounds of this group. The sweet tasting compounds of licorice, Glycyrrhiza spp., are saponins (Biochemical applications of mass spectrometry, 1972).

Saponins have been studied for their lipase-inhibiting potential with very satisfactory results (Zheng et al. 2007; Han et al. 2001; De la Garza et al. 2011).
CHOSEN METHOD: INCREASING SATIETY AND REDUCING FOOD INTAKE

It is a known fact that fat is a food product with high caloric density, playing a crucial role in weight gain/loss (Bray & Popkin, 1998; Lissner et al. 1987). This fact renders a major interest to the study of the mechanism for regulation of fat intake.

In our diet, fat (lipids) covers about 40% of our energy intake (Blundell et al. 1993). These dietary lipids are composed mainly of organic compounds including fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols (TG), phospholipids, sterols, vitamin A and E, carotenoids, and hydrocarbons (Galli et al. 2009).

Studies show that as long as fat stays in the intestine, satiety is promoted (Welch I et al. 1985). This occurs through the fat released peptide hormones, the best known being CCK, which is released by fatty acids (Beglinger & Degen, 2004). Hence, retarded fat digestion with prolonged time for delivery of fatty acids promotes satiety.

A. Satiety as a result of prolonged fat digestion

TGs are the major lipid component in the human diet (Umberto Bracco, 1994). They carry energy as well as essential fatty acids (TG consist of three fatty acids esterified to a glycerol backbone) (Umberto Bracco, 1994).

Pancreatic lipase (PL), together with its protein cofactor, colipase, is the main enzymatic system responsible for intestinal fat digestion (Lowe ME, 1994). Studies with the objective of discovering some way of inhibiting the lipase/colipase catalysed hydrolysis of TG have been largely undertaken (Tsujita et al. 2006; Bläckberg et al. 1979, Pafumi et al. 2002; Köhnke et al. 2009).

To better understand how this strategy may work, we must first understand part of the digestion mechanism, more specifically, the part which occurs when the chyme reaches the duodenum.
After a brief pre-digestion in the stomach, where some of the dietary fat is hydrolysed (Hamosh et al. 1979), the chyme is released into the duodenum.

Here is a simplified overview of the fat digestion mechanism (Fat Digestion and Absorption, 2000; Essentials of Medical Physiology, 2008):

- The chyme enters the first part of the small intestine which causes the secretion of a hormone called secretin that stimulates both the pancreas and the liver, the large fat droplets contained in the chyme stimulate the production of CCK,
- CCK causes the gallbladder to contract and secrete stored bile (produced in the liver) into the common bile duct,
- Bile salts emulsify fats into smaller particles, increasing the surface area and also lowering the particles’ surface tension, hence enhancing lipase activity (Borel et al. 1994),
- Lipase breaks down fat into monoglycerides and fatty acids,
- MGSs and fatty acids are absorbed through the intestinal villi and then reform into TGs.
The pancreatic enzymes are of immense importance because they can almost completely digest food in the absence of all other digestive secretions (Essentials of Medical Physiology, 2008). These enzymes can digest all three categories of foodstuffs (The Digestive System, 2001):

- Proteolytic enzymes for protein digestion, pancreatic amylase for carbohydrate digestion and PL for fat digestion.

The Pancreas secretes a pancreatic juice consisting of two components (Essentials of Medical Physiology, 2008):

- An aqueous alkaline secretion,
- Pancreatic enzyme-rich secretion.

The aqueous alkaline secretion is by far the largest component of pancreatic secretion and it serves a very important purpose. Pancreatic enzymes work best in a neutral or slightly alkaline environment but, upon reaching the duodenum, the chyme is highly acidic, which serves a required media for fat emulsion but, would not only inhibit enzyme activity but would also damage the duodenal mucosa. So, this secretion not only prevents acid damage to the intestinal walls but also ensures optimal functioning of the pancreatic enzymes by neutralizing the chyme’s acid content (The Digestive System, 2001).

On this work, focus will be given to PL and proteolytic enzymes.

**Pancreatic enzymes**

PL is the only enzyme present in the whole digestive system that is able to digest fat (in humans lingual and gastric lipase also exist but in negligible amounts) (Fat Digestion and Absorption, 2000; The Digestive System, 2001). It hydrolyzes TG into monoglycerides (first quickly into diglycerides and then slowly into monoglycerides) and free fatty acids, absorbable units of fat (Human Physiology: From Cells to Systems (7th Edition, 2008); Lauralee Sherwood; Cengage Learning).

PL is water-soluble but acts on insoluble substrates at oil-water interfaces. Even though it can bind alone to the surface of lipid emulsions, certain substances present in the duodenum, such as bile salts, phospholipids, dietary lipids, cholesterol esters and dietary carbohydrates inhibit PL (Erlanson-Albertsson 1992; Borgström and Erlanson-Albertsson 1982). Hence, it requires the presence of its protein cofactor, colipase, to restore its activity (Brockman 2000; Borgström et al. 1979). Colipase binds to PL in a 1:1 molar ratio to form an active-stable lipase on bile salt
Lipase is released as an inactive enzyme, a zymogen. In fact, most of the enzymes are secreted as inactive precursors or pro-enzymes (zymogens) (Biochemistry, 5th edition, 2002). The most important of all being trypsinogen (Chen et al. 2009).

Trypsinogen plays a central role in regulating all the other enzymes (Whitcomb et al. 2007). It converts to protein digesting trypsin by the release of a small peptide, in a reaction catalysed by enteropeptidase, an enzyme present in the epithelial cells of the small intestine (Bates et al. 1935; M. Kunitz, 1937). Once a small amount of trypsin has been produced it can catalyse the conversion of more trypsinogen to active trypsin.

Trypsin converts several other precursors or zymogens, such as prolipase, to their activated forms (Biochemistry, 5th edition, 2002), in this case, lipase.

**Figure 3.2. Zymogen Activation by Proteolytic Cleavage.** Enteropeptidase initiates the activation of the pancreatic zymogens by activating trypsin, which then activates other zymogens. Active enzymes are shown in yellow; zymogens are shown in tan. (Biochemistry. 5th edition, 2002)
Thylakoid membranes as natural satiety-inducing ingredients

(Albertsson et al. 2007).

Energy intake is a sine qua non condition for life to exist. We humans obtain our energy from nourishment but, green plants, algae and certain types of bacteria access this energy through sunlight, converting it into chemical energy. This process is named photosynthesis, and this term originates from two Greek words: photos = φως and synthesis = σύνθεσις, togethèrè The major chemical pathway in photosynthesis is the conversion of CO2 and H2O to carbohydrates and oxygen (Photosynthesis, 2008).

Figure 3.3. Plant cell scheme (Exploring Nature Educational Resource. © 2005 ñ 2012)

In higher plants and algae photosynthesis takes place in specialized organelles, called chloroplasts.

Figure 3.4. Green plant chloroplasts (Image: Kristian Peters ñ Fabelfroh, Evolutionary Routes ñ Wordpress.com)

These organelles present a highly complex structure and are made up of three distinct types of membrane systems:

- an outer membrane which is freely permeable to molecules,
- an inner membrane which contains many transporters,
- a network system of thylakoid membranes.
Thylakoids are flat, saclike structures located in the stroma and usually arranged in stacks called grana or grana.

