



Arbutus unedo L. and Ocimum basilicum L. as sources of natural ingredients for bread functionalization

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In the name of God, the Most Gracious, the Most Merciful

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TABLE OF CONTENT

<i>Acknowledgments</i> -----	2
<i>List of figures</i> -----	6
<i>List of tables</i> -----	7
<i>List of Abbreviations</i> -----	8
Abstract -----	10
Resumo -----	12
I. Introduction -----	14
The importance of using food additives -----	14
Classes of food additives -----	16
Preservatives-----	17
Nutritional agents-----	19
Coloring agents -----	19
Flavoring agents -----	20
Texturizing agent -----	21
Miscellaneous agents -----	21
Safety issues of synthetic food additives -----	21
Natural preservatives -----	23
Advantages of natural preservatives over the synthetic ones -----	23
Plants as sources of natural preservatives-----	25
Well succeeded examples -----	26
<i>Ocinium basillicum</i> as source of rosmarinic acid -----	28
<i>Arbutus unedo</i> as a source of catechins -----	29
II. Objectives -----	32
III. Material and Methods -----	33
Standards and reagents -----	33

Plants material extraction	33
<i>Arbutus unedo</i> – Catechin extraction	33
<i>Ocinium basillicum</i> – Rosmarinic acid extraction	34
Chemical characterization	35
Catechin and rosmarinic acid quantification	35
Evaluation of the antioxidant activity	36
DPPH radical scavenging activity	36
Reducing power	37
Inhibition of β -carotene bleaching	38
Thiobarbituric acid reactive substances (TBARS) assay	38
Evaluation of the hepatotoxicity	39
Evaluation of antimicrobial properties	40
Incorporation of <i>Arbutus unedo</i> fruits and <i>Ocinium basillicum</i> as natural ingredients in bread	42
Preparation of the bread samples	42
Evaluation of the nutritional properties	43
Evaluation of the antioxidant activity of the bread samples	44
Statistical analysis	45
IV. Results and Discussion	46
Biological activity of plant extracts	46
Characterization of different types of functionalized bread	49
Linear Discriminant Analysis	55
V. Concluding Remarks	58
References	59

List of figures

Figure 1. Groups and subgroups of food additives (Carocho et al., 2014).	17
Figure 2. Chemical structure of rosmarinic acid.	28
Figure 3. <i>Ocinium basilicum</i> L.	28
Figure 4. <i>Arbutus unedo</i> L.	30
Figure 5. Chemical structure of catechin.	30
Figure 6. Catechin extraction from <i>Arbutus unedo</i> L fruits.	34
Figure 7. Rosmarinic acid extraction from <i>Ocinium basilicum</i> leaves.	35
Figure 8. Microplate used in the evaluation of DPPH radical-scavenging activity	37
Figure 9. Microplate used in the evaluation of reducing power.	37
Figure 10. Tubes used in the β -carotene bleaching inhibition assay	38
Figure 11. Tubes used in the TBAS assay	39
Figure 12. Bread sample	42
Figure 13. DPPH scavenging activity of different bread types assayed in three consecutive periods.	54
Figure 14. Reducing power of different bread types assayed in three consecutive periods.	55
Figure 15. Canonical discriminant functions coefficients defined from the evaluated parameters to assess the effect of bread type	56

List of tables

Table 1. Advantages and disadvantages of natural and synthetic antioxidants commonly used for food preservation (Valenzuela and Nieto, 1996).	25
Table 2. Antioxidant activity (EC ₅₀ values, µg/mL extract) of the extracts of <i>A. unedo</i> fruit and <i>O. basilicum</i> leaves. Values are given as mean±standard deviation.	46
Table 3. Antibacterial activity (MIC and MBC values, mg/mL extract) of the extracts of <i>A. unedo</i> fruits and <i>O. basilicum</i> leaves.	47
Table 4. Antifungal activity (MIC and MFC values, mg/mL extract) of the extracts of <i>A. unedo</i> fruits and <i>O. basilicum</i> leaves.....	48
Table 5. Nutritional composition (g/100 g fw), energy (kcal/100 g fw) and pH values of different bread types (BT) and storage times (ST). Results are presented as mean±standard deviation. ¹	50
Table 6. Color parameters of different bread types (BT) and storage times (ST). Results are presented as mean±standard deviation. ¹	51
Table 7. Individual sugars (g/100 g) of different bread types (BT) and storage times (ST). Results are presented as mean±standard deviation. ¹	52
Table 8. Major fatty acids (relative percentage of different bread types (BT) and storage times (ST). Results are presented as mean±standard deviation. ¹	53

List of Abbreviations

ADI	Acceptable Daily Intake
BHA	Butylated hydroxyanisole
BHT	Butylated Hydroxytoluene
BT	Bread Type
CA	Codex Alimentarius
DAD	Diode Array Detector
DMEM	Dulbecco's Modified Eagle Medium
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EU	European Union
EFSA	European Food Safety Authority
EMM	Estimated Marginal Means
ESI	Electrospray ionization
EC₅₀	Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FeSO₄	Iron sulfate
JECFA	Joint FAO/WHO Expert Committee on Food Additives
HPLC	High Pressure Liquid Chromatography
IMP	Inosine monophosphate
INS	International Numbering System
INT	Iodonitrotetrazolium
GLM	General Linear Model
GMP	Guanosine Monophosphate
GC-FID	Gas Chromatography Coupled to Flame Ionization Detector
GI₅₀	Concentration that inhibited 50% of the net cell growth
K	Potassium
LDA	Linear Discriminant Analysis
MA	Malt agar
MBC	Minimum Bactericidal Concentration

MDA	Malondialdehyde
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
MH	Mueller-Hinton agar
MS	Mass Spectrometer
Na	Sodium
PDA	Photodiode Array Detector
PG	Propyl Gallate
RA	Rosmarinic Acid
RI	Reflexion Index
RP	Reducing Power
RSA	Radical Scavenging Activity
ROS	Reactive Oxygen Species
ST	Storage Time
TBA	Thiobarbaturic Acid
TBHQ	Tertiary Butylhydroquinone
TBARS	Thiobarbituric Acid Reactive Substance Assay
Tris-HCL	Hydrogen Chloride
USA	United State of America
UV	Ultra Violet
WHO	World Health Organization

Abstract

The overwhelming growth of the world population is causing direct consequences on food demands, which has forced the food industry to apply synthetic additives in order to meet the needs of the consumer and ensuring the global quality of the food products including aspect, texture and flavor. However, due to the publication of some studies describing adverse effects associated with these synthetic additives, the natural ones have been gaining increasing interest.

Arbutus unedo L. (strawberry tree) fruits and *Ocimum basilicum* L. (basil) leaves, are important sources of bioactive natural molecules. The aim of this work was to demonstrate the antioxidant potential (free radical scavenging effect, reducing power and inhibition of lipid peroxidation), antimicrobial (antibacterial using Gram-positive and Gram-negative strains and antifungal activity) and the absence of toxicity (porcine liver primary cells) of the ethanol extracts, obtained from the previously mentioned plants by using optimal conditions (23% of ethanol in *A. unedo* fruits; 80% of ethanol in *O. basilicum* leaves). Subsequently, the enriched extracts in catechin from *A. unedo* fruit and rosmarinic acid from *O. basilicum* leaves, were incorporated as natural ingredients in bread samples, with two objectives: to functionalize bread, introducing bioactive properties; and as natural ingredients with preservation capacity. Five groups of bread samples were prepared: i) control (without natural or synthetic additives); ii) bread incorporated with *A. unedo* fruit extract; iii) bread incorporated with *O. basilicum* leaves extract; iv) bread incorporated with ascorbic acid- E300 (natural additive); and v) bread incorporated with potassium sorbate - E202 (synthetic additive).

Both extracts (*A. unedo* fruit and *O. basilicum* leaves) presented antioxidant activity (EC₅₀ values lower the 595 and 351 µg/mL, respectively), without toxicity up to the maximal tested dose (400 µg/mL), and the main compounds present were catechin and rosmarinic acid, respectively.

The breads enriched with the extracts obtained from *O. basilicum* leaves revealed the highest DPPH scavenging activity, showing to be more effective than ascorbic acid and potassium sorbate. Regarding reducing power all bread type's revealed similar bioactivity. Some significant differences were observed for the color and nutritional parameters analyzed, and the bread with natural extracts showed lower values for color parameters (L^* and a^*) when compared to the breads with the chemical

additives. In relation to the nutritional parameters, higher values of water were observed in the breads with *A. unedo* fruits and ascorbic acid, whereas the higher values of fructose, energy and ash were attributed to the bread with *O. basilicum* leaves and potassium sorbate.

Overall, the obtained results demonstrate that these extracts could be used in the development of new bread replacing the synthetic preservatives, without interfering with the physical and nutritional characteristics.

Keywords: *Arbutus unedo* L.; *Ocimum basilicum* L.; Natural ingredients; Bread.

Resumo

O grande crescimento da população mundial tem efeitos diretos na procura de alimentos, o que obriga a indústria alimentar a aplicar aditivos sintéticos para atender às necessidades do consumidor e garantir a qualidade global dos produtos alimentares, incluindo aspeto, textura e sabor. No entanto, devido à publicação de alguns estudos que descrevem os efeitos adversos associados a aditivos sintéticos, o interesse pelos naturais têm vindo a aumentar.

Os frutos de *Arbutus unedo* L. (medronho) e folhas de *Ocimum basilicum* L. (manjeriço) são importantes fontes de moléculas naturais bioativas. O objetivo deste trabalho foi de demonstrar o potencial antioxidante (atividade captadora de radicais livres, poder redutor e inibição da peroxidação lipídica) e antimicrobiana (atividade antibacteriana utilizando bactérias Gram-positivo e Gram-negativo e antifúngica), e a ausência de toxicidade (células primárias de fígado porcino) de extratos etanólicos, obtidos a partir das plantas mencionadas anteriormente seguindo as condições otimizadas (23% de etanol para os frutos de *A. unedo*; 80% de etanol para as folhas de *O. basilicum*). Posteriormente, os extratos enriquecidos em catequina obtidos de frutos de *A. unedo* e em ácido rosmarínico obtidos de folhas de *O. basilicum*, foram incorporados como ingredientes naturais em amostras de pão, com dois objetivos: por um lado funcionalizar o pão introduzindo propriedades bioativas; e por outro lado verificar a capacidade de preservação destes ingredientes naturais. Para tal, foram preparados cinco grupos de amostras de pão: i) controlo (sem aditivos naturais ou sintéticos); ii) pão incorporado com extrato de frutos de *A. unedo*; iii) pão incorporado com extrato de folhas de *O. basilicum*; iv) pão incorporado com ácido ascórbico - E300 (aditivo natural); e v) pão incorporado com sorbato de potássio - E202 (aditivo sintético).

Ambos os extratos (frutos *A. unedo* e folhas *O. basilicum*) apresentaram atividade antioxidante (com baixos valores de EC₅₀ de 595 e 351 µg/mL, respetivamente), sem toxicidade até a dose máxima testada (400 µg/mL), e os principais compostos presentes foram catequina e ácido rosmarínico, respetivamente.

Os pães enriquecidos com extratos obtidos a partir de folhas de manjeriço e frutos de medronho revelaram maior capacidade antioxidante em comparação com os aditivos sintéticos.

Os pães enriquecidos com os extratos obtidos das folhas de *O. basilicum* revelaram a maior atividade captadora de radicais DPPH, mostrando ser mais eficaz do que o ácido ascórbico e sorbato de potássio. Em relação à redução do poder, todos os diferentes tipos de pães revelaram bioatividade semelhante. Observaram-se algumas diferenças significativas relativamente à cor e aos parâmetros nutricionais analisados, sendo que os pães com extratos naturais apresentaram valores mais baixos para os parâmetros de cor (L^* e a^*) quando comparados com os pães com os aditivos químicos. Em relação aos parâmetros nutricionais, os valores mais elevados de água foram observados nos pães incorporados com extratos de frutos de *A. unedo* e ácido ascórbico, enquanto os valores mais elevados de frutose, energia e cinzas foram atribuídos aos pães incorporados com extratos de *O. basilicum* e de sorbato de potássio.

No geral, os resultados obtidos demonstraram que os extratos utilizados podem ser utilizados no desenvolvimento de pão substituindo os conservantes sintéticos, sem interferir nas características físicas e nutricionais.

