UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Nutrición y Bromatología II

TESIS DOCTORAL

Desarrollo de nuevos productos alimenticios: incorporación de extractos de plantas como ingredientes funcionales y conservantes naturales
Development of new foods: incorporation of plant extracts as functional ingredients and natural preservatives

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Márcio Soares Carocho

Directores

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UNIVERSIDAD COMPLUTENSE DE MADRID
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Para optar al Grado de Doctor, con mención Europea,

Directoras:
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Madrid, 2016

UNIVERSIDAD COMPLUTENSE DE MADRID
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DEPARTAMENTO DE NUTRICION Y BROMATOLOGIA II
Bromatologia

Mª DOLORES TENORIO SANZ, PROFESORA TITULAR DEL ÁREA DE NUTRICIÓN Y BROMATOLOGÍA Y DIRECTORA DEL DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA II: BROMATOLOGÍA, DE LA FACULTAD DE FARMACIA, DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

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El presente trabajo de investigación titulado "Desarrollo de nuevos productos alimenticios: Incorporación de extractos de plantas como ingredientes funcionales y conservantes naturales" se ha realizado en este Departamento bajo la dirección de las doctoras Patricia Morales Gómez y Isabel Cristina Fernandes Rodrigues Ferreira, y constituye la Memoria que presenta Márcio Soares Carocho para optar al Grado de Doctor, con mención europea.

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CERTIFICAN QUE:

Márcio Soares Carocho, ha realizado bajo su dirección y en el Dpto. de Nutrición y Bromatología II, el trabajo de tesis doctoral que lleva por título **"Desarrollo de nuevos productos alimenticios: Incorporación de extractos de plantas como ingredientes funcionales y conservantes naturales"** y que constituye su Memoria de Tesis Doctoral. Dicho trabajo reúne las condiciones necesarias para su presentación y defensa para optar al grado de Doctor, con mención europea.

Y para que conste, a los efectos oportunos, firmo el presente certificado en Madrid a quince de febrero de dos mil dieciséis.

To my grandmothers,

Maria Augusta Cabral Dias Sampaio Carocho

Maria Fernanda de Jesus Cruz Castro

To my mother,

Ana Paula de Jesus Soares Carocho

Machhapuchchhre summit “Fish Tail”, in the Annapurna Himal in Nepal at 6993 meters high, taken from 4000 meters. It has never been climbed, for it revered as sacred to the God Shiva, known to be a limitless, transcendent, unchanging and formless deity. Mythology and Science share these attributes.

Photo by Paulo Antunes, 2014.

Equipped with his five senses, man explores the universe around him and calls the adventure Science.

Edwin Powell Hubble (1929)

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ABBREVIATIONS AND ACRONYMS

*a**	Greenness-redness
AAS	Atomic absorption spectrometry
Abs	Absorbance
ADI	Admissible daily intake
ANOVA	Analysis of variance
AOAC	Association of analytical communities
API	Atmospheric pressure ionization
ATCC	American type culture collection
*b**	Blueness-yellowness
BHT	Butylated hydroxytoluene
BL	Basil leaf
C	Control samples
CA	Codex alimentarius
Ca	Calcium
CAT	Catalase enzyme
CD	Chestnut decoction
CFU	Colony forming units
CP	Chestnut dehydrated plant
Cu	Copper
D50	Sample with 50 mg of chestnut flower decoction
D100	Sample with 100 mg of chestnut flower decoction
DAD	Diode array detector
D.O.	Denominación de orígen
DOP	Denominação de origem protegida
DPPH	2,2-diphenil-1-picrilhydrazil
DMEM	Dubelco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Concentration with 50% of antioxidant activity
EFSA	European food safety authority
EMM	Estimated marginal means
ESI	Electron spray ionization
EU	European Union
F200	Sample with 200 mg of dried chestnut flower
F400	Sample with 400 mg of dried chestnut flower

FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FDA	Food and drug administration of the United States of America
Fe	Iron
FID	Flame ionization detector
FW	Fresh weight
GAE	Gallic acid equivalents
GC	Gas chromatography
GI	Gastrointestinal tract
GI_{50}	Concentration that inhibits 50% of cell growth
GLM	General linear model
GRAS	Generally regarded as safe
HBSS	Hank's balanced salt solution
HHDP	Hexahydroxydiphenoyl
HPLC	High performance liquid chromatography
HP	Hewlett-Packard
HSD	Honest significant difference
I+D+I	Investigation, development and innovation
IDF	Insoluble dietary fiber
INT	Iodonitrotetrazolium
IS	Internal standard
IU	International unit
JD	"Judia" decoction
JI	"Judia" infusion
K	Potassium
*L**	Lightness
LD	"Longal" decoction
LI	"Longal" infusion
LDA	Linear discriminant analysis
MA	Malt agar
mA	Mili ampere
MBC	Minimum bactericidal concentration
MD	Decoction of *Melissa officinalis* (lemon balm)
MDA	Malondialdehyde
MDA-TBA	Malodialdehyde-thiobarbituric acid chromogen
MFC	Minimum fungicidal concentration
Mg	Magnesium
MIC	Minimum inhibitory concentration
mM	Milimoles

Mn	Manganese
MP	*Melissa officinalis* (lemon balm) dehydrated leaves
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
M-H	Deprotonation
NA	Natural additive
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NCI	National cancer institute
NCTC	National collection of type cultures
nd	Not detected
nm	Nanometer
OD	Decoction of *Ocimum basilicum* (basil)
OP	*Ocimum basilicum* (basil) dehydrated leaves
PDA	Photo-diode array detector
PDO	Protected designation of origin
PLP2	Porcine liver primary cell line
PNSI	Portuguese national statistics institute
PUFA	Polyunsaturated fatty acids
RI	Refraction index
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Reducing power
RPMI	Roswell park memorial institute medium
Rt	Retention time
SD	Standard deviation
SDF	Soluble dietary fiber
SPSS	Statistic package for social sciences
SRB	Sulforhodamine B
ST	Storage time
TAC	Total available carbohydrates
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TSB	Tryptic soy broth
SFA	Saturated fatty acids
SOD	Superoxide dismutase enzyme
UK	United Kingdom
USA	United States of America
UFLC	Ultra-fast liquid chromatography

UV	Ultra violet
v.	Version
Zn	Zinc
β-C	β-carotene bleaching inhibition assay
λmax	Wavelength of maximum absorption

INDEX

ABSTRACT

"Development of new food products: Incorporation of plant extracts as functional ingredients and natural preservatives"

Innovations and beneficial inputs to increase health effects of foodstuffs are an important task in I+D+I research in the food industry. Parallel to these needs, the consumers are becoming more aware of the food they eat and demand healthier products which should be minimally processed or not processed at all. Food additives are one of the most used ways of preserving the characteristics of food and have been used for dozens of years, with improvements being introduced routinely. The quest for better and healthier food additives allied to the demand of consumers have fostered the investment in natural food additives, that come from plants, animals or microorganisms. Extensive research has been carried out to find synergies between natural and synthetic additives, and to find the natural additive that is capable of carrying out various functions in food (coloring, preserving, antioxidant, etc.).

In the EU, some natural additives are approved for use in food, although many more are waiting authorization from the governing bodies. Natural additives can be individual compounds or groups of compounds that benefit from synergies. The use of plants and their extracts as additives has become one of the leading themes in research for natural food additives.

This dissertation focuses on the chemical characterization of plants and their extracts (decoctions and infusions), with subsequent incorporation into traditional Portuguese cakes "económicos" and traditional ewe cheese, "Queijo da Serra da Estrela". Three plants and their aqueous extracts were screened in terms of bioactivity, and subsequently incorporated into foods. The three plants used were chestnut flowers (*Castanea sativa* Mill.), leaves and stems of lemon balm (*Melissa officinalis* L.) and basil (*Ocimum basilicum* L.). For the flowers, both the infusions and decoctions of two cultivars (Judia and Longal) were screened for bioactive compounds and bioactivities. Phenolic compounds were determined by high performance liquid chromatography, coupled to a mass spectrometer, while the organic acids relied on ultra-fast liquid chromatography coupled to a diode array detector for determination. Tocopherols (only for lemon balm) and soluble sugars were both determined by high performance liquid

chromatography, coupled to a fluorescence and refraction index detectors, respectively. In terms of bioactivities, the antioxidant, antimicrobial (antibacterial and antifungal) and antitumor activities were screened. In terms of the antioxidant activity, the 2,2-diphenil-1-picrilhydrazil assay was tested, along with the reducing power, while two assays were used to determine lipid peroxidation, namely, β-carotene bleaching inhibition and inhibition of thiobarbituric acid reactive substances. The antimicrobial assays used different species of food contaminants, and the minimum inhibition concentration, minimum bactericidal and fungicidal concentration were determined for all plants. In terms of the antitumor assays, various human tumor cell lines were screened against the plants, while primary porcine liver cell lines were employed to determine the hepatotoxicity of the extracts.

For the chestnut flower extracts, the highest phenolic compounds were trigalloyl-HHDP-glucoside followed by pentagalloyl glucoside. Fructose, glucose, quinic and oxalic acids were the highest sugars and organic acids. The highest antioxidant activity was determined for the decoctions of Judia, which also proved to be the most efficient against the screened bacteria. The most sensitive tumor cell lines were liver and colon tumor cell lines, while no toxicity was detected for any of the extractions.

In terms of the screening of lemon balm, only the decoctions were screened, and the most abundant phenolic compounds were rosmarinic acid, followed by lithospermic acid A. Quinic acid, fructose and γ-tocopherol were the most abundant compounds within their bioactive group. The most sensitive microorganisms to the decoctions, with inhibition rations under the positive controls, were *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Penicillium funiculosum*. The most sensitive tumor cell lines were breast and liver, and no toxicity was detected for the lemon balm decoctions.

Finally, regarding basil, rosmarinic acid followed by chicoric acid were the most abundant polyphenols. Quinic acid and fructose were the most abundant organic acid and soluble sugar, respectively. Cervical and liver tumor cell lines were the most sensitive to the decoctions, and once again no toxicity was found. Thiobarbituric acid reactive substances assay and the reducing power were the antioxidant assays with the best results for this plant. Overall, the fungi seemed to be more susceptible to it that the bacteria, although it showed lower inhibition concentrations that the positive controls for *Listeria monocytogenes*, *Staphylococcus aureus, Enterobacter cloacae*, and *Salmonella typhimurium*.

The second part of the work plan was the incorporation of the best extracts in foodstuffs. The first incorporation was in traditional north-eastern Portuguese cakes, and only with the

decoctions of the chestnut flowers and the direct incorporation of dried flowers in two different lots. The quantity of incorporated decoction was calculated taking into consideration the antioxidant activity of the extract, relying of the EC_{50} (concentration at which the extract displays 50% of antioxidant activity) value, adjusted to the amount of batter used. The amount of dried flower incorporated relied on the extraction yield of the decoctions. In order to determine the changes and eventual preservation of the extracts and/or flowers, the cakes were analysed on the same day of manufacture, after fifteen and thirty days. To determine the influence of different concentrations of each incorporation, a two-fold concentration of each was incorporated to other lots of cakes, and subject to the same storage periods and analysis. The nutritional value (moisture, total available carbohydrates, dietary fibers, crude protein, crude fat, ash and energy) was measured relying on AOAC procedures. The fatty acids were determined by gas chromatography and mineral elements by atomic absorption spectrometry. Finally, the antioxidant activity of the cakes was also measured. Overall, and as expected, the moisture decreased over time, which lead to a raise of ash, total available carbohydrates, insoluble fiber and energy, although very slight changes were found among the incorporated samples. Succinic acid was the highest organic acid, with the highest values being detected in the cakes incorporated with the decoctions. α-tocopherol was the most abundant tocopherol, but detected in very small quantities, while sucrose was the most prevalent soluble sugar, but decreased during storage time, thus raising the amounts of fructose and sucrose. Sodium and potassium were the most abundant mineral elements. Palmitic acid was the most abundant fatty acid, and there was a continuous reduction in the polyunsaturated fatty acids along the storage time. To determine the influence of the storage time and the type of incorporation independently, 2-way ANOVAS were carried out, followed by linear discriminant analysis. Overall, and nutritionally, the cakes incorporated with the dried flowers seemed to be the best choices. In terms of the antioxidant activity of the incorporated cakes, storage time had a higher influence than the quantities incorporated, and the 2,2-diphenil-1-picrilhydrazil assay, along with the inhibition of thiobarbituric acid reactive substances, were the most improved parameters.

The second incorporated foodstuff was soft cheese (Serra da Estrela), which is one of Portugal's most appreciated and famous dairy products. Different lots were produced in order to incorporate the three selected plants and their decoctions in the same manner as for the cakes, although for the cheese only one concentration was tested. Analysing the different lots, in two different storage times, zero and six months. The external color was also determined. Calcium

and sodium were the most abundant mineral elements in all the cheeses, while palmitic and oleic acids were the most abundant fatty acids. Overall, the saturated fatty acids prevailed over the monounsaturated ones. Over time, moisture seemed to be lower in the incorporated samples, which could allow a quicker achievement of harder cheese (old cheese). The incorporated cheeses also showed higher antioxidant activity, both for radical scavenging activity and inhibition of lipid peroxidation. Overall, the dried chestnut flowers and lemon balm plants seemed to preserve and functionalize the cheeses better than their decoctions, but the basil decoctions proved to be better than the plants. In terms of external color, some changes were detected in the incorporated cheeses, especially for the ones incorporated with the dried plants. The findings of this work prove the efficacy of these plants and their aqueous extracts as functional ingredients and natural preservatives in these foodstuffs, by turning them into healthier products, with higher antioxidant potential while aiding their conservation, mainly in "Serra da Estrela" cheese.

RESUMEN

"Desarrollo de nuevos productos alimenticios: Incorporación de extractos de plantas **como ingredientes funcionales y conservantes naturales"**

La innovación y búsqueda de nuevas evidencias científicas que avalen el consumo de alimentos funcionales con contrastados beneficios para la salud de los consumidores, es hoy en día una tarea clave para la industria alimentaria. Paralelamente a estas necesidades, los consumidores son cada vez más conscientes de los alimentos que consumen y demandan productos más saludables. Los aditivos alimentarios son una de las formas más utilizadas por la industria alimentaria para mantener la calidad de los alimentos. Dentro de la mejora de los productos alimenticios, la industria alimentaria recurre a la búsqueda de aditivos más saludables, con menos efectos secundarios y más económicos, de ahí que se haya fomentado la investigación en aditivos naturales, que provengan de plantas, animales o microorganismos. Los aditivos naturales pueden ser compuestos, o grupos de compuestos que se benefician de las sinergias individuales. El uso de las plantas y sus extractos como aditivos se ha convertido en uno de los temas principales en la investigación de los aditivos alimentarios naturales. Así mismo, se ha llevado a cabo una amplia investigación para encontrar sinergias entre aditivos naturales y sintéticos, y para encontrar un aditivo natural que sea capaz de llevar a cabo diversas funciones en los alimentos (colorantes, conservantes, antioxidantes, etc.). En la Unión Europea, algunos aditivos naturales están aprobados para su uso en alimentos, como por ejemplo el extracto de romero, los glucósidos de la stevia, etc. habiendo muchos otros esperando la autorización. de gobierno.

El trabajo realizado en esta tesis doctoral se centra en la caracterización química de tres plantas tradicionalmente consumidas en Portugal y sus extractos acuosos (decocciones e infusiones), con la posterior incorporación en algunos alimentos tradicionales portugueses, como en los pasteles denominados "económicos" y el queso tradicional de oveja "Queijo da Serra da Estrela". Las plantas y sus extractos acuosos fueron seleccionados en función de su bioactividad, particularmente por su potencial antioxidante, escogiéndose las flores de castaño

(*Castanea sativa* Mill. variedad "Judia" y "Longal"), las hojas y tallos de melisa (*Melissa officinalis* L.) y la albahaca (*Ocimum basilicum* L.).

En todos los extractos se procedió al estudio de su contenido en compuestos bioactivos y evaluación de su potencial actividad biológica. Los compuestos fenólicos se determinaron por cromatografía de alta resolución acoplado a espectro de masas (HPLC-MS), mientras que los ácidos orgánicos se basaron en cromatografía de líquidos de ultra-rápido (UHPLC) acoplado a un detector diodarray para la determinación. Los tocoferoles (sólo caracterizados en las hojas de melisa) y azúcares solubles se determinaron por HPLC acoplado a detector de fluorescencia e índice de refracción, respectivamente. En términos de actividades biológicas, se evaluó la actividad antioxidante, antimicrobiana (antibacteriana y antifúngica) y la actividad antitumoral de los extractos acuosos. En términos de la actividad antioxidante, se emplearon diferentes métodos, para la valoración de la actividad antioxidante total se empleó el ensayo 2,2-diphenil-1-picrilhydrazil (DPPH) junto con el ensayo del poder reductor, mientras que para la evaluación de la peroxidación lipídica se emplearon el ensayo de inhibición de la decoloración del β-caroteno y el ensayo de inhibición de especies reactivas del ácido tiobarbitúrico (TBARS). En la evaluación de la actividad antimicrobiana, se determinó la concentración mínima inhibitoria, concentración mínima bactericida y la concentración mínima fungicida. Por último, en los ensayos antitumorales, se emplearon diversas líneas de células tumorales humanas, así como se testó la posible hepatotoxidad de los extractos mediante líneas celulares de hígado porcino primarios.

Para los extractos de la flor de castaño, los compuestos fenólicos más relevantes fueron el tri-galoil-HHDP-glucósido seguido del pentagalloyl glucósido. La fructosa, glucosa, el ácido quínico y oxálico fueron los azúcares y ácidos orgánicos mayoritarios, respectivamente. Los mejores resultados frente a los diferentes métodos de actividad antioxidante y antibacteriana se observaron en las decocciones de la variedad Judia. Las líneas de células tumorales más sensibles frente a estos extractos fueron las líneas de células tumorales hepáticas y de colon, no detectándose toxicidad hepática en ninguno de los extractos.

En relación a los extractos de hoja de melisa (decocciones), los compuestos fenólicos más abundantes fueron el ácido rosmarínico, seguido de ácido litospérmico A. Así mismo, se destacó por su contenido en ácido quínico, la fructosa y γ-tocoferol. Los microorganismos más sensibles, con valores de inhibición inferiores a los controles positivos, fueron *Pseudomonas aeruginosa*, *Salmonella typhimurium* y *Penicillium funiculosum*. Mientras que las líneas de

células tumorales más sensibles a estos extractos acuosos fueron las de mama e hígado, no detectándose toxicidad hepática en dicho extracto acuoso.

Por último, en relación con el extracto acuoso de albahaca (decocción), el ácido rosmarínico, seguido de ácido chicórico fueron los compuestos fenólicos más abundantes. El ácido quínico y fructosa fueron el ácido orgánico y azúcar soluble más abundantes, respectivamente. El ensayo del poder reductor y del TBARS fueron los ensayos de antioxidantes con los mejores resultados (menor EC_{50}). Mientras que se observaron mejores resultados de actividad antifúngica que antibacteriana, aunque el extracto acuoso presentó una concentración mínima inhibitoria menor que los controles positivos para *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterobacter cloacae*, y *Salmonella typhimurium*. Los mejores resultados de actividad antitumoral se observaron frente a líneas de células tumorales del cuello de útero e hígado. No detectándose hepatotoxidad de dichos extractos acuosos.

La segunda parte del plan de trabajo fue la incorporación de los mejores extractos en diferentes productos alimenticios tradicionales de Portugal. La primera incorporación se realizó en pasteles portugueses tradicionales del noreste ("economicos"), a los cuales se les adicionó las decocciones, así como la incorporación directa de flores secas, en dos lotes diferentes. La cantidad de decocción incorporada se calculó teniendo en cuenta la actividad antioxidante del extracto, apoyándose de su valor de EC_{50} (concentración a la que el extracto muestra 50% de la actividad antioxidante), ajustándolo a la cantidad de pastel. La cantidad de flores secas incorporadas dependió del rendimiento de la extracción de las decocciones. En ambos casos (extracto y planta) se incorporaron dos concentraciones diferentes, la anteriormente calculada y una concentración de dos veces superior a esta, para establecer la mejor dosis de incorporación en cada caso.

Con el fin de determinar los cambios producidos durante el periodo de conservación de los bollos, los diferentes lotes de bollos elaborados se analizaron en el mismo día de la elaboración, después de quince días, y después de treinta días de almacenamiento. Para determinar la influencia del tiempo de almacenamiento y el tipo de incorporación de forma independiente, se llevaron a cabo el estudio estadístico de ANOVA de 2 factores seguido por el análisis discriminante lineal. En todos los lotes y tiempos de almacenamiento se determinó el valor nutricional (humedad, hidratos de carbono disponibles, fibra alimentaria, proteína bruta, grasa, cenizas y valor energético) según los procedimientos oficiales de la AOAC. Así como se determinó el perfil de ácidos grasos mediante cromatografía de gases y el contenido en

elementos minerales por espectrometría de absorción atómica. Finalmente, también se evaluó la actividad antioxidante de los pasteles mediante los ensayos anteriormente descritos.

En general, y como era de esperar, la humedad disminuyó a lo largo del tiempo de almacenamientos, con el consecuente incremento del resto de macronutrientes como hidratos de carbono disponibles, fibra insoluble, cenizas y valor energético, no observándose modificaciones significativas entre los lotes de bollos incorporados. El ácido succínico fue el ácido orgánico mayoritario, especialmente en el lote de bollos a los que se les incorporó las decocciones de flor de castaño. La sacarosa fue el azúcar soluble más abundante, aunque su concentración disminuyó durante el tiempo de almacenamiento, degradándose en fructosa y glucosa. El α-tocoferol fue la isoforma más abundante, a pesar que se cuantificó en cantidades muy pequeñas. El ácido palmítico fue el ácido graso más abundante, probablemente debido al contenido en mantequilla con el que se elaboraron los bollos. Se observó una apreciable disminución del contenido en ácidos grasos poliinsaturados a lo largo del tiempo de almacenamiento, seguramente asociado a un proceso de peroxidación lipídica. Por otro lado, el sodio y el potasio fueron los elementos minerales predominantes.

En general, y particularmente en relación al valor nutricional, los lotes de los bollos a los que se les incorporó las flores secas de castaño fueron los que mostraron los mejores resultados. Mientras que, en relación a la capacidad antioxidante, los mejores resultados se observaron en los lotes de bollos con la incorporación de extracto y/o planta en comparación con los controles, mostrándose los mejores resultados frente al ensayo del DPPH y TBARS.

El segundo alimento al que se incorporó los extractos de las tres plantas anteriormente descritos fue el queso con D.O. “Serra da Estrela”, uno de los productos lácteos más apreciadas y conocidos de Portugal. Se elaboraron diferentes lotes a los que se les incorporó tanto la planta seca como sus extractos acuosos (decocciones) siguiendo el mismo procedimiento que para los bollos, analizándose los diferentes lotes tras un mes de maduración (T0) y tras 6 meses de almacenamiento. Analizándose los mismos parámetros que para los bollos, además del color externo de los quesos. Al igual que en la mayoría de los quesos, los nutrientes mayoritarios fueron las proteínas y la grasa. En general, como cabría esperar los ácidos grasos saturados fueron la fracción mayoritaria frente a los ácidos grasos monoinsaturados, destacando su contenido en ácido palmítico, seguido por el ácido oleico. El calcio y sodio fueron los elementos minerales más abundante en todos los quesos analizados. A lo largo del tiempo de almacenamiento se observó una disminución de la humedad, especialmente en los lotes de quesos a los que se les incorporó los extractos de plantas, cuestión podría favorecer el proceso

de maduración. Así mismo, se observaron los mejores resultados de actividad antioxidante (T0), tanto para el ensayo del DPPH como para los de inhibición de la peroxidación lipídica. Siendo en los lotes de queso con la incorporación de planta seca, los que mayores modificaciones experimentaran en la evaluación de color externo.

En general, podríamos afirmar, que los lotes de quesos a los que se les incorporó planta seca (flores secas de castaño y hojas secas de melisa) mostraron mejores resultados a la hora conservar el valor nutricional de los quesos y a la hora de funcionalizar en relación a su capacidad antioxidante. Mientras que las decocciones de las hojas de albahaca mostraron mejores resultados que su correspondiente planta seca.

Como conclusión final, podemos resaltar que los resultados de este trabajo corroboran la eficacia de las plantas seleccionadas y sus extractos acuosos como potenciales ingredientes funcionales y conservantes naturales para la obtención de productos alimenticios más saludables, con mayor poder antioxidante y contribuyendo a su conservación, especialmente en el caso del queso “Serra da Estrela”.

Part I. **INTRODUCTION**

Adding molecules to food, pros and cons

Since the dawn of man, our species searches for better ways to feed itself. There is still no way of living without eating, therefore, this commodity is of utmost importance for the well-being of every man, woman and child across the world. Although the need to feed has maintained itself immutable across the ages, the way we consume foodstuffs has seen deep changes. From the local gatherers in the Palaeolithic to the domestications of animals and vegetables there was a huge leap, only surpassed by the commercial trading of spices and other goods in the fifteenth century. Today, in developed countries, food is produced in specific facilities and then transported to markets that can be within the same country or even in distant ones (Atkins & Bowler, 2001). Delivering food in good conditions from the production site to the consumer requires a great load of energy, either by refrigeration, controlled packaging or the use of additives to avoid spoilage and reduce food alteration. In a competitive global market, the least expensive method of food preservation is always favoured, and in most cases, food additives are chosen over the others. Furthermore, food additives are essential to enable the food industry to make food meet the increasingly challenging market and legal demands (Saltmarsh et al., 2013). While the European Food Safety Authority's (EFSA) definition for food additive is “any substance not normally consumed as a food itself and not normally used as a characteristic ingredient of a food, whether or not it has nutritive value, the intentional addition of which to a food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may reasonably be expected to result, in it or its by-products becoming directly or indirectly a component of such food” (Council Regulation (EC) 1333/2008; Saltmarsh et al., 2013). The Food and Drug Administration of the United States (FDA) defines a food additive as “any substance the intended use of which results or may reasonably be expected to result – directly or indirectly – in its becoming a component or otherwise affecting the characteristics of any food. This definition includes any substance used in the production, processing, treatment, packaging, transportation or storage of food. The purpose of the legal definition, however, is to impose a premarket approval requirement. Therefore, this definition excludes ingredients whose use is generally recognized as safe (where government approval is not needed), those ingredients approved for use by FDA or the U.S. Department of Agriculture prior to the food additives provisions of law, colour additives and

pesticides where other legal premarket approval requirements apply" (FDA, 2015). On the other hand, the Codex Alimentarius (CA) defines a food additive as "any substance not normally consumed as a food 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. The term does not include contaminants, or substances added to food for maintaining or improving nutritional qualities, or sodium chloride" (Motarjemi et al., 2014; Codex Alimentarius 2015). Within the European Union (EU), food additives are divided into 26 functional classes, depending on their function in food: sweeteners, colourants, 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). The American approach of food additives narrows down the number of classes and allows additives to be mentioned in two or more classes. According to the FDA, there are more than 3000 food additives allowed in the United States, which are distributed into six groups: preservatives, nutritional additives, colouring agents, flavoring agents, texturizing agents, and miscellaneous agents (Figure 1). The preservatives group is divided into three subgroups, although some additives may serve more than one function in foods: antimicrobials, antioxidants, and antibrowning agents. Within the flavoring agents group, there are three subgroups: the sweeteners, the natural or synthetic flavors, and the flavor enhancers. The texturizing agents comprise emulsifiers and stabilizers. Finally, the miscellaneous agents are composed of many classes: chelating agents, enzymes, antifoaming agents, surface finishing agents, catalysts, solvents, lubricants, and propellants (Branen et al., 2001). Despite the various classes of additives and the different classifications used, they can be divided in four fundamental groups with regard to their origin and manufacture: natural additives (obtained directly from animals or plants); similar to natural additives (produced synthetically imitating natural ones); modified from natural (natural additives that are then modified chemically); and finally artificial additives (synthetic compounds). In the European Union, all food additives, whether approved or not, are labelled with the letter "E" (representing Europe) followed by a specific number. This nomenclature was extended to the Codex

Alimentarius Commission to easily identify food additives worldwide. A huge effort was put in motion to gather knowledge toward the creation of a single database of legal food additives to be used within the EU, and in the Regulation 1129, of 2011, all the approved additives as well as their acceptable daily intake (ADI) were listed (Council Regulation (EC) 1333/2008; Council Regulation (EC) 1129/2011). In the United States, as from 1961, the FDA determined that all food components were labelled as generally regarded as safe (GRAS). This term is still in use today, but for an additive to be considered in it, various toxicology assays must be performed, and the list has seen changes throughout the years.