Thylakoids consist of more than hundred different proteins (integral, peripheral and luminal proteins), pigments (i.e. chlorophylls), carotenes (i.e. β-Carotene), xanthophylls (i.e. zeaxanthin), membrane lipids (i.e. galactolipids), plastoquinones, tocopherols and phylloquinones (Juhler et al 1993; Dörzmierzak et al. 2009).

The thylakoid membranes, the most abundant biological membranes in nature, are the site of light-harvesting and adenosine triphosphate (ATP) synthesis (Photosynthesis, 2008).

Thylakoid membrane proteins, together with their bound pigments, contribute about 70% of whole thylakoid mass and the remaining 30% of mass made by membrane lipids, plastoquinones, tocopherols and phylloquinones. Integral membrane proteins of thylakoids play
**Chlorophyll a and b**

The basic constituents of all vegetation are the same: chlorophyll (the green pigment common to all photosynthetic cells) and other light-absorbing pigments, water, proteins, starches, waxes, and structural biochemical molecules such as lignin and cellulose (Elvidge, 1990). They all contribute to the reflectance, transmissivity and absorption spectra of each plant.

Green plants contain more than one type of chlorophyll: Blue chlorophyll (chlorophyll a), green chlorophyll (chlorophyll b), chlorofucin (chlorophyll c1, chlorophyll c2) and orange-yellow (xanthophyll) according to the pigment colours. These pigments have very specific absorbance properties, which facilitates their qualitative and quantitative analysis (Dere et al. 1997).

Studies have determined that the pigment level is influenced by factors such as amount and intensity of light, lack of nitrogen and limited nutrients (Greene et al. 1991; Collier et al. 1992) and have shown that chlorophylls have a maximum absorbance at different wavelengths depending on the chosen solvent.

For example, in pure acetone, chlorophyll a has a maximum absorbance at 661.6 nm and chlorophyll b at 644.8. (Lichtenthaler H.K., 1987)

Hence, chlorophyll a/b ratios change with the previously described factors, it is the organism’s way of adapting maintaining optimal rates of photosynthesis (Anderson et al., 1995).

![Figure 3.7 Absorbance spectra of chlorophylls a and b in acetone. (concentration 1 mg/L) (Arar et al. 1997)](image-url)
Studies show that thylakoid membranes, extracted from spinach leaves, partly inhibit the lipolytic activity of pancreatic lipase/colipase in vitro (Köhnke et al., 2009) and promote satiety in humans, as well as in mice (Köhnke et al., 2009).

The mechanism by which this takes effect has been studied and two complementary mechanisms have been proposed to explain this inhibition of lipolysis by biological membranes/membrane proteins (Emek et al., 2010):

1) Adsorption of the lipase/colipase complex onto the thylakoid membrane surface such that the substrate binding site of the enzyme complex is hindered at the thylakoid surface, preventing it from coming into contact with the lipid substrate.

2) Adsorption of the thylakoid membranes at the surface of the fat droplets (lipid/water interface) thereby covering the substrate surface and hindering the lipase/colipase to come in contact with lipid surface.

III) Phytochemicals as natural food intake inhibitors

Phytochemicals present several effects on human physiology, more specifically at digestive level, which may be harnessed to fight obesity. Studies have proven that phytochemicals present lipase inhibitory effect, suppressive effect on food intake, stimulatory effects on energy expenditure, regulatory effect on lipid metabolism, etc (Yun J.W., 2010).

Birari & Bhutani (2007) and de la Garza AL et al. (2011) have reviewed several extracts and several studies on natural extracts that present pancreatic lipase activity, without the side-effects caused by synthetic medication. The phytochemicals contained in these extracts mainly include saponins, polyphenols, flavonoids and some alkaloids such as caffeine (Kim and Kang, 2005; Han et al., 2006; Moreno et al., 2006; Shimoda et al., 2006). Some of the most widely-studied materials among the many natural sources of pancreatic lipase inhibitors are the different types of tea (green, black, oolong) (Han et al., 1999b; Lin and Lin-Shiau, 2006; Nakai et al., 2005; Thielecke and Boschmann, 2009), more specifically, compounds such as L-epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (Nakai et al., 2005).

Some of these extracts even present an effect similar to Orlistat (Chapter 1.C.III) (Tsujita et al., 2006; Birari and Bhutani, 2007), as they inhibit lipase activity. However, their inhibitory mechanisms differ from orlistat; some are reversible reaction inhibitors, while others, like orlistat, are irreversible inhibitors (Tsujita et al., 2006; Birari and Bhutani, 2007). Furthermore,
Nevertheless, as previously referred, several extracts have been studied for their pancreatic lipase inhibitory activity and for possible drug development (Birari & Butani, 2007), such as Japanese Ginseng (Han et al., 2005b), Chinese bellflower (Han et al., 2000), Salacia reticulata (Kishino et al., 2006), Indian lotus (Ono et al., 2006), Korean red ginseng (Kim et al., 2005), Camellia sinensis (Kao et al., 2000; Moon et al., 2007; Dulloo et al., 1999; Nagao et al., 2005; Wolfram et al., 2006), Caralluma fimbriata (Kuriyan et al., 2007), ephedra (Fleming, 2007), Citrus aurantium (Klontz et al., 2006), Phaseolus vulgaris (Baintner et al., 2003; Celleno et al., 2007), Robinia pseudoaccacia (Baintner et al., 2003), and sunflower oil (Ferrer-Lorente et al., 2007; Remesar et al., 2000; Romero et al., 2007; Salas et al., 2007).

Some of these phytochemical compounds have already been extracted and made available commercially. For example, natural (−)-hydroxycitric acid (HCA), prepared from Garcinia cambosia, a potential natural appetite suppressant, is currently commercially available under the names HCA-SX and Super CitriMax® (Ohia et al., 2002; Life Extension Vitamin Supplies and Life Extension Institute, Inc., Place Scottsdale, AZ, USA).

These compounds may also have a suppressive effect on food intake as they may affect satiety systems by altering hypothalamic levels of some hormones and neurotransmitters, hence suppressing appetite (Chantre and Lairon, 2002; Halford and Blundell, 2000; Wynne et al., 2005).

Naturally, the anti-nutritional effect varies with the compound involved, providing several mechanisms to fight obesity.

As practical example of a natural appetite suppressant is (−)-hydroxycitric acid (HCA), prepared from Garcinia cambosia. It acts by increasing the release/availability of serotonin, a neurotransmitter implicated in the regulation of eating behaviour and appetite control (Ohia et al., 2002). This extract has been made available commercially under the names HCA-SX and Super CitriMax® (Ohia et al., 2002; Life Extension Vitamin Supplies and Life Extension Institute, Inc., Place Scottsdale, AZ, USA).

Another similar natural appetite suppressant available on the market is CQR-300, an extract of Cissus quadrangularis (Oben et al., 2007; WellCrops International, LLC, San Francisco, CA, USA).

Eventough all this research has effectively identified several functional components in these extracts possessing appetite-suppressive capabilities (e.g. glycosides, saponin, and flavonoids), the ways in which these act to suppress appetite work (the mechanism depends on the plant type) still remain unclear, there is still insufficient clinical and laboratorial evidence
IV) Appetite suppression through Cholecystokinin release

There is still another mechanism through which satiety is promoted, direct stimulation of peptide hormone production, CCK for example.

Despite of multiple control systems existing for the regulation of satiety, the peptide CCK is the most important and most studied one (Näslund & Hellström, 2007).