Palavras-chave: *Arbutus unedo* L.; *Ocimum basilicum* L.; Ingredientes naturais; Pão.

I. Introduction

The importance of using food additives

There is an overwhelming growth of the world's population every decade, which has direct consequences on the consumption habits and the launching of new products by the food industry, which is always trying to meet the consumer's perspective (thelugarcenter.org/pp/area-8.pdf global food security). Consequently, the continuous population growth will have a direct effect on food demands (Godfray et al., 2010).

There is still the concern of production in large quantity to guarantee the satisfaction of the entire population. However, some of these products have to travel long distances and it is necessary to ensure that they reach their destiny in the desirable conditions (Motarjemi & Todd, 2014).

Many of the ingredients known as additives are not a recent introduction to the food industry but have been used in foods for hundreds of years. Once man had worked out to obtain more food than needed for immediate consumption, he started to think how to preserve them for longer times of storage, by using physical methods, or more recently, by adding some molecules (Carocho et al., 2014).

The need for food preservation led to the development of physical methods such as thermal processing, refrigeration, drying, concentration and the use of irradiation. In spite of this advancement in preservation methods, there are still some less developed countries where there is a shortage of infrastructures capable of preserving food using these technologies. As a consequence, they also resort to the use of cheaper chemical methods for the same preservation. Both chemical and physical method used as food preservatives can be used in combination so that allows the possibility of adding a lower concentration of chemical substances and, consequently, retaining the quality of the product (Surekha & Reddy, 2014).

For this propose, food additives have a crucial importance in order to satisfy consumers' demands by enhancing the appearance and flavor of food and prolong shelf life. Over the centuries, these have been used by the food industry to stabilize and preserve food or to add color or flavor, texturize, sweeten, thicken, soften, emulsify and so many other applications (Rasooli, 2007).

In 1995, Food and Agriculture Organization (FAO) and the World Health Organization (WHO) proposed an official definition for the term "additive", however, this definition has undergone some changes over the years and since 2012 it has been

established the definition “any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods” (Codex Alimentarius, 2015). The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities (Motarjemi & Todd, 2014; Carocho et al, 2015a).

There are currently two major regulators of food additives, the European Food Safety Authority (EFSA) for European Union (EU) and the Food and Drug Administration (FDA) for United States of America (USA).

In EU all food additives are identified with a specific number prefixed by the letter E (referred to Europe). This nomenclature was aimed to label additives whether approved or not in the EU. The Codex Alimentarius Commission put a huge effort to create a single database of labeled food additives to be accredited and used within the EU (Carocho et al., 2014).

So, most of countries, using either Codex Alimentarius, national or multinational systems, have a program of approval of food additives. Every food additive is assigned as a unique number in the International Numbering System (INS), adopted and extended by the Codex Alimentarius (Saltmarsh, 2013). A formal process exists for analyzing the test data on food additives. This safety evaluation is carried out at different levels. At a global level it is performed by a scientific advisory body of the FAO and WHO, namely the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In addition to countries, JECFA advises the Codex Alimentarius Commission, which has international responsibility for managing food safety and developing food standards (Codex Alimentarius, 2015). Despite of legislations, there are huge differences between countries on the definitions of food additives. For example, in the USA the term ‘food additive’ is not only limited to substances added for a technological purpose but also includes ingredients indirectly added for other purposes, such as boiler water additives and monomers from plastic food contact materials, and consequently some 3000 substances are approved for use as ‘additives.’ In contrast, Canadian regulations recognize only approximately 400 ‘technical’ food additives (Tomaska & Brook-Taylor, 2014).

Many of the food additives in use today have been assessed by JECFA as well as by various regional bodies, such as the European Food Safety Authority and countries such as the USA, Canada, Japan, and Australia and are regarded as safe, when used as specified. However, unfortunately, there is some illegal use of chemicals that are not approved as safe food additives especially in some developing countries, hence, human health continues to be at risk in these regions of the world (Tomaska & Brook-Taylor, 2014).

Classes of food additives

As previously mentioned, an additive may be defined as a substance or a mixture of substance, other than basic food compounds, which is present for reasons of production, processing, storage, or packaging so that the final product does not have any risk of contamination (Patel et al., 2010; Maqsood et al., 2013).

Due to great advances in food technology, there are a wide variety of additives. It is estimated that there are currently over 2500 different types of additives used by the food industry to achieve the desired effects in food (Branen et al., 2001).

The European standard 1333/2008 recognizes 26 classes of additives defined depending on their function in food: sweeteners, colorants, preservatives, antioxidants, carriers, acids, acidity regulators, anticaking agents, antifoaming agents, bulking agents, emulsifiers, emulsifying salts, firming agents, flavor enhancers, foaming agents, gelling agents, glazing agents, humectants, modified starches, packaging gases, propellants, raising agents, sequestrants, stabilizers, thickeners, and flour treatment agents (Council Regulation (EC) 1333/2008).

In turn, in the USA the food additives used in food are divided into 6 broad categories: Preservatives, nutritional additives, flavoring agents, coloring agents, texturizing agents, and miscellaneous additives (**Figure 1**).

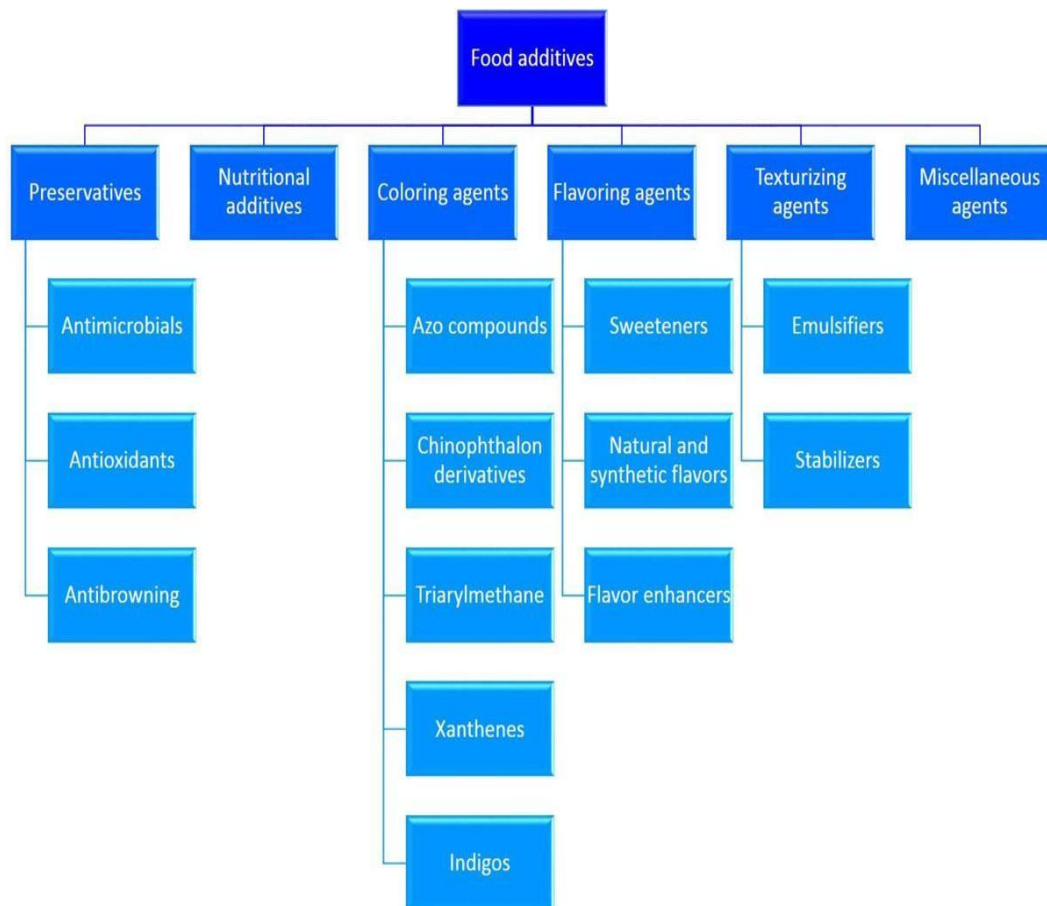


Figure 1. Groups and subgroups of food additives (Carocho et al., 2014).

Preservatives

Serving as an additive, preservative is a substance that is added to food to prevent the decomposition of food and to avoid any microbial growth or any undesirable chemical changes (Surekha & Reddy, 2014). Thus, preservatives are additives added to food to protect it from spoilage or becoming unconsumable.

The preservation of food is a habit that comes from ancestral times being drying, smoking, pickling, salting, and making of fruit conserves with sugar, some of the examples of the first techniques of food preservation against microbial decomposition, fattening and camouflaging unpleasant tastes and odors and used by consumer in order to extend the shelf life of food and to ensure protection of their physicochemical and organoleptic characteristics (Fletcher, 2014). As demands on processed and longer life food products increased, many chemical preservations agents have shown a great result under different fabrication conditions, which allows

food industry to select the most adequate agents for the desired function (Rai et al., 2016).

Several authors classify preservatives as the most important class of additives since they play such an important role in food safety to supply faraway populations (Quattrucci & Masci, 1992). In fact, the importance of food preservation is undeniable. There is a high degree of complementarity between processing and the use of preservatives in order to prolong shelf life of food. The use of preservatives in food stuff has significantly changed the production by the food industry and the habits of eating choices, either by increasing palatability of food but also by affording protection against reactive oxygen species that may have a pathological effect related with aging, cancer and cardiovascular diseases (Parke & Lewis, 1992).

The major roles of food preservatives are principally on preventing spoilage during processing, packaging or storage, distribution, retaining and use by the consumers. So that preservation purposes are summarized in ensuring food safety, preventing foodborne diseases and reducing microbial degradation that makes the food unacceptable for consumption (Fletcher, 2014).

Antimicrobial agents: Antimicrobial agents are widely used in preservation for two major reasons: to control natural spoilage of food and to avoid or control contamination by microorganisms, including pathogenic ones (Tajkarimi et al., 2010). The most common antimicrobial agents are: propionic acid, Na and K salts (E280); sorbic acid, Na, K and Ca salts (E200); Benzoic acid, Na and K salts (E210); *p*-hydroxybenzoate Me, Et, Pr, Bu esters (E219); nitrate, nitrite, Na and K salts (sodium nitrate E251 and potassium nitrate E252) (potassium nitrite E249; sodium nitrite E250); sulphites and sulphur dioxide (Parke & Lewis, 1992).

Antioxidant agents: Antioxidant agents are compounds which have the ability to inhibit or retard the oxidation of a variety of biomolecules including lipids. They prohibit the start of an oxidizing chain reaction by radicals or quench the propagation. This process of lipid peroxidation is readily catalyzed by minute traces of iron and other transition metals contained naturally in foodstuffs. These reactions can cause functional damage to the human body, like cancer or cardiovascular diseases. Antioxidants can prevent this process due to their redox properties like reductive behavior, the donation of hydrogen or quenching of singlet oxygen (Kahnonen, 1999). Hence, these interesting roles give antioxidant substances a good reason to be one of the major type of preservatives used in food industries.

Antioxidants are used in food industry to keep food safe from deterioration. The broad spectrum of antioxidant preservatives encompasses natural antioxidants such as tocopherols and ascorbic acid, as well as synthetic antioxidants such as propyl gallate (PG), tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA-E320), and butylated hydroxytoluene (BHT-E321) (Masgood et al., 2013).

Antibrowning agents: These agents play a major role on preventing both enzymatic and non enzymatic browning of food products, especially vegetables and fruits. Non enzymatic browning occurs in sugar caramelization and in the Maillard reaction between carbonyl and free amino groups, producing melanoid in pigments in various foodstuffs (Branen et al., 2001). Despite they are classified as antioxidant agents, citric acid (E330), vitamin C (E300) and sodium sulfite (E221) are the most common used in this category (Iyengar & McEvily, 1992).

Nutritional agents

Nutritional additives are utilized for the purpose of restoring nutrients lost or degraded during production, fortifying or enriching certain foods in order to correct dietary deficiencies, or adding nutrients to food substitutes. Vitamins and minerals are the most commonly added to many foods in order to enrich their nutritional value. For examples: the nutritional deficiency in the USA has been prevented by addition of vitamin D to milk and vitamin C to bread (Branen, 2001).