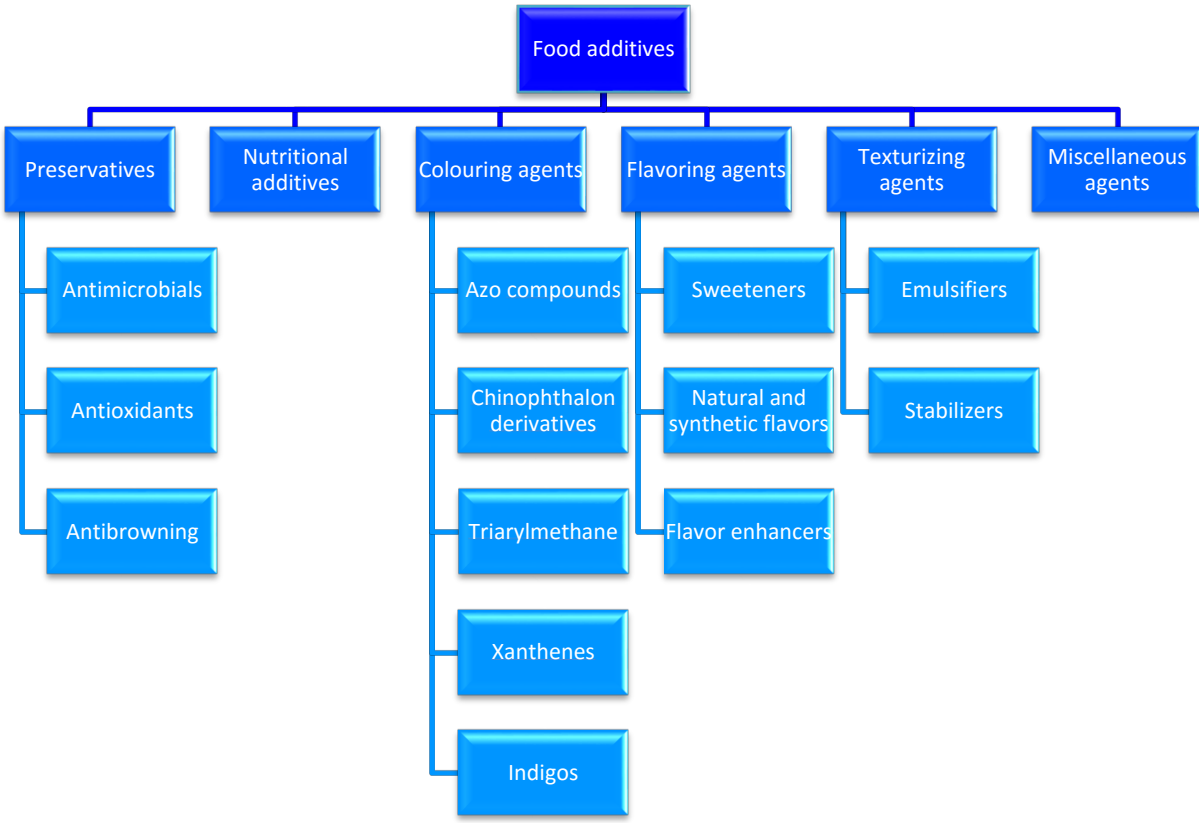


Figure 1. Principal groups and subgroups of food additives. Adapted from Carocho et al., 2014a

The ADI is the starting point to establish the maximum amount of a certain additive to be included in each foodstuff, which can vary from a few milligrams to "Q*uantum satis*", and expressed as milligrams of additive per kilogram body weight (mg/kg bw). The term *Quantum satis* is a Latin word employed by the EFSA which determines that there is "no maximum numerical level specified, and substances shall be used in accordance with good a manufacturing practice, at a level not higher than necessary to achieve the intended purpose and provided the consumer is not misled" (Council Regulation (EC) 1333/2008). The spread of

food additives reaches all kinds of foods, since the minimally processed until the highly processed and transformed foodstuff. The interaction between some food additives and the general public has not been peaceful. In the 80's food additives were considered dangerous to be consumed, which fuelled generalized scares and removal of some additives, namely colourants from processed food. Since then, the relation between additive and consumer has improved, although some distrust still lingers (Emerton & Choi, 2008). Today, some authors report health issues on the consumption of food additives, even though the authorities periodically review the data supporting the safety and correspondent ADI. Regarding the antimicrobials used in food, the most widespread are benzoates, sorbates, propionates, nitrites and parabens. Although studied for decades, some potential dangerous effects towards health are still found for many of them. Sodium benzoate, although regarded as safe, has yet to prove that it is not hazardous on long-term exposure (Lennerz et al., 2015). Depending on the dose used, sodium sorbate proves to be genotoxic on *in vitro* blood lymphocytes (Mamur et al., 2010), parabens have been proved to induce migratory and invasive activity in human breast cancer cells *in vitro*, while their dermic exposure has been overlooked, proving that this type of exposure can be added to the oral exposure, therefore increasing the overall intake (Karpuzoglu et al., 2013; Khanna et al., 2014). Extensive studies have been carried out through the years regarding sorbates and their health implications. Some of these studies describe these compounds as genotoxic and mutagenic, while others refer to this not being relevant. Still, controversy lingers, due to legislation, in which sodium sorbate is not allowed in the US, but legal to be used in food in the EU (Binstok et al., 1998; Mamur et al., 2012; Mpountoukas et al., 2008). Nitrates (E240-259) and nitrites (E249-250) are other antimicrobials that are used in foodstuffs. Nitrates have recently been restricted within the EU, and can now only be added to meat for slow curing. Nitrites can be found in nontreated fruit and vegetables, and are used in meat for colour formation, flavor enhancement and antimicrobial activity, being the only food additive to inhibit the *botulinum* toxin. They are also allowed in pickled herring, sprat and ripened cheese. Its use in the EU has been approved at the minimum possible dosage. (Council Regulation (EC) 1129/2011; Gøtterup et al., 2007; Honikel, 2008; Iammarino et al., 2013; Sebranek & Bacus, 2007; Sindelar & Milkowski, 2012; Watson & Preedy, 2010). Nitrites can take part in the formation of nitrosamines and have been shown to have carcinogenic effects, among other deleterious effects towards humans, namely the oxidation of oxyhemoglobin to ferrihemoglobin (Cammack et al., 1999). Sulphites or sulphiting agents are used in food, like wine, dried fruits, dehydrated biscuits, fish, among others, to avoid antimicrobial

contamination, to excerpt antioxidant and antibrowning activity. They are known to have cytotoxic and carcinogenic effects towards both rats and humans (Iammarino et al., 2012; Suh et al., 2007). Among the synthetic antioxidants, butylated hydroxyanisole, butylated hydroxytoluene, ethoxyquin, tert-butylhydroquinone and propyl gallate are the most common. Many studies have been carried out regarding these compounds, and while some studies point out hazardous effects such as toxicity and carcinogenic effects, others show the opposite, regarding them as tumor suppressant (Bauer et al., 2001; Botterweck et al., 2000; Carocho & Ferreira, 2013a; Ikezaki et al., 1996; Vandghanooni et al., 2013). Colourings like indigocarmine are found to be dangerous by producing superoxide dismutase during metabolisation in mice (Kohno et al., 2005). Safflower yellow and kokum red have shown to have clastogenic effects in mice bone marrow (Agarwal et al., 1994). Tartrazine, a widespread food colourant has been linked to irritability, restlessness and sleep disturbance in children (Rowe & Rowe, 1994). Many studies also deem colourings safe, within the ADI, but a study has proven that children could be consuming a higher quantity of dyes that initially thought, while adults are also exposed to other means of entrance into the body, are overlooked (*e.g.* shaving creams and after shave products) (Lucová et al., 2013; Stevens et al., 2015). Finally, among the sweeteners, mainly the "intensive sweeteners", such as saccharine, aspartame, sucralose and acesulfame K are the most common and widespread, used in the food industry mainly in low caloric food products. All of them provide the sweetening power at low doses. Saccharine and sucralose as regarded as safe to consume with restrictive maximum level (Council Regulation (EC) 1129/2011), aspartame still poses some controversial effects, namely by having deleterious effects on human babies during gestation, and by causing oxidative stress in wistar albino rats (Choudhary & Rathinasamy, 2014; Toigo et al., 2015). Acesulfame K has been proved to have clastogenic effects in mice and to induce allergies in humans (doses between 15 and 2250 mg acesulfame/kg body weight) (Mukherjee & Chakrabarti, 1997; Stohs & Miller, 2014). The concern of the ADI of synthetic additives their overconsumption has always been of great importance for the governing bodies. Back in 2001, the EU published a report regarding some member states, informing the consumption of additives in relation to their ADI. Although at the time, only preliminary results were given, and many additives were consumed under their ADI, some additives were clearly being overconsumed by the European population, namely, sulphites (E220-228), nitrites (E249-250), polysorbates (E432-436), sucrose esters and sucroglycerides (E473-474), stearoyl-2-lactylates (E481- 482), sorbitan monolaureate and sorbitan monoleate (E493-494), aluminium sulphates (E520-523), sodium aluminium phosphate (E541) and

aluminium silicates (E554-556/559). These additives were reported as being consumed in excess by the adult population, while many more were reported being consumed in excess by children. Although this report insisted that new studies should be carried out in the following years, including all the members of the EU, and with more accurate data being gathered, this did not occur. When the EFSA was formed, in 2002, it preferred to review the consumption and ADI estimations individually based on scientific committees and panels, and has been doing this until today. Many food additives have been re-evaluated by these panels and in some cases the ADI has changed, being all these reports published in the EFSA official journal and website. Furthermore, in 2010, concerned with food additives consumption, EFSA started a re-evaluation of all food additives gathering more studies and expert opinions, in order to find possible health implications and to adjust their ADI. This evaluation is gradual and is expected to end by 2020 (Lodi et al., 2011; Carocho et al., 2014a). Although some progress has been achieved in the past years in terms of harmonizing legislation regarding food additives across the world, there are still some big issues. Between the FDA and the EFSA, there are additives allowed in Europe that are not deemed safe for food in the USA, while the opposite also occurs. Some examples of the difference between legislations from the EFSA and the FDA are presented in Table 1. Furthermore, other legislative gaps can be found in the definition of food in terms of their calories. The scandals and fear of consumers, allied to these gaps in legislation has inspired researchers and companies to pursue natural food additives, in the hopes of lowering distrust and improving the benefits of these natural compounds.

Table 1. Examples of legislative differences for the same food additives. Adapted from Carocho et al., 2014a

Additive	E number	Type of Additive	EFSA Classification	FDA Classification	ADI
Sodium methyl *p*-hydroxybenzoate (paraben)	E219	Antimicrobial	EU Regulation No. 1129/2011	Banned	10 mg/kg bw
Sodium sorbate	E201	Antimicrobial	Banned in the EU	Code of Federal Regulations 21 Sec.182.3089	25 mg/kg bw
Carmoisine	E122	Azo compounds of dyes	EU Regulation No. 1129/2001	No permission sought in the USA	4 mg/kg bw
Amaranth	E123	Azo compounds of dyes	EU Regulation No. 1129/2011	Banned in the USA	0.8 mg/kg bw
Patent blue	E131	Triarylmethane compounds of dyes	EU Regulation No. 1129/2011	Banned in the USA	5 mg/kg bw
Brilliant green	E142	Triarylmethane compounds of dyes	EU Regulation No. 1129/2011	No permission sought in the USA	5 mg/kg bw
Fast green (DF&C Green No.3)	E143	Triarylmethane compounds of dyes	Banned in the EU	Code of Federal Regulations 21 Sec.74.203	25 mg/kg bw
Brilliant black	E151	Triarylmethane compounds of dyes	EU Regulation No. 1129/2011	No permission sought in the USA	5 mg/kg bw
Cyclamates	E952	Sweeteners	EU Regulation No. 1129/2011	Banned in the USA	11 mg/kg bw
Fructose (high fructose corn syrup)	-	Sweeteners	Not considered a food additive in the EU	Code of Federal Regulations 21 Sec.184.1866	No limitations
Sucrose	-	Sweeteners	Not considered a food additive in the EU	Code of Federal Regulations 21 Sec.184.1854	No limitations

Natural food additives or from natural origin as alternatives to synthetic ones

For some decades now, natural food additives have been gaining more interest both from the consumers and food manufacturers. Generally, the public will choose a food with no additives, but if these are not available, the same consumer will choose, if possible, a food containing natural compounds over synthetic ones (Carocho et al., 2014a). Depicted in Figure 2 are the main categories of natural food additives along with the most used compounds of each category. The endless amount of both synthetic and natural additives, added to the lack of knowledge of most of the population, when it comes to distinguishing natural from synthetic compounds does not help to clarify what is what in the label of most food products. The only way to overcome this limitation is to provide consumers with valuable information regarding these compounds purposely added to food. Different consumer studies have shown that consumers have recently

become more informed about food additives and always tend to choose the additives of natural origin than their synthetic analogues (Bearth et al., 2014; Devcich et al., 2007; Pokorný, 1991). Surprisingly there is no definition of natural preservatives, antioxidants, colours or sweeteners. Only natural flavorings have legislation both in the EU and the USA, and this is then transposed to the other classes of additives, leading to wrong interpretations and the confusion of what is natural or synthetic. There is a growing need for legislation regarding the natural additives, for they are of growing interest in developed countries (Baines & Seal, 2012). There are no defined categories for natural additives; in the EU, they are incorporated into the same "E" classification as all the other counterparts (Council Regulation (EC) 1129/2011). Still, the most researched natural additives are antioxidants, antimicrobials, colourings, and sweeteners (Figure 2).

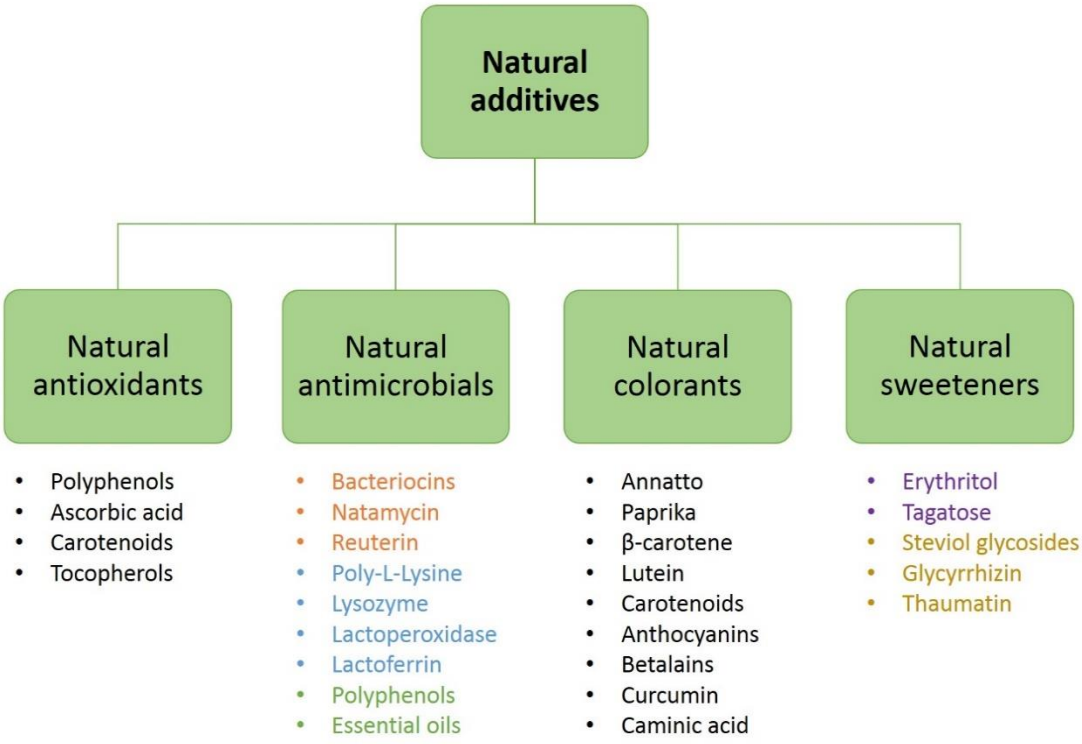


Figure 2. Depiction of the most common additives divided by categories. Represented in orange are the antimicrobials derived from microorganisms. In blue, the antimicrobials derived from animals and in green the ones derived from plants. The purple colour represents bulk sweeteners and in yellow, the high potency ones. In black, the antioxidants and colours derived from plants. Adapted from Carocho et al., 2015a

With regard to natural antioxidants, polyphenols are some of the most interesting groups in the vegetable kingdom, and due to their strong antioxidant capacity they display interesting effects towards human health, namely against cancer, osteoporosis, cataracts, cardiovascular dysfunctions, brain diseases, and immunological conditions (Carocho & Ferreira, 2013b). Due

to their high efficacy in preserving food and their wide acceptance from the general public, it is desirable to add them to food. In all the classes of polyphenols (phenolic acids-hydroxybenzoic or hydroxycinnamic acids, flavonoids including anthocyanins, tannins, lignans, stilbenes, and coumarins), some stand out with higher potential than others. They can be added as plant extracts, taking advantage of the synergistic effects between compounds, or by further purification to individual molecules, adding the most bioactive ones to the foodstuff. Polyphenolic extracts like rosemary and other extracts from plants have been used to act as antioxidants in food and, in terms of rosemary, it has been identified as a food additive in the Council Regulation (EC) 1129/2011, with the E number 392. Although the synergistic effect between the compounds is important for the extracts antioxidant activity, some industries seek specific molecules to carry out these effects. Among the individual polyphenols the most bioactive and used are carnosic and ferulic acid, and catechin (Ou & Kwon, 2004; Naveena et al., 2013; Kumar & Pruthi, 2014; Bitrić et al., 2015; Kaewprachu et al., 2015). Regarding other compounds with potential antioxidant activity, ascorbic acid (E300), also known as vitamin C, is a high oxygen scavenger used in various foodstuffs. It regenerates phenolic oxidants and tocopherols that have suffered oxidation, due to its higher oxidation potential. Ascorbic acid is particularly important to stabilize lipids and oils, but can be used in other food matrices (Baines & Seal, 2012). Carotenoids are also known for their antioxidant potential as food additives, although their use is always limited by being very susceptible to oxidation by light. Among these natural molecules, lycopene (E160d) and β-carotene (E160a (ii)) are the most widespread, being used in baked goods, eggs, and dairy products, among others, as singlet oxygen quenchers (Smith & Hong-Shum, 2011). Tocopherols (E306-309), which are the building blocks of vitamin E, are also known as very strong antioxidants. They can act isolated or in synergy with ascorbic acid, by regenerating it. Apart from this, their main antioxidant function is terminating free radicals in autoxidation reactions (Smith & Hong-Shum, 2011). In some cases, tocopherols are also used in films and coatings (Barbosa-Pereira et al., 2013; Lin & Pascall, 2014; Marcos et al., 2014). Many foodstuffs use mixes of carotenes, ascorbic acid and tocopherols to benefit from synergies between them.

Natural antimicrobials are also a very hot topic for food processing; they guarantee that the food is free of microorganisms and safe for its consumption. There are natural antimicrobials of three sources, derived from microorganisms, from animals and from plants. Ideally all natural antimicrobials should have a broad action, have bactericidal and fungicidal activities rather than only inhibitory ones, active at low concentrations, heat stable, unaffected by pH, impart no

flavor or colour, have no toxicity, easily assayable, have no pharmaceutical application, not susceptible to resistance from contaminants, label friendly, and finally, cost effective. Antimicrobials derived from microorganisms are molecules resulting from living organisms that have impact on others. Examples of this are bacteriocins; to date, about 300 bacteriocins have been discovered, with some of them having the potential to inhibit the growth of other proteins (Cotter et al., 2005; Hammami et al., 2007). Nisin (E234), pediocin (against *Listeria* microorganisms) and sakacin are the most used bacteriocins in food. They are used in dairy products (from 100 to 4000 IU/mL), beverages, eggs, meat (from 400 to 1000 IU/mL), cooked ham and cold cuts (from 12 to 35 mg/g) (Cleveland et al., 2001; Katla et al., 2002; Jofré et al., 2007; Millette et al., 2007; Nguyen et al., 2008; Santiago-Silva et al., 2009; Lacroix, 2011; Resa et al., 2014). Natamycin is also a widely used natural preservative. It is a polyene macrolide with antifungal activity, especially active against yeasts and moulds and virtually without effect on bacteria, protozoa and viruses. It has been used in a variety of foodstuffs, both as a free additive (E235), encapsulated and/or as a constituent of biofilms (Baines & Seal, 2012; Roller, 2003). Reuterin is an antimicrobial compound produced by the Gram positive lactic bacteria *Lactobacillus reuteri*, being effective against *Listeria* species. Reuterin has an application in food by adding the *Lactobacillus* into the foodstuff as starter cultures with glycerol, namely in cheese (Gómez-Torres et al., 2014; Langa et al., 2013), or by application of reuterin after extraction. Poly-L-Lysine is a homopolimer of the amino-acid lysine that has a GRAS status in the US and is allowed as a natural food additive in Japan, where it is used in staple foods for many years (Shih et al., 2006). Antimicrobials derived from animals are compounds like proteins and enzymes that are isolated from animals or are animal derived. Today, the only authorized natural antimicrobial derived from animals is lysozyme, which is used both in the US and the EU (E1105). The lysozyme used is derived from eggs (Baines & Seal, 2012). This enzyme's antimicrobial activity relies on the hydrolysis of the *b*-1,4 linkage site of the peptidoglycan in the bacterial walls, therefore yielding very high activity against Gram negative bacteria (which is constituted of 90% of peptidoglycan) and moderately effective against Gram positive bacteria (with much less peptidoglycan), but with no action against yeasts or fungi (Barbiroli et al., 2012). Lactoferrin, a GRAS compound, iron-binding glycoprotein very abundant in milk also displays antimicrobial activity. Although there are few reports of its use, researchers claim that it is potentially a molecule that can be used in the future to control microbes in food (Davidson et al., 2005; Baines & Seal, 2012). Antimicrobials derived from plants are usually compounds belonging to their secondary metabolism, which confer

protection from predators, code for signalling molecules and help the plant resist stress. Examples of compounds from this metabolism are terpenes, steroids, alkaloids and polyphenols. The polyphenols group encompasses various classes of molecules, and although they are attributed to have many biological effects on health, there are only few reports of some polyphenols with application in coatings and films showing antimicrobial activities (Sun et al., 2014; Giteru et al., 2015; Kaewprachu et al., 2015). Another very important group of molecules with antimicrobial activity are the essential oils. These compounds are complex mixtures of volatile compounds produced by living organisms. The most used essential oils are isolated through physical means from plants, and derive from the mevalonate, methyl-erithrytol and the shikimic pathways, with each one yielding different compounds (Başer & Buchbauer, 2010). Out of the 300 known essential oils of different plants, some have found their way into foodstuffs due to their antimicrobial activity; in fact, some have achieved the GRAS label in the US. Of these, oregano, thymol, carvacrol, clove, and cinnamon oil are some of the most important. There is a vast number of foodstuffs where essential oils have been applied, namely meat, fish, dairy products, vegetables, rice and fruit (Burt, 2004). Furthermore, recent developments have also been done in the packaging, with some films being impregnated with essential oils, namely with carvacrol and thymol (Ramos et al.,2012).

Food colours can be classified into three main groups; natural food colourants, which refer to ones that are synthesized naturally; nature-identical colourants, which although synthesized in industries, mime the natural ones, and finally the artificial/synthetic colourants (Msagati, 2013). There are many colourants used in the food industry, and even the natural or nature-identical represent an important amount, with some already allowed to be used and legislated. Annatto (E160b) is a permitted natural food colourant, extracted from the *Bixa orellana* L. tree. There are many foodstuffs where annatto is used, cakes, biscuits, rice, dairy products, flour, fish, soft drinks, snacks and meat products (Hendry & Houghton, 1996; Rao et al., 2005; Scotter, 2009). Paprika is another mixture of two carotenoids, capsanthin and capsorubin, it is also approved in the EU (E160c) and displays an orange to red colour (Hendry & Houghton, 1996). There are many other carotenoids used in food namely β-carotene, lutein, violaxanthin, neoxanthin, β-cryptoxanthin, fucoxanthin, lycopene and astaxanthin. They are extracted from plants, algae and even insects and represent a wide spectrum of colours in the food industry. The main applications of carotenoids in food are related to sauces, marinades, spice blends, coatings, beverages, milk, among others (Baines & Seal, 2012). Anthocyanins (E163) are responsible for pigments in nature, namely red, purple, violet and blue, and this can be transposed to food when

they are used as colourants. The main anthocyanins in nature are cyaniding, delphinidin, malvinidin, pelargonidin, peonidin, petunidin, being their main applications in soft drinks, confectionary products and fruit preparations (Hendry & Houghton, 1996; Baines & Seal, 2012). Chlorophylls (E140) are vegetable pigments that occur naturally in plants and confer colour. Among the five different chlorophylls that exist, only two (a and b) are used in the food industry as colourants. The used commercial colourants of chlorophylls are extracted from alfalfa, and have been employed in dairy products, soups, drinks and sugar confections (MacDougall, 2002). Curcumin (E100), a pigment purified from turmeric which is extracted from the dried rhizomes of the plant *Curcuma longa* L., is another widespread used food colourant. It confers an orange colour to food, and is used in mustard, yoghurt, baked goods, dairy industry, ice creams and salad dressings (Hendry & Houghton, 1996; MacDougall, 2002). Carminic acid (E120) is the main pigment present in the insect *Dactylopius coccus* Costa, which when complexed with aluminium, renders a brilliant red colour. This colouring agent is quite expensive when compared to other natural red ones, like anthocyanins, although it is considered technologically important due to its stability. It is used in jams, gelatines, baked goods, dairy products and non-carbonated drinks (MacDougall, 2002).

Natural sweeteners have the same objective as synthetic ones, to deliver a sweet taste while contributing with less or no calories at all to the diet. Regarding the bulk sweeteners, the two main compounds of this group are erythritol and tagatose. Erythritol (E968) is a sugar alcohol (polyol), which occurs naturally in some fruits and vegetables, although it is industrially produced through enzymes and osmophilic yeasts or fungi. It is allowed both in the US and in the EU, although in the latter with some restrictions in beverages. Being a bulk sweetener it only has around 65% of the sweetness of sucrose, but does not cause tooth decay, toxicity or carcinogenicity in the quantities used in food. The main foodstuffs where erythritol is used are baked goods, coatings, frostings, fermented milk, chocolate, low-calorie beverages, candy, chewing gums, among others (Nabors, 2001; Baines & Seal, 2012). Tagatose is a ketohexose, an enantiomer of fructose, and is also considered a prebiotic and a flavor enhancer. It occurs in very small quantities in fruits and heat treated dairy products. Its potency in relation to sucrose is about 92%, which makes it very similar in terms of taste (Nabors, 2001; Dobbs & Bell, 2010; Baines & Seal, 2012). Regarding high potency sweeteners, steviol glycosides (E960) are an example of natural compounds with a high dissemination around the world. These glycosides, mainly steviosides and rebaudiosides are also know just as stevia, stevioside or steviol, and are purified from the plant *Stevia rebaudiana* Bertoni leaves. Due to having various compounds in

its formula, steviol glycosides have different potencies, with the lowest ones being 30 times sweeter than sucrose (dulcoside A, rebaudioside C) and others, which are about 300 times more potent (rebaudioside A). Among the food industry, steviol glycosides are used in beverages, dairy products, ice cream, frozen desserts, sugar-free confectionary, mints, dried sea-foods and sauces (Baines & Seal, 2012; Brandle et al., 1998; Brusick, 2008; Nabors, 2001; Urban et al., 2015). Another high potency sweetener is glycyrrhizin (E958) (Barclay et al., 2014), a triterpene glycoside extracted from *Glycyrrhiza glabra* L., known as the liquorice plant. This compound, also known as glycyrrhizic acid, can act as a sweetener, with a potency of 50 times sweeter than sucrose, but also as a foaming agent and flavor enhancer. This compound is legally used in the US and EU under the form of monoammonium glycyrrhizinate and ammoniated glycyrrhizin. It is manly used in liquorice, baked goods, frozen dairy products, beverages, confectionery and chewing gum (Baines & Seal, 2012; Nabors, 2001; Spillane, 2006). Thaumatin (E957), a mixture of five proteins (taumatin I, I, III, a and b) are also used as a sweetener in many countries. Thaumatin is extracted from the fruit of *Thaumatococcus daniellii* Benth, a plant native to Africa. There is no conclusive value of its potency (Baines & Seal, 2012; Nabors, 2001).

Natural additives have come a long way from their beginnings as archaic additives to becoming in some cases the leading manner of conserving food. The controversy and ambiguity among the chemical additives allied to the sporadic scares have paved the way for natural additives to gain interest and funding.

Plant extracts as natural functional and preservative ingredients

Throughout the history of mankind, plant extracts have been used for many purposes. From beverages, to creams, ingredients to ointments and lotions, man has relied of plants and their extracts to maintain or regain health. Plant extracts are used regularly as functional ingredients in foods and drinks to either preserve, alter or confer taste to them. The incorporation of plants in food for bioactive properties started a few decades ago, when consumers became more aware of the food they were consuming and started seeking healthier foods with beneficial properties. The definition of plant extract is “the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids,

semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts" (Handa et al., 2008). Among the many ways of obtaining extracts of plants, the most used ones are maceration, percolation, infusion, decoction, distillation, solid-phase micro-extraction, supercritical fluid extraction, process-scale high performance liquid chromatography (HPLC) and flash chromatography. The bioactive properties of plants are endless, and every day new ones are discovered, converting plants in the primary substrate of drugs and functional ingredients. According to the website "Plant List", there are 350,699 identified plant species, but if taken into account the number of species left to be found and identified, it is easy to glimpse the potential of finding even more bioactivities in plants. Plant bioactivities are described for virtually every single plant on earth, from the smallest herb to the tallest sequoia, although the list narrows down when the extracts are to be consumed, due to possible toxic effects. Still, plant extracts can be consumed for their effect on the central nervous system, on different organ disorders, for common and non-lethal sicknesses (flu, cough, etc.), for relief of symptoms of chronic diseases, for disposition problems, among many others (Cechinel-Filho, 2012). From a food industry point of view, the most desirable bioactivities for plants are their antioxidant (to conserve the food and provide this activity to the consumer), antimicrobial (to avoid spoilage of the foodstuff) and antitumor activities.

1.1. Antioxidant properties

Antioxidants are molecules that fight free radicals, by stabilizing them. In terms of the human body, free radicals are produced in the normal metabolism, and neutralized by antioxidants produced in our bodies. Still, due to excess of physical exercise, radiation, smoking, unhealthy habits, inflammation processes and others, the quantity of free radicals can rise, and produce health issues (cardiovascular, neurological and pulmonary diseases, along with cancer and other problems), which is known as oxidative stress (Ferreira et al., 2009). These illnesses arise due to the constant attack of the free radicals towards lipids, sugars, DNA and RNA molecules, and proteins within the cells. Thus, the endogenous antioxidants are not enough to neutralise all the free radicals, and one depends on exogenous antioxidants, which can be absorbed through the diet. Foods that are rich in antioxidants will provide the human body with different compounds that fight oxidative stress. Figure 3 represents the different endogenous and exogenous antioxidants available.

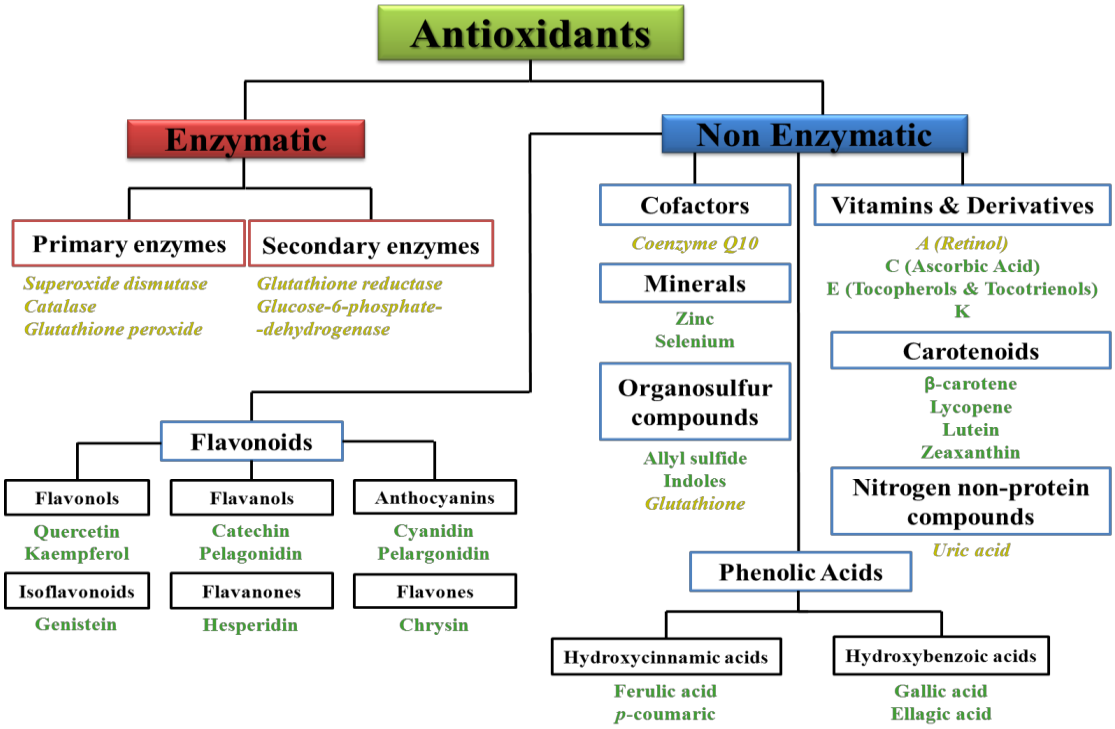


Figure 3 Natural antioxidants separated in classes. Green words represent exogenous antioxidants, while yellow ones represent endogenous antioxidants. Adapted from Carocho & Ferreira, 2013a

The richest foods in antioxidants are usually vegetables, fruits and cereals, which contain thousands of different compounds with antioxidant capacity, like polyphenols, carotenoids, tocopherols, ascorbic acid, some minerals, among others. Although not quite completely understood, the mechanisms of antioxidant activity are quite complex, and involve many reactions within the human body, although the one of the most important one is the neutralization of lipid peroxidation, in which the antioxidant molecule stabilizes the free radical that attacks the lipid molecules of the cell walls (Carocho & Ferreira, 2013a). In Figure 4, a representation of the various mechanisms of free radical damage, including lipid peroxidation.