Studies support that intestinal fat acts via the peptide CCK (long-chain free fatty acids by lipase-mediated hydrolysis, undigested triglycerides are not able to stimulate CCK release), released to blood from the intestine during digestion, as a physiological satiety pathway (Beglinger & Degen, 2004). CCK exerts various functions: stimulation of gallbladder contraction and exocrine pancreatic secretion, inhibition/delay of gastric emptying, and inhibition of appetite (Schwartz JG et al. 1994). Thus, CCK works as a positive feedback signal to stimulate digestive processes and as negative feedback signal to limit the amount of food consumed (Beglinger & Degen, 2004).

Evidence points to an increase in levels of circulating CCK through a trypsin-dependent mechanism (Hu et al. 2004; Komarnytsky et al. 2011).

Several studies have been developed on plant protease inhibitors (PPI) and their role as anti-nutritional agents. This class of protein compounds, commonly present in plants, present a natural defence mechanism against pests and infecting pathogens, help protect plant tissues from degradation by proteases and control sprouting (Plunkett G. et al., Arch Biochem Biophys 213, 1982).
These phytochemicals are present in several foods, soybean for example, the predominant trypsin inhibitors in soybeans and derived materials (tofu, flour, etc) are proteins (Anderson & Wolf, 1995)

But not only proteins showed trypsin inhibitory activity (TIA). Saponins and polyphenols have also presented this property. For example, green tea polyphenols (Huang et al., 2004; Ėś & Podsędek, 2004) wine polyphenols and tannins (Gonçalves et al. 2007), faba beans (Helsper et al. 1993), chickpea and pigeonpea (Singh U., 1988), white beans, coloured beans, black tea and quince (Ėś & Podsędek, 2004) have all presented a considerable TIA due to the presence of some of the previously referred phytochemicals.

Studies have shown that protease inhibitors promote CCK release through a trypsin-dependent mechanism. CCK secretion is inhibited by trypsin activity in the proximal small intestine (Chung Owyang et al., Gastroenterology 84, 1983; Schafmayer A. et al., Digestion, 1985; Spannagel AW et al., Physiology 93, 1995). The mechanism by which this occurs is still not clear but, by inhibiting trypsin activity, and therefore interfering with the protease-induced negative feedback that controls CCK secretion, the latter can be increased consequently increasing satiety and slowing/reducing gastric emptying (Schwartz JG. et al., Diabetes Care 17, 1994).

These protease inhibitors do not exert adverse effects in ruminants for example, because they are degraded in the rumen (Cheeke and Shull, 1985).

Thus, PPI as an agent that safely reduces appetite, potentially presents exceptional value and interest.
EXPERIMENTAL SECTION

A. Material

Acetic acid (FLuka, >99%), acetone (FLuka), ammonia (FLuka), aluminum chloride (AlCl₃) (FLuka), anisaldehyde (FLuka), antimony-III-chloride (FLuka), atropine (Sigma), bismuth nitrate (Merck), bovine serum albumin (BSA) (FLuka), Bradford reagent (Sigma), benzene (FLuka), caffeic acid (FLuka), chloroform (FLuka), catechin, dichloromethane (Sigma), diethyl ether (Sigma), diethylamine (Sigma), diphenylboric acid-β-ethylamino ester (=diphenylboryloxyethylamine) (Sigma), DMSO (FLuka), diosgenin, ethanol, ethyl acetate(FLuka), fast blue salt B (Sigma), ferrous sulphate (FeSO₄) (FLuka), Folin reagent (Panreac), formic acid, gallic acid (FLuka), glacial acetic acid (FLuka), hydrochloric acid (FLuka), iron-III-chloride (Sigma), lead (II) acetate (10%) (Sigma), lipase from porcine pancreas (Sigma), magnesium chloride (Sigma), methanol, n-Butanol, N-benzoil-DL-arginine-p-nitroanilide (BApNA) (Sigma), nylon mesh filters 20 µm, pentane (FLuka), phosphate buffer saline (Dulbecco’s PBS), potassium hexacyanoferrate (FLuka), potassium hydroxide (Sigma), protease inhibitor cocktail, polyethylene glycol-4000 (PEG) (Sigma), potassium iodide (Sigma), sodium chloride (NaCl) (Sigma), sodium citrate (Riedel-de-Haen), sodium hydroxide (NaOH) (Sigma), sodium sulphate (Merck), sulphuric acid (Merck), sodium phosphate buffer, sucrose (Sigma), Silica gel 60 F₂₅₄ i precoated TLC plates (Merck, Darmstadt), sodium dodecyl sulphate (SDS) (Sigma), tannic acid, lupeol, toluene (FLuka), tricine, trichloroacetic acid (FLuka), trypsin from bovine pancreas (Sigma), tris-base (FLuka), vanillin, Whatman filter paper, 3,5-dinitrobenzoic acid (FLuka).

Ultra-pure water (18.2 MΩ.cm) was obtained from a Millipore-Direct Q3 UV system (Millipore, USA).

B. Raw Materials

Fresh baby spinach, watercress and wild rucoła from Vitacress were purchased during the week and the extractions were performed according to Chapter C.

Prunes and tomato residues were obtained from juice industry by-products.

Prune was divided into three samples and was bombarded with 1.000 Watt microwave radiation for 0.5, 1 and 1.5 minutes, in order to determine if this pre-treatment would influence the extract’s efficiency in the several assays (e.g.: phenolic quantification). All three samples were extracted twice and sequentially.

Tomato residue was collected from two different points along the factory’s production line according to their granulometry. Those two samples were analysed separately, Tomato 0.6 and Tomato 0.9. The 0.9 matrix was collected further along the production line than the 0.6 matrix.
Opuntia ficus-indica cladodes were collected in Beja, Alentejo, Portugal, in October of 2010. Cladodes were sliced and were freeze-dried.

C. Extraction procedures

Different kinds of extractions were performed for the different matrixes, according to available information and objectives established for each of them.

PHYTOCHEMICAL EXTRACTION

The prune extraction, as previously described, was performed twice on the same solid samples. The initial prune matrix was homogenised using a grinder (UFESA, LC5005, China) and divided into 3 samples.

Several studies have been developed on how Microwave exposure can increase phenolic yield in an extraction (Singh et al. 2011; Zheng et al. 2011; Rafiee et al. 2011). Thus, a microwave pre-treatment was performed by the host laboratory team. Briefly, prune was divided into three samples and was exposed to 1.000 Watt microwave radiation for 0.5, 1 and 1.5 minutes, in order to determine if different pre-treatment times would influence the extract's efficiency in the several assays (e.g.: lipase inhibition).

The 3 samples were extracted sequentially twice, thus providing 6 different samples. The extractions were performed with a 50:50 ethanol:water (V:V) at a 1:4 ratio (mass:volume). Solvent, during 4 hours. Several solvents have been tested for phytochemical extractions in the laboratory and this particular one has yielded the best results up to date.

The ethanol:water 50:50 (V:V) solvent was chosen not only because of its low toxicity, but also because it is known to be an excellent phenolic extraction solvent (Nawaaz et al. 2006; Shi et al. 2003; Serpen et al. 2008). The ethanol is a good organic solvent and the water produces a swelling effect of plant tissue matrix, apart from solving some possible problems with some compounds' solubility in ethanol, hence rendering this mixture an efficient extraction solvent (Hemwimol et al. 2006).