There is a growing consumer interest in nutritional quality which has consequently led to an increase in the addition of nutritional additives. These nutritional enrichments are natural ones, and can be vitamins, amino acids, fibers, fatty acids, and polyphenols, among others. The sources can vary depending on plant, mushroom, animal, or even synthetic origin (Carocho et al., 2014).

Coloring agents

The processing of food such as heat treatment or canning can cause degradation or loss of natural pigments in the raw materials. In addition, some formulated products, such as soft drinks, confections, ice cream, and snack foods, require the addition of coloring agents. Colorants are often necessary to ensure consistency of color and to produce a uniform product from raw materials that vary in color intensity. Hence,

coloring agents are used to enhance the attractiveness and the visual decoration properties of many foods (Sarikaya et al., 2012).

Colorants used as food additives can be classified as natural or synthetic. Natural colorants are derived from plant, animal, and mineral sources; most natural colorants are extracts derived from plant tissues. The use of these extracts in the food industry has certain problems associated with it, including the lack of consistent color intensities, instability upon exposure to light and heat. While, synthetic colorants are primarily petroleum-based chemical compounds, they are water-soluble and are available commercially as powders, pastes, granules, or solutions (www.britannica.com/topic/food-additive).

Flavoring agents

The flavor of food results from the stimulation of the chemical senses of taste and smell by specific food molecules. Flavoring agents are used to enhance the taste or to improve the flavors of the food in which they are added (Carocho et al., 2014). Flavorings are the largest group of food additives, with more than 1700 compounds available for commercial use. In addition to the four basic tastes which are sweet, salty, bitter, and sour, the flavoring molecules in food stimulate specific olfactory (smell) cells in the nasal cavity. These cells can detect more than 10,000 different stimuli, thus fine-tuning the flavor sensation of a food. These agents are subdivided in three categories: sweeteners, natural and synthetic flavors, and flavor enhancers (www.britannica.com/topic/food-additive).

Sweeteners: this category confers sweetness to food. Sucrose or table sugar is the standard on which the relative sweetness of all other sweeteners are based. Because sucrose provides energy in the form of carbohydrates, it is considered a nutritive sweetener. Other nutritive sweeteners include glucose and fructose. There are also widespread non nutritive sweeteners such as saccharin (E954), cyclamates (E952), aspartame (E951), acesulfame K (E950), and sucralose (E955) (Branen , 2001).

Natural and synthetic flavors: A flavor additive is derived from natural or synthetic origin and provides all or part of the flavor impact of a particular food. These chemicals are added in order to replace flavor lost in processing and to develop new products (www.britannica.com/topic/food-additive). Natural flavorings are derived or extracted from plants, spices, herbs, animals, or microbial fermentations. Artificial

flavorings are mixtures of synthetic compounds that may be chemically identical to natural flavorings. Artificial flavorings are often used in food products because of the high cost, lack of availability, or insufficient potency of natural flavorings (Branen et al., 2001).

Flavors enhancers: they are used to improve the flavors of food to which they are added, being a substance that has no pronounced taste of their own. The best-known substance as a flavors enhancer is glutamic acid (E620) (Walker & Lupien 2000). Other compounds that are used as flavor enhancers include the 5'-ribonucleotides, inosine monophosphate (IMP), guanosine monophosphate (GMP), yeast extract, and hydrolyzed vegetable proteins. Flavor enhancers may be used in soups, broths, sauces, gravies, flavoring and spice blends, canned and frozen vegetables, and meats (Branen et al., 2001).

Texturizing agent

In order to modify the overall texture, hence, the mouth feel of foodstuffs, texturizing agents are added to food. There are two main groups of food texturizing agents, emulsifiers and stabilizers (Council Regulation (EC) 1129/2011). The purpose of these two agents are to facilitate the mixing of ingredients that would not mix (immiscible liquids) namely oils or fats and water and them maintain in good dispersion. These additives are essential in the production of many appreciate food stuffs like mayonnaise and chocolate product (Rohman et al., 2013).

Miscellaneous agents

This category contains additives that are added to food for specific objectives and they are not included in the other food additives groups. Chelating agent, enzymes, antifoaming agents, solvents and catalyst are knowing miscellaneous agents (Branen et al., 2001).

Safety issues of synthetic food additives

The first half of twentieth century has shown a significant increase in the use of food additives (Tomaska & Brook-Taylor, 2014). The initial uncontrolled use has raised many concerns regarding consumer safety. Since that, the safety of all food additives especially the synthetically produced ones, is rigorously tested and

periodically reassessed. Many organizations are developed to deal with this problematic; in the EU since 1985, an expert committees of the EFSA controls and panels food additives by evaluating their safety, and approve adequate ones with “E” number which indicates the European safety (Saltmarsh , 2013)

Many other individual countries have also similar procedures and at international level such as JECFA, FAO, WHO and Codex Alimentarius Commission. This world trade organization’s arrangement becomes increasingly important in recent years to insure the safety of food additives (Carocho et al., 2015a).

In additives’ evaluation, the national agencies allocate an “Acceptable Daily Intake” (ADI) which is the amount of substance that is acceptable, daily, throughout a life time without any serious health risk (Tomaska & Brook-Taylor, 2014). ADI is used to determine the amount of a particular additive or group of additives permitted in a specific food and expressed as mg per kg bodyweight per day. This value is usually based on the amount in which it can be given to animals or humans without producing adverse effects; the organizations called that “No Observed Adverse Effect Level” (NOAEL) (Saltmarsh, 2013).

Today, all new food additives undergo a safety assessment to minimize any potential adverse effects of food additive to human health. To assess the potential risk to human health, a number of tests are conducted including some using animals. These tests assess the potential for acute and chronic toxicity, genetic damage, or cancer and any possible effect on development and reproduction (Rasooli, 2007).

Despite the intensive risk assessment, it is argued that these tests do not address the entire spectrum of toxicities that can be caused by chemical additives. The incidence of foodborne disease has been increased significantly in the recent years. Moreover, joint action and interaction between different chemical additives that are present in food matrices can lead to an increase, in some cases, of the toxicity risk (Viator et al, 2015).

For example, a study about the effects of artificial food colors and other food additives in the behavior of children revealed that they have a hyperactive effect on 3-year-old and 8/9-year-old children (McCann et al, 2007).

Sun has reported a recent epidemiological study in 2012 by which looked at a possible link between nitrates/nitrites, which are two powerful chemical preservatives, and premature deaths. The result of this study highlighted that a regular consumption of processed meat, contributes substantially to premature death. Moreover, it showed

that eating any kind of red meat increased the chances of dying from heart disease by 16% and from cancer by 10%. In addition, processed red meat raised the risk of heart disease death by 21% and cancer death by 16% (Sun, 2012).

Food additives have been widely used in the last decades and their effective role became important with the increase of consumption because of the considerable changing in lifestyles of modernly civilization. Despite the improvement in legislation that controls food processing and the development of new technologies in order to reduce the excessive use of chemical additives, many problems still remain unsolved in terms of health safety of consumers. New sources of additives which can replace synthetics ones without being harmful and cause health perturbation could be the best way to eliminate this problematic.

Natural preservatives

Advantages of natural preservatives over the synthetic ones

Generally, food industry depends on chemicals for the preservation of foodstuff and to prolong the shelf life of food (Rai et al., 2016). Antimicrobials, antioxidants and antibrowning agents are among the additives mostly used by the food industry to preserve products for longer periods (Carocho et al., 2014).

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are some of the most popular synthetic antioxidants commonly used for food processing and pharmacological applications in the last century (Maqsood et al., 2013; Pasqualone et al., 2015).

However, this type of additives has undergone many toxicological tests due to some adverse effects that have been pointed out. Due to this possible toxicity and carcinogenic effects, some of these additives have been restricted by legislative rules (Reddy et al., 2005). For example, ascorbic acid (used as an antioxidant) and potassium sorbate (used as preservatives) are very common additives in food processing, but its use is restricted in terms of amounts in foods by the two major regulators of food additives, the FDA and EFSA (Branen, 2001).

Potassium sorbate (identified as E202) is one of the most widely used synthetic preservatives in the food industry, namely carbonated beverages, margarines, and some vegetables and fruits (Kitano et al., 2013). It has been used since 1940 when it proved to be an effective antimicrobial agent and has been used since then as a

preservative in food and beverage processing, but also to a lesser extent in cosmetic, pharmaceutical and tobacco products (Nikolov & Ganchev, 2011). However, despite being widely and legally used, several studies have reported a toxicity associated with excessive consumption of this agent causing skin, eye, respiratory and immune system problems (Kitano et al., 2002; Mamur et al., 2010). Potassium sorbate has been condemned by being both genotoxic and mutagenic to human DNA and blood cells especially on peripheral blood lymphocytes so that it has a direct effect on immunity system (Engel et al., 2015). Generally, potassium sorbate is used in combination with ascorbic acid in order to increase its effectiveness. However, this mixture led to the formation of mutagenic compound that damage the DNA structure (Kitano et al., 2002).

To overcome this problem, natural extracts appear as safer and healthier alternatives, guaranteeing not only additional value to the final products but also bioactive properties (Caleja et al., 2016a). Natural preservatives are found in the majority of plants, fungi, microorganism and even in some animal tissues (Carocho et al, 2015a). These types of compounds have gained increasing interest from the food industry for direct application or in synergy with other natural or chemical additives. Among the various effects studied, the high antioxidant and antimicrobial potential is the most widely reported (Carocho et al., 2014).

Natural preservatives seem to receive approval from consumers who increasingly care about their health and well-being and as such, prefer food labeled as healthier and more natural (Caleja et al., 2016b). Synthetic antioxidants are tested for carcinogenic or mutagenic effects which approve the need of natural food compounds. In addition, legislative approval seems to be easier with respect to synthetic additives (Gulcin et al., 2011).

The protective effects of natural antioxidants against oxidative-induced agents have received increasing attention in recent years, especially within biological, medical, nutritional and agrochemical fields (Magalhães et al., 2008). **Table 1** summarizes the main advantages and disadvantages of natural and synthetic antioxidants

Table 1: Advantages and disadvantages of natural and synthetic antioxidants commonly used for food preservation (Valenzuela & Nieto, 1996).

Synthetic antioxidants	Natural antioxidants
Inexpensive	Expensive
Widely applied	Usage of some products restricted
Medium to high antioxidant activity	Wide ranging antioxidant activity
Increasing safety concern	Perceived as innocuous substances
Usage of some of them banned	Increasing usage and expanding
Low water solubility	applications
Decreasing interest	Broad range of solubility
Some of them stored in adipose tissue	Increasing interest
	Completely metabolized

Natural antioxidants include tocopherols and phenolic compounds, such as flavonoids and phenolic acids. Flavonoids are very effective antioxidants with proven efficacy in protecting against cardiovascular disease, reducing the oxidation of low-density lipoproteins (Carocho & Ferreira, 2013).

Plants as sources of natural preservatives

Recently, the use of chemical substances as food additives is a topic which burden many studies. Due to the extensive utilization of preservatives, the safety, the nutritional values and the standard quality of food become insufficient for consumers that are looking for healthier lifestyle (Sanchez et al., 2015). The replacement of synthetics preservatives by plants-extracted natural substances that preserves food without being harmful or changing its quality becomes attractive for both consumers and industries (Caleja et al., 2015a; Wang et al., 2016).

The role of plant-origin preservatives in food and human health gain a growing importance in research area. Many studies recognized the beneficial influence of many foodstuffs and beverages including fruits, vegetables, tea, coffee, and cacao on human health (Gulcin et al., 2012).

They have been demonstrated that plant-products used in food processing contain a large amount of valuable biologically active compounds that could replace synthetic additives. In particular, secondary metabolites of plants such as phenolic compounds and carotenoids, which are a part of plant defense system, are good candidates. They have been intensely investigated and show antioxidant, antimicrobial and UV protecting activities. Hence, plant secondary metabolites and also some high- molecular-mass constituents of the plant cell wall may serve as natural preservatives that prevent food from oxidative deterioration (Carocho, et al., 2014; Caleja et al., 2015).

Plants, especially herbs and spices, are composed by many photochemical compounds which have antioxidant, anti-inflammatory and anticancer activities (Lee et al., 2004). Those types of compounds, for examples flavonoids, phenolics acids and tannins, present a potential source of natural antioxidants (Dawidowicz et al., 2006).

The interest on the use of natural antioxidant such as tocopherols, flavonoids and rosemary extract in food products is increasing in recent years (Bruni et al., 2004; Frutos & Hernandez-Herrero, 2005). These natural substances avoid toxicity problems which may arise from the use of synthetic antioxidants namely butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and propyl gallate (PG) (Amarowicz et al., 2000; Maqsood et al., 2013).