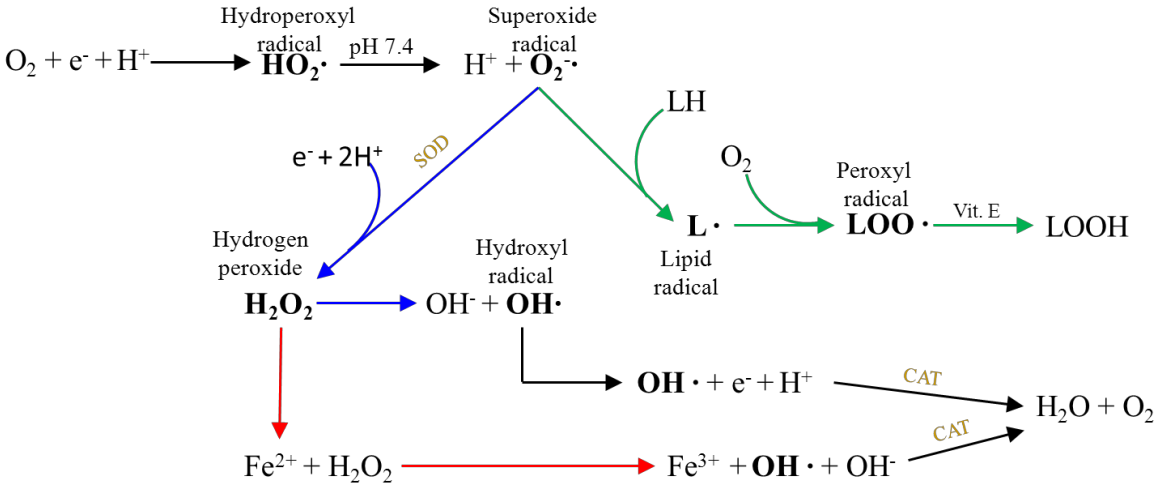


Figure 4. Overview of the reactions leading to the formation of ROS. Green arrows represent lipid peroxidation. Blue arrows represent the Haber-Weiss reactions and the red arrows represent the Fenton reactions. The bold letters represent radicals or molecules with the same behavior (H_2O_2). SOD refers to the enzyme superoxide dismutase and CAT refers to the enzyme catalase. Adapted from Carocho & Ferreira, 2013a

Although being healthy for consumers, antioxidants from plant extracts are added to food to avoid the oxidation of the food itself, mainly by halting the lipid peroxidation, and oxidation of other molecules, therefore extending its shelf life. In the EU, rosemary extract (E392) is allowed to be added to food as an antioxidant. Although no other extracts are legally added to food within the EU, many other extracts (rosemary, lemon, cinnamon, oregano, clove, pomegranate, grape) have proved to have antioxidant activity when added to food, namely minced meat, meat balls, cheese and biscuits (Fernández-López et al., 2005; Østerile & Lerfall, 2005; Reddy et al., 2005; Shan et al., 2011; Shahidi, 2015; Naveena et al., 2013; Bitrić et al., 2015).

1.2. Antimicrobial properties

Microorganisms are present throughout the food supply and can contaminate food in various ways, including at the farm level through irrigation water, field workers, insects, and fecal contamination by wild animals, as well as postharvest sources, such as handling by workers, transport vehicles, and processing equipment; wash water; and cross-contamination from other foods. They can be added to the actual food, to the packaging, contact surfaces, or food processing environments to either inhibit microbial growth or promote microorganism death (Zeuthen & Bøgh-Sørensen, 2003). These microorganisms pose two major problems to the food supply: the risk to human health from foodborne illness and the economic losses associated with food loss because of spoilage. Antimicrobials are added to food for two purposes; to

control natural spoilage of food (food control) and/or to avoid/control contamination by microorganisms, including pathogenic ones (of food safety concern) (Tajkarimi et al., 2010; Taylor, 2015). In the near future, very few synthetic antimicrobials are likely to appear in the market due to the strict requirements of international regulatory agencies, therefore widening the opportunity for natural ones to gain even more importance (Zeuthen & Bøgh-Sørensen, 2003). Plants are rich in many types of antimicrobial compounds, such as saponines, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpenes, lactones, terpenoids, and phorbol esters (Lewis & Ausubel, 2006; Tajkarimi et al., 2010). There are many published studies reporting the activity of these secondary metabolism compounds (Bajpai et al., 2008; Tajkarimi et al., 2010; Tiwari et al., 2009). These compounds excerpt their effects through different ways: (a) membrane disruption, (b) direct pH reduction, (c) inhibition of NADH oxidation, (d) interference of membrane permeability, (e) structural and functional damage to the microorganism's membrane, (f) attack towards the phospholipid membrane, (g) penetration of the cell membrane (Taylor, 2015). The most important natural extracts used as antimicrobials are phenolic extracts, essential oils extracts, dried plants (spices), aqueous extracts (infusions and decoctions). Some of the most effective plant extracts are derived from garlic, green tea, mint, thyme, meadowsweet, grape seeds, cranberry, coriander, lemongrass, citrus, among many others, which have been used in foods like shrimps, chicken meat, sausages, beef, bread, virgin olive oil, fish, dehydrated apple products, rice starch products, biscuits, milk formula, among many others (Taylor, 2015). Although the development of new natural antimicrobials has been rising consistently, there are still drawbacks to overcome, namely the toxic effects of some potential extracts and the cost to extract them from the source.

1.3. Antitumor properties

Cancer is a worldwide burden that causes millions of deaths each year. In fact, it is the leading cause of death in developed countries, it is estimated that it will affect 1.658.370 lives in 2015 in the USA alone (Siegel et al., 2015). The developing countries have begun to register increasing cases of cancer due to cancer-causing behaviours like smoking and unbalanced diets. Of the 7.6 million deaths in 2008, 64% were in developing countries (Jemal et al., 2011). Meng et al. (2012) reviewed the major theories that lead to cancer, available since the beginning of the century, and divided them into 8 categories: (a) cancer caused by viruses; (b) chromosomal abnormalities, (c) somatic mutations; (d) accumulated multiple mutations; (e) immunological

surveillances; (f) nonhealing wounds; (g) nonmutagenic mechanisms and (h) tissue organization field theories. Since ancient times, plants have been used as remedies to treat different types of illnesses and lesions with satisfying results. Today, more than 60% of anticancer drugs come from natural compounds or are derived from them, making these bioactive molecules become increasingly interesting for pharmaceutical industries, even as prototypes of final formulations for anticancer drugs (Rocha et al., 2001; Gordaliza, 2007). It is known that plant extracts can display interesting anticancer effects on cell lines and murine models, yielding higher effects when compared to pure natural or synthetic compounds. Despite this remarkable effect, it is difficult to assess which compounds are interacting with the cancer cells or if this orchestrated effect is due to synergy between different compounds. Most of the antitumor activity of plant extracts come from secondary metabolites, which comprehend phytosterols, acyl lipids, nucleotides, amino acids and organic acids. Secondary metabolites tend to accumulate in plant tissues; they play an important role in the plants adaptation process, acting as antibiotics, antifungals, antivirals, UV absorbing agents to reduce damage to tissues, signal molecules, allelopathic agents, attractants for pollinators and seed-dispersing animals as well as protecting the plant against herbivores diseases (Bougard et al., 2001; Crozier et al., 2006). For mankind, these metabolites are also important for providing dyes, fibers, glues, oils, waxes, flavoring agents, perfumes, insecticides, herbicides and are sources of new natural drugs for many diseases (Bougard et al., 2001; Crozier et al., 2006). The secondary metabolites are usually classified through their biosynthetic pathways, and are divided into three large families: alkaloids, terpenes and steroids, and phenolic compounds, all with antitumor activities (Bougard et al., 2001). They are mainly found in storage tissues rather than in vegetative ones, due to their high biologic potency (Walton & Brown, 1999). Terpenes and steroids are known for their lipophilic properties and their biosynthetic origin through the mevalonate and mevalonate-independent pathways. In terms of structure, they are unsaturated cyclic or linear hydrocarbons with varying degrees of oxygenation. They are subdivided according to the number of five carbon units (isoprene), which are the building blocks of these compounds (Walton & Brown, 1999; Tansey, & Shechter, 2000; Dewick, 2002; Crozier, et al., 2006). Regarding the alkaloids, as many as 12.000 alkaloids have been used as pharmaceuticals, stimulants, narcotics and poisons since the first alkaloid, morphine, was discovered (Crozier et al., 2006). Not all alkaloids derive from amino acids and, allowing a division into four groups: (a) alkaloids derived from amino acids (ornithine, arginine, lysine, histidine, phenylalanine/ tyrosine, tryptophan, anthranilic acid and nicotinic acid); (b) purine alkaloids (xanthine and

caffeine); (c) aminated terpenes (diterpene aconitine, triterpene solanine); (e) polyketide alkaloids (consisting of nitrogen introduced into a polyketide carbon skeleton) (Roberts, 1998). Phenolic compounds are widely dispersed throughout the plant kingdom representing about 8.000 different phenolic structures. As secondary metabolites they also display defensive, growth and development effects. They have at least one aromatic ring with one or more hydroxyl groups attached, being able to range from low molecular weight molecules to large and complex ones. The biosynthesis of phenolic compounds involves the shikimate, phenilpropanoid and flavonoid pathways (Roberts, 1998; Walton & Brown, 1999; Tansey, & Shechter, 2000; Dewick, 2002; Crozier, et al., 2006; Fraga, 2010). Phenolic compounds generally appear as esters and glycosides rather than as free compounds due to the conferred stability of these molecules. This family of compounds is one of the most studied worldwide and innumerous publications report beneficial effects in various aspects of human health and well-being (Lule & Xia, 2005; Boudet, 2007; Fraga, 2010). Figure 5 represents the various groups of molecules within the phenolic compounds family, while Table 2 displays their structure as well as the representative compounds.

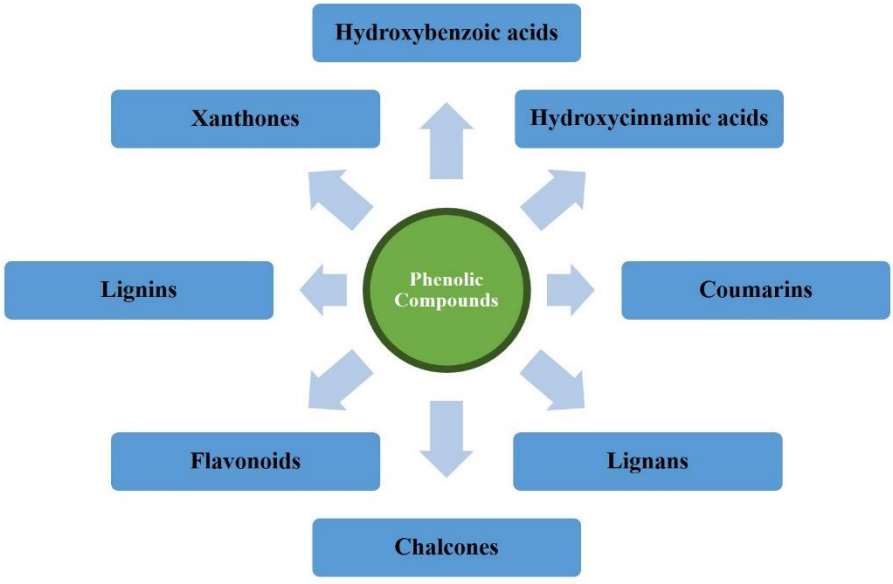
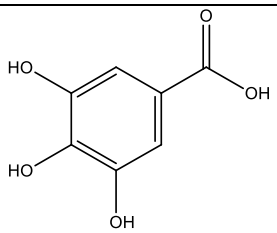
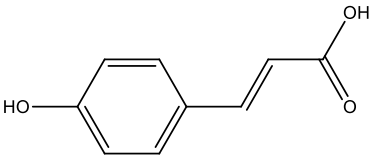
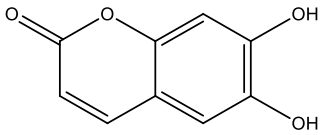
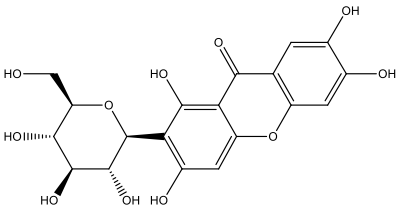
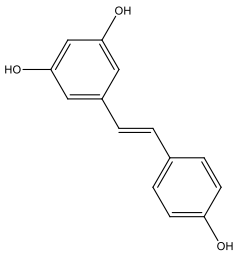
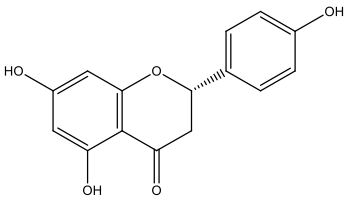


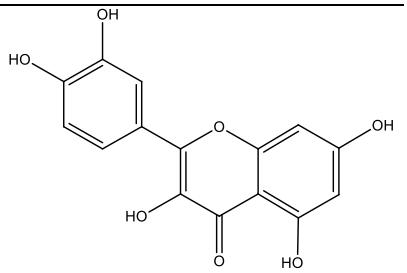
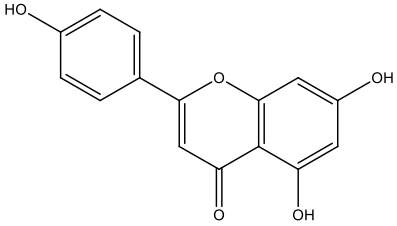
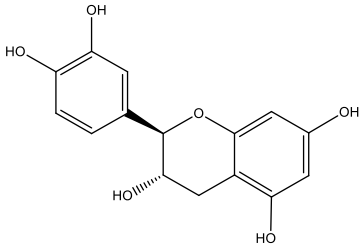
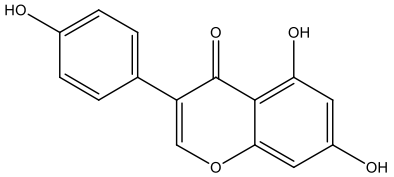
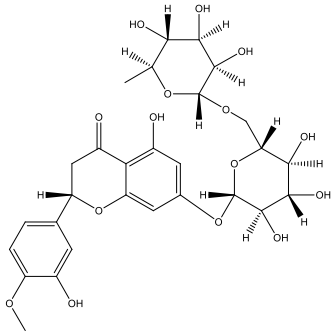
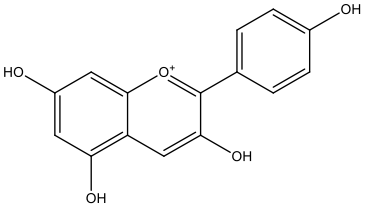
Figure 5. Depiction of the different subgroups within the phenolic compounds. Adapted from Carocho & Ferreira, 2013b

Table 2. Classification and representation of number of carbons of the phenolic compounds subgroups. Adapted from Carocho & Ferreira, 2013b

Classification	Example	Basic Structure
Hydroxybenzoic acids	Gallic acid	
Hydroxycinnamic acids	*p*-coumaric acid	
Coumarins	Esculetin	
Xanthones	Mangiferin	
Stilbenes	Resveratrol	
Flavonoids	Naringenin	

All these families of compounds are recognized as having some sort of antitumor properties (Carocho & Ferreira, 2013b). Within the flavonoids subgroup there are another 6 classes: (a) flavonols; (b) flavones; (c) flavanols; (d) isoflavones; (e) flavanones and (f) anthocyanins, as depicted on Table 3. These classes also display very active compounds with antitumor activity (Carocho & Ferreira, 2013b).

Table 3. Representation of the classes within the flavonoids subclass, examples of compounds and the basic structure

Classification	Examples	Basic Structure
Flavonol	Quercetin Kaempferol Myricetin	
Flavone	Apigenin Chrysin	
Flavanol	Catechin	
Isoflavone	Genistein	
Flavanone	Hesperidin	
Anthocyanin	Pelargonidin Cyanidin	

Although the individual compounds may display antitumor properties, in many cases the combined and synergistic effect of many compounds present in the plant extract have higher

and more efficient properties, although the pharmaceutical industry prefers isolated compounds or specific groups of compounds.

Many books and papers have been published regarding the effects of plant extracts and their antitumor and anticancer properties (Cragg & Newman, 2005; Ros, 2005; Slambrouck et al., 2007; Benzie & Wachtel-Galor, 2011; Karakaş et al., 2012; Juneja et al., 2013; Wiart, 2013).

A huge percentage of plants display antitumor and anticancer activities in cell lines or in murine models, but when scaled up into human trials, many extracts prove to not be effective, and only a fraction of the plant extracts shows effective antitumor or anticancer effects. Green tea extracts made from the herb *Camellia sinensis* L. are known for their preventive effects against many cancers (prostate, breast, skin, lung and liver) while also having interest in fighting these illnesses (Juneja et al., 2013; Ros, 2005). *Aloe vera* (L.) oral extracts have also been studied as metastasis reducing agents in patients with high metastasising cancers. Bilberry (*Vaccinum myrtillus* (L.)) aqueous extracts have shown interesting results in the reduction of cell proliferation in patients with colorectal cancer. Pomegranate fruit polyphenols (*Punica granatum* L.) extracts has antiproliferative and anticancer effects on prostate cancer. Turmeric dried extract (*Curcuma longa*) also has broad anticancer activity, namely against oral and stomach cancer (Benzie & Wachtel-Galor, 2011). Many other plant extracts are being screened against cell lines and murine models to determine their effects against tumors and then, if the results seem promising they are used in clinical trials. If these trials are successful, research is carried out to find the responsible compounds for the effect, which are then isolated. This pharmaceutical approach is not always successful due to the synergistic interaction among the compounds. A lot of research is being carried out with plants, with extracts being tested in humans. An example is a pomegranate extract pill which supresses tumor growth that is being tested at the National Cancer Institute (NCI). The project is now recruiting volunteers (NCI, 2015).

1.4. The particular cases of *Castanea sativa* Mill., *Melissa officinalis* L. and *Ocimum basilicum* L. due to their biological properties

Castanea sativa Mill., also known as chestnut, is a trees belonging to the *Fagaceae* family, a medium to large deciduous tree that can grow up to thirty-five meters high, and displaying a 2-meter crown with a deeply fissured bark (Lim, 2012). It has an important impact on the Portuguese economy, mainly in the north-eastern part of the country, where almost 85% of total

chestnut trees are grown, translating into a total revenue of 32 million Euros produced through the nut export (which shares the same name as the tree). There are four protected designation of origin (PDO) in the Trás-os-Montes area (north-eastern Portugal), "terra fria", "soutos da lapa", "padrela", and "Marvão-Montalegre". Some of the most important Portuguese cultivars are "Judia", "Longal", "Demanda", "Martaínha", "Aveleira", "Rebordã", "Trigueira" and "Zeive" (PNSI, 2012; Borges et al., 2007, 2008). Although the nuts are the most important commodity of this tree, the wood and honey are also very important. The flowers, depicted in Figure 6 (catkins) are by-products of the nut harvest, with no use after being fertilized and the development of the burr has started.

Figure 6. Detail of chestnut flowers (catkins) yellow. Author: M. Carocho

Ancestral claims report that "teas" of chestnut leaves and flowers are used for medicinal purposes, namely as mucolytic, antispasmodic, anti-dysenteric treatments, furthermore they can be used to help against coughs, colds, diarrhoea and high cholesterol (Neves et al., 2009; Lim, 2012). Recently, other studies have researched chestnuts leaves and flowers, finding interesting compounds in the flowers that can support the claims regarding the flowers (Barros et al., 2010a; Carocho et al., 2014b, c). Although very few reports can be found of the use of chestnut flowers in human diet, there are some patents that report their use in beverages like hot and cold teas and refreshments (Patents 1-3).

Melissa officinalis L., commonly known as lemon balm, is a member of the *Lamiaceae* family (Figure 7). It is an erect herbaceous perennial herb with opposite pairs of toothed, ovate leaves growing on square, branching stems (Tramte, 2007). It has been used for centuries for its outstanding traditional, medicinal effects on health and against illnesses. It is credited for its antibacterial, antifungal, antiviral, anticholinesterase, antiproliferative, antitumor, and anti-inflammatory effects, as well as being beneficial against Alzheimer.

Figure 7. Detail of lemon balm. Author: P. Ann

Furthermore, it acts as an expectorant, relieves digestion, headaches, diarrhea, ulcers, expectoration, hard digestions, and rheumatism (Carnat et al., 1998; Salah & Jäger, 2005; Carvalho, 2010; Martins et al., 2012; Barros et al., 2013a; Pereira et al., 2014; Carocho et al., 2015b). Recently, apart from some empirical claims of its applicability, other effects have been discovered for this plant, namely against neurodegenerative diseases, as an antitumor, antiproliferative, anticholinesterase, antioxidant and anti-Alzheimer (Martins et al., 2012; Pereira et al., 2014). Lemon balm is widely consumed in Portugal as well as all over the world, mainly as infusions and decoctions of the leaves, taking advantage of the heat to extract the bioactive compounds (Tramte, 2007).

Ocimum basilicum L., also known as basil, is native to the tropical regions of Asia, Africa, Central and South America, although it is now widespread throughout the world. It belongs to the *Lamiaceae* family, and is a green herb that can reach about 90 cm of height, displaying lanceolate leaves, which are glossy and fragrant, as depicted on Figure 8. One of the most known uses of this herb is as a spice and ingredient among the Italian and Southeast Asian cuisines (Wachtel-Galor, 2011).

Figure 8. Detail of the basil plant. Author: Swallowtail Garden Seeds

Still, many medicinal properties are attributed to this plant, among them, it can be used as pain and inflammation soother, nasal douche, calm cough, break fever, alleviate constipation and diarrhoea, wart remover, and kidney malfunctions (Javanmardi et al., 2002; Wiart, 2006; Benzie & Wachtel-Galor, 2011). Some of these ancient claims have been completely or partially confirmed though scientific studies, and other properties being found, namely as a diuretic, antipyretic, as a gastritis treatment and bronchodilatant (Boskabady et al., 2005). Its antimicrobial, antifungal, antimutagenic, and antioxidant activity have also been confirmed, especially in its essential oils, which are particularly active (Berić et al., 2008; Siddiqui et al., 2012; Govindarajan et al., 2013; Flanigan & Niemeyer, 2014; Abassy et al., 2015).

"Económicos" cakes and "Serra da Estrela" cheese as food incorporation targets

"Económicos" is the Portuguese name given to a typical cake, which is very famous in the north-eastern region. These cakes, depicted in Figure 9, are about ten centimetres in diameter and 5 in height. The name "económicos" translates to "economicals" due to their inexpensive ingredients, consisting on flour, milk, eggs, sugar and a small chalice of brandy, although the quantities and ingredients can vary between recipes. These cakes are now widespread across the country, and usually eaten as breakfast, as an afternoon or evening snack. Usually they are eaten with tea, or joined with marmalade and jams. The cakes are not eaten for their benefits to health, but rather to quench hunger whenever it strikes, therefore, their functionalization would bring benefits to consumers. Furthermore, they have a shelf life of about 15 days, after which they become crumbly and the taste becomes dull. The use of natural conservatives could increase their shelf life by avoiding rancidity and loss of flavour.

Figure 9. Top figure: External appearance of the cakes. Bottom figure: Detail of the inside colour of the cakes. Author: M. Carocho

"Serra da Estrela" cheese is one of the most well-known Portuguese cheeses across the world, being considered one of the most influential ambassadors of Portugal. Its name derives from the mountain, star mountain (Serra da Estrela) where the sheep that produce the milk for it, are bred (Figure 10). Since its production, the original recipe has not changed, and includes three ingredients, the unpasteurized milk from the ewe of the breeds "Churra Mondegueira" or "Bordaleira da Serra da Estrela", milk thistle (*Cynara cardunculus* L.) and salt (Figure 11). The milk thistle flowers have aspartic proteases that clot the milk, therefore transforming it into cheese (Shah, et al., 2014). This cheese has been referenced in a book by the Portuguese author "Gil Vicente", dating back to the XVI century. Back in 1996, the European Union awarded this cheese a PDO, where the ingredients, manufacturing methods and geographical limitations were certified (PDO, 1996). The cheese has ever since been produced by empirical means, relying on the producers' knowledge, that has been passed on from generation to generation. Recently, companies have conducted studies to improve yields and standardize the process of its fabrication (Macedo et al., 1993).

Figure 10. Serra da Estrela, the mountain where the Serra da Estrela cheese is produced. Author: P. Antunes

Figure 11. Top figure: Sheep of the "Churra Mondegueira" breed. Author: D. Crespo. Bottom figure: Milk thistle flower (*Cynara cardunculus*) flower. Author: J. Burt

In 2011, Serra da Estrela cheese was one of the winners of the 7 Wonders of the Portuguese Cuisine. This cheese can appear in two different forms, a very slight matured cheese (one moth of ripening) which is soft and can be eaten with a spoon, and a cured form, which has to mature at least four months, known as "old cheese" (queijo velho). The first is short and flat, displaying a regular cylinder with bulging sides and some bulging on the top and no defined edges. The rind must be soft or semi-soft, with a varying weight of 0.5 to 1.7 kg. The texture must be closed, slightly buttery, but must lose its shape upon cutting. Furthermore, it should be creamy

and smooth. The taste must be smooth, clean with a slight acidic bouquet. Finally, the colour should be white or slightly sallow. In terms of the matured cheese, the rind must be slightly wrinkled with a hard to extra hard consistency. The weight can vary between 0.7 to 1.2 kg with a texture that should be closed, only displaying some eyes, slightly dry, crumbly but smooth. A pleasant, lingering, clean, strong to slightly strong taste is desired, with a spicy and salty bouquet. The exterior colour can vary from yellowish to orange or light brown, becoming darker from the outside towards the center (Council Regulation (EC) 562/2013) (Figure 12).

Figure 12. Top figure: "Serra da Estrela" cheese after 1 month of ripening. Bottom figure: "Serra da Estrela" cheese after 6 months of maturation. Author: M. Carocho

The cheese has a high load of fats in its composition, and therefore, with high calories, not being considered a "healthy food", making its functionalization with plants an interesting step towards it acceptance by other consumers. This could be achieved by conferring bioactive properties, changing its appearance and flavour.

As a PDO cheese, the addition of other ingredients in its manufacture is not allowed, and should not be regarded as Serra da Estrela cheese, for the name is attached to the PDO, still, in order to simplify the reading of this document, it will be regarded as such.

Part II. **SCOPE AND OBJECTIVES //**
Parte II. ÁMBITO DE APLICACIÓN Y OBJECTIVOS

Scope and Objectives

The choices of consumers around the world are changing, with more and more concern regarding what they eat and their health status. The amount of unprocessed or minimally processed food is increasing in shelves of stores, along with food with natural ingredients, rather than chemicals to ensure conservation. Therefore, it is of great importance to study the use of plant extracts as natural ingredients that can conserve and confer bioactive properties to foodstuffs. In line with this, the main objective of this work was to evaluate the incorporation of plants and their extracts into traditional Portuguese foodstuffs in order to determine their capacity to act as natural conservatives, to functionalize them, and to create new food products.

Furthermore, the specific objectives were:

1) Chemically characterize the aqueous extracts of flowers of chestnut trees (infusion and decoction), lemon balm and basil plants (decoction) in terms of phenolic compounds, organic acids, soluble sugars.
2) Determine the bioactive properties of the aqueous extracts of these plants, namely the antioxidant, antimicrobial and antitumor potential.
3) Evaluate the incorporation effect of the chestnut flowers and their aqueous extracts (decoctions) in "económicos" cakes.
4) Evaluate the incorporation effect of chestnut flowers, lemon balm and basil leaves, as well as their aqueous extracts (decoctions) in different lots of "Serra da Estrela" cheese.
5) Evaluate the nutritional alterations conferred by the plants to the "económicos" cakes and "Serra da Estrela" cheese immediately after production.
6) Determine the bioactive changes to these foods in terms of antioxidant capacity.
7) Evaluate the nutritional alterations after specific periods of storage, in terms of proximate composition, dietary fiber, fatty acids and mineral modifications.
8) Evaluate the viability of these plants as functional and preservative ingredients.

Objectivos

La percepción de los consumidores, en relación a la alimentación ha ido cambiando mucho en todo el mundo a lo largo de los años, hoy en día los consumidores están más preocupados por los alimentos que consumen, que sean más sanos y naturales. Tanto es así que el volumen de negocio de alimentos crudos o mínimamente procesado con ingredientes naturales, en vez del uso de aditivos químicos, se ha incrementado exponencialmente. De modo que cada día adquiere mayor relevancia el estudio del uso de "extractos naturales" que puedan aportar propiedades funcionales y conservantes a los alimentos. En este sentido, el presente trabajo tiene como objetivo principal evaluar la incorporación de plantas y extractos acuoso en alimentos tradicionales de Portugal para determinar su capacidad como conservantes naturales, como ingredientes funcionales y crear "nuevos" productos alimenticios.

A partir de este objetivo principal se han propuesto los siguientes objetivos principales:

1) Caracterizar químicamente los extractos acuosos de flor de castaño (infusión y decocción), hoja de melisa y albahaca (decocción) en relación a su contenido en compuestos bioactivos como polifenoles, ácidos orgánicos y azúcares solubles.
2) Determinar las propiedades funcionales (actividad antioxidante, antimicrobiana y antitumoral) de los extractos acuosos de estas plantas.
3) Evaluar el efecto de la incorporación de la flor de castaño y su extracto acuoso (decocción) en los bollos "económicos".
4) Evaluar el efecto de la incorporación de la flor de castaño, hojas de melisa y albahaca y sus extractos acuosos (decocciones) en diferentes lotes de queso portugués con DOP "Serra da Estrela".
5) Evaluar las modificaciones en el valor nutricional y compuestos bioactivos de los bollos "económicos" y queso "Serra da Estrela", inmediatamente después de la incorporación de las plantas y sus extractos acuosos.
6) Evaluar los cambios en el contenido en compuestos biológicos y la actividad antioxidante en estos alimentos, tras la incorporación de las plantas y extractos anteriormente mencionados.

7) Evaluar las modificaciones en el valor nutricional, ácidos grasos y minerales de los bollos "económicos" y queso "Serra da Estrela" después de diferentes periodos de almacenamiento.
8) Valorar la viabilidad de estas plantas como ingredientes funcionales y conservantes.

Part III. **WORKING PLAN**

Working Plan

The main objective of this work was to evaluate the incorporation of different plants and their aqueous extracts into foodstuffs in order to determine their capacity to act as natural conservatives, to functionalize them and to create new food products. To achieve the goals of the study, the proposed working plan, depicted on Figure 13, was followed.

Task 1. Plant characterization

The selected plants were chosen based on their high antioxidant and antimicrobial potential, choosing three types of plants: One that is not currently and widely consumed as a beverage in the north-eastern region of Portugal, but given that the male flowers are by-products of the nut harvest (*Castanea sativa*), its use in food could benefit both the farmers that cultivate chestnuts, and also the consumer, due to its bioactivities. The other plant is commonly used as tea in the Portuguese diet, lemon balm (*Melissa officinalis*), and finally one that is consumed regularly in the Mediterranean diet as a spice or condiment, basil (*Ocimum basilicum*). It was imperative to carry out a thorough determination of the bioactive properties and compounds of the plants to evaluate the potential for the desired effects in the food and food products. Thus, chromatographic determinations of phenolic compounds, organic acids, soluble sugars and tocopherols, and bioactivity (antioxidant, antimicrobial and antitumor potential) evaluations were carried out in aqueous extracts, obtained by infusion and decoction procedures. Thus, having the decoctions proved to be the most bioactive extraction procedure for the flowers, and in order to keep coherence, for the lemon balm and basil plants, only decoctions were used as natural ingredients in the subsequent steps of the work.

Task 2. Cake incorporation and analysis

The first food to be incorporated were the "económicos" cakes, which were prepared following the recipe of the company "Pão de Gimonde", which is the biggest supplier of these cakes nationwide (Portugal). Its line of work includes baking traditional cakes, breads and biscuits of the Trás-os-Montes region. It is based in Bragança, Portugal. The decoctions of the chestnut flowers were incorporated into one lot, while another one was incorporated with the dried

flowers with no extraction procedure. Still, two concentrations of each ingredient were tested, based on the EC_{50} of the DPPH (2,2-diphenil-1-picrilhydrazil) assay (EC_{50} refers the concentration at which 50% of antioxidant activity is achieved) and the extraction yield, for the decoctions and flowers respectively. On the same day of manufacture, the cakes were frozen and lyophilized for further testing, while other lots of each were kept for 15 and 30 days to determine the changes in the nutritional (moisture, carbohydrates, protein, fat, ash, dietary fiber, fatty acids and mineral elements) and antioxidant properties of the cakes. For all storage times and assays, control samples were used in order to compare the changes.

Task 3. Cheese incorporation and analysis

Regarding the "Serra da Estrela" cheeses, the decoctions of the three plants were added to different lots, while other lots were incorporated directly with the dried plants. As for the cakes, control samples that were prepared and subject to the same conditions and assays. The cheeses were prepared at the facilities of the company "Queijos Casa Matias" which is a dairy based in Seia, Portugal, within the National Park of Serra da Estrela, the only region that can produce this kind of cheese. The extracts were added to the cheese, and after the normal month of ripening, the lots of each cheese were brought to the lab to be analysed, while other lots were kept for another 6 months at controlled temperature and humidity, once again to determine the changes in the nutritional properties (moisture, protein, fat, ash, fatty acids and mineral elements). The antioxidant capacity and external color determination were only carried out for the cheese with one month of ripening. Finally, a thorough statistical analysis was carried out to determine the functionalization and conservation capacities of each plant during the different storage times.