The first extraction was performed on the 3 pre-treated samples, 0.5, 1 and 1.5 minute radiation exposure, with a 50:50 water-ethanol solvent (V:V) The mixture was stirred for 4 hours at room temperature with a magnetic stirrer. After this, the mixture was filtered, the liquid extract was freeze-dried and the solid remains were collected for further extraction.

The second extraction was performed in exactly the same conditions as the first one on the solid remains collected after the first extraction.

In order to facilitate result presentation, the following designations will be attributed to the prune samples:
THYLAKOID EXTRACTION

Method was performed for the 3 green plant matrixes: Spinach, Watercress and Rucola. Protocol was adapted from Emek et al. (2009). Matrixes were homogenized using a grinder (UFESA, LC5005, China) with a matrix to water ratio of 1:1,25 for 5 minutes. The slurry was filtrated with 20 µm pore size nylon mesh filters under vacuum. Filtrate was 10 fold diluted in water and acidified to pH 4.7 with HCl. The solution was cooled to 4ºC for 4 hours. A green deposit was obtained. The yellowish supernatant was discarded carefully not to break the sediment which was 10x diluted in water afterwards. After pH adjustment to 4.7, solution was left to settle at 4ºC for 4h. Again, yellowish supernatant was discarded and the green pellet was collected. Pellet was immediately lyophilized.

The whole procedure was conducted with minimal light exposure to minimize damage to photosensitive cells.

PROTEASE INHIBITOR EXTRACTION

This method was applied to the Opuntia ficus-indica cladodes and the 2 tomato samples.

Protease inhibitors were extracted according to Komarnytsky et al. (2011). Matrixes were homogenized in Extraction Buffer Solution (EBS i sodium chloride, acetic acid, water (1:1:9)(W:V:V)) with a matrix-EBS ratio of 1:2 (W:V), using a grinder (UFESA, LC5005, China) intermittently for 10 minutes. Slurry was centrifuged for 10 min at 13.000g (Avanti J-26 XPI, Beckman Coulter, USA). Supernatant was filtrated through Whatman filter paper. Filtered was heated to 70ºC and rapidly cooled to 30ºC to precipitate thermosensitive protein. Precipitated protein was removed through centrifugation for 10 min at 13.000g.
Supernatant was collected and thermostable proteins were precipitated through addition of sodium chloride. Solution was centrifuged at 13,000g for 10 min to isolate the precipitate, PIC (Protease Inhibitor Concentrate). PIC was collected and lyophilized.

In table 4.1 is summarized the extracts used in this work.

<table>
<thead>
<tr>
<th>Extract Designation</th>
<th>Raw Material</th>
<th>Procedure</th>
<th>Target Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>Spinach</td>
<td>Thylakoid extraction</td>
<td>Thylakoids</td>
</tr>
<tr>
<td>Rucola</td>
<td>Rucola</td>
<td>Thylakoid extraction</td>
<td>Thylakoids</td>
</tr>
<tr>
<td>Watercress</td>
<td>Watercress</td>
<td>Thylakoid extraction</td>
<td>Thylakoids</td>
</tr>
<tr>
<td>Tomato 0.6</td>
<td>Tomato</td>
<td>Protease inhibitors extraction</td>
<td>Proteins</td>
</tr>
<tr>
<td>Tomato 0.9</td>
<td>Tomato</td>
<td>Protease inhibitors extraction</td>
<td>Proteins</td>
</tr>
<tr>
<td>Plum I30</td>
<td>Plum</td>
<td>Microwave Irradiation 30s + Phytochemical extraction</td>
<td>Phytochemicals</td>
</tr>
<tr>
<td>Plum I60</td>
<td>Plum</td>
<td>Microwave Irradiation 60s + Phytochemical extraction</td>
<td>Phytochemicals</td>
</tr>
<tr>
<td>Plum I90</td>
<td>Plum</td>
<td>Microwave Irradiation 90s + Phytochemical extraction</td>
<td>Phytochemicals</td>
</tr>
<tr>
<td>Plum II30</td>
<td>Plum</td>
<td>Microwave Irradiation 30s + Phytochemical extraction</td>
<td>Phytochemicals</td>
</tr>
<tr>
<td>Plum II60</td>
<td>Plum</td>
<td>Microwave Irradiation 60s + Phytochemical extraction</td>
<td>Phytochemicals</td>
</tr>
<tr>
<td>Plum II90</td>
<td>Plum</td>
<td>Microwave Irradiation 90s + Phytochemical extraction</td>
<td>Phytochemicals</td>
</tr>
<tr>
<td>Opuntia</td>
<td>Opuntia ficus-indica cladodes</td>
<td>Protease inhibitors extraction</td>
<td>Proteins + Phytochemicals</td>
</tr>
</tbody>
</table>
In order to maximize result accuracy, all assays were performed with sample triplicates.

TOTAL PHENOLIC CONTENT

Total Phenolics Quantification was performed using the Folin-Ciocalteau colorimetric Method (Singleton & Rossi, 1965).

In short, 20μL of an extract dilution was added to 1480μL of distilled water and oxidized with 100μL of Folin Ciocalteau reagent.

The reaction was then neutralized with 300μL of sodium carbonate and, after a 30 minute incubation at 40ºC, sample absorption was measured at 765 nm using a Genesys 10 UV spectrophotometer (Thermo Spectronic, New York, USA).

Result analysis is based on a standard curve, built by values from a standard curve obtained from tests performed on solutions with known concentrations of a standard substance, gallic acid.

Results were expressed by means of a standard curve (mg of gallic acid equivalents per g dry extract – mg GAE/g dry extract). Samples were analysed in triplicate.

PROTEIN DETERMINATION

Total protein quantification was performed according to the Bradford protein assay (Bradford, 1976).

In short, 1mL of Bradford reagent and 20μL of sample were added in a cuvette. The solution was homogenized and was allowed to sit at room temperature for 5 minutes.

Sample absorbance was then measured at 595 nm using a Spectrophotometer (Genesys™ 10UV).

Bovine serum albumin was used as standard and results were expressed as mg of BSA per mL (mg BSA/mL).

THIN LAYER CHROMATOGRAPHY (TLC)

Extract chemical characterization was performed using TLC (Plant Drug Analysis, 2001).
A specific solvent was chosen for each sample and Standard solution. Specific solvent systems were prepared for each class of compounds, using up to 2 different ones of each for a single class if required to ensure proper compound identification.

The sample solution was applied to the silica-gel plate near one edge, as 4 colinear small spots (1 Pattern, 3 Solutions). After the solvent evaporated, the sheet was placed inside the chromatography chamber with the edge to which the spot was applied down. When the solvent front nearly reached the top of the adsorbent coating, the plate was removed from the container and left to dry. After the eluent evaporated, the plate was sprayed with the developing solution and left to dry, followed by compound identification in UV or Vis.

**CHLOROPHYLL QUANTIFICATION**

Chlorophyll quantification was adapted from Lichtenhaler (1987). The chloroplast lyophilized suspension was shaken in 80% aqueous acetone. Solution absorbance was measured at 750, 662, 645 and 470 nm.

**E. Enzymatic assays**

**PANCREATIC LIPASE ACTIVITY MEASUREMENT**

Pancreatic lipase activity was measured according to Sugiyama et al. (2007).