Well succeeded examples

Recently, food industry is focused on the development of novel functional foods containing health promoting natural ingredients, avoiding the potential harm of synthetic food additives. Plants are recognized as sources of a wide range of bioactives, including phenolic compounds which are characterized by their antioxidant properties. An example of this is *Foeniculum vulgare* Mill (fennel), a biennial plant that belong to the family Apiaceae (Umbelliferaceae), distributed in central Europe and Mediterranean region. Fennel showed both antioxidant (Barros et al., 2009) and antimicrobial (Dadalioglu & Evrendilek, 2004) properties.

The use of fennel decoction as a natural ingredient in cottage cheese in order to increase shelf life and provide bioactive properties has been proved by Caleja et al. (2015). Those properties are attributed to phenolic compounds namely di-caffeoyl-2,7-anhydro-3- deoxy-2-octulopyranosonic acid and luteolin-*O*-glucuronide in fennel (Caleja et al., 2015).

Another study was conducted by Carochó et al (2016a) with the objective to evaluate the effects of natural additives extracted from *Castanea sativa* Mill. (chestnut flowers), *Melissa officinalis* L. (lemon balm) dry material and their decoctions on the nutritional properties of a famous cheese in Portugal. Chestnut flowers have an outstanding antioxidant capacity, also displaying antimicrobial and antitumor effects. This activity could be related to their phenolic fraction, which could also contribute to other described medicinal effects, namely as mucolytic, antispasmodic and anti- dysenteric (Neves et al., 2009). Lemon balm, also display many medicinal effects, especially when consumed as an infusion or decoction. These effects encompass expectorant, digestion relief, ease of headaches and rheumatism, prevention of neurodegenerative diseases, antitumor, antiproliferative, anticholinesterase, antioxidant and anti-Alzheimer properties (Martins *et al.*, 2012, Barros *et al.*, 2013). Regarding all these beneficial properties, chestnut flowers and lemon balm dry plants showed to be good preserving agents during the maturation process of “Serra da Estrela” cheese (Carochó et al., 2016a).

Among the various plants and spices used in alternative medicines, *Rosmarinus officinalis* L. (rosemary) has focused particular attention on presenting a pattern of phenolic compounds that defines its high antioxidant potential (Del Bano et al., 2003). Rosemary is originated from the Mediterranean region, categorized as a famous aromatic plant. Its leaves extract is extensively used in traditional medicine against rheumatic, dyspepsia and dysmenorrhea diseases due to its properties as antioxidant, anti-inflammatory, antimicrobial, antidiabetic and hepaprotective. These medicinal applications, as well as the uses in food preservation are attributed to rosemary’s high content in phenolic compounds, namely caffeic acid derivatives such as rosmarinic acid, and related bioactive properties (Ribeiro et al., 2016).

The structure of rosmarinic acid (RA) (**Figure 2**) shows that it has four phenolic hydroxyl groups separated into two pairs located in ortho-positions of the benzene rings (Zhang et al., 2015). RA (α -*O*-caffeoyl-3,4-dihydroxyphenyllactic acid) is a naturally hydroxylated compound derived from caffeic acid and (R)-(+)-3-(3, 4-dihydroxyphenyl) lactic acid represent one of the most common caffeic esters in plant material. It is a well-known natural product present in many plants like rosemary, lemon balm (*Melissa officinalis*), *Artemisia capillaris*, *Callendula officinalis*, *Salvia officinalis* and other several Lamilaceaes medicinal plant families (Solomon et al., 2015).

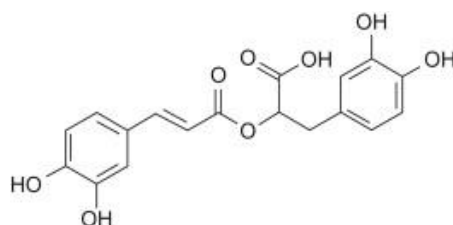


Figure 2. Chemical structure of rosmarinic acid.

Different biological activities are attributed to RA, as antioxidant astringent, antimutagenic, antitumor, anti-inflammatory, antidepressive, antiangiogenic, antibacterial, and antiviral (Pereira et al., 2005; Furtado et al., 2008; Sanchez-Campillo et al., 2009). It is also used for food preservation regarding its antioxidant properties. In Japan, the perilla extracts, which is a medicinal plant, is rich in rosmarinic acid and used to garnish and improve the shelf life of fresh seafood (Park et al., 2008).

***Ocinium basilicum* as source of rosmarinic acid**

Ocinium basilicum L. (**Figure 3**) known as sweet basil represents one of the most popular aromatic plants, mainly used in flavoring and in traditional medicine because of its great variation of components. This plant, native to the tropical region of Asia, Africa, Central and South America belongs to the Lamilaceae family. It is a green herb that can reach about 90 cm of high, displaying lanceolate leaves, which are glossy and fragrant (Jayasinhe et al., 2003).



Figure 3. *Ocimum basilicum* L.

([http://www.palmbeachmedicinalherbs.com/assets/images/ProductImages/basil-greek-dwarf-seeds/basil-ocimum-basilicum-greek-dwarf-seeds-palm-beach-medicinal-herbs%20%20\(2\).jpg](http://www.palmbeachmedicinalherbs.com/assets/images/ProductImages/basil-greek-dwarf-seeds/basil-ocimum-basilicum-greek-dwarf-seeds-palm-beach-medicinal-herbs%20%20(2).jpg))

Although, *O. basilicum* is used as a culinary and ornamental plant, it is also considered medicinal due to various properties like inflammation soother, nasal douche, cough suppressor, antipyretic, constipation and diarrhea alleviation, wart removal, kidney malfunction treatment, diuretic and gastritis treatment (Crocho et al., 2016). Its antimicrobial, antifungal, antimutagenic and antioxidant activities have also been confirmed, especially in its essential oils, which were particularly utilized extensively in the food industry as a flavoring agent, and in perfumery and medical industries. It is also considered as a source of aromatic compounds, and it possesses a range of biological activities such as insect repellent, nematocidal, antibacterial, antifungal and antioxidant (Lee et al. 2005; Flanigan & Niemeyer, 2014).

Rosmarinic acid is the most abundant caffeic acid esters present in *Ocimum* spp. The presence of this compound is highly investigated and reported in *O. basilicum* as quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside (Vlase et al., 2014; Carocho et al. 2015b). Hence, in *O. basilicum*, as well as other Lamiaceae, rosmarinic acid is supposed to act as an accumulated defense compound. The presence of rosmarinic acid in medicinal plants, herbs and spices has beneficial and health promoting effect (Zhang et al., 2015).

***Arbutus unedo* as a source of catechins**

Arbutus unedo L. (**Figure 4**) or strawberry tree is a native species from Mediterranean plant with an extension throughout the European Atlantic Coast.

It belongs to the Ericaceae family and Arbutoideae or Vaccinioideae subfamilies. The small tree of strawberry can reach normally between 1.5 to 3 m tall (Sulusoglu et al., 2011). In Portugal, *A. unedo* is widely distributed, from Atlantic climate areas in the North to dry arid areas in the South, occupying about 15,500 ha (Malheiro et al., 2012).



Figure 4. *Arbutus unedo* L.

(<https://encrypted-tbn0.gstatic.com/images?q=tbn:ANd9GcTOXNbGIw6pF63x7daLBzvxGDuypU4gajuQW8GSqihu0texXKS>)

It produces an edible reddish sweet and tasty berry which is, when fully matured rich in nutritional properties and have several medicinal effects, as astringent, diuretic and antiseptic properties and especially effects on cancer and cardiovascular disease. Regarding antioxidants, the *A. unedo* fruit could be considered an important dietary source of ascorbic acid and a lesser source of fat-soluble antioxidants such as α -tocopherol and carotenoids (β -carotene and lutein) (Oliveira et al., 2011). Some literature reports the traditional use of the leaves also as diuretic, antiseptic, antidiarrheal, astringent, depurative, and against blennorrhagia, diabetes and as antihypertensive (Barros et al., 2010).

The protective effects have been primarily attributed to antioxidants, such as vitamin C, vitamin E, β -carotene and, lately, to phenolic compounds (Soobrattee, et al., 2005). In particular, among a variety of phenolic compounds, some authors focused on catechins (**Figure 5**) which are flavan-3-ols that have attracted attention particularly due to their relative high antioxidant activity.

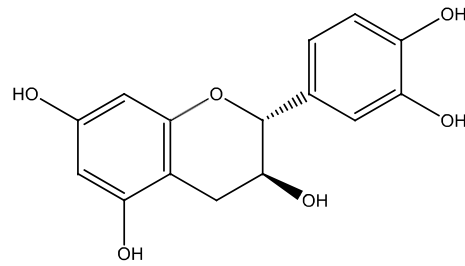


Figure 5. Chemical structure of catechin.

Catechin has proved to be potent antioxidants in different *in vitro* models, and in some *in vivo* intervention studies due to its properties and including principally the different mechanisms implicated on cardiovascular conditions or problems, such as hypertension, inflammation, cellular proliferation, thrombogenesis, hyperglycaemia and hypercholesterolemia (De Pascaual-teresa et al., 2010).

Additionally, the presence of high levels of phenolic compounds also leads to the hypothesis that antimicrobial effects could be displayed by *A. unedo* (Barros et al., 2010). The *A. unedo* fruit is less frequently eaten fresh but more used for preparing alcoholic beverages (wines, liqueurs and brandies), jams, jellies and marmalades (Santos, et al., 2012). Also, it can be added to yoghurt either in pieces or as flavors and be used, like other berries, in confectionery, such as for pie and pastry fillings and cereal products (Miguel et al., 2014).

An important study carried out by Albuquerque et al. (2017) indicate the viability of using *A. unedo* fruits as a productive source of catechin extracted by maceration, microwave and ultrasound extraction techniques. The production of *A. unedo* fruits in industrial scale could serve as a source of bioactive compounds to be used as natural additives in functional foods.

II. Objectives

The main objectives of this work are focused on:

- i) Chemical characterization of the ethanol extracts of *Arbutus unedo* (strawberry tree) fruits and *Ocimum basilicum* (basil) leaves obtained through the ideal conditions;
- ii) Evaluation of the bioactive properties such as, antioxidant (free radical scavenging activity, reducing power assay, β -carotene bleaching and lipid peroxidation inhibition) and antimicrobial (antibacterial and antifungal) properties and the toxicity of the referred extracts;
- iii) Incorporation the ethanol extracts (*Arbutus unedo* fruits and *Ocimum basilicum* leaves) in bread samples with two different aims: functionalize the bread by adding bioactive properties and increase the product shelf life. For this purpose, the physical appearance, nutritional parameters and antioxidant activity were compared between bread incorporated with: i) potassium sorbate (synthetic additive - E202); ii) ascorbic acid (natural additive -E300); iii) *A. unedo* fruit extract rich in catechin and *O. basilicum* leaves extract rich in rosmarinic acid; and iv) control sample (without any incorporation). These samples were evaluate along three different storage times, 0, 3 and 7 days;
- iv) Finally, it was demonstrate that the extracts can be used in the food industry in the substitution of synthetic additives.

III. Material and Methods

Standards and reagents

HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards were bought from Extrasynthèse (Genay, France). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, MO, USA), as also other individual fatty acid isomers, nitric acid, formic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Ellipticine and sulforhodamine B. Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Fetal bovine serum (FBS), l-glutamine, nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and Dulbecco's Modified Eagle Medium (DMEM) media were acquired from Thermo Fischer Scientific (Waltham, MA, USA). Sulphuric, hydrochloric and nitric acid were obtained from Fisher Scientific (Waltham, MA, USA). All other reagents were purchased from specialized retailers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Plants material extraction

Arbutus unedo – Catechin extraction

The fruits of *Arbutus unedo* L. (strawberry tree) were gathered in the Natural Park of Montesinho territory, in Trás-os-Montes, North-eastern Portugal. The botanical identification was confirmed by Dr. Ana Maria Carvalho (School of Agriculture, Polytechnic Institute of Bragança, Portugal) according with a previous report of the authors (Guimarães et al. 2013). The fruits were lyophilized (FreeZone 4.5, Labconco, Kansas Kansas City, MO, USA) and stored in a deep-freezer at - 20 °C for subsequent analyses.