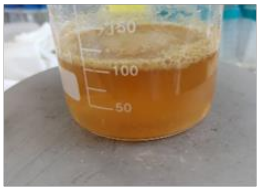
TASK 1
Plant characterization

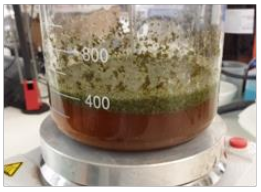
Chestnut flower | Lemon balm | Basil





Dried samples





Infusion
Decoction

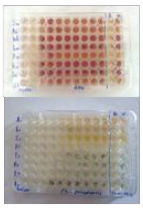
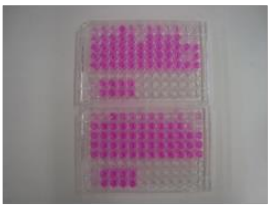
- Phenolic compounds
- Soluble sugars
- Organic acids

Decoction

- Phenolic compounds
- Soluble sugars
- Organic acids
- Tocopherols

Decoction

- Phenolic compounds
- Soluble sugars
- Organic acids
- Tocopherols

Antioxidant activity

Antimicrobial activity

Antitumor activity

TASK 2
Cake incorporation and analysis

Chestnut flowers

Dried flowers

Decocted flowers

Incorporation

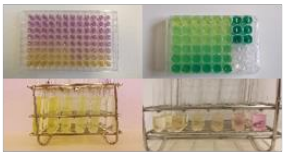
Cakes with dried flowers

Control cakes

Cakes with decocted flowers

3 storage periods

T0 days
T15 days
T30 days



Antioxidant activity

Nutritional profile

Fatty acids
Minerals
Tocopherols
Organic acids
Soluble sugars

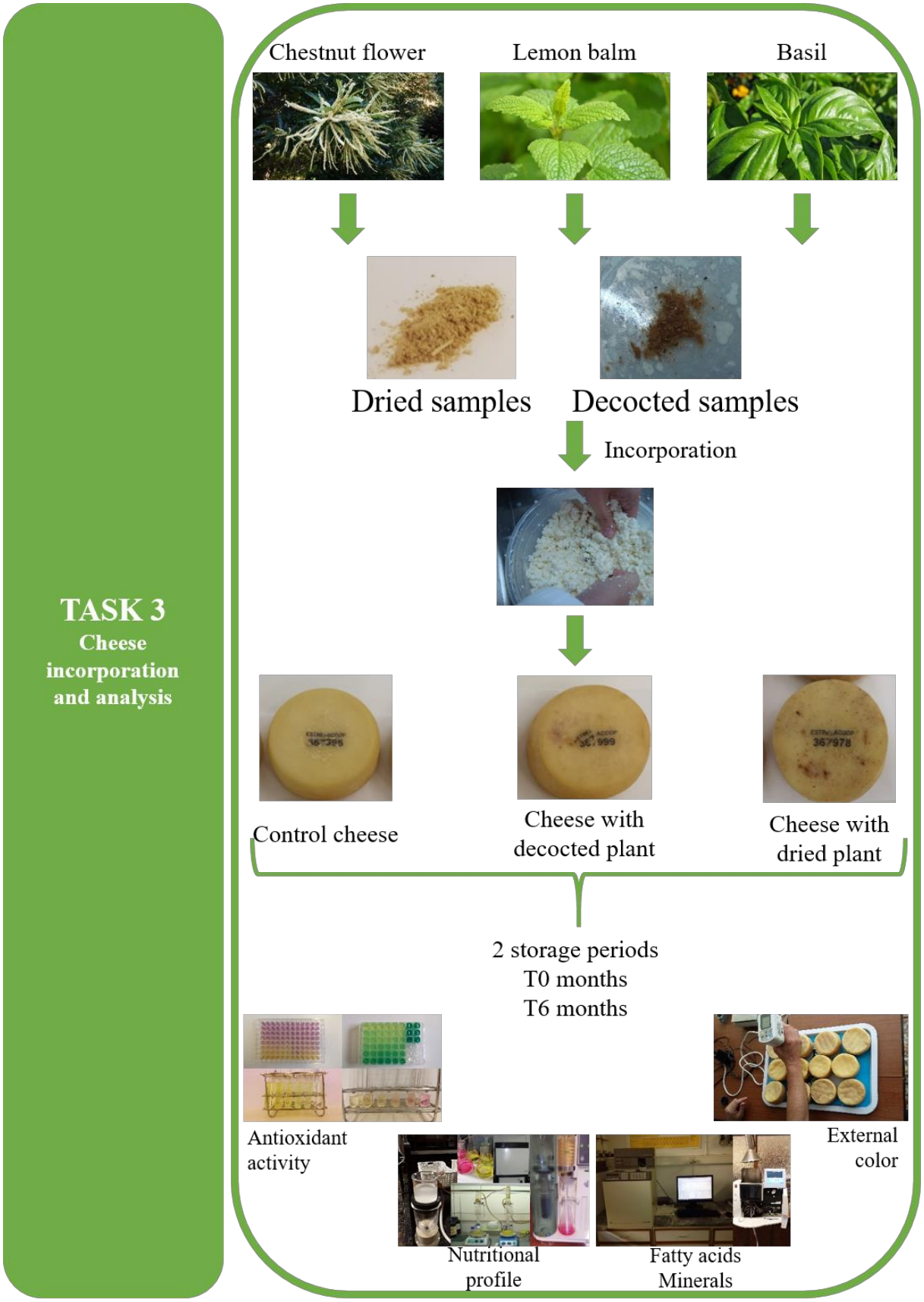


Figure 13. Depiction of the different tasks

Part IV. **MATERIALS AND METHODS**

Natural Preservers/Bioactive Ingredients

The natural preservers or bioactive ingredients were the dried flowers of chestnut trees, leaves of lemon balm and basil, as well as their decoctions and/or infusions. Both infusions and decoctions were analysed for two cultivars of chestnut flowers as well as basil. The decoctions of lemon balm were analysed in this work, while the infusions were previously studied by other members of the BioChemCore research group (Dias et al., 2012; Barros et al., 2013a; Pereira et al., 2015a; b). Furthermore, the infusions of basil were not presented in this thesis, given that the decoctions of chestnut flowers and lemon balm had been used in the incorporations due to their higher activities, and to maintain coherence, only the decoctions of lemon balm are presented.

1.1. Plant samples

All plant samples were from Portuguese origin. The chestnut flowers (*Castanea sativa*) were picked from the ground in groves from Trás-os-Montes (41º51'02''N, 6º49'54''W) and belonged to two of the most disseminated cultivars in Portugal, "Judia" and "Longal". Lemon balm (*Melissa officinalis*) was provided by the company "Mais Ervas", which is dedicated to the trade of dried plants, and based in Alfândega da Fé, Portugal. Finally, basil (*Ocimum basilicum*) was provided by the company "Cantinho das Aromáticas", which is also dedicated to the commercialization of dried aromatic plants, and is based in Vila Nova de Gaia, Portugal. After reaching the lab, the plants were milled down to a powder, as depicted on Figure 14.

Figure 14. Dried plants milled down, just before being subject to extraction procedures. Author: M. Carocho

Bioactive Compounds and Bioactivity of the Plant Extracts

To determine the bioactive properties of the plant extracts, different determinations of individual groups of compounds were carried out, namely polyphenols, organic acids, tocopherols (only for lemon balm) and soluble sugars. Furthermore, three types of bioactivities were also carried out, the antioxidant, antimicrobial and antitumor activity.

1.2. Preparation of the plant extracts

In order to prepare the extracts for the incorporation of the cakes and cheeses, infusions and decoctions were carried out in the same manner for all tested plants.

Infusions

- 1 g of the plant was added to 200 mL of boiling deionized water
- The heat was turned off and the solution was left to stand for 5 minutes
- After the 5 minutes it was filtered through a Whatman filter paper
- The filtered solution was frozen and finally lyophilized for subsequent analysis

Decoctions

- 1 g of the plant was added to 200 mL of cold deionized water
- The heat was turned on
- When the solution started boiling the heat was turned off and the solution was left to stand for 5 minutes
- After the 5 minutes, it was filtered through a Whatman filter paper
- The filtered solution was frozen and finally lyophilized for subsequent analysis

1.3. Phenolic compounds

1.3.1. Method principles

Phenolic compounds are some of the most bioactive group of molecules, and their determination and quantification can provide an idea of the highest contributing compounds to the different bioactivities of the plants extracts.

The detection of phenolic compounds was carried out by HPLC coupled to a photo diode array detector (PDA) with electron spray ionization (ESI) and mass spectrometry (MS) detection. The analysis of phenolic compounds was carried out for all three plant samples.

1.3.2. Solvents, reagents and materials

Acetonitrile was acquired from Fisher Scientific (Porto Salvo, Portugal), formic acid was acquired from Panreac (Barcelona, Spain) and the phenolic compounds standards (catechin, gallic acid, isorhamnetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, myricetin, quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside, caffeic acid and rosmarinic acid) were purchased from Extrasynthese (Genay, France). Water was treated using a Milli-Q water purification system (TGI Pure Water Systems, Greenville, USA). All other solvents and reagents were acquired from scientific retailers.

1.3.3. Methodology

- 10 mg of each extract was added to 2 mL of deionized water with a final concentration of 5 mg/mL
- They were then dissolved with the help of ultrasounds and finally filtered through a Whatman (0.2 μm) filter, into an HPLC vial

The followed chromatographic methodology had been previously reported by Barros et al. (2013b). All conditions are described on Table 4. Double online detection was carried out with a PDA using 280nm and 370nm as the preferred wavelengths and a mass spectrometer connected to the HPLC system via the PDA cell outlet. The mass spectrometric detection was performed by means of an API 3200 (Applied Biosystems, Darmstadt, Germany) triple quadrupole-ion trap analyser equipped with an ESI source. Spectra were recorded in negative ion mode between m/z 100 and 1700. The phenolic compounds were characterized according

to their UV (ultra violet spectra), mass spectra, retention times, and comparison with authentic standards when available. For the quantitative analysis of phenolic compounds, a baseline to valley integration with baseline projection mode was used to calculate peak areas. For quantification, calibration curves were generated by injection of known concentration (2.5-100 μg/mL) of standard compounds: catechin (y=132.76x−59.658; R^2=1); gallic acid (y=556.94x−738.37; R^2=0.999); isorhamnetin-3-*O*-glucoside (y=262.31x−9.8958; R^2=1); kaempferol-3-*O*-glucoside (y=190.75x−36.158; R^2=1); kaempferol-3-*O*-rutinoside (y=175.02x−43.877; R^2=0.999); myricetin (y=778x−1454.3; R^2=0.999); quercetin-3-*O*-glucoside (y=316.48x−2.9142; R^2=1.000); quercetin-3-*O*-rutinoside (y=222.79x−243.11; R^2 =0.999); caffeic acid (y=359x−488.4; R^2=0.999) and rosmarinic acid (y=312.2x−424.06; R^2=0.999). The results were expressed in mg per g of lyophilized decoction or infusion.

Table 4. Chromatographic conditions for phenolic compounds analysis

HPLC-PDA-ESI/MS operating conditions for phenolic compounds	
System	Hewlett-Packard (HP) 1100 Chromatograph with a quaternary pump (Agilent Technologies, Santa Clara, California, USA)
Column	Waters Spherisorb S3 ODS-2 C18, 3 µm (4.6 mm × 150 mm) (Milford, Connecticut, USA)
Detectors	PDA coupled to an HP Chem Station (rev. A.05.04) data-processing station; API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany)
Mobile phase/Gradient	(A) 0.1% formic acid in water and (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15-25% B over 5 min, 25-35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column
Flux	0.5 mL/min
Wavelength	280 and 370 nm
Temperature	35 ºC
Software	HPLC - Chemstation, Agilent Technologies; MS - Analyst®, Applied Biosystems

1.4. Organic acids

1.4.1. Method principles

Organic acids are small compounds, known to be any carboxylic acid with a general R-COOH structure (Dibner & Buttin, 2002; Jones, 1998). In terms of their properties, citric, succinic, fumaric and malic acids are play important roles in the Krebs cycle, making them essential for plants and human metabolism. Furthermore, citric acid is known to ameliorate ketosis and can

protect against the development of diabetic complications in murine models with type 1 diabetes (Nagai et al., 2010). It is also an interesting antimicrobial agent that is used as food preservative (E330), in association with other organic acids or alone (Brul & Coote, 1999; Kim & Rhee, 2015). Succinic acid derivatives (succinic acid monoethyl ester) are known to help in diabetes treatment (Pari & Saravanan, 2007). Fumaric acid alleviates psoriasis and inflammatory processes and can be used as a neuro and chemoprotector. It is also used as an antimicrobial in foodstuffs, being attributed the additive number E297 (Baati et al., 2011). Malic acid has bactericidal effects (Raybaudi-Massilia et al., 2009), while shikimic acid is a main compound in the shikimate pathway, making it essential for vegetable metabolism, therefore producing l-phenylalanine and l-tryptophane, essential amino acids for humans (Krämer et al., 2003). Finally, ascorbic acid, one isoform of vitamin C, is a naturally occurring organic acid, which is essential against scurvy, a powerful antioxidant and quite effective against hypertension (Duffy et al., 1999). Thus, the quantification of these compounds is important, given that they can confer some bioactivities to the plant extracts.
Organic acid determination was carried out using UFLC (ultra-fast liquid chromatography) coupled to a PDA detector. The analysis of these compounds was carried out for all three plants.

1.4.2. Solvents, reagents and materials

Sulphuric and metaphosphoric acid were acquired from Fisher Scientific (Porto Salvo, Portugal), the standards of organic acids (L(+)-ascorbic acid, citric acid, malic acid, oxalic acid, shikimic acid, succinic acid, fumaric acid and quinic acid) were purchased from Sigma (St. Louis, Missouri, USA), while all other reagents and used solvents were acquired from scientific retailers. Water was treated using the previously mention purification system.

1.4.3. Methodology

- 3 mL of metaphosphoric acid at 4.5% were added to 50 mg of the extracts and diluted with the help of ultrasounds
- They were then filtered through Whatman (0.3μm) filters into HPLC vials

The followed chromatographic methodology was previously described by Pereira et al., (2013), with the conditions described in Table 5. Analysis was performed on a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) coupled to PDA detector (Shimadzu), using 215nm and 245nm (for ascorbic acid) as the preferred wavelengths. Separation was achieved

on a SphereClone (Phenomenex, Torrance, California, USA) reverse phase C18 column (5 μm, 250mm × 4.6mm i.d) thermostatted at 35°C. Analytes were eluted with 3.6mM sulphuric acid at a flow-rate of 0.8 mL/min. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm or 245 nm (for ascorbic acid) with calibration curves obtained from commercial standards of each compound: oxalic acid (y=1x107x+96178; R^2=0.999); quinic acid (y=601768x+8853.2; R^2=1); malic acid (y=952269x+17803; R^2=1); shikimic acid (y=8x107+55079; R^2=0.999); ascorbic acid (y=80000000x+55079; R^2=1). The results were expressed in mg per g of lyophilized decoction or infusion.

Table 5. Chromatographic conditions for organic acids detection

UFLC-PDA operating conditions for organic acids	
System	Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan)
Column	SphereClone (Phenomenex, Torrance, California, USA) reverse phase C18 column
Detectors	PDA Shimadzu detector
Mobile phase/Gradient	3.6mM sulphuric acid
Flux	0.8 mL/min
Wavelength	215 and 245 (ascorbic acid)
Temperature	35 ºC
Software	LabSolutions, LCsolutions Version 1.25

1.5. Tocopherols

1.5.1. Method principles

Tocopherols are the isoform of vitamin E, there are 8 isoforms, α-, β-, γ-, and δ-tocopherols, and α-, β-, γ-, and δ-tocotrienols. These compounds are quite important in the overall antioxidant capacity of plants and extracts, due to their capacity to halt lipid peroxidation by donating their phenolic hydrogen to unstable molecules. Furthermore, they also work in synergy with ascorbic acid, which regenerates them to continue scavenging free radicals (Carocho & Ferreira, 2013a). Tocopherols were analysed through HPLC coupled to a fluorescence detector using the internal standard (IS) method. They were analysed for lemon balm and basil extracts.

1.5.2. Solvents, reagents and materials

Methanol, hexane (HPLC grade) and ethyl acetate (HPLC grade) were acquired from Fisher Scientific (Porto Salvo, Portugal), butylated hydroxytoluene (BHT) from Panreac (Barcelona, Spain) and Tocol from Matreya (State College, Pennsylvania, USA). All other reagents and used solvents were acquired from scientific retailers. Water was treated using the previously mentioned purification system.

1.5.3. Methodology

- All the methodology was carried out with the least light possible in the room and at the lowest temperatures possible, using ice to maintain the extracts cold, given the high sensibility to light and temperature
- 500 mg of the extracts were added to 400 µL of tocol (IS) at 50 µg/mL and 100 µL of BHT (antioxidant to protect the tocopherols) in a falcon tube
- 4 mL of Methanol and hexane, and 2 mL of aqueous solution of NaCl were added sequentially to the falcon tube with followed by a vortex shake for 30 seconds after every addition
- The falcon tubes were centrifuged (Centurion, K2OR-2003, West Sussex, UK) at 4000g for 5 minutes at 10 ºC
- The top phase was removed to a vial covered in aluminium foil. Another 4 mL of hexane were added and the tubes re-centrifuged twice with the top phase being removed into the same vial once again
- 2 small spoons of anhydrous sodium sulphate were added to the vial to remove moisture
- The hexane in the vials was evaporated using nitrogen current
- The remaining residue was re-dissolved in 2 mL of hexane (HPLC grade), filtered through Whatman HPLC filter (0.2µm) into HPLC vials and further analysed, following the methodology by Barros et al. (2011), with the system depicted on Table 6.

The equipment consisted of a pump (Knauer, Smartline System 1000, Berlin, Germany), a degasser (Smart line Manager 5000), an autosampler (AS-2057, Jasco, Easton, MD, USA), and a fluorescence detector (FP-2020, Jasco, Easton, Maryland, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a polyamide II normal-phase column (5 µm, 250 mm × 4.6 mm i.d., YMC Waters,

Milford, Connecticut, USA), operating at 35 °C. The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. The tocopherols were quantified by comparison of the area of their peaks recorded at 290 nm for excitation and 330 for emission with calibration curves obtained from commercial standards of each compound: α-tocopherol (y=1.44764x; R^2=0.999) and γ-tocopherol (y=0.30665x; R^2=0.999). Quantification was based on the fluorescence signal response, using the IS method. The tocopherol contents in the samples were expressed in µg per g of lyophilized decoctions.

Table 6. Chromatographic conditions for tocopherols detection

HPLC-Fluorescence operating conditions for tocopherols	
System	Pump (Knauer, Smartline System 1000, Berlin, Germany), degasser (Smart line Manager 5000), autosampler (AS-2057, Jasco, Easton, MD, USA)
Column	Polyamide II normal-phase column (5 µm, 250 mm × 4.6 mm i.d., YMC Waters, Milford, Connecticut, USA)
Detectors	Fluorescence detector (FP-2020, Jasco, Easton, Maryland, USA)
Mobile phase/Gradient	Hexane and ethyl acetate (70:30 v/v)
Flux	1 mL/min
Wavelength	Excitation at 290 nm and emission at 330 nm
Temperature	35 ºC
Software	DataApex Clarity, Version 2.4.1.43

1.6. Soluble sugars

1.6.1. Method principles

Soluble sugars are natural compounds of plants; therefore, they are also interesting to detect when carrying out a full chemical characterization. Furthermore, reducing sugars and aminoacids are responsible for the formation of the Maillard reaction compounds that could display antioxidant activity. Some soluble sugars (*e.g.* galactinol, mannitol, sucrose and raffinose) are also responsible for antioxidant activity in plants (Tuteja & Gill, 2013). Soluble sugars determination was carried out by HPLC-RI for all three plants.

1.6.2. Solvents, reagents and materials

Acetonitrile of HPLC grade was acquired from Fisher Scientific (Porto Salvo, Portugal). The sugar standards (D(−)-fructose, D(+)-glucose anhydrous, and D(+)-sucrose, trehalose

and melezitose) were acquired from Sigma Chemical Co. (Saint Louis, Missouri, USA). All other reagents and used solvents were acquired from scientific retailers. Water was treated using the previously mentioned purification system.

1.6.3. Methodology

- 3 mL of deionized water were added to 50 mg of each extract and 75 μL of melezitose (IS) and thoroughly dissolved with the aid of ultrasounds
- After filtration through a Whatman HPLC filter (0.2 μm) into a vial they were analysed by HPLC

The followed procedure had been previously reported by Barros et al. (2010b), depicted on Table 7. The HPLC equipment used was the same as the one for tocopherols, with changes to the column and detector. The column was an Eurospher 100-5 NH_2 column 5 μm, 250 mm × 4.6 mm i.d. (Knauer, Berlin, Germany) and the detector was a refraction index (RI) Smartline 2300 (Knauer, Berlin, Germany). The detected sugars were quantified by comparison of the area of their peaks with calibration curves obtained from commercial standards of each compound: fructose (y=0.98177x; R^2=0.999); glucose (y=1.00778x; R^2=0.999); sucrose (y=0.95604x; R^2=0.999); trehalose (y=0.90995x; R^2=0.999). Quantification was carried out through the internal standard method, and the results are expressed in mg per g of lyophilized decoction or infusion.

Table 7. Chromatographic conditions for sugars detection

HPLC-RI operating conditions for sugars	
System	Pump (Knauer, Smartline System 1000, Berlin, Germany), degasser (Smart line Manager 5000), autosampler (AS-2057, Jasco, Easton, MD, USA)
Column	Eurospher 100-5 NH2 column 5 μm, 250 mm × 4.6 mm i.d. (Knauer, Berlin, Germany)
Detectors	Refraction index Smartline 2300 (Knauer, Berlin, Germany)
Mobile phase/Gradient	Acetonitrile and deionized water (70:30 v/v)
Flux	1 mL/min
Wavelength	-
Temperature	35 ºC
Software	DataApex Clarity, Version 2.4.1.43

1.7. Antioxidant activity

1.7.1. General

Due to the different types and behaviour of free radicals, four types of antioxidant activity evaluation assays were conducted in the plant extracts, in order to acquire a comprehensive knowledge of their overall antioxidant activity. The used methods were DPPH (2,2-diphenil-1-picrilhydrazil) radical scavenging activity, reducing power ferricyanide/Prussian blue assay (RP), β-carotene bleaching inhibition and inhibition of thiobarbituric reactive species (TBARS).

The lyophilized extracts were diluted in water, at a concentration of 10 mg/mL and sequential dilutions were made from this stock solution. The results are expressed in EC_{50} values, which corresponds to the concentration at the extract is able to inhibit 50% of any oxidative process, involving free radicals or lipid peroxidation (Sánchez-Moreno et al., 1998). In order to calculate this parameter, Equation 1 was used:

$$EC_{50} = \frac{(x_2 - x_1)}{(y_2 - y_1)} \times (50 - y_1) + x_1$$

Equation 1. Ec_{50} equation

Where:

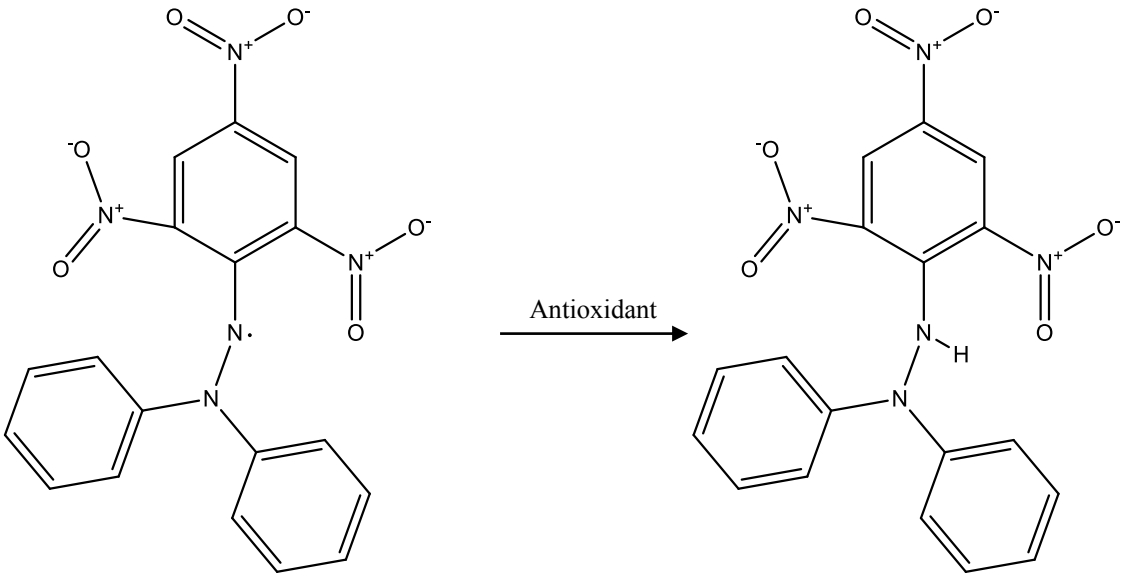
- x_1 is the concentration of the extract whose inhibition percentage is under 50%
- x_2 is the concentration of the extract whose inhibition percentage is over 50%
- y_1 inhibitory percentage under 50%
- y_2 inhibitory percentage over 50%

1.7.2. DPPH scavenging activity

1.7.2.1. Method principles

The DPPH method is based on the premise that a hydrogen donor is an antioxidant (Figure 15). This colorimetric assay uses the DPPH radical, which is a nitrogen compound that is very stable due to the relocation of the free electron and confers to it a purple colour. It

reacts easily with molecules that can donate hydrogen atoms, changing to yellow when it accepts them (Figure 16). Is widely used as a preliminary antioxidant study (Antolovich et al., 2002; Amarowicz et al., 2004; Moon & Shibamoto, 2009).



2,2-diphenyl-1-picrylhydrazyl
Purple

2,2-diphenyl-1-picrylhydrazine
Pale yellow

Figure 15. Depiction of the reduction of the DPPH radical

1.7.2.2. Solvents, reagents and materials

Methanol was acquired from Fisher Scientific (Porto Salvo, Portugal) and the DPPH radical was acquired from Alfa Aesar (Ward Hill, Massachusetts, USA). Trolox was bought from Sigma-Aldrich (St. Louis, Missouri, USA). All other solvents and reagents were acquired from scientific retailers.

1.7.2.3. Methodology

- For this assay, 96 well plates were used
- 30 μL of each dilution (in triplicate) were pipetted to the plate
- 270 μL of the solution of DPPH was pipetted into each well
- The plate was placed in a dark place for 45 minutes
- The absorbance was measured at 515 nm in an ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, Vermont, USA)
- Trolox was used as a positive control
- The percentage of DPPH discolouration was calculated according to Equation 2

$[(A\text{DPPH}-AS)/A\text{DPPH}]\times 100$

Where AS is the absorbance of the solution containing the sample at 515 nm, and ADPPH is the absorbance of the DPPH solution.

Equation 2. DPPH discoloration formula

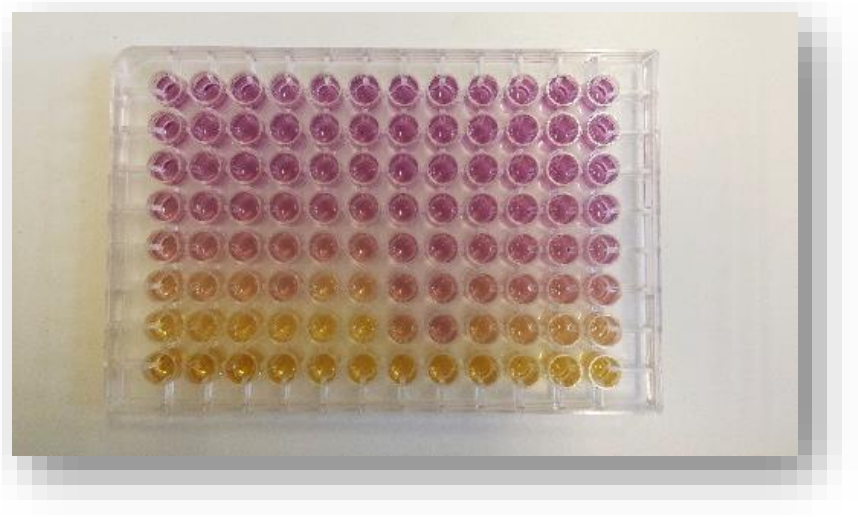


Figure 16 Depiction of a DPPH scavenging activity assay with the stronger (more antioxidant) concentrations showing a yellow tone. Author: M. Carocho

1.7.3. Reducing power

1.7.3.1. Method principles

The reducing power method is characterized by the reduction of complex Fe(III)/ferrcyanide [$FeCl_3/K_3(CN)_6$] to Fe (II) (Fe^{3+} to Fe^{2+}) depending on the available reducing species. This reduction is accompanied by a change in colour from yellow to prussian blue. When the oxidant species is either Fe(III) or $Fe(CN)_6^{3-}$ in the composite ferricyanide reagent, either Fe(II) or $Fe(CN)_6^{4-}$ forms, respectively, as the reduction product with the antioxidant, and combines with the other reagent component to produce prussian blue, $KFe[Fe(CN)_6]$, as the coloured product. In other words, when Fe^{3+} is used along with $Fe(CN)_6^{3-}$ as the oxidizing agent (in the modified ferricyanide assay), either one of the two reaction pairs occur, which end up with the same product, which is prussian blue (Figure 17) (Antolovich et al., 2002; Berker et al., 2007).

Fe^{3+} + antioxidant ⇄ Fe^{2+} + oxidized antioxidant, $Fe^{2+} + Fe(CN)_6^{3-} \rightleftarrows Fe[Fe(CN)_6]^-$

or

$Fe(CN)_6^{3-}$ + antioxidant ⇄ $Fe(CN)_6^{4-}$ + oxidized antioxidant, $Fe(CN)_6^{4-} + Fe^{3+} \rightleftarrows Fe[Fe(CN)_6]^-$

Equation 3. Two reaction pairs for the reducing power assay

1.7.3.1. Solvents, reagents and materials

Potassium ferricyanide and iron chloride were acquired from Sigma-Aldrich (St. Louis, Missouri, USA) and tricholoroacetic acid from Panreac (Barcelona, Spain). All other solvents and reagents were acquired from scientific retailers.