4-methylumbelliferyl-oleate (MUO) was used as the substrate. Lipase degrades 4MUO releasing 4-methylumbelliferone which can be measured using a fluorescence reader at an excitation wavelength of 320 nm and an emission wavelength of 450 nm.

In brief, Twenty-five microliters of the sample solution were dissolved in water, and 25 µL of the pancreatic lipase solution (1 mg.mL⁻¹) were mixed in the well of a microtiter plate. Fifty microliters of 4MUO solution (0.1 mM) dissolved in Dulbecco’s phosphate buffered saline was then added to initiate the enzyme reaction. After incubation at 23 °C for 20 min, 100 µL of 0.1M sodium citrate (pH 4.2) was added to stop the reaction. The amount of 4-methylumbelliferone released by lipase was measured using a fluorescence microplate reader at an excitation wavelength of 320 nm and an emission wavelength of 450 nm.

The inhibitory activity was expressed as a percentage of the control.
Trypsin activity was measured according to Gonçalves et al. (2007).

BApNA was used as substrate; temperature was 37°C (physiological temp.); pH 7.0 (duodenal pH). Trypsin degrades BApNA (in DMSO, 2.5 g.L\(^{-1}\)) releasing p-nitroaniline which is spectrophotometrically detectable at 410 nm.

Briefly, 20 µL of BApNA solution were mixed with 20 µL of sample solution in a 96-well plate. 220 µL of phosphate-buffer solution were added to each well. Absorbance was read for 3 minutes. 20µL of Trypsin solution were added (1 g.L\(^{-1}\) in phosphate buffer, pH 7.0, 50 mM). Absorbance was read for 15 minutes in a UV-Vis plate reader at 410 nm.
In this work several matrixes and their capacity to induce satiety by enzyme inhibition mechanisms were studied.

Concerning the several matrixes and why they were chosen for this work:

Spinach was chosen because studies have proved it to be a great source of lipase inhibiting thylakoids (Köhnke et al. 2009a), and furthermore not only they were the subject of the studies that initially inspired this work (Albertsson et al. 2007; Köhnke et al. 2009b) but there is currently a company being established that will commercialize spinach thylakoids as weight-controlers (http://www.naturalproductsinsider.com/news/2011/06/spinach-satiety-product-to-drop-in-2012.aspx).

Rucola and most of the vegetables belonging to the same family, have presented major phytochemical content, including phenolics and flavonoids in all different tissues, from flowers to roots, compounds such as several quercetin compounds, isorhamnetin and kaempferol (Martínez-Sánchez et al. 2007). Polyphenolic compounds are known for presenting lipase inhibitory activity (Birari and Bhutani, 2007), quercetin specifically has presented very high anti-lipase properties (Zheng et al. 2010).

Watercress has presented hypocholesterolemic and fat reducing potential (Manesh et al. 2012), its plant family phytochemical analysis has revealed the presence of flavonoids, saponins, tannins, cardiac glycosides, etc (Bhasin et al. 2011), watercress itself has been screened for phytochemicals and presented a high quantity of flavonoids, glycosides, terpenoids and saponins and a lower amount of alkaloids (Penecilla and Magno, 2011). The presence of all these compounds, aside from watercress being available as a food industry residue, as a green plant, its study was found to be interesting.

Furthermore, also rucola is a green plant, thus containing thylakoid membranes.

Studies regarding other varieties of Opuntia cactus have produced interesting results on the presence of protease inhibitors in its seeds and cladodes (Torres-Castillo J.A. et al. Acta Horticulturae 811; Torres-Castillo J.A. et al 2009), thus, as studies on Opuntia Ficus-indica were already being developed in the host laboratory, assays on the cladodesânti-trypsin capacity gained high interest.

Plums have been proved to contain high amounts of flavonols, phenolic acids, anthocyanins and other phytochemicals (Slimestad et al. 2009) as well as being able to inhibit pancreatic lipase activity (Slanc et al. 2009). Traceable amounts of powerful lipase inhibitor quercetin were found in several cultivars of plum (Slimestad et al. 2009). This alone would be more than enough to render it interesting to study the anti-lipase potential of plum extracts.
Tomato is reported as one of the two vegetables with the highest amount of protease inhibitors (Dana & Lewis, 2007). Several studies have been developed on proteinase inhibitors from tomato, mainly focusing on the production of protease inhibitors as a response to external stress (Plunkett et al. 1982; Graham et al. 1985; Jongsma et al. 1994) so, it is possible that the completely mowed tomato residue obtained from the food industry, may present a reasonable amount of inhibitors due to its processed state. Apart from this, tomato’s Lycopene has anti-inflammatory and anti-oxidant properties that can naturally be beneficial for an obese individual (Markovits et al. 2009).

As previously described, the extraction solvent for phytochemical extraction was ethanol:water 50:50 (V:V). Not only because of its low toxicity, but also because it is known to be an excellent phenolic extraction solvent (Nawaz et al. 2006; Shi et al. 2003; Serpen et al. 2008). The ethanol is a good organic solvent and the water produces a swelling effect of plant tissue matrix, apart from solving some possible problems with some compounds’ solubility in ethanol, hence rendering this mixture an efficient extraction solvent (Hemwimol et al. 2006).

Upon the analysis stage of this work, 12 samples (6 matrixes) were ready for compound and activity screening (matrixes and samples), namely spinach, watercress, rucola, opuntia cladodes, the 2 tomato samples and the 6 prune samples (Chapter 4.C.).

In this work, thylakoid extracts were prepared as previously described (Experimental Section. Methods). This method had only been assayed for Spinach matrixes, it was applied to rucola and watercress for the first time.

Apart from the properties that were previously described, matrix choices were also based in a "Green perspective" industrial waste exploit, because they are available in large quantities and are a very cheap to obtain. If any functional ingredient could be harnessed from them and made available, the commercial potential of this concept would be extremely interesting.

Obtained extracts were characterized for their total phenolic and protein content (figures 5.1 to 5.4), screened for other bioactive compounds using TLC, chlorophyll content (figure 5.15) and their inhibitory activity of lipase (figures 5.16 to 5.28) and trypsin (figures 5.29 to 5.34) was analysed.
I) Total Phenolic Content

All 12 samples were screened for total polyphenolic content (TPC) by means of the Folin Ciocalteau colorimetric method. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract and are presented in figure 5.1.

The prune extracts presented high concentrations of phenols, with the II90 extract presenting the highest value at 36,18 mg GAE / g Dry Extract.

These results indicate that not all functional components were extracted in the first extraction. In fact, it points to the 2nd extraction not yet being able to remove all the components from the matrix, as it presented the highest value of phenolic content. This could probably be due to the...
Further extractions would be required in order to determine how much more material could be extracted from the initial matrix.

The results for the first extraction were apparently independent from the radiation pre-treatment. However, the 2nd extraction values indicate that total phenolic content increases with the time the matrixes were exposed to the 1.000 Watt radiation. Further studies could probably prove if there is a direct connection between radiation exposure time and total phenolic content of an extract. Using different radiation powers would also be interesting in order to determine if this would have any effect on TPC. The fact that this relation between radiation and phenolic content is only apparent for the second extraction can also be due to the high sugar content of prune.

Spinach presented the highest value out of the three green plant matrixes (spinach, watercress and rucola), 26,061 mg GAE / g Dry Extract.