The catechin extraction (**Figure 6**) was optimized by a study of Albuquerque et al. (2017): the lyophilized powdered fruit samples (5 g) were placed in a beaker with 100 mL of ethanol:water 23:77(v/v) . The beaker was placed in a thermostatic water bath at 80 °C under continuous electro-magnetic stirring for 93 minutes. The collected

extracts were filtered through a Whatman paper filter n°4. Then, the filtered material was evaporated to remove the ethanol at 40 °C in a rotary evaporator (Büchi R-210, Flawil, Switzerland). Afterwards, the samples were frozen and then lyophilized.



Figure 6. Catechin extraction from *Arbutus unedo* L fruits.

***Ocinium basilicum* – Rosmarinic acid extraction**

Commercial samples of *Ocinium basilicum* (basil) were bought in a local supermarket. The dried samples were powdered (~20 mesh; Ultra Centrifugal Mill ZM 200, Porto, Portugal). For the rosmarinic acid extraction (**Figure 7**) 1 g of the sample was extracted with 30 mL of ethanol:water 80:20 (v/v) at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman n° 4 paper. The residue was then re-extracted twice with additional 30 mL portions of ethanol:water 80:20 (v/v). The combined extracts were evaporated at 35 °C (rotary evaporator Büchi R-210) to remove ethanol. Afterwards, the samples were frozen and then lyophilized.

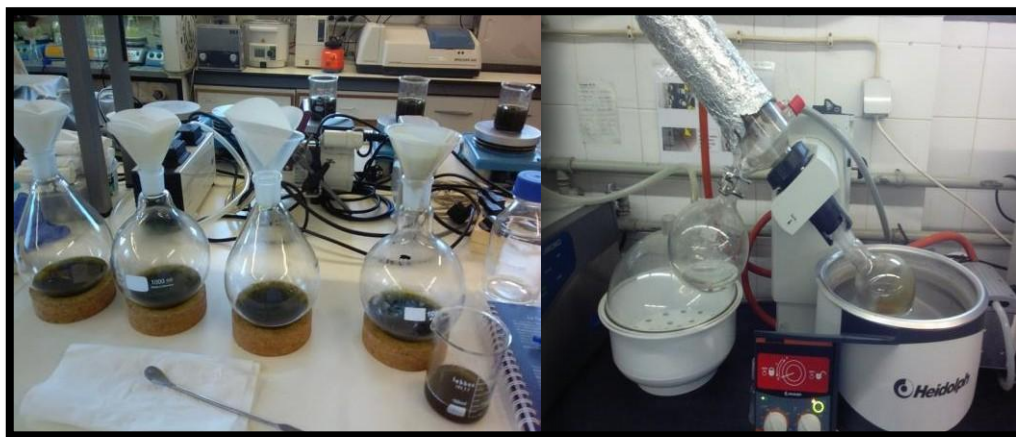


Figure 7. Rosmarinic acid extraction from *Ocimum basilicum* leaves.

Chemical characterization

Catechin and rosmarinic acid quantification

The extracts obtained with were dissolved in 20% aqueous ethanol at 5 mg/mL and filtered through a 0.22- μ m disposable LC filter disk. Chromatographic analysis were performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to a electrospray ionization mass detector (LC-DAD-ESI/MSn), a quaternary pump, an auto-sampler (kept at 5 °C), a degasser and an automated thermostat column compartment. Chromatographic separation was achieved with a Waters Spherisorb S3 ODS-2 C18 (3 μ m, 4.6 mm \times 150 mm, Waters, Milford, MA, USA) column working at 35 °C. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% B (5 min), 15% B to 20% B (5 min), 20-25% B (10 min), 25-35% B (10 min), 35-50% B (10 min), and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 5 kV, a source temperature of 325 °C, a capillary voltage of -20 V. The tube lens offset was kept at a voltage of -66 V. The full scan covered the mass range from m/z 100 to 1500. The collision energy used was 35 (arbitrary units). Data

acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

The catechin and rosmarinic acid were identified by comparing their retention times, UV-vis and mass spectra with those obtained from standard compounds. For quantitative analysis, a calibration curve for catechin and rosmarinic acid was constructed based on the UV signal. The results were expressed as µg/g of extract.

3.4. Evaluation of the antioxidant activity

To obtain stock solutions of 5 mg/mL, the lyophilized extracts of two plants were redissolved in water. The mentioned stock solutions were successively diluted into sequential concentrations until determination of EC₅₀ values (sample concentration providing a value of 50% in the DPPH, β-carotene bleaching and TBARS assays or 0.5 absorbance in the reducing power assay). Trolox was used as positive control in all the assays.

DPPH radical scavenging activity

In this assay, the deep violet chromogen DPPH radical is reduced to slight yellow color in the presence of hydrogen donating antioxidants leading to the formation of non-radical form. This assay is widely used as a preliminary antioxidant study (Amarowicz et al., 2004; Moon & Shibamoto, 2009). The reaction mixture consisted of one of the different concentrations of the diluted ethanoic extracts (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10⁻⁵ mol/L). The microplate (**Figure 8**) was stored in the dark at 25 °C for 1h. Absorbances were recorded using ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) at 515 nm. The results were expressed in mg/mL as the extract concentration responsible for 50% of DPPH radical scavenging activity (EC₅₀), calculated by interpolation from the graph of the radical scavenging activity (RSA) percentage against extract concentration and expressed in mg/mL of extract:

$$\%RSA = [(A_{DPPH} - A_S) / A_{DPPH}] \times 100$$

(A_S- absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH}- absorbance of the DPPH solution).

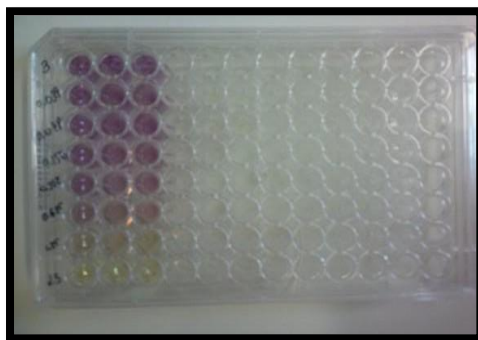


Figure 8. Microplate used in the evaluation of DPPH radical-scavenging activity.

Reducing power

This methodology is based on the ability to reduce yellow ferric form (Fe^{3+}) to blue ferrous form (Fe^{2+}) by the action of electron-donating antioxidants (Pinela et al., 2012). Different concentrations of the ethanolic extracts (0.5 mL), sodium phosphate buffer (0.5 mL, 200 mmol/L, pH 6.6) and potassium ferricyanide (0.5 mL, 1% w/v) were mixed in eppendorf tubes. The tubes were incubated in a water bath (50 °C) for 20 min. After the addition of trichloroacetic acid (0.5 mL, 10% w/v), the mixture (0.8 mL) was transferred into 48 well microplates (**Figure 9**) as also deionized water (0.8 mL) and ferric chloride (0.16 mL, 0.1% w/v). Absorbance was then read at 690 nm in the microplate reader mentioned above. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance against extract concentration and expressed in mg/mL of extract.

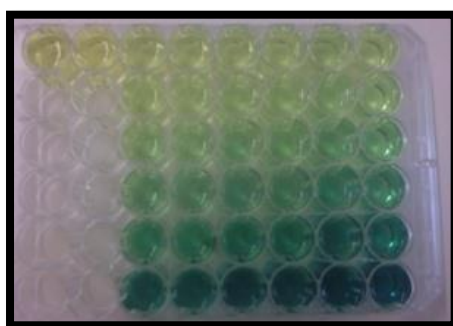


Figure 9. Microplate used in the evaluation of reducing power.

Inhibition of β -carotene bleaching

This assay is based on the capacity of antioxidants to neutralize the linoleate free radical. This neutralization is detected by the discoloration of the yellowish color of β -carotene (Amarowicz et al., 2004; Kaur & Geetha, 2006). The reagent is prepared by dissolving 2 mg of β -carotene in 10 mL of chloroform in a round-bottom flask. This solution was concentrated in a rotary evaporator to remove the chloroform, and then 800 mg of Tween 80 emulsifier, 4 drops of linoleic acid and 200 mL of distilled water were added to the flask with vigorous agitation. About 4.8 mL of this mixture were added to 0.2 mL of each dilution distributed in test tubes. After reading zero time absorbance at 470 nm, the tubes (**Figure 10**) were incubated in a water bath at 50 °C with agitation (100 rpm) for 2h, and the absorbance was measured for the second time at 470 nm (UV–Vis spectrophotometer SPECORD 200, Analytik Jena AG, Jena, Germany). β -carotene bleaching inhibition was calculated through the equation:

$$(\text{absorbance after 2 h of assay}/\text{initial absorbance}) \times 100$$

and further converted to EC50 (extract concentration responsible for 50% of β -carotene bleaching inhibition), expressed in mg/mL of extract.

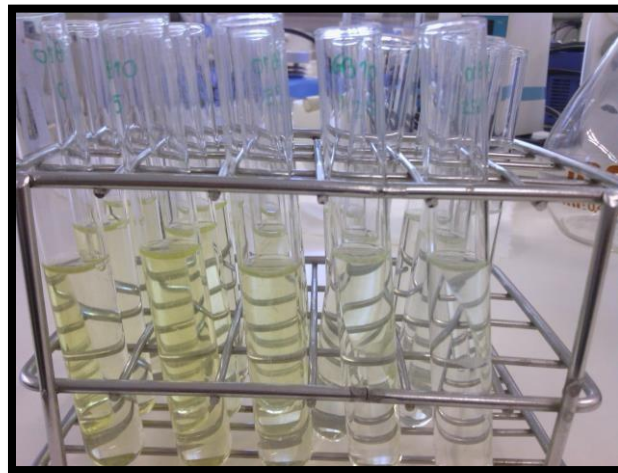


Figure 10. Tubes used in the β -carotene bleaching inhibition assay.

Thiobarbituric acid reactive substances (TBARS) assay

TBARS is a colorimetric assay in which lipid peroxidation produces malondialdehyde (MDA) as secondary breakdown product, and reacts with the thiobarbituric acid (TBA) to form MDA-TBA complex with the production of a pink pigment (Kaur & Geetha, 2006). The inhibition of lipid peroxidation in porcine (*Sus scrofa*) brain

homogenates in the presence of antioxidant is detected by measuring the absorbance of MDA-TBA complex at 532 nm (**Figure 11**). Porcine brains were purchased from a local slaughter house, dissected and dissolved in Tris-HCl buffer (20 mmol/L, pH 7.4) in a proportion of 1:2 *w/v* brain tissue homogenate which was centrifuged at 3000 *g* for 10 min. Each dilution of the sample solutions (200 μL) was pipetted into test tubes, adding also 100 μL of ascorbic acid (0.1 mmol/L), 100 μL of FeSO_4 (10 mmol/L) and 100 μL of the supernatant of brain tissue homogenate. The tubes were incubated at 37 °C for 1h. Trichloroacetic acid (500 μL , 28% *w/v*) was added to stop reaction, together with 380 μL thiobarbituric acid (TBA, 2% *w/v*), and the mixture was then incubated at 80 °C for 20 min. The mixtures were centrifuged at 3500 rpm for 5 min to eliminate the precipitated protein; the absorbance of the supernatant samples was measured at 532 nm. The percentage of inhibition was calculated through this equation: inhibition ratio (%) = $[(A-B)/A] \times 100$; (A- absorbance of the control and B- absorbance of the sample solution) and converted to EC_{50} values (extract concentration responsible for 50% of lipid peroxidation inhibition) expressed in mg/mL of extract.

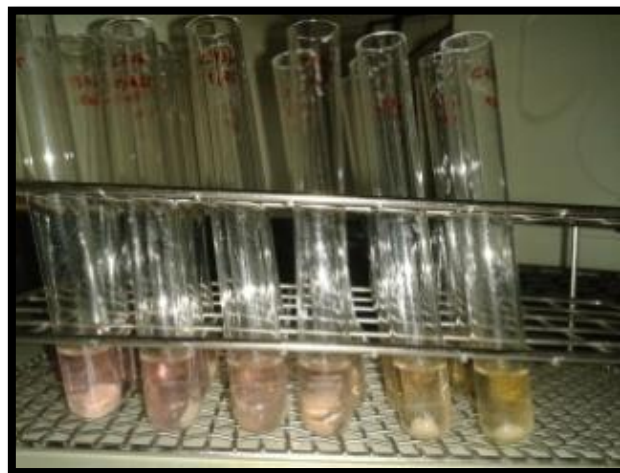


Figure 11. Tubes used in the TBAS assay.