1.7.3.2. Methodology

- For this assay 48 well plates were used
- 500 µL of each dilution was added to different eppendorfs
- 500 µL of phosphate buffer was added (200 mM, pH 6.6)
- 500 µL of potassium ferricyanide was added (10 mg/mL)
- The eppendorfs were kept at 50 ºC for 20 minutes
- After removing the heat, 500 µL of tricholoroacetic acid (10%) was added
- After centrifuging, 800 µL of the eppendorfs were pipetted to the wells (duplicates)
- 800 µL of deionized water were pipetted in the well
- Finally, 160 µL of iron chloride were pipetted (1 mg/mL)
- The absorbance was measured at 690 nm in the same microplate reader as section 2.5.1.2. of this part
- Trolox was also used as a positive control

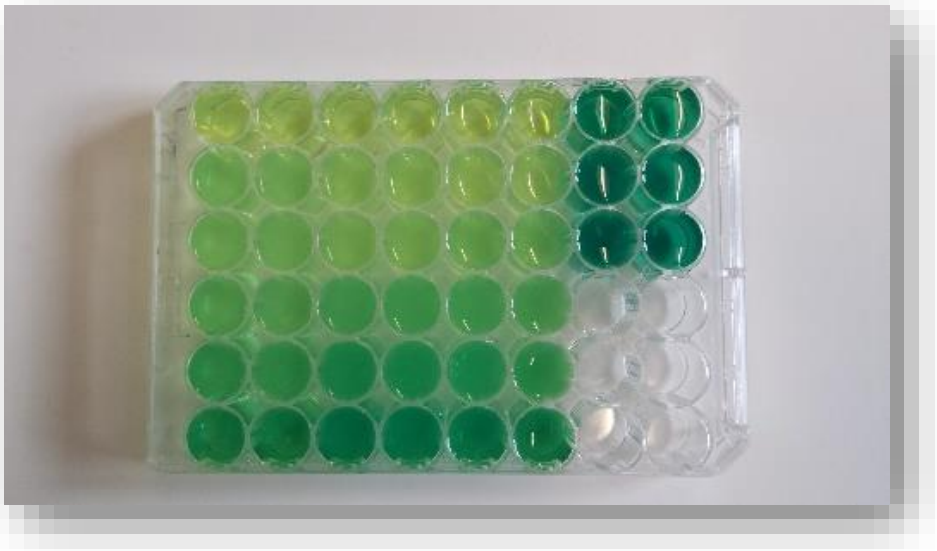


Figure 17. Depiction of the reducing power assay with the stronger (more antioxidant) concentrations showing a prussian blue tone. Author: M. Carocho

1.7.4. β-carotene bleaching inhibition

1.7.4.1. Method principles

The antioxidant activity for this assay is measured by the system β-carotene-linoleate. It is based in the spectrophotometric measures of the discolouration of β-carotene, evaluated by the inhibition of free radicals by the antioxidants in the extracts, which are formed by peroxidation of the linoleic acid, leading to the radical linoleate that attacks the double bonds of the β-carotene (Figure 18), discolouring it. The underlying reactions are patent in Equation 4.

$$\beta\text{-carotene - H (orange colour)} + ROO\cdot \rightarrow \beta\text{-carotene}\cdot \text{ (discoloured)} + ROOH$$
$$\beta\text{-carotene - H (orange)} + ROO\cdot + AH \rightarrow \beta\text{-carotene - H (orange)} + ROOH + A\cdot$$

Equation 4. β-carotene bleaching inhibition reaction

This mechanism is involved in the discolouration of carotenoids through a thermal oxidation, which can be reduced by the action of antioxidants within the extracts (Figure 19) (Mi-Yae et al., 2003; Amarowicz et al., 2004; Kaur & Geetha, 2006).

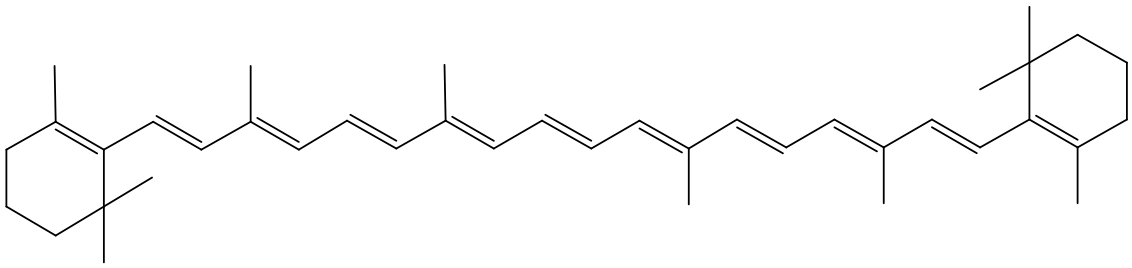


Figure 18. Depiction of the β-carotene molecule

1.7.4.2. Solvents, reagents and materials

β-carotene and linoleic acid were acquired from Sigma-Aldrich (St. Louis, Missouri, USA) and Tween 80 from Panreac (Barcelona, Spain). All other solvents and reagents were acquired from scientific retailers.

1.7.4.3. Methodology

- 2 mL of a β-carotene solution in chloroform (0.2 mg/mL) were pipetted to a round flask end evaporated in a rotary evaporator R210 (Büchi, Flawil, Switzerland)
- 400 mg of Tween 80 were pipetted into the flask, along with 2 drops of linoleic acid
- The dried β-carotene was scraped off the flask and mixed with the Tween
- 100 mL of deionized water were added
- The different concentrations of the extracts were pipetted into test tubes
- 4.8 mL of the β-carotene solution was transferred into each test tubes
- Each test tube was measured in a spectrophotometer at 470 nm (Analytikjena 200, Jena, Germany)
- After measuring, the test tubes were placed at 50 ºC for 2 hours
- After the 2 hours they were once again measured at the same wavelength
- Trolox was once again used as a positive control
- The inhibition was calculated based on Equation 5

$$\frac{Abs470\ after\ 2hours}{Abs470\ beginning} \times 100$$

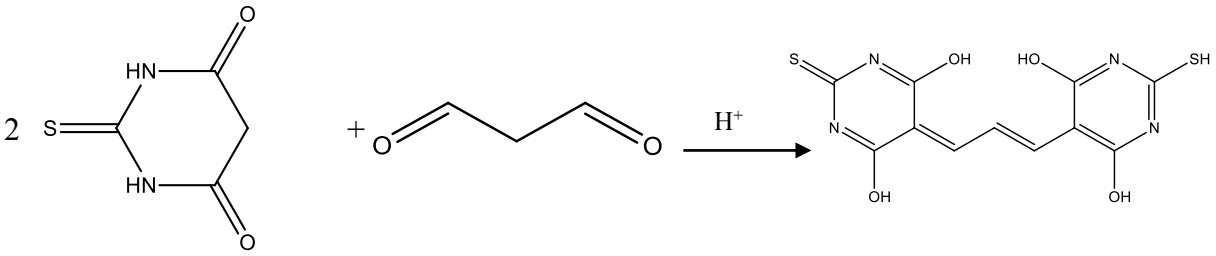
Equation 5. β-carotene bleaching assay equation

Figure 19. Test tubes with different discolouration. Author: M. Carocho

1.7.5. TBARS formation inhibition

1.7.5.1. Method principles

This method relies on the fact that thiobarbituric acid (TBA) and malodialdehyde (MDA) are biomarkers of lipid peroxidation. This lipid peroxidation can be determined by the products of oxidation that react with TBA to create pink compounds that are known as thiobarbituric acid reactive species (TBARS), that can be measured. The TBA in association with MDA, and in presence of H^+ ions form a chromogen (MDA-TBA) (Figure 20). There are two crucial steps in this reaction, first, the solution with the antioxidant is oxidised due to the addition of a metallic ion (iron or copper), then, the extension of this reaction is dictated by the thiobarbituric acid, in which solutions with a high number of antioxidants will stop the oxidation by inhibiting the formation of the chromogen (less pink) (Figure 21) (Gutteridge, 1995; Ng et al., 2000; Kaur & Geetha, 2006).



TBA MDA MDA-TBA

Figure 20. TBARS assay reaction

1.7.5.2. Solvents, reagents and materials

Ascorbic and tricholoroacetic acid were acquired from Panreac (Barcelona, Spain), iron sulphate from Fisher Scientific (Porto Salvo, Lisbon), Tris HCl buffer from Molekula (Newcastle, UK) and thiobarbituric acid from Acros (Geel, Belgium). All other solvents and reagents were acquired from scientific retailers.

1.7.5.3. Methodology

- About 5 g of porcine brains (acquired from official slaughter houses) were cut in small pieces and placed in a falcon tube with double the amount of Tris-HCl buffer
- The solution was centrifuged at 3500g for 10 minutes at 10 ºC (Centurion, K2OR-2003, West Sussex, UK)
- 200 µL of Each concentration of the extracts was pipetted into test tubes
- 100 µL of a Solution of ascorbic (0.1 mM) was added, followed by 100 µL of iron sulphate (10µM) and finally 100 µL of the brain homogenate solution
- The test tubes were heated to 37.5 ºC for 1 hour
- After heating, 500 µL of tricholoroacetic acid (28%) were added to each tube
- 380 µL of thiobarbituric acid (2%) was pipetted to each tube
- The test tubes were heated to 80 ºC for 20 minutes
- They were centrifuged (Labofuge 200, Hanau, Germany) at 3000 rpm for 5 minutes and finally measured in a spectrophotometer as 5232 nm (Analytikjena 200, Jena, Germany)
- Trolox was used as a positive control

Figure 21. Test tubes with different shades of pink, as a result of the inhibition of the MDA-TBA chromogen. Author: M. Carocho

1.8. Antimicrobial activity

1.8.1. Method principles

The antimicrobial activity was carried out to verify if the extracts had any activity towards possible contaminants, when eventually added to the food. Thus, both the antibacterial and the antifungal activity were analysed. For each one, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) were determined. These methods place the bacteria or fungi in direct contact with the extracts, and if it has any type of activity, the microorganisms will either stop developing (inhibition) or die (cidal). For this, various dilutions of the extracts are prepared. The MIC's are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). MBC or MFC is the lowest concentration of an antibacterial or antifungal agent required to kill a particular bacterium or fungi, respectively. The MBC/MFC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by ≥99.9%. Antibacterial agents are usually regarded as bactericidal if the MBC or MFC is no more than four times the MIC (French, 2006; Brooks et al., 2013). What is desired in these assays if for the used extracts to show a lower inhibitory or cidal concentration, which should be below the concentration of the positive controls.

1.8.2. Antibacterial activity

The bacteria used are listed on Table 8, which were a mixture of negative and positive Gram specimens. Their provenance was from the American type culture collection (ATCC), clinical isolates or national collection of type cultures (NCTC-Serbia). The followed methodology, in which 96 well plates were used instead of test tubes (Figure 22) had been previously reported (Petrović et al., 2014; Vieira et al., 2016).

Table 8 Bacteria species used in the antibacterial activity evaluation assays

Bacteria	Gram	Provenance
Escherichia coli	Negative	ATCC 35210
Pseudomonas aeruginosa	Negative	ATCC 27853
Salmonella typhimurium	Negative	ATCC 13311
Enterobacter cloacae	Negative	ATCC 35030
Staphylococcus aureus	Positive	ATCC 6538
Bacillus cereus	Positive	Clinical isolate
Micrococcus flavus	Positive	ATCC 10240
Listeria monocytogenes	Positive	NCTC 7973

1.8.2.1. Solvents, reagents and materials

Streptomycin, ampicillin, dimethyl sulfoxide (DMSO) and tryptic soy broth (TSB) were acquired from Sigma-Aldrich (St. Louis, Missouri, USA), and iodonitrotetrazolium chloride (INT) from Biochemica (Panreac, Barcelona, Spain). All other solvents and reagents were acquired from scientific retailers.

1.8.2.2. Methodology

- The used microorganisms belonged to the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" at the University of Belgrade in Serbia
- A fresh overnight culture of bacteria corresponding to a bacterial suspension was adjusted with a spectrophotometer (625 nm) (Agilent/HP 8453, Santa Clara, California, USA) to a concentration of 1×105 CFU/mL (colony forming units)

- 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 TSB, with 10 µL of the inoculum being added to all the wells
- The microplates were incubated for 24 h at 37 °C (Memmert INC153, Schwabach, Germany)

MIC (minimum inhibition concentration)

- The MIC of the samples was determined by adding 40 µL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL) and by 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 the tested samples were also determined by a colorimetric microbial viability assay based on the reduction of INT colour and compared with the positive control for each bacterial strain

MBC (minimum bactericidal concentration)

- MBC was determined by serial sub-cultivation of 10 µL into microplates containing 100 µL 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% DMSO was used as a negative control
- The results of MIC and MBC were expressed in mg per mL of the aqueous solutions of the lyophilized decoctions

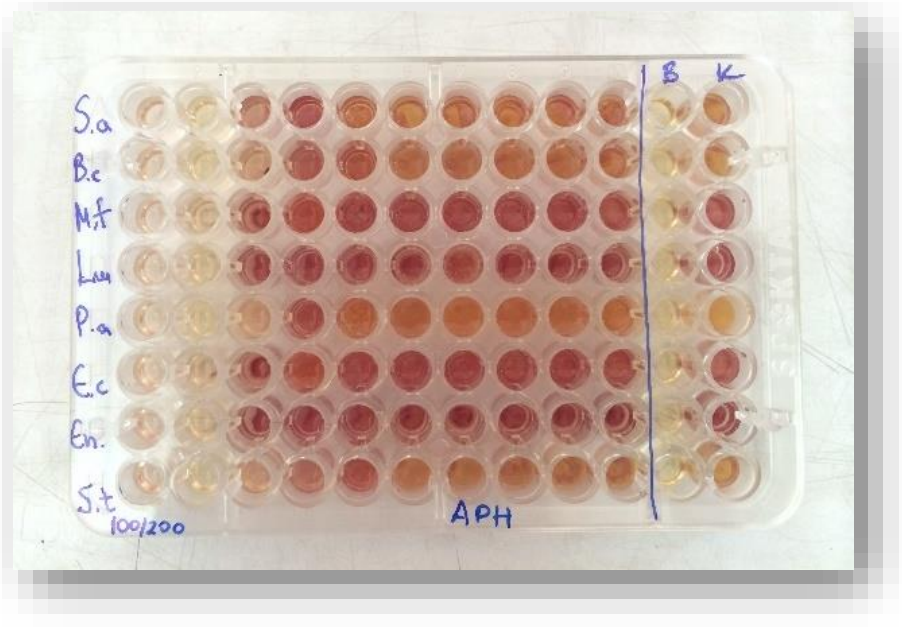


Figure 22. Microplate with different bacteria in the assay for MIC and MBC determinations. Author: S. Heleno

1.8.3. Antifungal activity

The fungi used are listed on Table 9. Their provenance was also from the American type culture collection (ATCC), but also from the culture collection of the Centre for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan (IAM) and a food isolate. The followed methodology, in which 96 well plates were used instead of test tubes (Figure 23) was the same as the one followed for the antibacterial activity, in section 2.6.1.1. of this part).

Table 9 Fungi species used in the antifungal activity evaluation assays

Fungi	Provenance
Aspergillus fumigatus	ATCC 1022
Aspergillus ochraceus	ATCC 12066
Aspergillus versicolor	ATCC 11730
Aspergillus niger	ATCC 6275
Trichoderma viride	IAM 5061
Penicilium funiculosum	ATCC 36839
Penicillium ochrochloron	ATCC 9112
Penicillium verrucosum var. *cyclopium*	Food isolate

1.8.3.1. Solvents, reagents and materials

The solvents used for this assay were the same as the ones used in the antibacterial activity evaluation assay. Ketoconazole was acquired from Sigma-Aldrich (St. Louis, Missouri, USA), bifonazole from Srbolek (Belgrade, Serbia), tween 80 from Sineks (Belgrade, Serbia) and malt agar (MA) from Torlak (Belgrade, Serbia). All other solvents and reagents were acquired from scientific retailers.

1.8.3.2. Methodology

- The 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 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 1.0×105 in a final volume of 100 μL per 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

MIC (minimum inhibition concentration)

- The MIC determination was performed by a serial dilution technique using 96-well microplates
- The sample solutions were added to the broth malt medium with the fungal inoculum
- The microplates were incubated for 72 h at 28 °C
- The lowest concentrations without visible growth (using a binocular microscope) were defined as the MIC

MFC (minimum fungicidal concentration)

- The MFC's were determined by serial sub-cultivation of 2 μL in microtiter plates containing 100 μL of malt broth per well and further incubated 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 bifonazole and ketoconazole were used as positive controls
- The results of MIC and MFC were expressed as mg per mL of the aqueous solutions of the lyophilized decoctions

Figure 23. Microplate with different fungi used in the determination of MIC and MFC determinations. Author: S. Heleno

1.9. Antitumor potential

1.9.1. General and method principle

The antitumor activity was carried out in human tumor cell lines, as depicted in Table 10. The used procedure was the sulforhodamine B (SRB) assay, reported previously (Vichai & Kirtikara, 2006). This *in vitro* cytotoxicity screening relies on the fact that SRB can bind to protein components of cells that have been previously fixed. SRB is a bright-pink aminoxanthene dye that can be spectrophotometrically measured at 515 nm. The intense colour of this dye allows the assay to be carried out on 96-well plates, reducing expenses and time. It is an indirect method to measure cell proliferation, given that what is measured is the amount of protein produced by cells, therefore implying that the higher amount of

proteins, the more cells have grown and the more intense pink colouration should appear (Figure 24). What is desired in this assay is for the used extracts to reduce the proliferation of the tumor cells. Furthermore, hepatotoxicity studies were carried out on porcine liver cell lines (PLP2) following the procedure described by Abreu et al. (2011). In this manner, the eventual hepatotoxicity of the extracts can be measured by the inhibition of the growth of these normal cells. The use of porcine cells is due to the fact that there are less ethic and legal setbacks than the use of human ones, and due to the high similarity of human and pig cell physiology.

Table 10 Used human tumor cell lines

Name	Type
HCT-15	Dukes' type C, colorectal adenocarcinoma
HeLa	Cervix adenocarcinoma
HepG2	Hepatocellular carcinoma
MCF7	Mammary gland adenocarcinoma
NCI-H460	Large cell lung carcinoma

1.9.2. Solvents, reagents and materials

Roswell Park Memorial Institute medium (RPMI), glutamine, Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, aminoacids, SRB and ellipticine were acquired from Sigma-Aldrich (St. Louis, Missouri, USA), while Fetal Bovine Serum (FBS) and Hank's balanced salt solution were obtained from Hyclone (GE Healthcare Life Sciences, Logan, Utah, USA). All other solvents and reagents were acquired from scientific retailers.

1.9.3. Methodology

SRB assay

- Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine at 37 °C, in a humidified air incubator, containing 5% CO_2
- Each cell line was plated at an appropriate density (7.5×10^3 cells per well for MCF7 and NCI-H460, or 1.0×104 cells per well for HCT-15, HeLa and HepG2) in 96-well plates and allowed to attach for 24 hours

- Following this incubation period, the adherent cells were fixed by adding cold 10% tricholoroacetic acid (100 µL) and incubated for 60 min at 4 °C
- Plates were then washed with deionised water and dried
- SRB solution (0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature
- Unbound SRB was removed by washing with 1% acetic acid
- Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 µL) and the absorbance was measured at 515 nm in the microplate reader (ELX800, Bio-Tek Instruments, Winooski, USA)
- Ellipticine was used as a positive control
- The results were expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell growth) in µg/mL of the aqueous solutions of the lyophilized decoctions

Hepatotoxicity

- A cell culture was prepared from a fresh porcine liver obtained from a local slaughter house, it was designated as PLP2
- Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL of penicillin, 100 µg/mL of streptomycin and were divided into $1\times1\times1$ mm^3 explants
- Some of these explants were placed in 25 cm^2 tissue flasks with DMEM medium supplemented with 10% FBS, 2 mM nonessential amino acids and 100 U/mL of penicillin, 100 mg/mL of streptomycin and incubated at 37 °C under a humidified atmosphere containing 5% CO_2
- The medium was changed every two days. The cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope
- Before the confluence was reached, the cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells per well, and cultivated in DMEM medium with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin
- Finally, the cells underwent the same treatment as the cancer lines for the final steps of the SRB assay (starting from the fixation with tricholoroacetic acid)

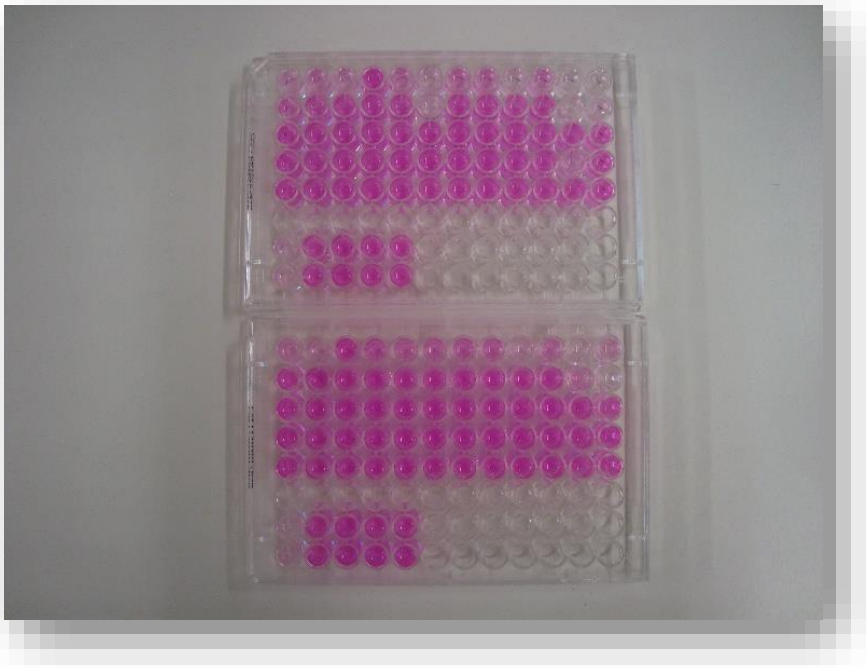


Figure 24. Microplate with different tones of pink resulting of the SRB colouration. Author: M. Carocho

Incorporation of the Natural Ingredients in Food Matrices

The incorporation of the extracts in the "económicos" cakes and "Serra da Estrela Cheese" was carried out manually during the production process taking into consideration all the hygienic precautions to avoid contamination through hand contaminants.

1.10. **Incorporation in "económicos" cakes**

1.10.1. Recipe and manufacture

The recipe for the cakes was the following:

- 6 eggs
- 500 g of sugar
- 45 g of margarine
- 30 g of heated olive oil
- 35 g of orange zest
- 230 mL of pure orange juice
- 250 mL of milk

- 45 mL of brandy (firewater)
- 25 g of cinnamon powder
- 1.05 kg of flour
- 22 g of ROYAL® baking powder
- 25 g of sodium bicarbonate

The procedure was as follows:

- The oven was pre-heated at 170 ºC
- The eggs were beaten with the sugar
- The margarine and the olive oil were added to the mixture
- After thorough beating, the zest, orange juice, milk, brandy and cinnamon were sequentially added
- Finally, the flour and baking powder were also added
- When a wooden spoon could stand vertically in the batter, or when the batter was placed on a tray and would not lose its shape, the ideal consistency had been reached
- The batter was then divided into 5 portions of 500 g each
- The extracts were added manually, followed by vigorous mixing
- Scoops of the batter (the size of a table spoon) were placed on an oven tray and baked at 170 ºC for 15 minutes

1.10.2. Details of the incorporated concentrations

The chestnut flowers and respective decoctions were added to the cakes at 2 different concentrations, resulting in a total of 5 lots. Given that there were 3 storage times to be analysed, the total lots came to 15.

The incorporated concentrations were based on the antioxidant capacity (EC_{50} values) of the best extract (decoctions), namely through the DPPH scavenging activity assay. The EC_{50} value corresponds to the needed concentration of the extract to scavenge 50% of the radicals. Thus, for the decoctions, the best DPPH EC_{50} value was 0.09947 mg/mL. To 500 g of batter, the extract was added at 0.090 mg/mL, (49.735, which were then rounded to 50mg), and for the second lot, a 2-fold quantity was added (100 mg). Regarding the cakes incorporated with the dried flowers, the incorporation was based on the decoction yield. Thus, the yield of 1 g of dried

flower was calculated and came to 20%. Given that the EC_{50} of the extract (0.09947 mg/mL) corresponded to a yield of 31%, 0.49735 mg corresponded to a yield of 100%. Thus, for 500 g of batter, 248.675 mg (rounded to 200 g) of dried flower were needed, leading to the incorporation of 200 mg for one lot of the batter, and once again, the double amount (400 mg) for the other batter.

1.10.3. Preparatory procedures

On the same day of manufacture, after cooling down, 2 cakes from the control lot and from each of the incorporated lots were brought to the lab for the T0 analysis. Initially, the cakes were frozen and lyophilized. After lyophilization they were milled down to a fine powder and thoroughly mixed with each other. The remaining cakes were kept for 15 and 30 days respectively, in closed plastic bags, protected from light and with temperatures ranging from 18 to 23 ºC. Finally, after the due time, they were removed from storage and subject to the same preparatory treatment as the T0, before undergoing further chemical and nutritional assays.

1.11. **Incorporation in "Serra da Estrela" cheese**

The cheeses were produced at a certified dairy (Queijos Casa Matias), therefore the incorporation and ripening of the cheese also took place there. As a PDO cheese, the addition of other ingredients in its manufacture is not allowed, and therefore it should not be regarded as "Serra da Estrela" cheese, for the name is attached to the PDO, still, in order to simplify the reading of this document, it will be regarded as such.

1.11.1. Recipe and manufacture

The cheese ingredients consisted solely on ewe milk, milk thistle extract for coagulation and salt. Initially the fresh milk (milked during early hours of the same day) arrived at the dairy in certified containers, and the temperature and pH were recorded (5 ºC and 6.88). After being transferred to a vat, the milk thistle was added to induce clotting, along with salt. After the clotting, the cheese was produced by means of automated machinery, which pressed it and individually placed it in forms that were then placed on a conveyor belt to be collected by personnel for a second pressing at a higher pressure. After 2 hours of the pressing stage the cheese was embalmed with a cloth around its sides to maintain its format, sprinkled with salt and placed in maturing chambers. The first chamber had a relative humidity of 95 to 100% and

a constant temperature ranging from 7 to 9 ºC. The cheeses were kept here for 15 days, after which they were transferred to the second chamber, kept between 80 to 82% of relative humidity, 11 to 13 ºC for another 15 days. The cheeses were washed every second week with water to remove possible surface contaminations.

1.11.2. Details of the incorporation

All three plants (dry material and decoctions) were incorporated in different lots of cheese. As for the cakes, the incorporation of the plants extracts in the cheese also took into consideration the antioxidant capacity of each plant, and the dried plants also relied on the yield of the extractions. Following the same logic for the incorporation of the cakes, and using the same value of the EC_{50} in the DPPH assay for the flowers (0.09947 mg/mL), and assuming that 2.5 L of milk are needed for one cheese, 248 mg of extract (rounded to 250 mg) were added to each. Regarding the dried flowers incorporation, it was also based on the decoction yield. Thus, the yield of 1 g of dried flower was calculated and came to 31%. Given that the EC_{50} of the extract (0.09947 mg/mL) corresponded to a yield of 31%, 0.3197 mg/mL corresponded to a yield of 100%. Thus, for 2.5 L (one cheese), 0.799 mg (rounded to 800 g) of dried flower were needed. Unlike the cake incorporation, no double fold lots were made. For the other plant samples, the same logic and methodology was used, the only differences were the amounts incorporated based on the EC_{50} and the extraction yield, that are detailed on Table 11.

Table 11. Important values followed in the incorporation of the natural ingredients into the cheese

	Chestnut	Lemon balm	Basil
DPPH value (EC_{50})	0.09947 mg/mL	0.06 mg/mL	0.141 mg/mL
Yield	31%	39%	25.7%
Amount of lyophilized decoction per cheese	250 mg	150 mg	352.5 mg
Amount of dried plant per cheese	800 mg	386 mg	1370 mg

1.11.3. Preparatory procedures

The incorporation of the extracts and plants was done manually, just after the first pressing by mixing the extracts in the cheese. Then, they were subject to a second pressing to allow them to achieve their final shape. This pressing took about 2 hours. Finally, they were placed in ripening chambers, detailed in section 3.2.1. of this part. When the maturing was complete, approximately after 30 days, the cheeses were brought to the laboratory. Each lot was sub-

divided into 2 lots, with 3 cheeses each. One sub-lot of each was then placed in a refrigerator with controlled temperature (ranging from 3.5 to 5 ºC, and average humidity of 54%) for 6 months (T6) to mature the cheese into "old cheese", while the other sub-lot was immediately analysed (T0). For the analysis, the cheeses were peeled, cut into pieces (5 cm^3), frozen, lyophilized, milled down and thoroughly mixed. When the 6 months were over, these old cheeses suffered the same preparatory procedures as the T0.

1.12. Determination of nutritional parameters in the final products

The nutritional composition encompassed the moisture, total available carbohydrates (TAC), proteins, crude fat, ash and dietary fiber. Furthermore, fatty acids and mineral elements content were also analysed by gas chromatography and atomic absorption spectroscopy, respectively.

1.12.1. Moisture

Moisture was analysed both for the cakes and the cheeses with aid of an oven. It is based on the evaporation of water from the sample, and calculating its amount by difference.

1.12.1.1. Methodology

The methodology followed was in accordance with AOAC procedures (AOAC 925.09).

- 2 g of the sample were placed in a metal dish that was closed and weighed
- The dish was placed in an oven (Scientific Series 2000, Contherm, New Zealand) at 100 ºC for 5 hours
- After the 5 hours the dish was placed in a desiccator until it reached room temperature
- It was weighed once again
- The moisture was calculated by subtracting the final weight from the initial one (g/100g FW)

1.12.2. Total available carbohydrates

Total available carbohydrates were only determined for the cakes, as none were detected in the cheese. Their detection followed the anthrone method (Osborne & Voogt, 1986). It is a colorimetric method, in which anthrone reagent (9,10-dihydro-9-oxoanthracene) gives off a green colour in the presence of products of the destruction of carbohydrates, when reacted with sulphuric acid and heat is applied.

1.12.2.1. Methodology

- 0.3 g of each lyophilized sample was placed in a 100 mL flask
- 13 mL of perchloric acid (52%) and 10 mL of distilled water were added to the flask and gently shaken
- The flasks were left overnight
- The solution was filtered through a pleated paper filter into a 100 mL and filled with distilled water and shaken
- A calibration curve of D-glucose (10-100 μg/mL) was prepared, in order to be compared to the samples
- For each sample, 3 test tubes were identified "sample", "blank" and "standard", and 1 mL of the sample solution was added to the first, 1 mL of distilled water to the second and 1 mL of the standard solution (0.1%) to the third
- To each test tube, 5 mL of anthrone solution (0.01%) were added
- Laboratory parafilm was placed on the top to seal the tubes
- The test tubes were placed in boiling water for 12 minutes, after which they were quickly cooled down with ice
- Finally, the quantity of carbohydrates was measured at 630 nm in a spectrophotometer (EZ210 Perkin Elmer, Waltham, Massachusetts, USA), reading the blank, standard and sample test tubes sequentially
- The total available carbohydrates were expressed as g/100 g of FW

1.12.3. Dietary fibers

Dietary fibers were only determined for the cakes, for they were not detected in the cheese. The analysis relied on an enzymatic-gravimetric method that allows the detection of both soluble dietary fiber (SDF) and insoluble dietary fiber (IDF).

1.12.3.1. Methodology

The methodology followed was in accordance with the AOAC procedures (AOAC 993.21).

- For this method, 4 crucibles with 0.5 g of celite were used for each sample
- 0.3 g of each lyophilized sample was weighed into 250 mL erlenmeyers in quadruplicate
- 50 mL of phosphate buffer (0.08M pH6) was added to each erlenmeyer and they were placed in a hot bath at 100 ºC

- 100 µL of α-amylase, heat stable (Sigma, St. Louis, Missouri, USA) was added to each erlenmeyer, kept in these conditions for 15 minutes with mechanical agitation
- After cooling 10 mL of NaOH (0.275N pH7.5) and 5mg of protease (Sigma, St. Louis, Missouri, USA) was added to the erlenmeyers, then left for 30 minutes at 60 ºC under agitation
- After, 10 mL of HCl (0.325N pH4.5) and 100 µL of amyloglucosidase was added. The erlenmeyers were once again sealed and left for 30 minutes at the same temperature
- The liquid solution of the erlenmeyers was filtered through the previously prepared crucibles under vacuum filtration and the insoluble dietary fiber (IDF) residue was trapped in the crucible. The filtrate liquid was placed in a 500 mL erlenmeyer with ethanol 96º and left overnight for soluble dietary fiber (SDF) precipitation.
- This liquid was then re-filtered through other crucibles, previously prepared with celite. All the crucibles with the fiber residue were placed in an oven and kept overnight.
- After the crucibles were weighed, the ash and protein procedure was carried out with the residue, in order to avoid weight interferences due to proteins and ash in the insoluble and soluble dietary fiber.
- The IDF and SDF was expressed as g/100g of FW

1.12.4. Crude protein

The protein determination relied on the Kjeldahl method, and was carried out for both foodstuffs (cakes and cheeses). The nitrogen conversion factor for the cakes was 6.25, while for the cheese it was 6.38, according to AOAC (2000). This method is based on the amount of nitrogen present in a sample that can indicate the amount of crude proteins. It relies on the destruction of all organic matter by addition of a strong acid (sulphuric acid) that retains nitrogen under the form of $(NH_4)_2SO_4$. After the addition of NaOH, the nitrogen is released as NH_3 (steam distillation) and collected by H_2SO_4 N/10, and after a titration with NaOH N/10 using methyl red as an indication, a colour shift is observed.