Tomato presents nearly no phenolic content, which contradicts other studies that report high phenolic content for tomato (Kaur & Kapur, 2002; Dewanto et al, 2002; Kähkönen et al. 1999; Luthria et al. 2006). Those studies however involved extremely longer extraction times (up to 24 hours of stirring, which is immense when compared to the 10 minutes of homogeneization the tomato matrix was submitted to or the 5 minute homogeneization applied to the 3 green plant samples), different extraction methods (mostly using toxic salts or solvents, which goes against one of the prime ideas of this work, extracting natural functional ingredients by only using solvents that do not render the extract toxic for ingestion) and other TPC quantification methods. Thus, no conclusions can be reached as no comparisons can be performed (Luthria et al. 2006; Marığı et al. 2009).

The result for the *Opuntia ficus-indica* cladodes was 8,02 mg GAE / g Dry Extract. Studies characterize an ethanolic extract of the stem of *Opuntia ficus-indica* as containing a high amount of phenolics (180.3 mg/g) (Lee et al. 2002). However, the extraction procedure was different, the matrix was collected in a different place and analysis methods were different, rendering it impossible to compare obtained values.

### II) Protein Quantification

The colorimetric method developed by Bradford was employed using bovine serum albumin (BSA), as a standard.

Results are presented in figure 5.3 for all 12 samples, expressed as milligrams of BSA per gram of Dry Extract.
As expected, the thylakoid membrane extracts presented the highest values (Spinach 87.01; Watercress 53.53; Rucola 13.27) as these membranes present very high protein content (Rayner et al. 2010). Amongst them, rucola presented the lowest and spinach the highest amount of proteins (figure 5.2).

Concerning the tomato samples: Again, none of the protein contents found in the literature could be compared with these values, either because the extraction methods were different, or because no similar units could be obtained. Nevertheless, two values were found for pure tomato pulp and peel puree (Ajayi and Olasehinde, 2009), 8.00 and 8.25 mg of protein/ml, a bit higher than the obtained values for both tomato matrixes (2.95 and 4.03 mg of BSA/g dry extract), nevertheless, the samples analysed in this work were extracts and not pure matrix.

Prune presented very similar values for the two extractions and three radiation times. If the error bars are considered, one can even go as far as to assume the values are the same for all samples, thus not indicating any improvement in protein yield with radiation exposure.

Values found in other studies were in the vicinity of 10g of protein per gram of dry extract (Wehmeyer, 1966), therefore, the values obtained in this study can be considered average.
TLC analysis was performed for all extracts in order to identify the presence of several compounds.

Sample volume applied to the TLC plate was the same for all classes of compounds, 20µL. Sample concentration was always 10 mg Dry Extract / mL Solvent.

Standard solution concentration varied. In order to find the optimal standard concentration for each solvent system-standard combination, before each compound class TLC analysis, several concentrations were spotted on the TLC plates.

Table 5.1 presents a summary of the obtained results.

**Table 5.1** Summary of TLC compound identification in all 12 samples

[++] many easily viewable bands; [+] viewable bands; [-] no bands were viewable

<table>
<thead>
<tr>
<th>Sample</th>
<th>Saponins</th>
<th>Tannins</th>
<th>Bitter principles</th>
<th>Alkaloids</th>
<th>Flavonoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Watercress</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rucola</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Plum I30</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Plum I60</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Plum I90</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Plum II30</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Plum II60</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Plum II90</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Tomato 0.6</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tomato 0.9</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Opuntia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
(a) Analysis of Saponins

The standard used was diosgenin 10mg/ml in ethanol. The extract solutions were developed in one solvent system (chloroform-glacial acetic acid methanol-water), and sprayed either with Anisaldehyde-Sulphuric acid reagent or with Vanillin-Sulphuric acid reagent. Using this solvent system and the two spraying reagents, clear separation of saponins was identified in the prune extracts (which was more obvious for the first extraction). The tomato extracts also presented mildly viewable bands, which is in accordance to other studies that prove that tomato contains saponins such as Esculeoside A and Ú-Tomatine (Moco et al. 2007). Despite of the migration than can be viewed in the 3 thylakoid and the opuntia sample, no bands were perceivable, leading us to believe that no saponins may be found in those matrixes. At least one study has proved that Opuntia contains saponins (Lin et al, 2003) so, either the TLC methods that were used did not have enough sensitivity to detect saponins, or the extraction method that was implemented did not allow for saponin extraction.

(b) Analysis of Tannins

The standard used was Tannic Acid 50mg/ml in ethanol.

The extract solutions were developed in one solvent system (ethyl acetate-acetic acid), and sprayed with FeCl₃.

Despite of evident migration of compounds for all extracts, except tomato and Opuntia, no bands were visible, which points to the inexistence of tannins in all twelve extracts.

Some tannins are soluble in water and alcohols, but are not soluble in organic solvents. Others are simply not soluble in any of these solvents. We know that at least Tomato (Jiang et al. 2012) and prune have tannin content so, a possible justification for these results would be that the extraction procedure and solvents did not remove tannins from the initial matrixes or that TLC does not have enough resolution to allow naked-eye detection of tannins in these matrixes.

(c) Analysis of Bitter Principles

Lupeol (triterpene) at a concentration of 15 mg/ml in chloroform was used as standard.

The extract solutions were developed in one solvent system (ethyl acetate-methanol-water), and sprayed either with Vanillin-Sulphuric acid reagent or with Liebermann-Burchard reagent.
Using this solvent system and the two spraying reagents, and with only mild migration viewable, no bands were perceivable, leading us to believe that no bitter principles may be found in all 12 samples.

Spinach has been reported to contain bitter principles (Drewnowski and Gomez-Carneros, 2000), no information was found for the remaining matrixes. A heavy migration of compounds occurred but, no bands were viewable so, the use of another method for bitter principle detection would be advisable in order to confirm these results.

TLC bitter principle screening with Liebermann-Burchard Reagent yielded different results. Clear bands were viewed in the plates for all the plum samples. Again, heavy compound migration could be seen in the thylakoid extracts. The opuntia and tomato samples did not yield any bands.

(d) **Analysis of Alkaloids**

Standard was atropine 20 mg/ml in ethanol.

The extract solutions were developed in one solvent system (Toluene-ethyl acetate-diethylamine), and sprayed either with Drangendorff reagent or with Iodoplatinate reagent. With this solvent system and the two spraying reagents, no bands were viewable and migration was only present with iodoplatinate reagent for the 3 thylakoid samples, evidence points to the absence of alkaloids in all 12 samples.

This is in alignment with several studies that indicate that alkaloids mainly occur in plants instead of fruits or vegetables.

(e) **Analysis of Flavonoids**

Standard was catechin 15 mg/ml in ethanol.

The extract solutions were developed in one solvent system (Ethyl acetate formic acid glacial acetic acid water), and sprayed either with Fast blue salt reagent (FBS) or with NP-PEG reagent. Plates were inspected in UV 365nm. With this solvent system and the two spraying reagents, the presence of flavonoids was observed in all samples.

Several Flavonoids have been identified in spinach, tomato, watercress (Haytowitz et al. 2002; Cho et al. 2008), opuntia (Cai et al. 2010), rucola and plum (USDA Database for the Flavonoid Content of Selected Foods). This presence of flavonoids in all matrixes is confirmed by the TLC analysis. Clear bands could be seen in all plates.
Chlorophyll content was determined, as previously described in material and methods section, in the thylakoids extracts since it has been used previously and an indicator of thylakoid extraction efficiency (Arnon, 1949). Results are presented in figure 5.3.