Evaluation of the hepatotoxicity

A cell culture was prepared from a fresh porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and divided into $1 \times 1 \text{ mm}^3$ explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine

serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO₂. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

Sulphorhodamine B assay was performed according to a procedure previously described by Guimarães et al. (2013). Ellipticine was used as positive control. The results were expressed as GI₅₀ values (sample concentration that inhibited 50% of the net cell growth) in µg per mL of the aqueous solutions of the lyophilized extract.

Evaluation of antimicrobial properties

Antibacterial activity. The following Gram-negative bacteria were used: *Escherichia coli* (ATCC (American type culture collection) 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC (National collection of type cultures) 7973). These microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research “Siniša Stanković” at the University of Belgrade in Serbia. Fresh overnight culture of bacteria was adjusted with a spectrophotometer to a concentration of 1×10^5 CFU/mL. The requested colony forming units (CFU)/mL corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. The sample solutions were pipetted into the wells containing 100 µL of Tryptic Soy Broth (TSB), with 10 µL of inoculum being added to all the wells. The microplates were incubated for 24 h at 37 °C. The MIC (minimal inhibitory concentration) of the samples was determined by adding 40 µL of iodinitrotetrazolium chloride (INT) (0.2 mg/mL) and incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The MICs obtained from the

susceptibility testing of various bacteria to tested samples were also determined by a colorimetric microbial viability assay based on reduction of INT color and compared with positive control for each bacterial strain (CLSI, 2009). MBC (minimal bactericidal concentration) was determined by serial sub-cultivation of 10 μ L into microplates containing 100 μ L of TSB. The lowest concentration that showed no growth after this sub-culturing was regarded as the MBC. Streptomycin and ampicillin were used as positive controls, while 5% dimethyl sulfoxide (DMSO) was used as negative control. The results of MIC and MBC were expressed in mg per mL of *A. unedo* and *O. basilicum* extract

Antifungal activity. The following microfungi were used: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Aspergillus flavus* (ATCC 9643), *Cladosporium cladosporioides* (ATCC 11278), *Penicillium funiculosum* (ATCC 36839), and *Penicillium verrucosum var. cyclopium* (food isolate). These organisms were also obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research “Siniša Stanković” at the University of Belgrade in Serbia. The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4 °C and sub-cultured once a month. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μ L/well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. The MICs determination was performed by a serial dilution technique using 96-well microplates. The sample solutions were added to broth malt medium with the fungal inoculum. The microplates were incubated for 72 h at 28 °C (Espinel-Ingroff, 2001). The lowest concentrations without visible growth (using a binocular microscope) were defined as the MIC. The minimum fungicidal concentrations (MFC) were determined by serial sub-cultivation of 2 μ L in microtiter plates containing 100 μ L of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. 5% DMSO was used as a negative control, while bionazole and ketoconazole were used as positive controls. The results of MIC and MFC were expressed in mg per mL of *A. unedo* and *O. basilicum* extract.

Incorporation of *Arbutus unedo* and *Ocimum basilicum* as natural ingredients in bread

Preparation of the bread samples

The bread samples (**Figure 12**) were prepared with a bread maker (Tefal home bread XXL, model: 573902) and by following the recipe: 325 mL of water, 18 g of oil, 11 g of salt, 20 g of sugar, 25 g of milk, 600 g of flour and 4 g of yeast. The synthetic and natural antioxidants were added to the mixture at 1.2 g for each bread (1 kg), which is the maximum quantity permitted for sorbate potassium (synthetic additive).

Five groups of bread samples were prepared: i) control bread (bread without any incorporation of natural or synthetic ingredient); ii) bread with incorporation of *O. basilicum* leave extract rich in rosmarinic acid; iii) bread with incorporation of *A. unedo* fruit extract rich in catechin; iv) bread with incorporation of ascorbic acid (E300); and v) bread with incorporation of potassium sorbate (E202). All samples were prepared in triplicate in order to be able to evaluate three different storage times. All the samples were submitted to an evaluation of color, pH, nutritional composition, and antioxidant activity, immediately after preparation and after 3 and 7 days of storage at 4 °C.



Figure 12. Bread sample.

Evaluation of color and pH of the bread samples along storage time

The samples color was measured in three different points on the top, bottom and inside the bread, using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The illuminate C was used and a diaphragm aperture of 8 mm that was

previously calibrated against a standard white tile. The CIE L^* (lightness), a^* (greenness/redness), b^* (blueness/yellowness) color space values were registered using a data software “Spectra Magic Nx” (version CM-S100W 2.03.0006) (Fernandes et al., 2012).

The pH of the samples was also measured in three different points directly in the samples with a HI 99161 pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA).

Evaluation of the nutritional properties

Following the AOAC methods (AOAC, 2012), the contents of protein, fat, carbohydrates and ash, were determined. Total energy was calculated following the equation: Energy (kcal) = $4 \times (\text{g proteins} + \text{g carbohydrates}) + 9 \times (\text{g lipids})$.

Total protein content ($\text{N} \times 5.70$) was calculated as nitrogen content by the Kjeldahl method, while crude fat relied on the extraction of dried samples with petroleum ether using a Soxhlet apparatus. Ash content was determined by incineration at 550 ± 15 °C, and carbohydrates were calculated by difference. (Barros et al., 2013).

Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column, after trans-esterification procedure. Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v:v), during at least 12 h in a bath at 50 °C and 160 rpm; then 3 mL of deionised water were added, to obtain phase separation; the FAME were recovered with 3 ml of diethyl ether by shaking in vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 µm nylon filter from Whatman (Barros et al., 2013). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 °C) and a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15

min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI). Dried sample powder (1.0 g) was spiked with the melezitose as internal standard (IS, 5 mg/mL), and was extracted with 40 mL of 80% aqueous ethanol at 80 °C for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000g for 10 min. The supernatant was concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 µm nylon filters from Whatman (Barros et al., 2013). The equipment of analysis consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD) and an RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH₂ column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 °C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

Evaluation of the antioxidant activity of the bread samples

All lyophilized samples were extracted as previously mentioned in 3.2.2. Afterwards, the extracts were dissolved in ethanol in a final concentration of 200 mg/mL. The antioxidant activity evaluation was performed using two in vitro assays: 2,2- Diphenyl-1- picrylhydrazyl (DPPH) radical-scavenging activity and reducing power (RP), following the experimental methodologies described previously.

3.8. Statistical analysis

All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA).

Except for antimicrobial assays results, data were expressed as mean±standard deviation, maintaining the significant numbers allowed by the magnitude of the standard deviation.

The results obtained in the biological activity of *A. unedo* and *O. basilicum* extracts were compared by t-Student test, while an analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model (GLM) procedure, to compare all parameters assayed in the prepared breads. The dependent variables were analyzed using 2-way ANOVA with the factors “bread type” (BT) and “storage time” (ST). When a statistically significant interaction was detected for these two factors, they were evaluated simultaneously by the estimated marginal means plots for all levels of each factor. Furthermore, if no statistical significant interaction is found, the means were compared using Tukey’s multiple comparison test, with a previous assessment of the equality of variances through a Levene’s test.

In addition, a linear discriminant analysis (LDA) was used to compare the effect of BT and ST over all assayed parameters. A stepwise technique was applied, considering the Wilks’ λ test with the usual probabilities of F (3.84 to enter and 2.71 to be removed) for variable selection. This procedure is based in sequential forward selection and backward elimination steps, where the inclusion of a new variable requires verifying the significance of all previously selected variables (Zielinski et al., 2014). In general, it was intended to estimate the relationship between single categorical dependent variables (bread types) and the quantitative independent variables (results obtained in laboratorial assays). The generated outputs allowed determining which independent variables contributed more to the differences in the average score profiles of the different bread samples. To verify the significance of the canonical discriminating functions, Wilk’s λ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

IV. Results and Discussion

Biological activity of plant extracts

As previously explained, it was intended to evaluate the effects of incorporating the extracts of *Ocimum basilicum* leaves (enriched extract in rosmarinic acid) and *Arbutus unedo* fruit (enriched extract in catechin) in the preservation and functional properties of bread. The potential functionalizing effect of both extract types became evident in previously published research reports (Albuquerque et al., 2017; Andrade et al., 2009; Barros et al., 2010; Carcho et al., 2016b; Fortalezas et al., 2010). Owing to current consuming trends, which progressively require longer shelf-lives for bread products, those effects were evaluated in three different periods (baking day, after 3 days of storage and after 7 days of storage).

The extracts were obtained using ethanol:water mixtures (23% of ethanol in *A. unedo* fruits; 80% of ethanol in *O. basilicum* leaves). Before proceeding to their incorporation, extracts were individually characterized regarding antioxidant (**Table 2**), antimicrobial (**Table 3**) and cytotoxic (using a PLP2 cell line) activities. Furthermore, the quantities of rosmarinic acid (45.3 ± 0.6 mg of rosmarinic acid/g of extract) and catechin (25.7 ± 0.2 mg of catechin acid/g of extract) obtained from the extracts *O. basilicum* leaves and *A. unedo* fruit, respectively, were also determined.

Table 2. Antioxidant activity (EC₅₀ values, µg/mL extract) of the extracts of *A. unedo* fruit and *O. basilicum* leaves. Values are given as mean±standard deviation.

	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS formation inhibition
<i>Arbutus unedo</i> fruit	595±10	328±2	2169±25	347±17
<i>Ocimum basilicum</i> leaves	261±4	136±1	1008±53	351±20
<i>p</i> -value Homoscedasticity ¹	0.023	0.024	0.003	0.871
(n = 18) <i>t</i> -student test ²	<0.001	<0.001	<0.001	0.624

¹Homoscedasticity plant species was tested by Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. ² $p < 0.05$ indicates that the mean value of both species differ from each other significantly. EC₅₀ values – concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power. Trolox, was used as positive control for the antioxidant activity (EC₅₀ values: 41 ± 1 and 18 ± 1 for DPPH scavenging activity and reducing power, respectively).

Table 3. Antibacterial activity (MIC and MBC values, mg/mL extract) of the extracts of *A. unedo* fruits and *O. basilicum* leaves.

	Gram positive bacteria							
	<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>		<i>Salmonella typhimurium</i>		<i>Enterobacter cloacae</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Arbutus unedo</i> fruit	0.60	0.90	0.15	0.30	0.30	0.45	0.20	0.45
<i>Ocimum basilicum</i> leaves	0.45	0.60	0.20	0.30	0.15	0.30	0.30	0.45
Streptomycin	0.20	0.30	0.15	0.30	0.25	0.50	0.20	0.30
Ampicilin	0.40	0.50	0.30	0.60	0.40	0.75	0.25	0.50
	Gram negative bacteria							
	<i>Staphylococcus aureus</i>		<i>Bacillus cereus</i>		<i>Micrococcus flavus</i>		<i>Listeria monocytogenes</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Arbutus unedo</i> fruit	0.45	0.60	0.10	0.15	0.30	0.45	0.45	0.60
<i>Ocimum basilicum</i> leaves	0.20	0.30	0.10	0.15	0.20	0.30	0.20	0.30
Streptomycin	0.04	0.10	0.10	0.20	0.20	0.30	0.20	0.30
Ampicilin	0.25	0.45	0.25	0.40	0.25	0.40	0.40	0.50

The extracts obtained from *O. basilicum* leaves showed significantly higher antioxidant activity, as indicated by DPPH scavenging activity, reducing power and β -carotene bleaching inhibition; on the other hand, the ability to inhibit TBARS formation was similar in both extracts.

Besides their high antioxidant activity, the extracts from *A. unedo* and *O. basilicum* showed also a high antibacterial activity, often comparable to that achieved with the standard antibiotics streptomycin and ampicillin. Among Gram positive bacteria, *Pseudomonas aeruginosa* and *Salmonella typhimurium* were the most sensitive to the extracts of *A. unedo* fruit and *O. basilicum* leaves, respectively. In both cases, MIC

values were lower than those obtained with ampicillin (and also with streptomycin in the case of *O. basilicum* leaves). In general, the antibacterial activity of *O. basilicum* leaves extracts was higher than that of *A. unedo* fruit extracts (except regarding MIC in *Pseudomonas aeruginosa* and *Enterobacter cloacae*).