1.12.4.1. Methodology

The methodology followed was in accordance with the AOAC procedures (AOAC 984.13). Crude protein procedure consists in three steps: organic matter digestion, ammonium distillation and sulphuric acid titration.

- Organic matter digestion: 0.5 g of each lyophilized sample were placed in test tubes with 1 tablespoon of catalysing agent (K_2SO_4/$CuSO_4$) and 25 mL of sulphuric acid (99%)
- Then, the test tubes were placed in a Kjeldahl digester (Büchi, Flawil, Switzerland) at 150 ºC for 3 h or until the destruction of organic matter was achieved
- Ammonium distillation: Alkaline steam distillation took place in a Kjeldahl distiller (Büchi Distillation Unit k-350, Flawil, Switzerland) The ammonium was distilled into and erlenmeyer (500 mL) with 25 mL of sulphuric acid (N/10) and a few drops of methyl red as an indicator
- Titration step: Liquids ($(NH_4)_2SO_4$ and excess of sulphuric acid) were titrated with sodium hydroxide (N/10) to a colorimetric endpoint
- The crude proteins (%Kjeldahl N × F) were expressed as g/100g of FW

1.12.5. Crude fat

Crude fat was determined for both the cakes and cheeses. The soxhlet extraction method was followed, which relies on the non-polarity of petroleum ether to extract the fat from the samples.

1.12.5.1. Methodology

The methodology followed was in accordance with the AOAC procedures (AOAC 983.283).

- 3 g (lyophilized sample) of each sample was weighed into a cartridge of filter paper, and covered with cotton
- The cartridges were placed in a soxhlet apparatus, relying of petroleum ether as the solvent
- The heat was turned on and after 30 cycles the round bottom beaker containing petroleum ether and fat was removed
- The petroleum ether was evaporated by means of a rotary evaporator R210 (Büchi, Flawil, Switzerland)
- After returning to room temperature the erlenmeyer was weighed once again to determine the fat content
- The crude fat was expressed as g/100 g of FW

1.12.6. Ash

Ash content was analysed for both the cakes and the cheeses using a muffle. This method relies on the destruction of all organic matter by means of high temperature.

1.12.6.1. Methodology

The methodology followed was in accordance with AOAC procedures (AOAC 923.03).

- 0.3 g (lyophilized sample) of the samples were weighed into a dried porcelain crucible which was then weighed again and both values recorded
- The crucibles were placed in a muffle (Lenton ECF 12/22, Hope Valley, UK) at 550 ºC
- The crucibles were removed from the muffle and placed in a desiccator until they reached room temperature
- The crucibles were then weighed once more and the ash content calculated by difference, and the ash was expressed as g/100g of fresh weight (FW)

1.12.7. Energy

Energy value was calculated according to Equation 6, based on the European Parliament and Council Regulation, No. 1169.The energy was expressed in kcal/100g of fresh cake or cheese.

- Cakes
  - Energy (kcal/100g) = 4 × (g protein + g total available carbohydrate) + 2 × (g dietary fiber) + 9 × (g crude fat)
- Cheese
  - Energy (kcal/100g) = 4 × (g protein) + 9 × (g fat) (due to not having any total available carbohydrates or dietary fiber)

Equation 6. Equations used to calculate energy

1.12.8. Fatty acids

Fatty acids are a reliable source of information to explain changes during storage period, therefore individual fatty acids determination was carried out for both the cakes and the cheese by gas chromatography (GC), coupled to a flame ionization detector (FID). The conditions are depicted on Table 12.

1.12.8.1. Solvents reagents and materials

Methanol and sulphuric acid were acquired from Fisher Scientific (Porto Salvo, Portugal), toluene and ethyl ether from Sigma (St. Louis, Missouri, USA) and sodium sulphate from Carlo

Erba Reagents (Peypin, France). The fatty acids methyl esters (FAME) reference standard mixture 37 (standard 47885-U) and the other individual fatty acid isomers were purchased from Sigma (St. Louis, Missouri, USA), while all other reagents and used solvents were acquired from scientific retailers. Water was treated using the previously mentioned purification system.

1.12.8.2. Methodology

- After the procedures of the crude fat extraction (section 3.4.4. of this part), the fat inside the test tubes was analysed
- The transesterification of the fat was achieved by adding 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v/v/v) to the test tubes
- They were then sealed with laboratory parafilm and placed in an agitating 50 ºC hot water bath for 12 hours
- Then, to obtain phase separation, 3 mL of deionized water and 3 mL of ethyl ether were added to the test tubes, being the ethyl ether recovered to a vial containing sodium sulphate
- Finally, the solution was filtered through a HPLC filter (0.2 μm) into another vial, and finally injected in a GC-FID

This equipment, a DANI model GC 1000 (Contone, Switzerland) gas chromatograph, with a split/splitless injector, and a FID. The used column was a 30 m × 0.32 mm i.d., 0.25 μm, 50% cyanopropyl-methyl-50% phenylmethylpolysiloxane (Macherey-Nagel, Düren, Germany). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 10 °C/min ramp to 240 °C and held for 11 min. The carrier gas (hydrogen) flow rate was 4 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 DataApex Clarity, version 2.4.4.43 software and expressed in relative percentage of each fatty acid.

Table 12. Chromatographic conditions for fatty acids detection

GC-FID operating conditions for organic acids	
System	DANI model GC 1000
Column	30 m × 0.32 mm i.d., 0.25 μm, 50% cyanopropyl-methyl-50% phenylmethylpolysiloxane
Detectors	Flame ionization detector
Carrier gas	Hydrogen
Flow rate	4 mL/min
Temperature	Variable
Software	DataApex Clarity, version 2.4.4.43

1.12.9. Mineral elements

The mineral elements content of the samples was detected through atomic absorption spectroscopy, and was carried out in both the cakes and cheese, following the AOAC procedures (AOAC 930.05). The studied minerals were macroelements, namely: sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), and microelements, namely: copper (Cu), iron (Fe), and zinc (Zn).

1.12.9.1. Methodology

Mineral element analysis was performed on freeze-dried samples. After dry ash mineralization at 550 ºC the residue of incineration was extracted with HCl (50% v/v) and HNO_3 (50% v/v) and made up to an appropriate volume with distilled water, where Fe, Cu, Mn, and Zn were directly measured. An additional 1/10 (v/v) dilution of the sample extracts and standards was performed to avoid interferences between different elements in the atomic absorption spectroscopy: for Ca and Mg analysis, in 1.16% La_2O_3/HCl (leading to $LaCl_2$); for Na and K analysis in 0.2% CsCl. The methodology followed was previously reported by Fernández-Ruiz et al., 2011), in which an Analyst 200 Perkin Elmer (Perkin Elmer, Waltham, Massachusetts, USA) atomic absorption spectroscope (AAS) with air/acetylene flame was used. The conditions used are displayed on Table 13.

Table 13. Conditions for mineral determination

Mineral element	Wavelength (nm)	Aperture (cm)	Type of flame
Ca	422.7	0.7/0.2	Oxidant
Mg	285.2	0.2/0.2	Oxidant
Fe	248.33	1.8/1.35	Oxidant
Cu	324.75	2.7/2.8	Oxidant
Mn	279.48	1.9/0.6	Oxidant
Zn	213.86	2.7/1.8	Oxidant

As an emitting source, dry cathode lamps were used, with an intensity of 25 mA for the multiple lamp (Fe, Mn and Zn) and 15 mA for Ca and Mg. As an excitation source of the atomizing system, an air/acetylene oxidant flame was used.

1.13. Evaluation of antioxidant activity of the final products

The evaluation of the antioxidant capacity was carried out for both the cakes and cheeses, although for the latter, only for the samples that were not subjected to storage. The employed assays to determine the antioxidant capacity (DPPH, reducing power, β-carotene bleaching inhibition and TBARS assay) were previously described in section 2.6. of this part. The only extra method used was the Folin-Ciocalteu for primary detection of phenolic content.

1.13.1. Folin-Ciocalteu assay

1.13.1.1. Method principles

The Folin-Ciocalteu method is used to determine the presence of phenols in samples. This test, although being widely used is not free of interferences and limitations, and is not specific for phenols. It can be used as an indicator of the reducing power of a sample, and, therefore, capable of evaluation antioxidant capacity. This is due to the mixture of phosphotungstenic and phosphomolybdic acids that react with both phenolic and non-phenolic compounds (aminoacids and sugars for instance). Both acids constitute the Folin-Ciocalteu's reagent, and are oxidized in alkaline solutions, generating O_2 that then reacts with molybdate, finally forming molybdenum oxide (MoO^{4+}), which has a high absorbance at 750 nm (Kellie & Hayball, 2002; Molyneux, 2004).

1.13.1.2. Methodology

- 500 µL of one dilution were pipetted to a test tube
- 2.5 mL of Folin-Ciocalteu's (1:9) reagent were pipetted into the test tube
- 2 mL of calcium carbonate were pipetted into the test tube
- After vortexing the test tubes they were placed at 40 ºC for 30 minutes
- Finally, their absorbance was measured at 765 nm
- Trolox was also used as a positive control
- The results were expressed as equivalents of gallic acid (GAE) per mL of extract

1.14. External colour determinations

For the incorporation of the cheese, the colour was determined at T0. A Konica Minolta spectrophotometer (Konica Minolta, Chroma Meter CR-400, Tokyo, Japan) was used to determine the colour of the cheeses, with six readings on the top and bottom part. Illuminant C at 2º was used, with an 8mm opening of the diaphragm. The CIE colour L*, a* and b* values were reported through the Spectra Magic Nx software (version CM-S100W 2.03.0006, Konica Minolta). L* measures the lightness, and varies between black (L*=0) and white (L*=100). a* corresponds to the greenness-redness, where the positive values correspond to a red colour, and the negative to green. Finally, b* refers to yellow and blue, in which positive values refer to yellow and negative to blue. The instrument was previously calibrated with standard white tiles.

Statistical Tools

In order to allow an objective interpretation of the results, various statistical studies were carried out, which are depicted in the following sections. The software used was Statistics Package for Social Sciences (SPSS) version 22 (IBM Corporation, New York, USA). All results were expressed as mean values ± standard deviations (SD), maintaining the decimal places allowed by the magnitude of standard deviation. All tests were performed at a 5% significance level. For all methods, three samples were mixed to have a representative pool; furthermore, all assays were carried out in triplicate.

1.15. Analysis of variance (ANOVA)

ANOVA is a statistical technique used to analyse differences among mean values for different datasets. Prior to carrying out the ANOVA test, some preliminary assumptions were verified, namely i) the homoscedasticity of variances (using a Levene's test); ii) the normality of distributions (by applying the Shapiro Wilks' test); and iii) the existence of statistically significant differences (through the Welch's statistics). The ANOVA results were classified according to the results obtained in the previous tests. Tukey's test or a Tamhane's T2 test were applied, for homoscedastic or heteroscedastic samples, respectively. Furthermore, when a specific factor was studied using only two levels, a simple student's *t*-test was used to classify the results.

1.16. 2-way ANOVA

In some cases, the differences observed among the studied parameters resulted from combined effects of more than one factor; *e.g.*, storage time or different formulations (dried plant or decoction) of the incorporated plant. Accordingly, a simple 1-way ANOVA would not be enough to understand the true effect of each single factor *per se*. Accordingly, the results were analysed by means of a 2-way ANOVA, which allows evaluating the contribution of each factor independently of the potential changes induced by the other, and simultaneously verifying the potential interaction among factors. Every time this interaction was significant, the results were evaluated simultaneously by the estimated marginal means plots for all levels of each factor. If no statistically significant interaction was found between the two defined factors, the means were compared using a Tukey's test if the differences caused individually by the corresponding factor were statistically significant.

1.17. Linear discriminant analysis (LDA)

The use of a 2-way ANOVA is a useful help in order to understand the contribution of each factor for differences detected among the samples, but the outputs are sometimes lagging to interpret, given the magnitude of the generated information. Furthermore, the number of studied variables was often too high to allow a step-by-step analysis. Thus, LDA was applied in those cases, in order to find the variables that suffer the most relevant changes in result of the effect

of a determined factor level. Besides the dimensionality reduction effect, LDA clusters the most similar samples in easy-to-interpret outputs, while defining the studied variables that mostly contribute to define the discriminant functions distributing each clustered marker. The significant independent variables were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only those with a statistical significant classification performance (p<0.050) were kept in the analysis.

Part V. **RESULTS AND DISCUSSION**

Article 1: *Castanea sativa* Mill. flowers amongst the most powerful antioxidant matrices: A phytochemical approach in decoctions and infusions

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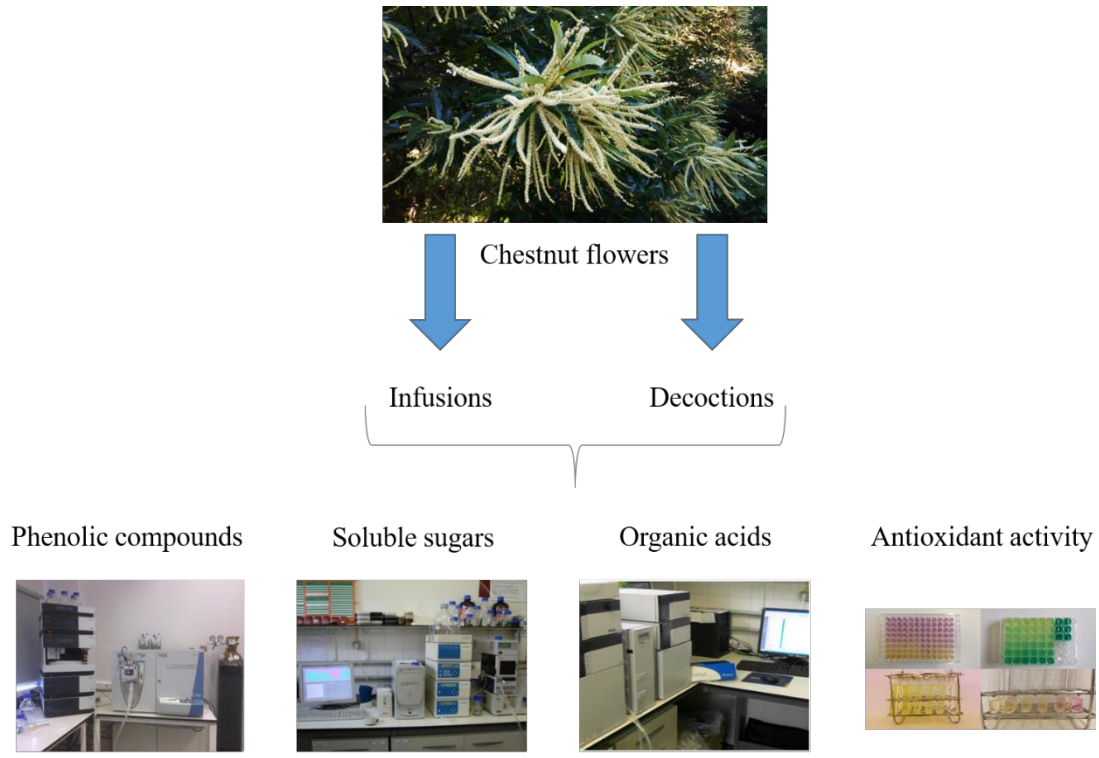

Figure 25 Graphical abstract article 1

Abstract

Infusions and decoction of chestnut tree flowers have been used for different medical purposes, but their phytochemical profile and antioxidant activity are still mostly unknown. Herein, decoctions and infusions of flowers from the two most appreciated chestnut cultivars ("Longal" and "Judia") in Trás-os-Montes, Portugal, were prepared and characterized with regard to their content in soluble sugars, organic acids, and phenolic compounds, such as flavonoids and hydrolysable tannins, and their antioxidant activity. Overall, the decoction of the cultivar

"Judia" was the sample with both the highest quantity of flavonoids and antioxidant activity. The phenolic compound with the highest abundance in all samples was trigalloyl-HHDP-glucoside, followed by pentagalloyl glucoside. The sample with the highest quantity of total phenolic compounds was "Judia infusion", closely followed by "Longal decoction", which also gave the highest quantities of ellagitannins. Regarding sugars and organic acids, the profiles were more similar. These results corroborate ancestral claims of the health benefits of infusions and decoctions of chestnut flowers.

Article 2: Infusions and decoctions of *Castanea sativa* flowers as effective antitumor and antimicrobial matrices

Carocho, M., Calhelha, R.C., Queiroz, M.R.P., Bento, A., Morales, P., Soković, M., Ferreira, I.C.F.R. (2014). *Industrial Crops and Products*, 62, 42-46.
DOI: http://dx.doi.org/10.1016/j.indcrop.2014.08.016

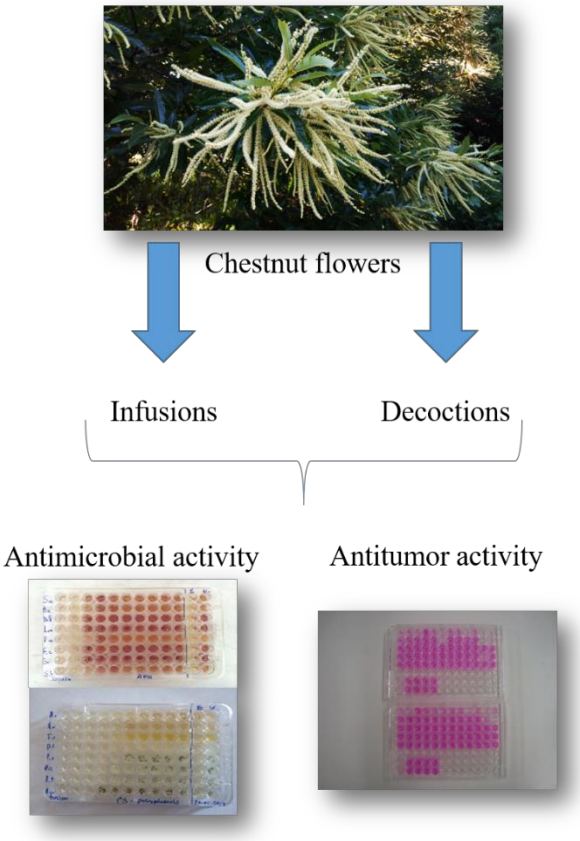

Figure 26 Graphical abstract article 2

Abstract

Chestnut trees are one of the most important crops in the north-eastern part of Portugal, representing millions of euros of yearly income. There are many ancestral claims of the health benefits of the consumption of chestnut flowers in infusions that remain unproven. In this manuscript, the antitumor and antimicrobial potential of chestnut flowers from two cultivars,

"Judia" and "Longal", extracted through infusions and decoctions are reported. In terms of antitumor activity, the most sensitive cell lines were HepG2 and HCT15 with the cultivar "Judia" showing higher activity for HCT15 and "Longal" for HepG2, regardless of the extraction methods. Regarding the antibacterial activity of the extracts, decoctions proved to be more effective with lower minimum inhibition concentrations, while infusions were better in terms of antifungal activity. The good overall antimicrobial activity could justify the inclusion of the flowers in food chain processing to act as a natural antimicrobial. Furthermore, the results corroborate some of the ancestral claims of the consumption of these flowers.

Article 3: *Melissa officinalis* L. decoctions as functional beverages: A bioactive approach and chemical characterization

Carocho, M., Barros, L., Calhelha, R.C., Ciric, A., Soković, M., Santos-Buelga, C., Morales, P., Ferreira, I.C.F.R. (2015). *Food & Function*, 6, 2240-2248.

DOI: http://dx.doi.org/10.1039/c5fo00309a

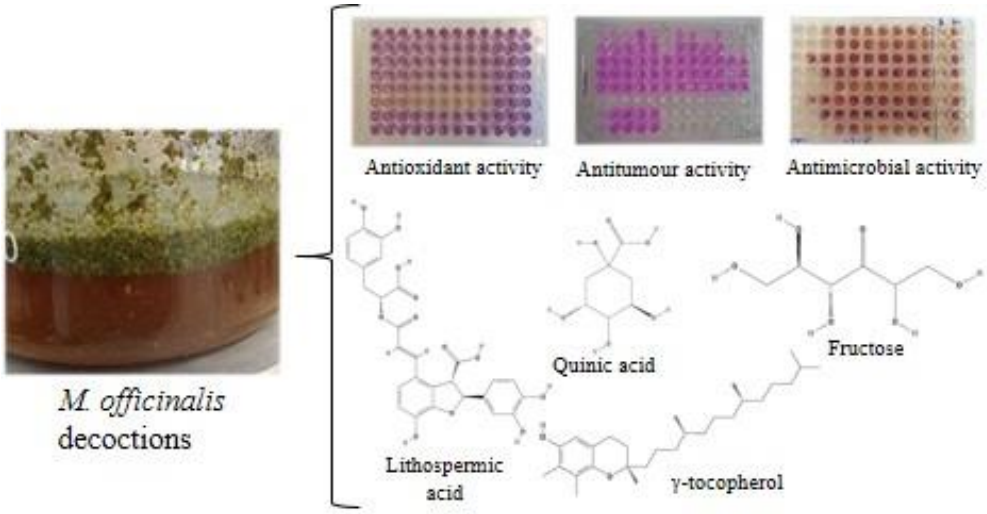

Figure 27 Graphical abstract article 3

Abstract

Lemon balm (*Melissa officinalis* L.) is a member of the Lamiaceae family with a long story of human consumption. It has been consumed for decades, directly in food and as a decoction or an infusion for its medicinal purposes. In this manuscript, a detailed chemical characterization of the decoction of this plant is described, encompassing antimicrobial, antioxidant and antitumor activities. Rosmarinic acid and lithospermic acid A were the most abundant phenolic compounds. Quinic acid, fructose, glucose and γ-tocopherol were the most abundant within their groups of molecules. *M. officinalis* decoctions were active against a wide range of microorganisms, *Pseudomonas aeruginosa* and *Salmonella typhimurium*, and *Penicillium funiculosum* being the most sensitive bacteria and fungi, respectively. The growth inhibition of

different human tumor cell lines (mainly MCF-7 and HepG2) was also observed, as also high free radical scavenging activity and reducing power. This manuscript highlights some beneficial effects of these functional beverages.

Article 4: Traditional pastry with chestnut flowers as natural ingredients: An approach of the effects on nutritional value and chemical composition

Carocho, M., Barreira, J.C.M., Barros, L., Bento, A., Cámara, M., Morales, P., Ferreira, I.C.F.R. (2015). *Journal of Food Composition and Analysis*, 44, 93-101.

DOI: http://dx.doi.org/10.1016/j.jfca.2015.08.003

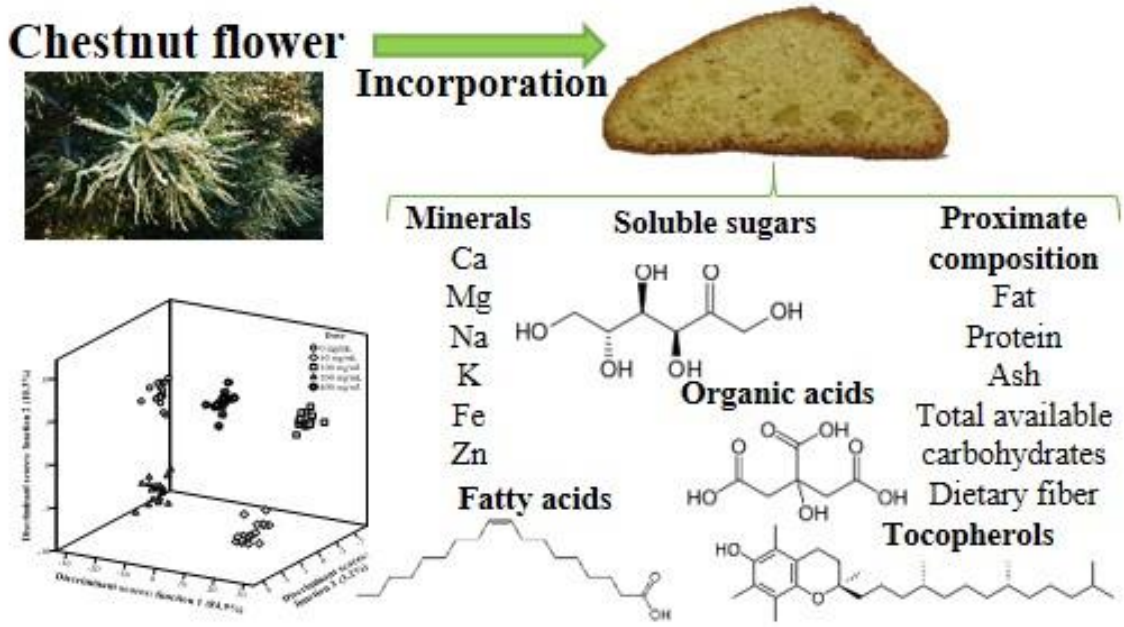

Figure 28 Graphical abstract article 4

Abstract

Portuguese traditional pastry known as económicos with satiating qualities have been elaborated with chestnut (*Castanea sativa* Mill.) flowers and their decoctions. The complete nutritional profile, mineral content, free sugars, organic and fatty acids, and tocopherols were determined immediately after baking and also after 15 and 30 days of storage. The results were processed through a 2-way ANOVA, followed by a linear discriminant analysis, to conclude that only slight effects were detected even in the assayed parameters, after 30 days. The amount of water decreased with time, resulting in a raise of ash, carbohydrates, energy and insoluble fiber over time. In terms of organic acids, succinic acid was the most abundant molecule, with

the samples incorporated with the decoction showing the highest amounts of these acids. Sucrose was the highest sugar, although a decrease was detected overtime. Sodium and potassium were the most abundant minerals while zinc was the least. Finally, α-tocopherol was the most abundant isoform of tocopherols and palmitic acid the most abundant fatty acid. Polyunsaturated fatty acids tended to decrease along storage time. The use of dried flowers seemed to better preserve the original profile (control) of the "económicos" in comparison with the decoctions.

Article 5: Chestnut flowers as functionalizing agents to enhance the antioxidant properties of highly appreciated traditional pastry

Carocho, M., Barreira, J.C.M., Bento, A., Morales, P., Ferreira, I.C.F.R. (2014). *Food & Function*, 5, 2989-2995.

DOI: http://dx.doi.org/10.1039/c4fo00552j

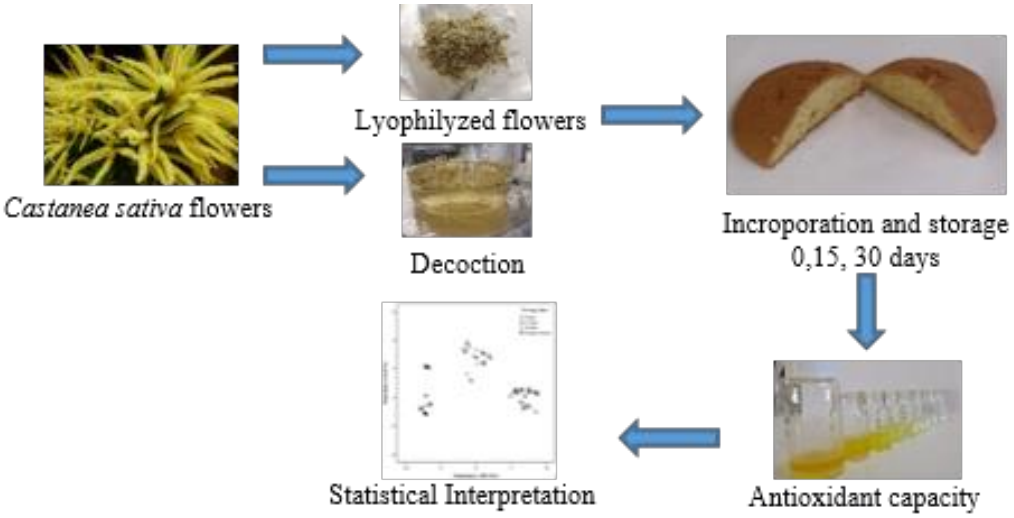

Figure 29 Graphical abstract article 5

Abstract

Some studies have proven the antioxidant and antimicrobial potency of chestnut flowers both in the raw matrix and after extraction, and the consumption of their decoctions has been related to beneficial effects towards health. In recent years, due to controversy and ambiguous legislation of chemical conservatives, plant extracts have been successfully used as functionalizing agents in different matrixes by displaying their various beneficial effects towards the foodstuff and/or the consumer. In this paper, decoctions of chestnut flowers as well as the dried flower were added to Portuguese traditional cakes that were then stored for 15 and 30 days, after which they were analysed for their antioxidant potential. The results were analysed by means of a 2-way ANOVA and a linear discriminant analysis, concluding that

storage time had a slightly higher influence on alteration of the antioxidant activity. DPPH and TBARS were the most improved parameters, regardless of the concentration added.

Article 6: Chestnut and lemon balm based ingredients as natural preserving agents of the nutritional profile in matured "Serra da Estrela" cheese

Carocho, M., Barreira, J.C.M., Bento, A., Fernández-Ruiz, V., Morales, P., Ferreira, I.C.F.R. (2016). *Food Chemistry*, 204, 185-193.

DOI: http://dx.doi.org/10.1016/j.foodchem.2016.02.136

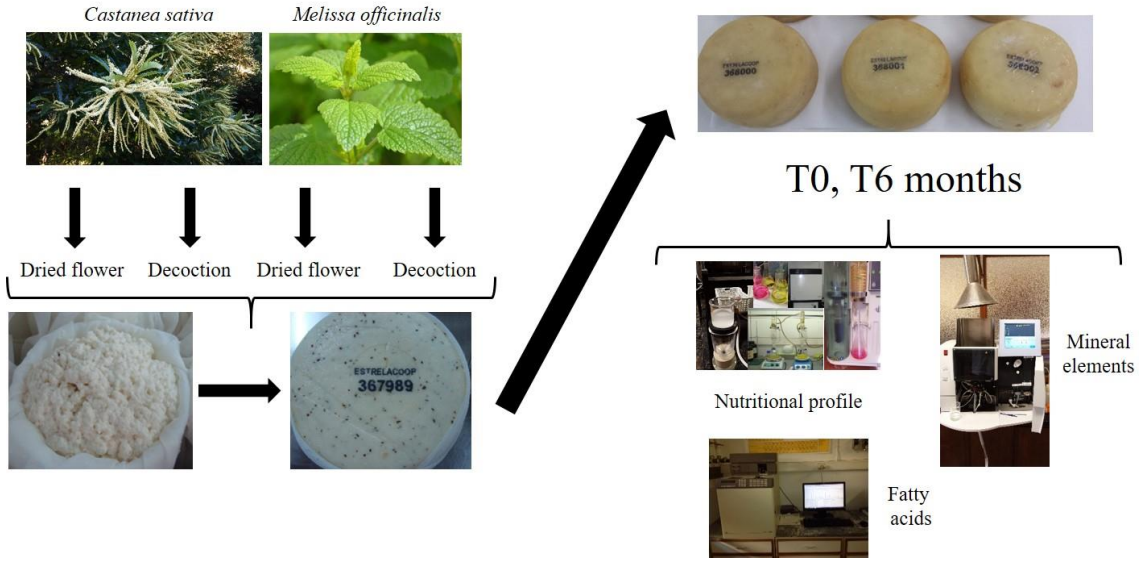

Figure 30 Graphical abstract article 6

Abstract

Chestnut flowers and lemon balm plants and their decoctions were incorporated into "Serra da Estrela" cheese, to assess their potential to preserve its nutritional properties and provide new foodstuffs. The analyses were carried out after the normal ripening period of 1 month and after 6 months of storage. The most abundant nutrients were proteins and fat. The most abundant minerals were Ca and Na, while C16:0 and C18:1 were the main fatty acids. Saturated fatty acids were the most abundant, followed by the monounsaturated. Moisture seemed to be lower in the samples incorporated with the plants. The dried plants when incorporated seemed to be more efficient as preservers then the decoctions, although these better preserved the proteins.