**Figure 5.3** Chlorophyll a and b concentration in Watercress, Spinach and Rucola extracts. Results are expressed in µg of chlorophyll per milligram of dry extract. (Chl a į Chlorophyll a ; Chl b į Chlorophyll b)

Values for chlorophyll were 26,94 µg Chl a + 12,37 µg Chl b (total 39,31 µg) per gram of dry extract for Rucola, 22,52 µg Chl a + 12,97 µg Chl b (total 35,49) for Watercress and 17,38 µg Chl a + 11,87 µg Chl b (total 29,25 µg) for spinach. All 3 extracts presented very similar values for chlorophyll b, a slight difference can be easily seen for chlorophyll a.
The 3 Green Plants and 6 prune extracts were screened for their potential as lipase inhibitors. For the 3 green plant extracts, results were presented as Inhibition percentage per µg of chlorophyll and per mg of dry extract. For plum extracts, results are presented as inhibition percentage per mg of dry extract.

**Figure 5.4** Lipase inhibitory activity of spinach extract. Results are expressed as Inhibition percentage per µg of chlorophyll.

**Figure 5.5** Lipase inhibitory activity of watercress extract. Results are expressed as Inhibition percentage per µg of chlorophyll.
Figure 5.6: Lipase inhibitory activity of rucola extract. Results are expressed as inhibition percentage per µg of chlorophyll.

For the inhibition-chlorophyll graphics, spinach presents the highest value of inhibition with the lowest chlorophyll concentration (94.67% for a total 292.5 µg Chl / ml). The results show that the Watercress thylakoids are the 2nd most effective lipase inhibitors (total 322.14 µg Chlorophyll / ml cause a 95.56% inhibition) and the Rucola thylakoids shall be the third best matrix (372.14 µg / 96.82%).
Figure 5.7 | Lipase inhibitory activity of spinach, watercress and rucola extract. Results are expressed as Inhibition percentage per different extract concentrations.

However, the classification is reversed for extract efficiency when results are presented as a function of dry extract weight. A 10 mg dry extract/mL Rucola extract causes a 96.82% inhibition of pancreatic lipase (the highest value), while for the same concentration, the Watercress extract exerts a 95.56% inhibition and the Spinach extract a 94.67% suppression of lipase activity.
Figure 5.8  Lipase inhibitory activity of plum 1st extract samples. Results are expressed as Inhibition percentage per different extract concentrations.

Figure 5.9  Lipase inhibitory activity of plum 2nd extraction samples. Results are expressed as Inhibition percentage per different extract concentrations.
All plum extracts present a very high inhibitory activity. In the absence of thylakoid membranes, this effect is caused by the high amount of phenolic compounds the extracts contain (de la Garza AL et al. 2011), as figure 5.2 demonstrates.

Figure 5.28 alerts to the relative strength of the Orlistat pill when compared with the natural extracts. A very small amount of Orlistat is needed to cause the same inhibitory effect as the studied matrices. However, as previously stated, Orlistat is reported as having several side effects, so, the study of natural products as an alternative remains a subject of great interest.

The following table contains the IC50 (half maximal inhibitory concentration) values for all 9 extracts. The IC50 is used as a measure of an extract or drug effectiveness.
### Table 5. IC50 values for the lipase inhibitory extracts

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC50 (mg dry extract/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watercress</td>
<td>1.173</td>
</tr>
<tr>
<td>Spinach</td>
<td>0.912</td>
</tr>
<tr>
<td>Rucola</td>
<td>1.094</td>
</tr>
<tr>
<td>Plum</td>
<td></td>
</tr>
<tr>
<td>I30</td>
<td>0.275</td>
</tr>
<tr>
<td>I60</td>
<td>0.437</td>
</tr>
<tr>
<td>I90</td>
<td>0.361</td>
</tr>
<tr>
<td>II30</td>
<td>0.264</td>
</tr>
<tr>
<td>II60</td>
<td>0.302</td>
</tr>
<tr>
<td>II90</td>
<td>0.372</td>
</tr>
</tbody>
</table>

#### VI) Trypsin Activity Inhibition

Trypsin inhibition was assessed as previously described. Results were presented relating inhibition percentage with both sample concentration (mg Dry Extract / mL) and Protein Concentration (mg BSA / mL).

The maximum screened sample concentration was 400 mg of dry extract / ml. This was the maximum amount of sample that could be diluted, thus, we weren't able to reach superior inhibition values.
Tomato 0.9 extract has the highest inhibitory activity, 42.05% for 400mg of Dry Extract / mL solution. It also presents higher inhibitory activity for every other sample concentration. The difference between each value however, for the three matrixes considering the error bars, is not enough to prove that Tomato 0.9 is the strongest protease inhibitor. Nevertheless, opuntia presented the 2nd best results, in front of Tomato 0.6. It would be however more accurate to say that the 3 samples present very similar, if not even equal trypsin inhibitory activity.
Figure 5.12 ̅ Trypsin inhibitory activity of tomato 0.6 extract. Results are expressed as Inhibition percentage per different extract concentrations.

Figure 5.13 ̅ Trypsin inhibitory activity of tomato 0.9 extract. Results are expressed as Inhibition percentage per different extract concentrations.
When the results are presented relating protein content with inhibitory activity, the order completely changes. Opuntia is now the strongest inhibitor, followed by Tomato 0.6, with Tomato 0.9 presenting the lowest Inhibitory Activity – Protein Concentration ratio.

Other studies have been developed for tomato’s trypsin inhibitory activity evaluation. In another study for example (Komarnytsky et al. 2011), a single dose of 100 mg/kg of rat body weight, decreased trypsin-like activity in the duodenum by 47.3% one hour after administration to Male Wistar rats, weighing 180–200 g. No effective comparison may be established however, eventhough the extraction method was extremely similar, the trypsin inhibitory activity assay was completely different (nondenaturing PAGE gels were used instead of 96-well plates, N-Acetyl-phenylalaninephthylester was used as substrate, instead of N- Benzoyl-DL-arginine 4-nitroanilide hydrochloride).

Looking at Table 6.1, some interesting information can be found and some relations may be established between the several results.

The first thing that pops out is the extremely similar IC50 values for spinach and rucola, eventhough the phenolic and proteic content is extremely higher for spinach. As explained earlier, several studies have proven that phenolic compounds present lipase inhibitory activity (Birari & Bhutanii 2007; de la Garza AL et al. 2011; Kim and Kang, 2005; Han et al., 2006; Moreno et al., 2006; Shimoda et al., 2006; Han et al., 1999b; Lin and Lin-Shiau, 2006; Nakai et al., 2005; Thielecke and Boschmann, 2009), and others have pointed out that membrane proteins account for approximately 70% of the thylakoid mass (Rayner et al. 2010). Hence, taking both these facts into account, we can safely say that rucola is a very powerful lipase inhibitor. Either the type of phenolic compounds present in the matrix (although being few of them) are extremely powerful lipase inhibitors (Gos J. and Podsňdek A. (2004) suggested that
low molecular weight polyphenols present lower inhibitory activity than high molecular weight type present different modus operandi from the ones extracted from spinach. The very high chlorophyll concentration can be another fact that points to this conclusion. Further analysis of the rucola extract could give out important information as to exactly why this effect is this powerful.