On the other hand, the extracts obtained from *A. unedo* fruits achieved higher antifungal activity, demonstrating particularly high activity against *Aspergillus ochraceus*, *Aspergillus flavus* and *Cladosporium cladosporioides* (**Table 4**). In fact, the results obtained for the indicated species were comparable, or even better, than those produced by the tested standards.

Table 4. Antifungal activity (MIC and MFC values, mg/mL extract) of the extracts of *A. unedo* fruits and *O. basilicum* leaves.

	<i>Aspergillus fumigatus</i>		<i>Aspergillus ochraceus</i>		<i>Aspergillus versicolor</i>		<i>Aspergillus niger</i>		<i>Penicillium funiculosum</i>		<i>Aspergillus flavus</i>		<i>Cladosporium cladosporioides</i>		<i>Penicillium verrucosum</i>	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
<i>Arbutus unedo</i> fruit	0.20	0.45	0.15	0.30	0.20	0.30	0.30	0.45	0.30	0.60	0.10	0.30	0.15	0.30	0.30	0.45
<i>Ocimum basilicum</i> leaves	0.60	0.90	0.30	0.60	0.45	0.90	0.60	0.90	0.45	0.60	0.20	0.30	0.30	0.45	0.45	0.90
Ketoconazole	0.25	0.50	1.50	2.00	0.20	0.50	0.20	0.50	0.20	0.50	0.30	0.50	0.075	0.15	0.20	0.30
Bifonazole	0.15	0.20	0.15	0.20	0.10	0.20	0.15	0.20	0.20	0.25	0.15	0.30	0.05	0.10	0.10	0.20

The higher antioxidant and antibacterial activities measured in the extracts of *O. basilicum* leaves might be associated to the high contents in phenolic acids, particularly in rosmarinic acid (Carocho et al., 2016b), whilst the activity of *A. unedo* fruits should be explained, in part, by the presence of significant levels of catechin (Albuquerque et al., 2017). None of the assayed extracts showed cytotoxicity up to the maximal assayed concentrations (400 µg/mL).

Characterization of different types of functionalized bread

Bread represents a staple food all over the world, thereby being a suitable food matrix to assay different incorporation processes (e.g. preservative ingredient, functionalization/fortification approaches) (Bolarinwa et al., 2017; Singh et al., 2012). Concomitantly, people are progressively receptive to food products prepared with natural ingredients in substitution to general synthetic compounds, which are often associated with unwanted effects (Carocho et al., 2014). In addition to the potential health effects conveyed by the functionalizing agents, the rheological and technological properties of food products themselves might also be improved (Berdahl et al., 2010; Caleja et al., 2015; Carocho et al., 2015; Sciarini et al., 2012).

In the present work, five bread types (BT) were prepared: i) control (bread without any type of incorporated extract); ii) bread with *A. unedo* fruit extract (enriched in catechin); iii) bread with *O. basilicum* leaves extract (enriched in rosmarinic acid); iv) bread with ascorbic acid; and v) bread with potassium sorbate. In addition to the evaluation of each extract type on different chemical and bioactive parameters, it was also intended to verify how these effects evolved throughout time, specifically considering 3 periods: preparation day, 3 days after preparation and 7 days after preparation.

From a practical point of view, the effect of each extract should be evaluated independently of storage time (ST). The interaction among factors (BT×ST) was evaluated to assess possible differentiated effects, i.e., if the influence of one factor showed different variations depending on the level of the other factor. When a significant interaction was found ($p < 0.050$), no multiple comparisons can be performed. In those cases, the effect of each factor was assessed by interpreting the estimated marginal means (EMM) plots.

Regarding nutritional composition (**Table 5**), the interaction among factors was significant in all cases, showing that the variation in water, fat, protein, ash, carbohydrates and energy throughout time depended of BT, thereby hindering the possibility of identifying general tendencies. Comparing individual factors, BT had a higher effect than ST, as the variations induced by BT were significant in all cases, while ST produced a significant effect only in water, protein and ash.

Table 5. Nutritional composition (g/100 g fw), energy (kcal/100 g fw) and pH values of different bread types (BT) and storage times (ST). Results are presented as mean±standard deviation.¹

		Water	Fat	Protein	Ash	Carbohydrates	Energy	pH
BT	Control bread loaf	30±1	1.7±0.2	6.9±0.5	1.9±0.2	59±2	280±7	5.7±0.3
	Bread with <i>A. unedo</i>	32±1	1.8±0.2	6.9±0.2	1.7±0.2	57±1	274±4	5.8±0.3
	Bread with <i>O. basilicum</i>	30±1	2.3±0.1	7.2±0.3	2.0±0.4	59±1	285±5	5.7±0.4
	Bread with ascorbic acid	32±1	2.1±0.2	6.8±0.2	1.9±0.1	57±1	274±5	5.6±0.3
	Bread with potassium sorbate	29±1	2.1±0.2	7.0±0.5	2.2±0.3	60±1	286±5	5.7±0.2
	ANOVA <i>p</i> -value (n = 27) ²	<0.001	<0.001	0.013	<0.001	<0.001	<0.001	0.157
ST	0 days	31±2	2.1±0.3	6.9±0.2	2.1±0.3	58±2	278±7	5.3±0.1
	3 days	31±2	2.0±0.3	6.8±0.5	1.7±0.2	58±2	279±7	5.8±0.1
	7 days	30±2	2.0±0.3	7.1±0.4	1.9±0.2	59±1	282±7	6.0±0.2
	ANOVA <i>p</i> -value (n = 45) ³	0.021	0.436	0.005	<0.001	0.063	0.082	<0.001
BT×ST	<i>p</i> -value (n = 135) ⁴	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

¹Results are reported as mean values of each bread type (BT), including results from 0, 3 and 7 days, and mean values of each storage time (ST), considering all bread types in each period. ²If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one bread type. ³If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one storage time. ⁴If $p < 0.05$, the interaction among factors is significant; in this case, no multiple comparisons can be performed.

Owing to the relevancy of the visual appearance of bread, its color parameters (L^* : lightness, a^* : redness and b^* : blueness) were evaluated in the top crust, interior and bottom crust (**Table 6**). The cooperative effect of BT and ST was also significant in most cases, except L^* and b^* in the bottom crust. Among color parameters, L^* showed the most marked differences, with higher values in control bread, regarding the effect of BT, but specially with lower values in samples stored during 7 days, in what concerns ST effect. In either case, the effects over color parameters were

more noticeable in bread interior, as crust color is especially dependent on baking temperature and time (Debonne et al., 2017), which were rigorously maintained in the study reported herein.

Table 6. Color parameters of different bread types (BT) and storage times (ST). Results are presented as mean±standard deviation.¹

		Top crust			Bottom crust			Bread interior		
		<i>L</i> *	<i>a</i> *	<i>b</i> *	<i>L</i> *	<i>a</i> *	<i>b</i> *	<i>L</i> *	<i>a</i> *	<i>b</i> *
BT	Control bread	58±4	13±1	33±3	40±4 a	14±1	24±3 ab	68±3	-0.7±0.2	17±2
	Bread with <i>A. unedo</i>	52±3	15±2	33±2	40±4 ab	14±1	25±2 a	61±5	0.6±0.1	17±2
	Bread with <i>O. basilicum</i>	55±4	14±1	31±3	38±3 bc	14±1	23±3 b	62±5	0.6±0.2	21±2
	Bread with ascorbic acid	55±4	12±2	32±4	36±3 d	14±1	23±2 b	65±4	1.2±0.3	21±2
	Bread with potassium sorbate	55±5	16±2	33±4	37±3 cd	15±1	24±3 ab	69±5	1.0±0.3	21±1
	ANOVA <i>p</i> -value (n = 27) ²	<0.001	<0.001	0.163	<0.001	<0.001	0.042	<0.001	<0.001	<0.001
ST	0 days	56±4	14±2	33±3	40±3 a	14±1	25±2 a	68±4	0.5±0.5	19±3
	3 days	56±4	14±3	32±3	40±3 a	15±1	25±2 a	66±5	0.5±0.5	19±3
	7 days	52±4	15±2	32±3	35±2 b	14±1	22±2 b	61±5	0.5±0.5	20±3
	ANOVA <i>p</i> -value (n = 45) ³	<0.001	0.006	0.289	<0.001	<0.001	<0.001	<0.001	0.552	0.921
BT×ST	<i>p</i> -value (n = 135) ⁴	<0.001	<0.001	<0.001	0.125	<0.001	0.221	<0.001	<0.001	<0.001

¹Results are reported as mean values of each bread type (BT), including results from 0, 3 and 7 days, and mean values of each storage time (ST), considering all bread types in each period. ²If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one bread type. ³If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one storage time. ⁴If $p < 0.05$, the interaction among factors is significant; in this case, no multiple comparisons can be performed.

The profiles in individual sugars (**Table 7**) and fatty acids (major compounds are presented in **Table 8**) were also assessed in order to verify their integrity along ST. Both components are prone to oxidation, representing one of the main indicators of adequate conservation of food products

(Barreira et al., 2010; Pereira et al., 2016). Nevertheless, and despite the existence of significant differences resulting from BT and ST, the profiles in sugars and fatty acids were maintained nearly unchanged inclusively in control bread. In addition, the interaction among factors was significant in all cases, thereby hampering the possibility of obtaining general conclusions.

Table 7. Individual sugars (g/100 g) of different bread types (BT) and storage times (ST). Results are presented as mean±standard deviation.¹

		Fructose	Glucose	Sucrose	Maltose	Trehalose	Total sugars
BT	Control bread loaf	1.6±0.1	0.56±0.02	1.1±0.1	3.4±0.3	0.15±0.01	6.9±0.4
	Bread with <i>A. unedo</i>	1.6±0.1	0.57±0.03	1.2±0.1	3.7±0.2	0.16±0.02	7.3±0.2
	Bread with <i>O. basilicum</i>	1.7±0.1	0.58±0.05	1.1±0.1	3.5±0.1	0.16±0.02	7.0±0.2
	Bread with ascorbic acid	1.3±0.1	0.58±0.02	1.2±0.1	3.2±0.1	0.13±0.01	6.4±0.2
	Bread with potassium sorbate	1.7±0.1	0.68±0.04	1.2±0.1	3.5±0.1	0.14±0.01	7.2±0.2
	ANOVA <i>p</i> -value (n = 27) ²	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST	0 days	1.7±0.2	0.58±0.05	1.1±0.1	3.5±0.3	0.15±0.02	7.1±0.5
	3 days	1.6±0.1	0.59±0.05	1.2±0.1	3.5±0.2	0.16±0.02	7.0±0.3
	7 days	1.5±0.1	0.61±0.05	1.1±0.1	3.4±0.1	0.15±0.01	6.8±0.2
	ANOVA <i>p</i> -value (n = 45) ³	<0.001	0.014	0.114	0.003	0.063	<0.001
BT×ST	<i>p</i> -value (n = 135) ⁴	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

¹Results are reported as mean values of each bread type (BT), including results from 0, 3 and 7 days, and mean values of each storage time (ST), considering all bread types in each period. ²If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one bread type. ³If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one storage time. ⁴If $p < 0.05$, the interaction among factors is significant; in this case, no multiple comparisons can be performed.

Table 8. Major fatty acids (relative percentage of different bread types (BT) and storage times (ST)). Results are presented as mean±standard deviation.¹

		C16:0	C18:0	C18:1	C18:2	C22:0	SFA	MUFA	PUFA
BT	Control bread	15.1±0.2	5.2±0.5	37±2	26±1	3.5±0.4	28±1	40±2	32±3
	Bread with <i>A. unedo</i>	15.0±0.1	5.0±0.2	36±1	26±1	3.5±0.1	28±1	36±5	36±5
	Bread with <i>O. basilicum</i>	15.0±0.2	4.9±0.1	37±1	26±1	3.6±0.1	28±1	39±1	33±1
	Bread with ascorbic acid	15.4±0.5	5.2±0.1	36±1	26±1	3.7±0.1	29±1	39±1	33±1
	Bread with potassium sorbate	15.1±0.5	5.1±0.3	36±2	26±1	3.7±0.2	28±1	39±1	33±3
	ANOVA <i>p</i> -value (n = 27) ²	0.004	0.007	<0.001	0.691	0.035	0.084	<0.001	<0.001
ST	0 days	15.1±0.2	5.2±0.5	37±2	26±1	3.7±0.2	29±1	37±4	34±5
	3 days	14.9±0.3	4.9±0.2	36±1	27±1	3.6±0.2	28±1	38±1	34±1
	7 days	15.4±0.5	5.1±0.2	37±1	26±1	3.5±0.3	29±1	39±1	32±2
	ANOVA <i>p</i> -value (n = 45) ³	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.004
BT×ST	<i>p</i> -value (n = 135) ⁴	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

¹Results are reported as mean values of each bread type (BT), including results from 0, 3 and 7 days, and mean values of each storage time (ST), considering all bread types in each period. ²If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one bread type. ³If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one storage time. ⁴If $p < 0.05$, the interaction among factors is significant; in this case, no multiple comparisons can be performed.