These plants can be regarded as promising natural preservers in foodstuffs cheese, given the preservation of key parameters and the slight impact on the nutritional value.

Article 7: **The incorporation of plant materials in "Serra da Estrela" cheese** improves antioxidant activity without changing the fatty acid profile and visual appearance

Carocho, M., Barreira, J.C.M., Antonio, A.L., Bento, A., Morales, P., Ferreira, I.C.F.R. (2015). *European Journal of Lipid Science and Technology*, 117, 1607-1614.

DOI: http://dx.doi.org/10.1002/ejlt.201500018

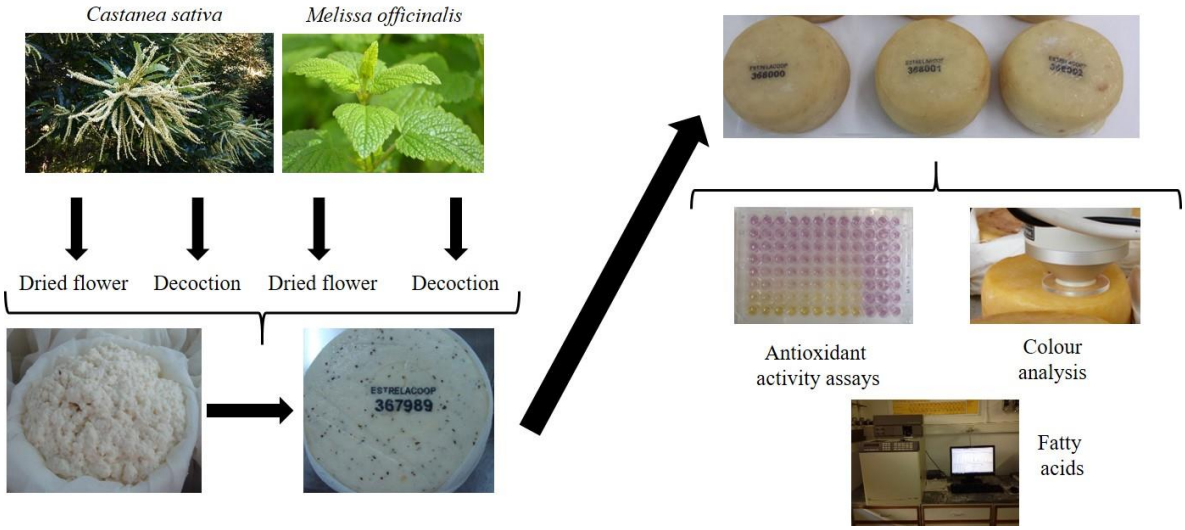

Figure 31 Graphical abstract article 7

Abstract

'Serra da Estrela' cheese is a Portuguese delicacy, which has been produced for centuries from the milk of cattle pasturing in the protected Serra da Estrela natural park. Transforming this cheese into a functional food would be a huge benefit for the market and the consumer. Decocted extracts, dried chestnut flowers and lemon balm plants were incorporated into the cheese to functionalise it, granting antioxidant activity to this foodstuff. The functionalised cheeses showed higher antioxidant activity, especially lipid peroxidation inhibition. The incorporation of dried plants appeared to be more effective than using decoctions, but the influence of the plant species was less observable. Furthermore, the fatty acids profile of the cheeses was also determined through gas chromatography. C18:1 and C16:0 were the most

abundant fatty acids; saturated fatty acids prevailed over the unsaturated ones. Between the control and the incorporated samples no significant differences were found. In addition, the external colour was measured through a spectrophotometer for lightness, yellowness and redness. There were some differences recorded for each samples' colour. In general, the results indicated that the functionalisation of this exquisite dairy product with natural plant extracts provided beneficial characteristics, both for consumers (healthier product) and producers (added-value products).

Article 8: **Basil as functional and preserving ingredient in "Serra da Estrela" cheese**

Carocho, M., Barros, L., Barreira, J.C.M., Calhelha, R.C., Soković, Fernández-Ruiz, V., Santos-Buelga, C., Morales, P., Ferreira, I.C.F.R. (2016). Basil as a functional and preserving ingredient in "Serra da Estrela" cheese. (Submitted to Food Chemistry)

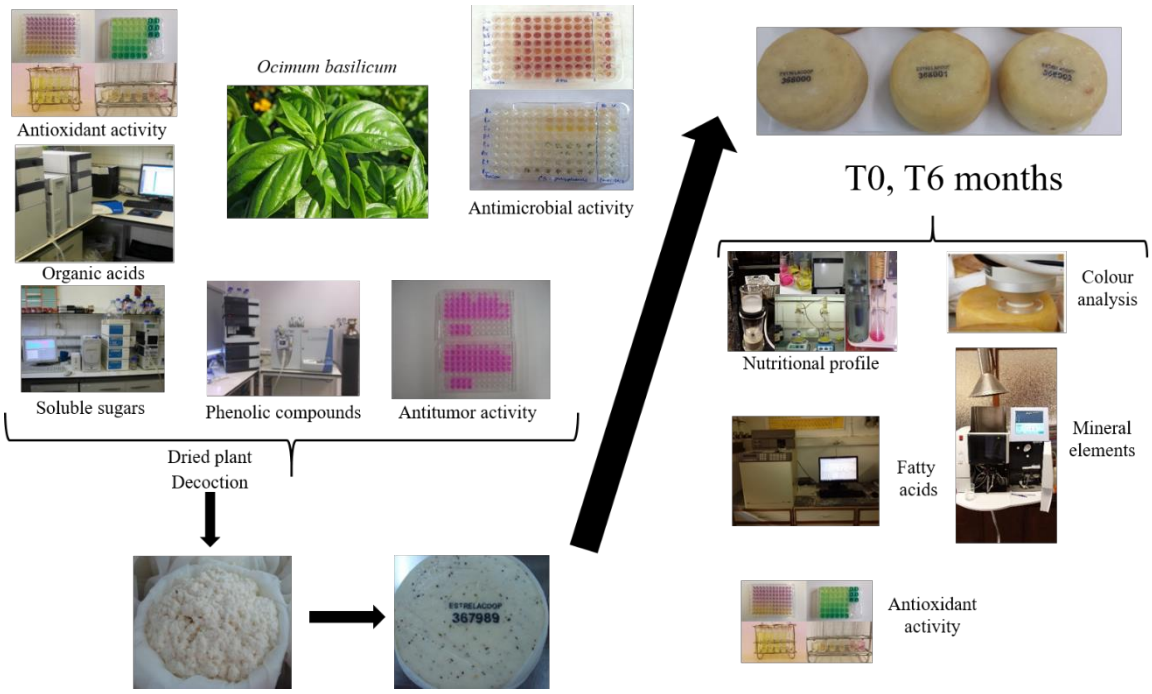

Figure 32 Graphical abstract article 8

Abstract

Antitumor, antimicrobial and antioxidant activities of basil were studied, along with its characterization in phenolic compounds, organic acids and soluble sugars. The results placed basil as a valuable candidate for functionalization and conservation of food products, maintaining their nutritional properties, while increasing their shelf life and potential health effects. Herein, basil leaves were incorporated in the high-valued "Serra da Estrela Cheese", either in their dehydrated form or as a decoction. The cheeses were then subject to a nutritional evaluation, being characterized for their fatty acids, minerals and CIE color parameters. To assess the combined effects of plant incorporation and storage time, a 2-way ANOVA was used

to process the results, further analysed through a linear discriminant analysis. Overall, basil leaves provided antioxidant activity to the cheeses, reduced the moisture, and preserved the unsaturated fatty acids and proteins. Comparing both incorporation types, the decoctions had a higher functionalizing and conservation effect.

Part VI. **INTEGRATIVE DISCUSSION**

Plant Samples

The plant extracts used in this work, chestnut flower infusions and decoctions, lemon balm and basil plant decoctions were analysed for their content in phenolic compounds, organic acids, and soluble sugars, as well as their bioactivities, namely antioxidant, antimicrobial and antitumor. In the next sections, the contents in each compounds and bioactivities are detailed for all these samples, and correspond to articles 1, 2, 3 and 8.

1.1. Phenolic compounds

Given the diversity of phenolic compounds found in each plant sample, no comparisons could be carried out for individual phenolic compounds, therefore, only the total flavonoids and total phenolic compounds of all plant extractions were compared (Table 14).

Table 14. Total flavonoids and total phenolic compounds in all samples, represented in mg/g of lyophilized extract

	Total flavonoids	Total phenolic compounds
Decoction of "Judia" chestnut flower (JD)	14.26±0.14^{e}	68.04±0.18^{b}
Decoction of "Longal" chestnut flower (LD)	8.38±0.01^{c}	69.88±0.81^{cb}
Infusion of "Judia" chestnut flower (JI)	12.94±0.17^{d}	72.20±0.14^{c}
Infusion of "Longal" chestnut flower (LI)	7.79±0.35^{b}	68.10±1.52^{b}
Decoction of lemon balm (*Melissa officinalis*) (MD)	nd	223±1^{d}
Decoction of basil (*Ocimum basilicum*) (OD)	4.21±0.05^{a}	62.46±1.13^{a}

In each column, different letters mean statistical significant differences among the samples. Significance level was set at $p<0.05$

Upon interpreting the results present on Table 14, it is clear that the highest amount of flavonoids was detected in the decoction of "Judia" chestnut flowers (JD) and the lowest for the decoctions of basil (OD). Furthermore, the decoction of lemon balm (MD) did not display any flavonoid and that the cultivar "Judia" had the highest amount of flavonoids regardless of the extraction method. In terms of total phenolic compounds, the MD showed the highest amounts, and the OD the least. Even among the chestnut cultivars, significant differences were only obtained for the infusions of "Judia" chestnut flowers (JI), that displayed a higher amount

when compared to the other flowers decoctions and infusion. The most abundant phenolic compounds in the chestnut flowers were trigalloyl-HHDP-glucoside, pedunculagin isomer (bis-HHDP-glucoside), and pentagalloyl glucose (article 1 of part V). For lemon balm the most abundant ones were *trans*-rosmarinic acid, lithospermic acid A isomer, and sagerinic acid (article 3 of part V). Finally, for basil, the most abundant phenolic compounds were rosmarinic acid, chicoric acid and yunnaneic acid E derivative (article 8 of part V).

1.2. Organic acids

The organic acids characterized in the plant samples are displayed on Table 15. Regarding the organic acids present in the analyzed plant extracts, overall the OD had the highest amounts for all the different compounds. Succinic and fumaric acids were only detected in the MD, as citric acid, which was also detected in OD, with the highest values. The least amounts of oxalic acid were detected in the MD, although the decoctions did not show differences ($p<0.05$). Regarding quinic acid, the lowest amounts were detected for the decoctions of "Longal" chestnut flowers (LD), but for this compound, there were no statistical differences for the infusions. Lemon balm decoctions showed the second largest concentration of this compound. Malic acid had the lowest amounts for the MD, but among the infusions there were no statistical differences, as well as among the decoctions of the chestnut flower cultivars. Finally, for shikinic acid, the least contents were detected in the chestnut flowers, but the IJ, which did not present differences with the MD.

Table 15. Organic acids in all samples, represented in mg/g of lyophilized extract

	Oxalic	Quinic	Malic	Shikinic	Citric	Succinic	Fumaric
JD	72.91±1.82^{d}	84.61±0.64^{c}	27.17±1.36^{c}	1.83±0.02^{b}	nd	nd	nd
LD	71.85±0.52^{d}	52.59±3.14^{a}	22.94±0.10^{cba}	1.26±0.08^{a}	nd	nd	nd
JI	43.42±0.75^{b}	69.04±2.81^{b}	25.03±0.52^{cb}	1.35±0.02^{a}	nd	nd	nd
LI	55.84±1.82^{c}	61.63±4.40^{b}	20.82±0.70^{ba}	1.35±0.07^{a}	nd	nd	nd
MD	11.84±0.1^{a}	96.9±0.2^{d}	18.84±0.1^{a}	1.8±0.01^{b}	24.1±0.1^{a}	26±1	0.032±0.001
OD	98.6±4.5^{e}	179.91±4.8^{e}	54.33±3.2^{d}	2.92±0.03^{c}	38.3±1.3^{b}	nd	nd

In each column, different letters mean statistical significant differences among the samples. Significance level was set at $p<0.05$

1.3. Soluble sugars

The soluble sugars, analysed for all plant extracts, are present in Table 16. Trehalose was only detected for MD, which also had no sucrose. Basil decoction (OD) was the extraction with the least overall amount of soluble sugars. Regarding fructose, the highest amounts were detected for JD, being overall higher in the chestnut flowers, although no evident statistical difference ($p<0.05$) was observed. Glucose was highest in the decoction of the chestnut flowers, this time for the cultivar "Longal" (JD). Once again, the chestnut flowers proved to have the highest amounts. Finally, sucrose was detected in a higher content in the JI, with no statistical differences allowed between the decoctions of the chestnuts flowers and the infusions of "Longal" chestnut flowers (LI).

Table 16. Soluble sugars in all samples, represented in mg/g of lyophilized extract

	Fructose	Glucose	Sucrose	Trehalose
JD	160.41 ± 0.01^{e}	149.09 ± 0.04^{cb}	27.01 ± 0.45^{b}	nd
LD	152.08 ± 6.52^{ed}	191.91 ± 7.35^{d}	25.69 ± 0.99^{b}	nd
JI	148.94 ± 4.60^{d}	145.71 ± 5.63^{dc}	35.68 ± 1.45^{c}	nd
LI	123.58 ± 1.76^{c}	164.07 ± 2.31^{dc}	26.67 ± 0.5^{b}	nd
MD	49 ± 4^{b}	47 ± 1^{ba}	nd	198±0.2
OD	12.1 ± 1.1^{a}	5.8 ± 0.4^{a}	11.7 ± 0.4^{a}	nd

In each column, different letters mean statistical significant differences among the samples. Significance level was set at $p<0.05$

1.4. Antioxidant activity

The antioxidant activity for all the samples is present in Table 17. The antioxidant capacity recorded for all plant extraction revealed that for the DPPH scavenging assay, the best result was observed in the MD, and the worst, for this method, was the OD. Between the decoctions of the chestnut flowers, no statistical difference could be achieved ($p<0.05$), neither for their infusions. In terms of the RP assay, once again, the MD was the most active extract, followed by both the decoctions of the chestnut flowers, while the least antioxidant extracts were the infusions. In terms of lipid peroxidation inhibition, for the β-C bleaching inhibition assay, the best result was observed for JD extract, and given the poor activity of both the MD and OD, all

the chestnut extracts were better. The same happened for the TBARS assay. Overall, the chestnut flower extracts proved to be better antioxidants than the lemon balm and basil extracts.

Table 17. Antioxidant activity for all samples presented in EC_{50} values (μg/mL of decoction or infusion)

	DPPH	RP	β-C	TBARS
JD	99 ± 6^{b}	68 ± 1^{b}	48 ± 2^{a}	39 ± 1^{a}
LD	100 ± 10^{b}	76 ± 1^{c}	185 ± 1^{a}	49 ± 1^{a}
JI	126 ± 5^{c}	91 ± 1^{e}	177 ± 4^{a}	15 ± 2^{a}
LI	133 ± 5^{dc}	99 ± 1^{f}	195 ± 1^{a}	19 ± 3^{a}
MD	49 ± 5^{a}	35 ± 1^{a}	6073 ± 348^{b}	271 ± 2^{b}
OD	144 ± 5^{d}	86 ± 1^{d}	7327 ± 72^{c}	40 ± 1^{a}

In each column, different letters mean statistical significant differences among the samples. Significance level was set at $p<0.05$

1.5. Antimicrobial activity

Figure 33 to Figure 36 represent the minimum inhibition concentration (MIC) for bacteria and fungi, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) for the chestnut flowers infusions and decoctions, as well as lemon balm and basil decoctions. By interpreting Figure 33, reports to the antibacterial MIC's, for *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus*, and *Listeria monocytogenes*, all but MD had lower concentrations, and better activity than the positive control ampicillin. Still, for *Staphylococcus aureus*, streptomycin positive control presented better results than all the analysed extracts, which was surpassed by JI for *Bacillus cereus*. In the *Micrococcus flavus* assay, JD, LD and JI proved to have lower MIC values that streptomycin, while for *Listeria monocytogenes* this occurred for OD. Interestingly, for *Pseudomonas aeruginosa*, all extracts proved better results than ampicillin, but worse than streptomycin. Regarding *Salmonella typhimurium* and *Escherichia coli*, the same behaviour as *Bacillus cereus* was obtained for ampicillin, although LD and MD were the only samples over the concentration of streptomycin in the *Salmonella*, while for *Escherichia coli*, all the chestnut flower samples proved to be better (lower MIC values) than streptomycin. For *Enterobacter cloacae*, all the extractions except MD were better that both positive controls. The sample which inhibited a higher number of bacteria, under both positive controls, was the infusion of "Judia" chestnut flower, which was the sample that also

showed the highest amounts of polyphenols and the high number of ellagitannins, could be correlated and have contributed to this good antibacterial activity.

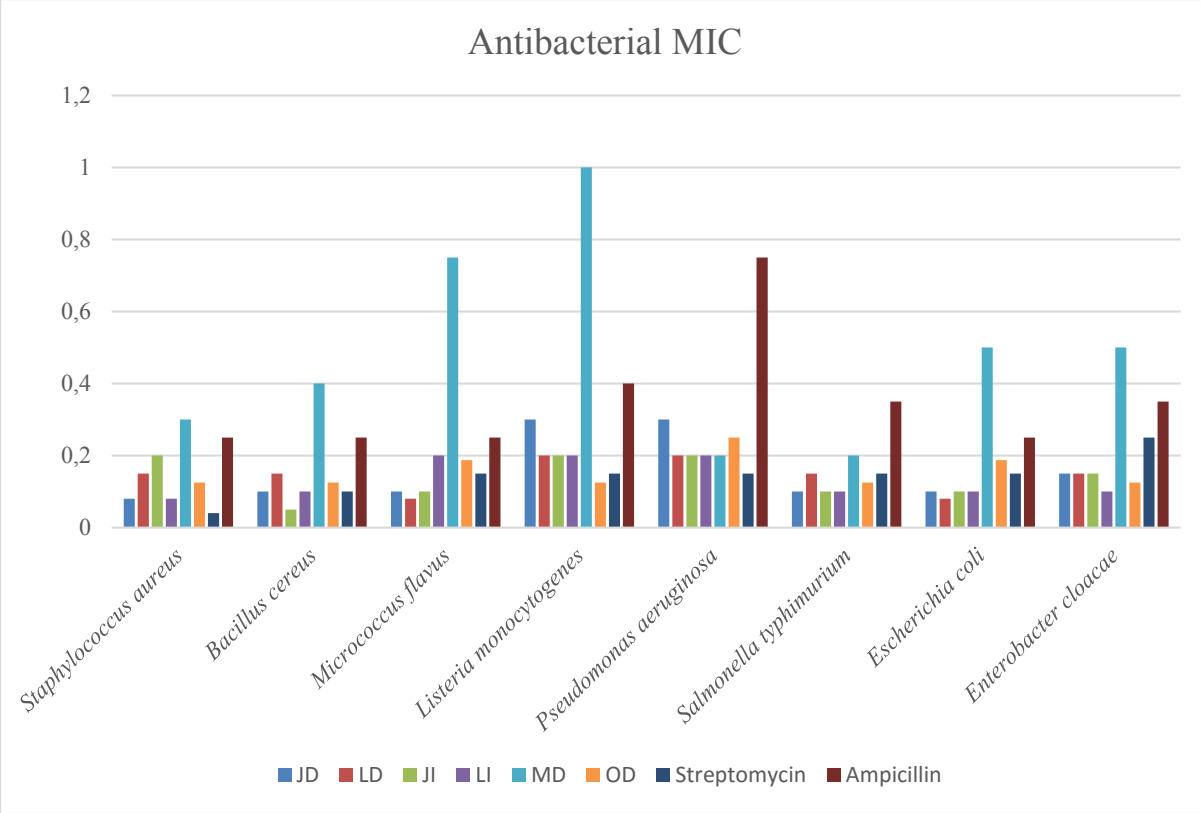

Figure 33. Antibacterial MIC's (mg/mL of extract)

Figure 34 represents the antibacterial MBC's; for *Staphylococcus aureus*, streptomycin had lower bactericidal concentrations than all the samples, but JD, LD, LL and OD had lower ones when compared to ampicillin. For *Bacillus cereus*, all plants but MD proved to be better than ampicillin, but only JI proved better than streptomycin. Regarding *Micrococcus flavus*, all extracts but LI and MD presented better results than both positive controls. For *Listeria monocytogenes*, all the chestnut samples and OD proved to be better bactericidal than ampicillin, while streptomycin had lower bactericidal activity than all the samples. For *Pseudomonas aeruginosa*, streptomycin was better than all the tested extracts, but ampicillin had higher concentrations than all the extracts. In *Salmonella typhimurium*, only MD was not better than the positive controls, this also occurred for *Escherichia coli* and *Enterobacter cloacae*. Once again the infusion of "Judia" chestnut showed lower bactericidal concentrations when compared to both positive controls.

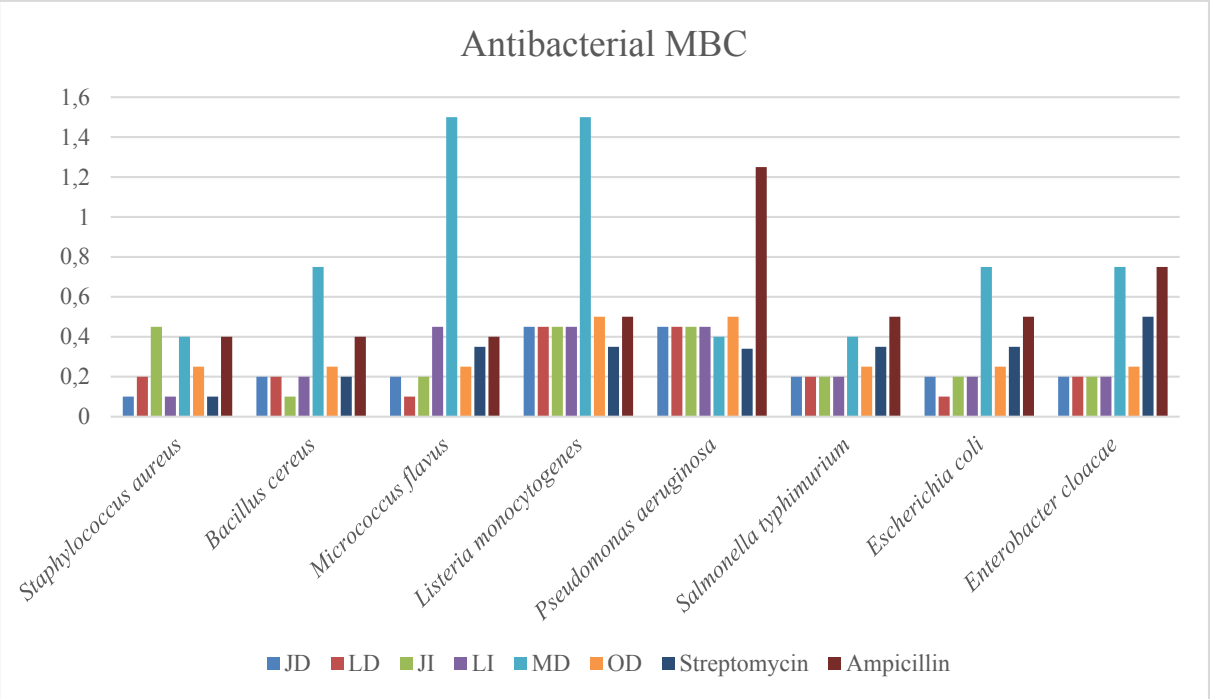

Figure 34 Antibacterial MBC's (mg/mL of extract)

In terms of the antifungal MIC's (Figure 35), *Aspergillus fumigatus* and *Aspergillus versicolor* showed the same behaviour, with both positive controls being better antifungals than the plant samples. In *Aspergillus ochraceus*, ketoconazole presented worse results than all plant samples evaluated, while only JI, LI and OD were better than bifonazole. For *Aspergillus niger*, LI and OD had lower inhibition concentrations than both positive controls. In *Trichoderma viride*, all the plant samples were better than ketoconazole, while only LD, JI, LI and OD were better antifungals than bifonazole. Regarding the *Penicillium* genera, *P. verrucosum* proved to be more sensible to the positive controls, while *P. ochrochloron* was more sensible to the plant samples than to ketoconazole, while only OD remained better than both positive controls. In *funiculosum*, all but OD were more effective than the positive controls. For the antifungal MIC's, the decoction of basil showed an inhibition under both positive controls for a higher number of different fungi species. This plant showed the highest quantities of organic acids, which are also known for their antioxidant and antimicrobial activity (Berić et al., 2008; Siddiqui, et al., 2012; Govindarajan et al., 2013; Flanigan & Niemeyer, 2014; Abassy et al., 2015), and could have contributed to this behaviour.

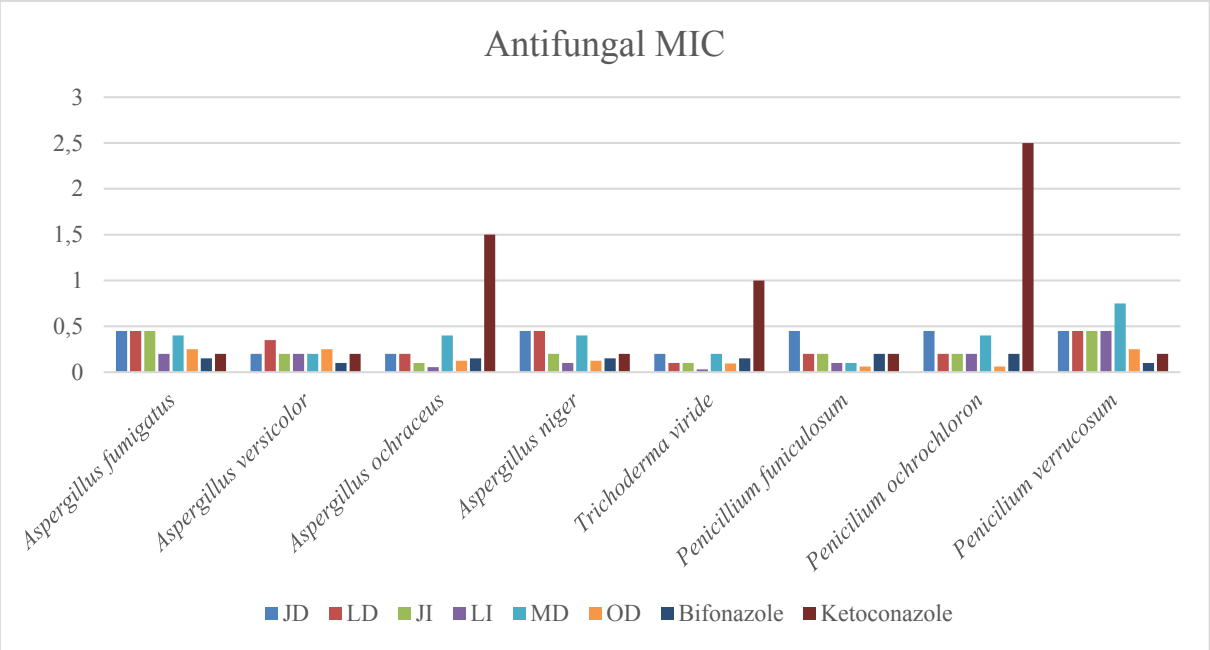

Figure 35 Antifungal MIC's (mg/mL of extract)

Figure 36 represents the antifungal MFC's; for *Aspergillus fumigatus*, both controls (bifonazole and ketoconazole) were better that the plant samples, while for the A. *versicolor* species, OD did not follow the plant tendencies and have a lower fungicidal control under the ketoconazole concentration, but in terms of bifonazole, all plant samples proved to be better. In terms of *Aspergillus ochraceus*, all plant samples were better than ketoconazole, but only LD, JI, and LI equalled the concentration of bifonazole. For the *Aspergillus niger* species, only LI extract managed to equal bifonazole, while LD, LI and OD remained under ketoconazole's concentration. In the *Trichoderma viride* assay, all plants proved better than ketoconazole in destroying this fungi, although only the decoctions of chestnut had lower concentrations than bifonazole. All but JD remained under ketoconazole for *Penicillium funiculosum*, but LD, LI and MD equalled bifonazole. In terms of *Penicillium ochrochloron*, all plant samples proved better than ketoconazole, but only OD was better than bifonazole. Finally, for *Penicillium verrucosum*, both the positive controls proved better than the plant samples analysed. Once again, the decoction of basil showed the highest fungicidal activity against the most fungi species.

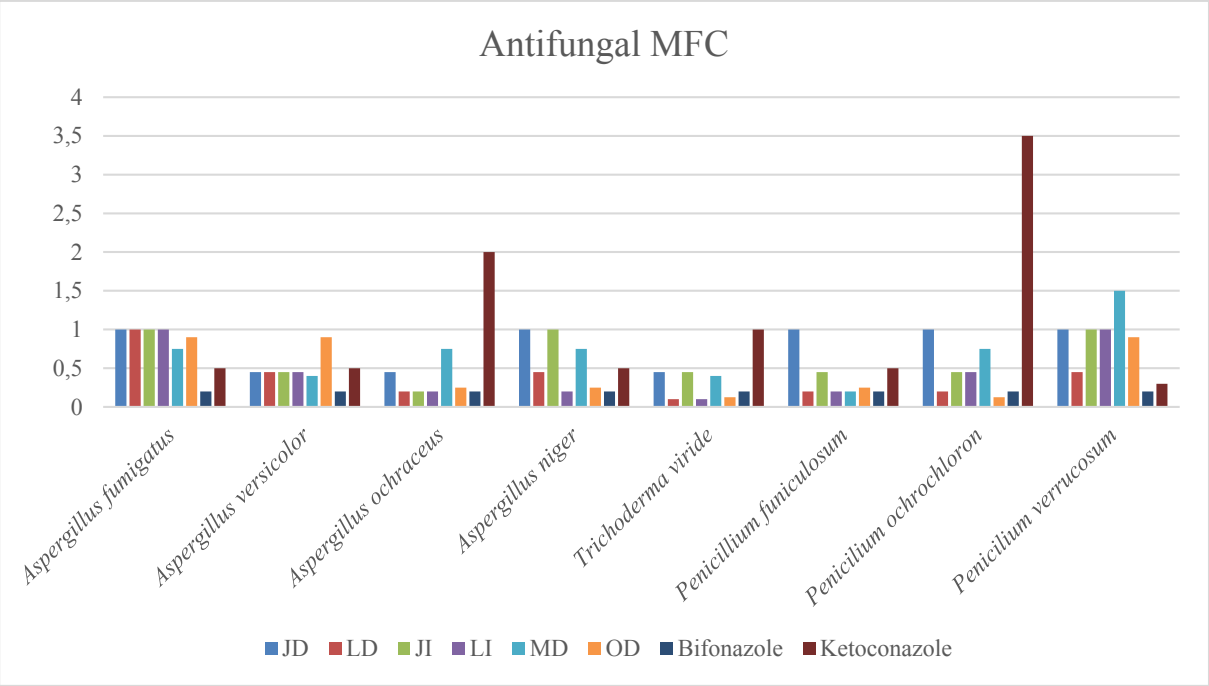

Figure 36 Antifungal MFC's (mg/mL of extract)

1.6. Antitumor activity

The results of the antitumor activity are displayed on Table 18, and its interpretation allows to understand that no plant was able to have a lower antitumor concentration then ellipticine, a potent antitumoral compounds, although it also displayed inhibition of porcine liver cell lines (PLP2) as can be seen on articles 2, 3 and 8 of Part V, with an inhibition between 2.06 and 2.29 µg/mL, corresponding to hepatotoxicity against normal cells. Thus, no plant extraction displayed inhibitory effect against PLP2, as can be seen in the same articles, placing them as non-hepatotoxic. Regarding the plant extracts (infusions and decoctions), it is also clear that the MD extract was the most effective against the majority of the tumor cell lines. Regarding MCF7 cell line, no statistical difference ($p<0.05$) was found for the chestnut flower extractions, being MD responsible for the lowest GI_{50} values. For HCT15 cell lines, only the chestnut flowers were analysed, but no statistical difference ($p<0.05$) was found between them. In the HeLa cell line, the chestnut flowers had no inhibitory effect, being the most effective, once again, the MD extract. Regarding HepG2, all plants and extractions methods had statistical differences, with lemon balm proving to be the most effective after ellipticine. The decoctions of lemon balm were better than all the chestnut flowers extracts. Finally, for NCI-H460, the chestnut extractions were not analysed, thus, the OD did not display any effects, and, once again lemon

balm being the most effective. Overall, lemon balm proved to be the best sample in all the tumor cell lines it was analysed. This could be correlated with the contents in phenolic compounds and the antioxidant activity, as observed for this plant, that showed the highest quantities in total polyphenols and the best antioxidant capacity in the DPPH and RP assays.