In regard to protein content, no actual conclusions can be obtained from these results, Trypsin inhibition however appears to be related to the protein content of the studied extracts. This conclusion is in accordance to what we expected to obtain, as studies have shown that trypsin inhibitors are a class of protein compounds (Dana & Lewis, 2007).

<table>
<thead>
<tr>
<th>Table 5.3</th>
<th>Summary of some results for the several matrixes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Phenolic Content (mg GAE/g dry extract)</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
</tr>
<tr>
<td></td>
<td>Watercress</td>
</tr>
<tr>
<td></td>
<td>Rucola</td>
</tr>
<tr>
<td>Plum</td>
<td>I30</td>
</tr>
<tr>
<td></td>
<td>I60</td>
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<td></td>
<td>I90</td>
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<td>II30</td>
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<td></td>
<td>II60</td>
</tr>
<tr>
<td></td>
<td>II90</td>
</tr>
<tr>
<td>Tomato 0,6</td>
<td>0,00</td>
</tr>
<tr>
<td>Tomato 0,9</td>
<td>0,15</td>
</tr>
<tr>
<td>Opuntia</td>
<td>8,02</td>
</tr>
</tbody>
</table>

57
In summary:

All extracts were screened for Total Phenolic Content (Folin-Ciocalteau) and Protein Content (Bradford), only the 3 green plants, Spinach, Watercress and Rucola were screened for Chlorophyll Content, Presence of Bioactive Compounds (TLC), Lipase Inhibitory Activity (LIA) and Trypsin Inhibitory Activity (TIA).

The highest values of Total Phenolic Content were obtained for the 6 prune samples (ranging from 36.18 to 25.45 mg of GAE / g Dry Extract), followed by spinach (26.06 mg of GAE / g Dry Extract).

With respect to Protein Quantification, the 3 Green Plant extracts presented the highest values (Spinach 87.01; Watercress 53.53 and Rucola 13.27 mg of BSA / g of Dry Extract). This would be expected as thylakoid membranes contain a very large amount of different proteins and these 3 extracts were basically crude thylakoid membranes.

The TLC screening for compounds confirmed that most of the extracts contained bioactive compounds that can be very helpful in fighting obesity such as saponins, polyphenols, terpenes (Birari & Butani, 2007). The prune extracts were the most promising, for they contained mostly all of the compounds we expected to find, e.g.: polyphenols and saponins, which present lipase inhibitory activity (Birari & Butani, 2007).

Regarding chlorophyll quantification, Rucola had the highest chlorophyll:dry extract mass ratio, followed by watercress, with spinach having the lowest amount of chlorophyll per mass unit of dry extract.

Chlorophyll quantity is indicative of the amount of thylakoid membranes present in an extract. Having the highest value for chlorophyll, rucola extract should, therefore, theoretically, be the strongest inhibitor. In spite of taking the second place, after the spinach extract, their IC50 values are very similar. Rucola however, in spite of having a higher IC50 value, it has considerably lower phenolic and proteic contents than spinach. This allows us to present rucola as being the most effective pancreatic lipase inhibitor of the 3 thylakoid extracts. Spinach actually has the best IC50 value (lowest), which can probably be due to its very high proteic content, indicating a very high thylakoid concentration.

The prune matrixes presented the lowest IC50 value of all matrixes (the best ones). The presence of elevated amounts of phenols and saponins would account for this fact.

Still, all matrixes presented a considerably low lipase inhibitory activity when compared to Orlistat. Naturally this would be expected as we are comparing a synthetic drug to natural extracts, but it raises the necessity of further studies being undertaken in order to try and improve the lipase inhibitory activity of the extracts. The extraction process time could be increased well over the few minutes that it took to perform all extractions apart from prune. This one alteration could probably render much better results.
In what refers to trypsin inhibition, the extract that presented the highest trypsin inhibitory activity expressed as percentage of inhibition of Dry Extract mass / solution volume, was tomato 0.9, followed by the Opuntia ficus-indica cladodes extract. The Tomato 0.6 extract presented the lowest TIA.

As previously described, tomato and potato are stressed as the vegetables with the largest quantity of protease inhibitors (Dana & Lewis, 2007), so, it would be interesting to apply this method to a potato matrix in order to compare results.

When extract concentration was expressed as percentage of inhibition per protein quantity however, this order changed. Opuntia ficus-indica is now the extract with the highest trypsin inhibitory activity (TIA), Tomato 0.6 is the 2nd best and Tomato 0.9 presents the lowest TIA.

The results for the prune matrixes, regarding the exposure to radiation, were very promising. As explained earlier, no apparent increase in yield was caused by 1.000 Watt radiation exposure in the three 1st extraction matrixes, probably due to the high viscosity of the extract caused by very high sugar content. The matrixes for the 2nd extract however, referring to Total Polyphenolic Content, presented a clear increase in yield with extended radiation exposure time. The next steps here would be to try different exposure times and different radiation power in order to assess if this would enhance extraction efficacy.
Obesity represents a major health issue nowadays. Several factors contribute to the appearing of this disease and, depending on the person’s level of obesity treatment can comprehend behavioural therapy, surgery and/or medication. However, none of these methods are neither safe nor do they ensure the desired results.

Natural products represent a safe and effective alternative to these methods and, not only can they be regarded as a treatment but also as a prevention strategy. Amongst the many natural products are phytochemicals presents an interesting alternative to counteract this epidemic. The main objective of this work was to evaluate the potential of some natural extracts derived from fruits and vegetables, namely spinach, watercress, rucola, tomato, plum and Opuntia ficus-indica cladodes, for their use against obesity.

The capacity of these extracts to induce satiety was evaluated by their ability to inhibit pancreatic lipase activity (in the case of extracts from plum and green vegetables) and trypsin activity (for extracts of tomato and Opuntia cladodes).

The plum samples presented the best lipase inhibitory activity. This result would be expected as these matrixes presented the highest phenolic content (ranging from 36.18 to 25.45 mg of GAE / g Dry Extract) and contain bitter principles, saponins and flavonoids, namely quercetin.

In regard to the 3 green plant matrixes (spinach, rucula and watercress), they all presented a relatively similar IC50 value, relatively to their inhibitory enzymatic effect. It is however interesting to correlate with their phenolic content and proteic content. Spinach, that showed the lowest IC50 (0.91mg dry extract) compared with the other green plants, had the highest phenolic (26.06 mg GAE / g dry extract) and highest proteic content (87.01 mg of BSA / g of Dry Extract), which indicates a high amount of phenolic lipase inhibitors and a high amount of thylakoid membranes. Rucola, that contains quercetin, was the second most active extract between the green plants, despite it had the lowest amount of phytochemicals and protein. Quercetin is a very powerful lipase inhibitor.

In what refers to trypsin inhibition, the extract that presented the highest trypsin inhibitory activity (TIA) expressed as percentage of inhibition per Dry Extract mass / solution volume, was tomato 0.9, followed by the Opuntia ficus-indica cladodes extract. The Tomato 0.6 extract presented the lowest TIA. As previously described, tomato and potato are stressed as the vegetables with the largest quantity of protease inhibitors (Dana & Lewis, 2007), so, it would be interesting to apply this method to a potato matrix in order to compare results. When extract concentration was expressed as percentage of inhibition per protein quantity however, this order changed. Opuntia ficus-indica is now the extract with the highest trypsin inhibitory activity (TIA), Tomato 0.6 is the 2nd best and Tomato 0.9 presents the lowest TIA.


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