Finally, the antioxidant activity of different BT was evaluated to compare the effects of each incorporated extract. More than evaluating improvements in comparison to control bread, which would be easily anticipated, it was intended to verify potential differences among natural extracts and commercial compounds. Regarding DPPH scavenging activity (**Figure 13**), bread prepared with *O. basilicum* extract achieved the best results, showing to be more effective than ascorbic acid and potassium sorbate.

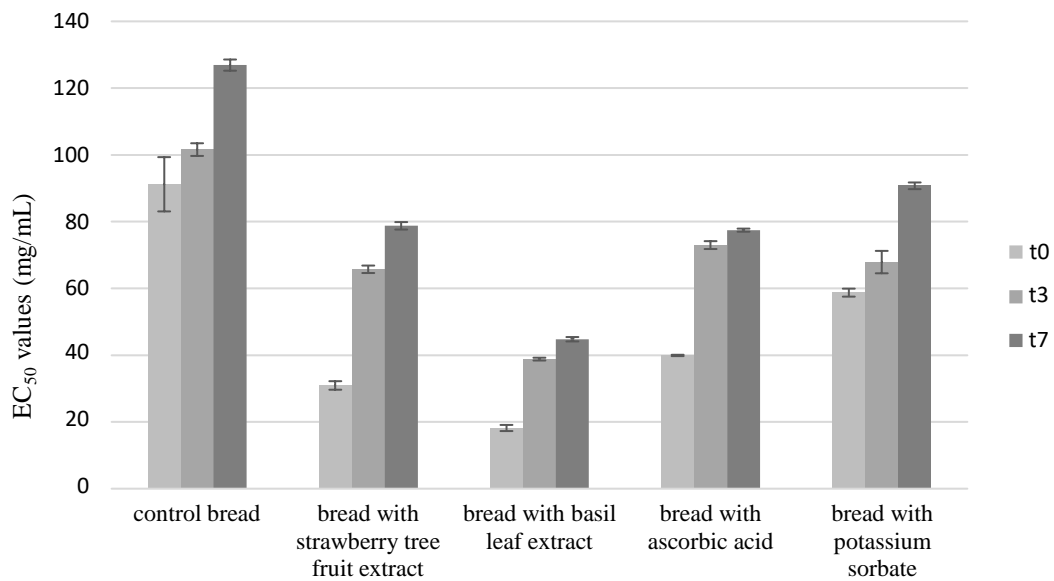


Figure 13. DPPH scavenging activity of different bread types assayed in three consecutive periods.

However, the ability to scavenge DPPH radical decreased along time in all BT. On the other hand, no significant differences were found in the reducing power of all functionalized breads (**Figure 14**), which however maintained similar activity throughout ST.

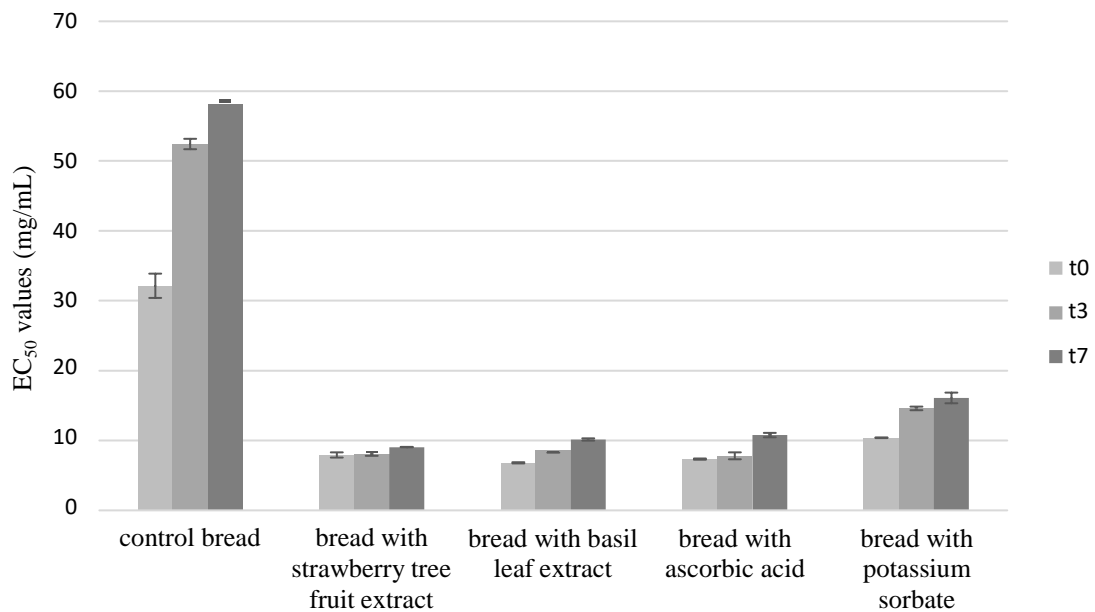


Figure 14. Reducing power of different bread types assayed in three consecutive periods.

Furthermore, none of the functionalized breads showed cytotoxicity up to the maximal assayed concentrations (400 $\mu\text{g/mL}$) and the quantities of rosmarinic acid (bread with *O. basilicum* leaves extract) and catechin (bread with *A. unedo* fruit extract) were also maintained along ST.

Linear Discriminant Analysis

In the previous section, several statistically significant differences were found in different assayed parameters (either in result of BT or ST). However, it would be useful to have a thorough characterization of each BT, considering its effects on all parameters simultaneously, in that way verifying which of them had higher contribution to the detected differences. Accordingly, a linear discriminant analysis (LDA) was performed to evaluate the linkage between BT (categorical dependent variables) and the matrix of obtained results (quantitative independent variables). The significant independent variables were selected following the stepwise method of LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.050$) were maintained by the statistical model.

The first three defined discriminant functions included 96.3% (first function: 76.5%; second function: 12.9%; third function: 6.9%) of the observed variance (**Figure 15**).

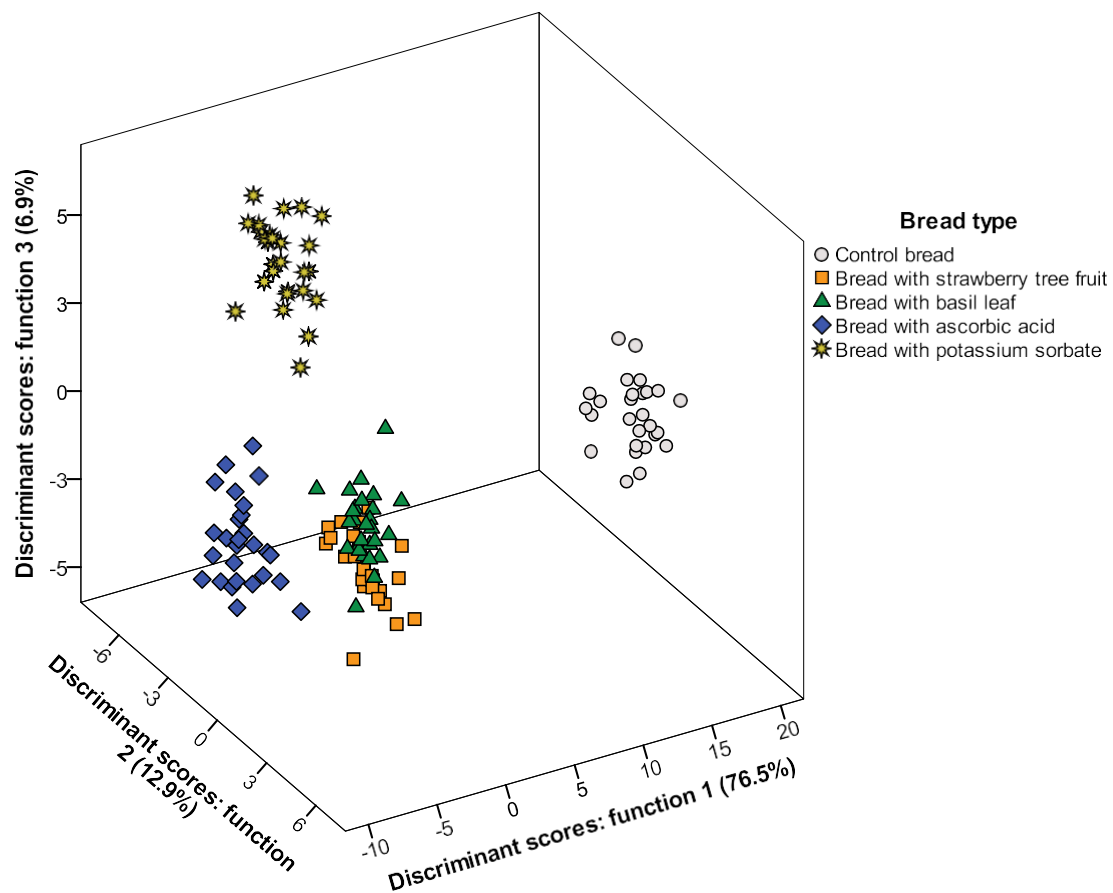


Figure 15. Canonical discriminant functions coefficients defined from the evaluated parameters to assess the effect of bread type.

Among the 32 inserted variables, the model selected water, fat, ash, energy, pH, a^* (top crust), L^* (interior), a^* (interior), b^* (interior), fructose, glucose, trehalose, sugars, DPPH scavenging activity and reducing power as the ones with the highest discriminant effect. Function 1, which was mostly correlated (data not shown) with DPPH scavenging activity and reducing power, separating markers corresponding to control bread and a second group formed by all functionalized breads, specifically by the lower EC_{50} values of the last. Function 2 was particularly useful to separate markers corresponding to bread functionalized with *A. unedo* fruit and *O. basilicum* leaves from those functionalized with the commercial compounds (ascorbic acid and potassium sorbate), mainly due to the differences in L^* , a^* (lower in breads with natural extracts) and trehalose (higher in breads with natural extracts), the three variables with the highest correlation with function 2. To finish, function 3 was more highly correlated to (ordered by correlation factor) water, fructose, energy and ash, defining two distinct groups: one constituted by the markers corresponding to bread

with *A. unedo* fruit extract + bread with ascorbic acid (with higher water values), and a second one formed by markers corresponding to bread with *O. basilicum* leaves extract + bread with potassium sorbate (with higher fructose, energy and ash contents).

In the performed LDA, the classification performance was 100% accurate, either for original grouped cases, as well as for the cross-validated grouped cases.

V. Concluding Remarks

The extracts obtained from *O. basilicum* leaves and *A. unedo* fruits showed high antioxidant and antimicrobial activity without revealing cytotoxicity. This ability may be associated with high levels of phenolic compounds, especially rosmarinic acid in the case of the extracts obtained from leaves of *O. basilicum* and catechins in the case of the extracts obtained from fruits of *A. unedo*.

The antioxidant activity evaluation revealed a greater capacity for the functionalized bread with the extract obtained from *O. basilicum* leaves. Both natural extracts showed lower EC₅₀ values than the synthetic additives, thus revealing a higher antioxidant activity.

Regarding the appearance and nutritional analysis, it was possible to observe significant differences for the analyzed parameters (additive effect and the storage time effect) and, therefore, an LDA was made for a more correct analysis. This analysis presented lower values of L^* and a^* for the breads incorporated with extracts obtained from fruits of *A. unedo* and from leaves of *O. basilicum*, in relation to the breads incorporated with ascorbic acid and potassium sorbate. On the other hand, bread with extracts of *A. unedo* fruits and bread with ascorbic acid showed the highest values of water, whereas bread with *O. basilicum* and with potassium sorbate had the highest values of fructose, energy and ash content.

Overall, it was possible to verify that the studied natural extracts might represent effective replacers of ascorbic acid or potassium sorbate, depending on the intended preservation effect.

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