Table 18. GI_{50} values of the plant samples against tumor cell lines, represented in μg/mL

	MCF7	HCT15	HeLa	HepG2	NCI-H460
JD	293.14±4.15^{c}	259.85±13.79^{b}	>400^{d}	361.14±7.84^{f}	-
LD	289.75±6.80^{c}	296.54±26.47^{b}	>400^{d}	278.09±7.73^{d}	-
JI	287.95±6.80^{c}	276.84±17.10^{b}	>400^{d}	311.15±2.99^{e}	-
LI	292.64±0.81^{c}	292.00±11.79^{b}	>400^{d}	266.01±20.44^{d}	-
MD	51±4^{b}	-	155±10^{b}	67±2^{b}	258±17^{b}
OD	>400^{d}	-	254±5^{c}	225.74±5^{c}	>400^{c}
Ellipticine	0.91±0.04^{a}	1.91±0.06^{a}	1.14±0.21^{a}	3.22±0.67^{a}	0.91±0.11^{a}

In each column, different letters mean statistical significant differences among the samples. Significance level was set at $p<0.05$

"Económicos"

The "económicos" cakes were incorporated with the decoctions of the chestnut flowers as well as with the dried flowers in two concentrations each and analysed at three points in time, being the first, the day of manufacture, the second, after 15 days, and finally after 30 days of storage. In the next sections, the results of the antioxidant activity, nutritional value, mineral elements composition, fatty acids, tocopherols, organic acids and soluble sugars are presented, in line with articles 4 and 5 of Part V.

1.7. Nutritional profile

The nutritional profile for all the cakes samples in the three storage times is presented on Table 19. Given the very low quantity of moisture in the cake preparation, few changes were expected along the storage time, being the main objective to functionalize the cakes and not alter their nutritional properties. Regarding T0, the sample with the highest moisture was the control cake, being the lowest the cakes incorporated with the decoctions of chestnut flowers. Among F200 and F400 no statistical differences could be sought. In terms of proteins, the control sample

displayed the least, while the highest were recorded for D50, D100 and F200. In the total available carbohydrates (TAC), which were the overall most abundant nutrient in the cakes, only D100 was statistically different ($p<0.05$) from the other samples, displaying a higher quantity. Dietary fiber is a very important bioactive compound, which is essential for optimal digestive health, and can be important to reduce the risk of chronic diseases like cardiovascular illnesses, type 2 diabetes and obesity (Brownawell et al., 2012) was also present in both soluble and insoluble forms within the cakes. The EFSA (2010) recommends an intake of about 25g/day of dietary fiber in healthy adults, and the cakes, which weigh on average about 80 to 100 g each can confer nearly half the amount of that that bioactive compound, especially after the 30 days of storage. The plant incorporated cakes (F200 and F400) seem to be richer in total dietary fiber in all the storage times. Furthermore, following the same organization, the cakes could be defined as high in fiber, given they have "at least 6 g of fiber per 100 g or at least 3 g of fiber per 100 kcal" (Council Regulation (EC) 1924/2006).

For T15, the sample with the least moisture was D100, and the ones with the highest were F200 and F400. For the energy value at this storage time, the least amount was recorded for D100 and F400, while all the other had a higher quantity, but not significantly different between them. For the final storage period, T30, the lowest moisture was recorded for both cakes incorporated with the decoctions, and the highest, once again for the dried flower incorporated cakes, proving that the dried plant incorporations could have preserved the moisture content in the samples, therefore maintaining the "spongy-like" texture of the cakes through the storage time. No differences were recorded among the insoluble dietary fibers for all the samples. The lowest energy value was recorded for the control sample, while the highest was detected for D50. Regarding the variations among the storage times, fat, total available carbohydrates and total dietary fibers presented statistical variations ($p<0.05$), but not relevant for the study, and no general tendencies could be drawn. Moisture had no statistical difference for all samples from T0 to T15, but a statistical ($p<0.05$) one from T15 to T30. Soluble dietary fibers decreased over time for all the samples, although with very few effects on the total dietary fiber quantity. Finally, the energy value had no statistical variations among the storage times for the control samples, while the incorporated ones had some little variations. Overall, as desired, no major changes were induced by either incorporations, although, to preserve the moisture in the cakes, which is desirable for them not to become excessively dry during the stored time, the dried flower incorporations seemed to be the most suitable choice.

Table 19. Nutritional value of the cakes samples across the different storage times, represented in g/100g of fw

	Moisture	Fat	Protein	Ash	Total available carbohydrates	Total dietary fiber	Insoluble dietary fiber	Soluble dietary fiber	Energy kcal/100g
					T0				
C	18.63±0.4c, B	6.61±0.05b, B	13.54±0.09a, B	2.13±0.02c, B	47.88±3.5a, A	9.53±0.5ba, A	7.5±0.5a, B	2.1±0.2b, A	324.22±15.1ba, A
D50	14.79±0.4ba, B	5.41±0.06a, B	14.86±0.06c, C	2.16±0.2c, B	48.54±1.9a, A	9.43±0.2a, B	6.83±0.2a, B	2.58±0.07c, C	321.2±3.9a, A
D100	14.22±0.5a, B	5.45±0.06a, B	15.06±0.01c, C	2.02±0.2cb, A	52.01±1.9b, B	9.6±0.6ba, A	8.22±0.4b, A	1.81±0.09a, B	336.58±6.5b, B
F200	15.98±1.1b, B	7.24±0.1d, B	14.81±0.2c, C	1.78±0.2a, A	50.32±0.4ba, B	12.27±0.7c, A	8.95±0.09c, A	2.79±0.03d, B	350.23±1.6c, B
F400	16.16±1.7b, B	6.97±0.1c, B	14.34±0.4b, B	1.84±0.04ba, A	48.38±0.7a, B	10.41±0.6b, A	6.98±0.5a, A	3.44±0.09e, C	334.41±5.2ba, A
					T15				
C	18.07±0.4d, B	7.18±0.04d, C	13.97±0.4b, B	2.04±0.04a, A	46.56±0.2ba, A	9.43±0.7b, A	5.46±0.5a, A	3.61±0.2d, C	325.58±2.6b, A
D50	15.39±0.1b, B	4.91±0.04a, A	14.14±0.1cb, B	2.09±0.04a, A	51.91±2.1b, B	8.05±0.9a, A	5.17±0.1a, A	2.24±0.03b, B	324.48±8.5b, A
D100	14.65±0.09a, B	5.22±0.07b, A	14.5±0.1c, B	2.2±0.03b, B	40.91±9.4a, A	12.88±0.13d, C	10.89±0.1b, B	2±0.04a, B	294.37±37.8a, A
F200	16.52±0.3c, B	7.65±0.1e, C	12.24±0.2a, A	2.23±0.01b, B	46.92±0.7ba, A	12.79±0.3d, A	9.72±0.3a, BA	3.12±0.2c, C	331.06±4b, A
F400	16.63±0.09c, B	6.75±0.02c, A	14.25±0.1cb, B	2.32±0.05c, B	45.61±1.9ba, A	11.47±0.5c, B	9.22±0.5a, B	2.25±0.02d, A	323.19±7.2a, B
					T30				
C	12.8±0.3b, A	5.44±0.04a, A	12.93±0.2b, A	2.37±0.02b, C	51.6±1b, B	13.57±0.2b, B	10.95±0.2[a], C	2.58±0.2c, B	334.24±3.9a, A
D50	10.87±0.9a, A	5.53±0.06ba, B	12.4±0.5a, A	2.51±0.03c, C	55.87±0.9d, C	12.5±0.4a, C	10.49±0.4[a], C	2±0.03b, A	347.8±2.6c, B
D100	10.59±0.5a, A	5.63±0.06b, C	13.2±0.2b, A	2.49±0.02c, C	53.93±0.6c, B	12.22±0.1a, B	10.84±0.1[a], B	1±0.4a, A	343.84±2.6cb, B
F200	12.71±1.15b, A	7.03±0.07d, A	12.94±0.2b, B	2.35±0.07ba, B	49.56±1a, B	12.4±0.9a, A	10.73±1.1[a], A	1.22±0.08a, A	338.05±7ba, A
F400	13±0.8b, A	6.67±0.1c, A	12.84±0.1ba, A	2.3±0.04a, B	50.8±0.6ba, C	12.6±0.2a, C	10±0.1[a], C	2.64±0.2c, B	340±3.5ba, A

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

1.8. Mineral composition

The mineral content for all the cake samples is presented on Table 20. Four macro elements (Ca, Mg, Na and K) and two micro elements (Fe and Zn) were detected, being clear that the major mineral element is Na, and the least abundant is Zn. In terms of mineral elements intake, these values vary from each one. According to EFSA (2006), Ca has an admissible intake of 900 to 1200 mg/day, while Mg has differences between men and woman; men should consume between 400 and 420, while woman should only consume 310 to 320 mg/day. In terms of Na, the admissible quantity varies between 1.5 and 2 g/day. K and Fe vary between 3.1 to 3.5 g/day and 8 to 10 mg/day, respectively. Finally, Zn has an admissible amount of 9.5 mg/day for men and 7 mg/day for women. Given the profile in mineral elements of the cakes, and the fact that one cake weighs about 100 g, the consumed minerals are generally under 10% of the daily intake.

Regarding the comparison among samples for T0, no interesting trends could be found, although F400 was the highest for all samples, apart from Fe and Zn, in which D50 had the highest amount. Furthermore, regarding T15, the cake sample with the highest amount of each mineral was D50, except for Fe. For T30, the highest sample in each mineral was either D50 or D100, except for Ca, in which F400 was the highest. Regarding the comparison of each sample over time, there was an increase in Ca. For all the other minerals, significant variations occurred along the storage period, but no trends or behaviours worthy of remark took place. The ambiguous behaviour over time of the minerals could be due to the uneven distribution in the batter, for they were not affected in a great manner with the incorporations, which was desired.

In terms of the admissible daily intake for each mineral, the consumption of 100 g of the cakes with the highest quantity of each mineral represents 3.22% for Ca, 4.66% for Mg, 53.2% for Na, 4.75% for K, 31% for Fe, and 12% for Zn. Only Na has a high percentage in 100 g of the cakes, whilst the other mineral elements are far from the daily admissible dose. Thus, an excessive consumption of these cakes is not advisable for people with hypertension problems. EFSA (2005) estimates the daily intake of sodium between 3 to 5 g daily, when the dietary needs are fixed at 1.5 g.

Table 20. Mineral composition of the cake samples, presented in mg/100 fw, across the storage times

	Ca	Mg	Na	K	Fe	Zn
			T0			
C	5.33±0.07$^{a, A}$	15.26±0.8$^{b, B}$	656.73±3.9$^{b, A}$	110.32±4.8$^{b, A}$	2.2±0.1$^{a, B}$	0.16±0.01^{a}
D50	5.29±0.4$^{a, A}$	12.14±0.4$^{a, A}$	613.38±35.3$^{a, A}$	140.17±5$^{d, B}$	1.89±0.06$^{c, B}$	0.37±0.01$^{b, B}$
D100	8.53±1.4$^{b, A}$	14.24±0.5$^{b, B}$	594.26±12.1$^{a, A}$	131.82±10.7$^{cd, B}$	1.12±0.3$^{b, A}$	nd
F200	5.39±0.2$^{a, A}$	11.42±1$^{a, A}$	594.98±16.0$^{a, A}$	78.33±8.9$^{a, A}$	0.47±0.3$^{a, A}$	0.43±0.04$^{c, B}$
F400	15.52±0.5$^{c, A}$	16.6±0.3$^{c, B}$	649.79±23.6$^{b, A}$	124.62±8$^{c, B}$	0.55±0.01$^{a, A}$	nd
			T15			
C	9.68±0.9$^{a, B}$	12.17±0.8$^{a, A}$	636.42±20.5$^{a, A}$	134.3±4.6$^{b, B}$	2.52±0.07$^{e, C}$	nd
D50	29.02±1$^{e, C}$	17.04±0.2$^{b, C}$	735.93±17.51$^{b, B}$	130.31±7.8$^{b, A}$	1.82±0.08$^{c, B}$	1.12±0.02$^{d, C}$
D100	21.12±1$^{b, B}$	17.59±1.1$^{b, A}$	778.85±21.5$^{c, C}$	111.52±1.1$^{a, A}$	1.48±0.1$^{b, B}$	0.34±0.02
F200	13.05±0.3$^{b, B}$	10.44±3.4$^{a, A}$	610.24±9.6$^{a, A}$	119.07±3.9$^{a, B}$	2.26±0.08$^{d, C}$	nd
F400	15.7±1.4$^{c, A}$	13.1±0.1$^{a, A}$	609.6±36.4$^{a, A}$	137.13±6.3$^{b, A}$	1.15±0.05$^{a, C}$	0.16±0.01$^{b, B}$
			T30			
C	18.09±0.7$^{a, C}$	16.22±0.4$^{b, B}$	707.91±36.7$^{a, B}$	139.04±1.5$^{ba, B}$	0.93±0.02$^{a, A}$	nd
D50	20.61±0.2b$^{c, B}$	14.93±1.4$^{a, B}$	719.28±54.2$^{a, B}$	142.44±1.6$^{b, B}$	1.71±0.02$^{b, A}$	0.2±0.01$^{c, A}$
D100	24.57±0.7$^{cd, C}$	18.64±0.4$^{c, A}$	733.548±10b$^{a, B}$	147.34±3.4$^{b, C}$	0.86±0.2$^{a, A}$	nd
F200	21.63±3.2$^{cb, C}$	16.39±0.22$^{b, B}$	687.06±66.3$^{a, B}$	129.71±10.9$^{a, B}$	0.96±0.1$^{a, B}$	nd
F400	26.23±2$^{d, B}$	17.28±0.2$^{b, C}$	798.8±28.8$^{b, B}$	130.19±3.6$^{a, AB}$	1.03±0.1$^{a, B}$	0.11±0.01$^{b, A}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

1.9. Fatty acids

Table 21 represents the major fatty acids profile for the different cheeses across the different storage times, in relative percentage. It is clear that the most abundant individual fatty acids are C18:1 and C18:2, and that the monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) largely exceed the saturated fatty acids (SFA). Other fatty acids were detected in the samples, but their contribution was insignificant to the overall profile (<1%). Analysing the samples for T0, for the saturated fatty acids, the cake with D50 decoctions showed the highest values, while the lowest were recorded for F400 and F200. For the unsaturated fatty acids, F400 was the highest, being the least abundant in the SFA. After 15 days of storage, once again F200

and F400 were the least for the saturated fatty acids and SFA, while being the highest for the unsaturated ones, as well as for MUFA and PUFA. In terms of T30 the same behaviour was recorded, only for the MUFA, F200 was the less abundant. Comparing each individual sample along the storage times, for palmitic acid (C16:0), there was a significant increase ($p<0.05$) for all samples from T15 to T30. In C18:0 the variations did not provide tendencies. For C18:1, C18:2, C18:3 there was a general decrease over time, which was expected as the lipid peroxidation reactions take place. In terms of SFA, the samples D50 and D100 rose from T0 to T15, but then maintained the values from T15 to T30, while F400 maintained the quantities from the first period to the second and then rose from the second to the third. In terms of MUFA, from T0 to T15 all but D100 raise in quantity, probably due to the breakdown of PUFA, but then from T15 to T30 the values decrease. Finally, for PUFA, D50 and D100 reduce their quantities in the first period and then maintain the quantities, with no significant statistical changes ($p<0.05$). The control sample sequentially decreases the amounts, while F200 maintains in the first period and then decreases. Interestingly F400 reduces the total PUFA relative percentage at first but then increases in the second period. Regarding other bioactive compounds involved in lipid peroxidation inhibition, mainly tocopherols and polyphenols due to their antioxidant capacity, the dried flower incorporations (F200 and F400) showed better preserving capacities, which was also observed in lipid peroxidation inhibition (β-C and TBARS). For the fatty acids, it seems that the decoctions (D50 and D100) showed a slightly better activity in avoiding rancidification, by maintaining the levels of PUFA after the 15 days, given their interlinked behaviour. Still, after 30 days F400 maintained the amount of MUFA.

Table 21. Main fatty acids of the cake samples, presented in relative percentage, across the three storage times

	C16:0	C18:0	C18:1	C18:2	C18:3	SFA	MUFA	PUFA
				T0				
C	9.3±0.1$^{a, A}$	1.38±0.05$^{a, B}$	59.46±0.1$^{d, B}$	19.49±0.02$^{d, C}$	6.29±0.02$^{c, B}$	12.67±0.1$^{b, B}$	61.29±0.1$^{c, B}$	26.03±0.01$^{d, C}$
D50	11.97±0.09$^{c, A}$	2±0.04$^{c, B}$	58.95±0.02$^{b, B}$	18.01±0.02$^{b, B}$	4.72±0.01$^{a, B}$	15.93±0.07$^{d, A}$	60.99±0.01$^{b, B}$	23.08±0.06$^{b, B}$
D100	12.77±0.2$^{d, A}$	1.96±0.2$^{bc, A}$	58.48±0.03$^{a, B}$	17.74±0.02$^{a, B}$	4.69±0.01$^{a, B}$	16.69±0.01$^{e, A}$	60.55±0.04$^{a, B}$	22.77±0.03$^{a, B}$
F200	10.04±0.04$^{b, B}$	1.47±0.5$^{ba, B}$	58.68±0.3$^{a, B}$	19.25±0.1$^{c, B}$	6.23±0.04$^{b, A}$	13.68±0.5$^{c, B}$	60.62±0.3$^{a, B}$	25.7±0.2$^{c, B}$
F400	9.22±0.03$^{a, A}$	1.37±0.4$^{a, B}$	59.23±0.1$^{d, A}$	19.62±0.2$^{d, C}$	6.47±0.06$^{d, B}$	12.24±0.2$^{a, A}$	61±0.2$^{b, A}$	26.7±0.02$^{e, C}$
				T15				
C	9.53±0.1$^{b, A}$	0.22±0.05$^{a, A}$	60.39±0.2$^{c, C}$	19.18±0.03$^{d, B}$	6.55±0.02$^{d, C}$	11.86±0.2$^{b, A}$	62.24±0.2$^{c, C}$	25.9±0.01$^{d, B}$
D50	12.91±0.3$^{d, C}$	1.51±0.3$^{b, A}$	56.51±0.1$^{b, A}$	17.9±0.08$^{b, A}$	4.73±0.01$^{b, B}$	16.53±0.01$^{c, B}$	61.4±0.03$^{b, C}$	22.88±0.01$^{b, A}$
D100	12.89±0.09$^{d, A}$	2.03±0.2$^{c, A}$	58.2±0.1$^{a, A}$	17.65±0.08$^{a, A}$	4.63±0.03$^{a, A}$	17.06±0.2$^{c, B}$	60.35±0.1$^{a, A}$	22.59±0.1$^{a, A}$
F200	8.41±0.05$^{a, A}$	0.17±0.01$^{a, A}$	61.28±0.1$^{e, C}$	19.43±0.04$^{e, BA}$	6.65±0.03$^{c, B}$	5.43±3$^{a, A}$	63.1±0.1$^{d, C}$	26.32±0.07$^{e, B}$
F400	9.98±0.2$^{c, B}$	0.13±0.03$^{a, A}$	60.98±0.2$^{d, B}$	18.73±0.04$^{c, A}$	6.02±0.01$^{c, A}$	12.11±0.2$^{b, A}$	62.89±0.1$^{d, B}$	25±0.05$^{c, A}$
				T30				
C	12.19±0.3$^{c, B}$	2.95±0.01$^{d, C}$	58.37±0.2$^{b, A}$	17.48±0.05$^{a, A}$	4.63±0.02$^{a, A}$	17.2±0.3$^{c, C}$	60.4±0.2$^{b, A}$	22.4±0.07$^{a, A}$
D50	12.35±0.08$^{c, B}$	2.22±0.06$^{c, B}$	58.46±0.08$^{b, A}$	17.84±0.04$^{a, A}$	4.69±0.01$^{a, A}$	16.62±0.1$^{c, B}$	60.54±0.09$^{b, A}$	22.84±0.06$^{a, A}$
D100	13.21±0.3$^{d, B}$	1.87±0.2$^{b, A}$	58.2±0.1$^{b, A}$	17.71±0.05$^{a, AB}$	4.63±0.01$^{a, A}$	17.03±0.03$^{c, B}$	60.3±0.09$^{b, A}$	22.67±0.05$^{a, AB}$
F200	10.85±0.8$^{b, C}$	1.83±0.2$^{b, B}$	56.57±1.7$^{a, A}$	19.92±0.7$^{c, B}$	6.29±0.2$^{c, A}$	14.85±0.7$^{b, B}$	58.67±1.6$^{a, A}$	22.48±0.9$^{b, A}$
F400	10±0.4$^{a, B}$	1.55±0.2$^{a, B}$	59.04±0.3$^{b, A}$	19.04±0.1$^{b, B}$	6.03±0.08$^{b, A}$	13.65±0.5$^{a, B}$	61.02±0.3$^{b, A}$	25.33±0.2$^{c, B}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

1.10. Organic acids

On Table 22, the composition in organic acids is displayed for all the cake samples, across the three storage times. It can be seen that the most abundant organic acid in the cakes was citric acid followed by succinic, probably due to the incorporation of the orange juice and zest.

Regarding T0, no statistical difference ($p<0.05$) was registered for oxalic acid, while for quinic acid, the sample D50 was the highest, and all the others did not show statistical differences. Malic and citric acid showed the same profile. The highest content of succinic acid was recorded for the control cakes. Moreover, for fumaric, no differences were found among the samples. For T15, oxalic and fumaric acid did not have any statistical differences among samples, this also occurred for fumaric acid. For succinic acid, the control cakes showed the highest quantity. In the final storage time, oxalic acid was lowest in F400, being the highest, F200. Quinic, malic and citric acids had similar profiles among the samples, with the control cakes the samples with least of these acids, and also F400 for citric. The highest quantities of these compounds was recorded for D50 and D100, and still, F200 for quinic acid. Succinic acid showed a higher quantity in D50 and the lowest for D100. Regarding each sample individually along the three storage times, for fumaric acid, control cakes, D100 and F400 did not show any changes during the storage times. This also happened in all the samples of quinic acid, while for malic and succinic acid all samples decreased over time. Finally, for fumaric acid, this compound only rose from T15 to T30 for the control cakes. Overall, neither the dried flowers or their decoctions seemed to have any significant impact on the organic acid profile. There is an established ratio of oxalic acid/Ca that should be below 2.5, which is difficult to reach in a normal diet, although due to some plant foods that are rich in this organic acid it could be achieved. Thus, a lethal dose of oxalic acid was established at 5 g/day. Oxalic acid is known to reduce the availability of dietary Ca and can also lead to the formation of kidney calculus (Guil et al., 1996). In all samples, this ratio was calculated and was always below 0.5, therefore, the consumption of these cakes is risk free in terms of kidney formation or bone decalcification due to oxalic acid consumption.

Table 22. Organic acids profile of the cake samples, presented in g/100 fw, across the storage times

	Oxalic acid	Quinic acid	Malic acid	Citric acid	Succinic acid	Fumaric acid
			T0			
C	0.07±0.02$^{a, A}$	0.19±0.05$^{ba, B}$	0.14±0.01$^{b, C}$	0.45±0.01$^{b, C}$	0.74±0.04$^{b, C}$	0.01±0.01$^{a, A}$
D50	0.08±0.02$^{a, B}$	0.25±0.04$^{b, A}$	0.13±0.01$^{b, B}$	0.13±0.06$^{b, B}$	0.56±0.03$^{a, C}$	0.01±0.01$^{a, A}$
D100	0.06±0.01$^{a, A}$	0.2±0.010.1$^{ba, B}$	0.13±0.01$^{b, B}$	0.44±0.01$^{b, B}$	0.59±0.06$^{a, B}$	0.01±0.01$^{a, A}$
F200	0.06±0.01$^{a, A}$	0.16±0.03$^{a, A}$	0.1±0.01$^{a, C}$	0.34±0.01$^{a, A}$	0.56±0.04$^{a, B}$	0.01±0.01$^{a, A}$
F400	0.05±0.02$^{a, A}$	0.17±0.04$^{a, A}$	0.1±0.01$^{a, B}$	0.35±0.04$^{a, B}$	0.56±0.04$^{a, B}$	0.01±0.01a$^{, A}$
			T15			
C	0.06±0.01$^{a, A}$	0.16±0.02$^{a, A}$	0.09±0.01$^{c, B}$	0.39±0.01$^{d, B}$	0.57±0.06$^{b, B}$	0.01±0.01$^{a, A}$
D50	0.05±0.01$^{a, A}$	0.21±0.02$^{b, A}$	0.08±0.01$^{b, A}$	0.36±0.01$^{c, A}$	0.44±0.04$^{a, B}$	0.01±0.01$^{a, A}$
D100	0.05±0.01$^{a, A}$	0.19±0.03$^{ba, BA}$	0.08±0.01$^{b, A}$	0.36±0.01$^{c, A}$	0.46±0.04$^{a, A}$	0.01±0.01$^{a, A}$
F200	0.05±0.01$^{a, A}$	0.18±0.02$^{ba, A}$	0.08±0.01$^{b, B}$	0.34±0.02$^{b, A}$	0.49±0.06b$^{a, BA}$	0.01±0.01$^{a, A}$
F400	0.04±0.01$^{a, A}$	0.17±0.02$^{ba, A}$	0.07±0.01$^{a, A}$	0.29±0.01$^{a, A}$	0.42±0.04$^{a, A}$	0.01±0.01$^{a, A}$
			T30			
C	0.08±0.01$^{bc, A}$	0.13±0.02$^{a, A}$	0.06±0.01$^{a, A}$	0.31±0.02$^{a, A}$	0.43±0.04$^{b, A}$	0.04±0.02$^{b, B}$
D50	0.09±0.01$^{dc, B}$	0.24±0.01$^{c, A}$	0.08±0.01$^{c, A}$	0.36±0.01$^{c, A}$	0.34±0.01$^{a, A}$	0.01±0.01$^{a, A}$
D100	0.06±0.01$^{ba, A}$	0.18±0.01$^{c, B}$	0.09±0.01$^{c, A}$	0.36±0.01$^{c, A}$	0.51±0.01$^{c, A}$	0.01±0.01$^{a, A}$
F200	0.1±0.01$^{d, B}$	0.17±0.03$^{c, A}$	0.07±0.01$^{b, A}$	0.33±0.01$^{b, A}$	0.44±0.05$^{b, B}$	0.01±0.01$^{a, A}$
F400	0.05±0.01$^{a, A}$	0.16±0.02$^{ba, A}$	0.07±0.01$^{b, A}$	0.31±0.01$^{a, A}$	0.45±0.05$^{bc, A}$	0.01±0.01$^{a, A}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

1.11. Tocopherols

Table 23 represents the tocopherol profile of the cakes across the three storage times. The major form of vitamin E present in plant tissues is α-tocopherol. Moreover, it has also been considered the most active form in humans due to a preferential absorption and distribution of this compound in the human body (Caretto et al., 2010). Furthermore, compared to α-tocopherol (100%), β-tocopherol would provide 50% of vitamin E activity, γ-tocopherol 10%, δ-tocopherol 3%, α-tocotrienol 30%, and β-tocotrienol 5% of vitamin E activity. The content of vitamin E was calculated based on Equation 7.

$$Vitamin\ E = \alpha - tocopherol + 0.5\beta - tocopherol + 0.1\gamma - tocopherol$$

Equation 7. Vitamin E equation

A deficiency of vitamin E results in a variety of pathological conditions that affect the muscular, cardiovascular (atherosclerosis, ischemic heart disease), reproductive, and central nervous systems (cerebella ataxia), as well as the liver, kidney, and red blood cells, and development of different types of cancer (Zingg & Azzi, 2004). To avoid these disorders, a daily intake between 1.5-10 mg/day (FAO/WHO) is advisable in the diet.

The main tocopherol found in the samples was α-tocopherol, being the least β-tocopherol. Regarding T0, the highest quantities of tocopherols were detected in the cakes incorporated with F400, while the least were found for D100 for all isoforms. D50 was also the least abundant for α- and γ-tocopherol. For T15 and T30, F200 was the cake with the highest content for all isoforms. For T30, the least detected was D100 for α-tocopherol, F400 for β-tocopherol and D50 and D100 for γ-tocopherol. It seems that once again, the dried flower incorporations seemed to be the best option to add these very strong antioxidants. In terms of the individual incorporations across the storage times, for α-tocopherol, all plants and plant extracts except F200 decreased their quantities. Then, from T15 to T30, this vitamin E isoform rose in all samples, but F400.

In terms of β-tocopherol, from T0 to T15 all reduced their amounts, only D100 maintained them, but then from T15 to T30 all rose. The same behaviour was recorded for γ-tocopherol, where D50, D100 and F400 decreased but control and F200 rose for the first interval, and all rose in the second time interval. With regard to the calculated vitamin E values (Table 23), throughout the whole storage time, the highest amounts of this vitamin were recorded for F200. Interestingly, the consumption of a cake portion of 100 g could provide 17.9 to 35.4% of the daily requirements of this vitamin, as in the case of the D50 (T15) and F200 (T30), respectively. Given the higher amounts in F200 and F400, the use of these extracts could be useful to functionalize this basic and daily used foodstuff in terms of vitamin E and tocopherol composition. Being helpful to cardiovascular disease prevention as well as to avoid cake rancidity over the storage time.

Table 23. Tocopherol composition of the cake samples, presented in mg/100 fw, across the storage times

	α-tocopherol	β-tocopherol	γ-tocopherol	Calculated Vitamin E
		T0		
C	2.42±0.08$^{b, B}$	0.19±0.01$^{c, B}$	1.42±0.07$^{b, A}$	2.66±0.09$^{b, A}$
D50	1.99±0.01$^{a, B}$	0.18±0.01$^{b, B}$	0.68±0.01$^{a, B}$	2.15±0.02$^{a, B}$
D100	2.01±0.01$^{a, C}$	0.16±0.01$^{a, A}$	0.72±0.02$^{a, C}$	2.15±0.01$^{a, B}$
F200	2.57±0.05$^{c, A}$	0.19±0.01$^{c, B}$	1.36±0.01$^{b, A}$	2.8±0.05$^{b, A}$
F400	2.81±0.1$^{d, B}$	0.33±0.01$^{d, C}$	1.54±0.04$^{c, C}$	3.13±0.1$^{c, B}$
		T15		
C	2.33±0.03$^{b, A}$	0.14±0.01$^{a, A}$	1.52±0.01$^{c, B}$	2.55±0.04$^{b, A}$
D50	1.66±0.01$^{a, A}$	0.16±0.01$^{b, A}$	0.54±0.02$^{a, A}$	1.79±0.02$^{a, A}$
D100	1.7±0.04$^{a, A}$	0.16±0.01$^{b, A}$	0.5±0.01$^{a, A}$	1.83±0.05$^{a, A}$
F200	2.51±0.01$^{c, A}$	0.18±0.01$^{c, A}$	1.61±0.02$^{d, B}$	2.76±0.02$^{c, A}$
F400	2.37±0.05$^{b, A}$	0.16±0.01$^{b, A}$	1.3±0.03$^{b, A}$	2.58±0.06$^{b, A}$
		T30		
C	2.73±0.01$^{d, C}$	0.2±0.01$^{b, C}$	1.86±0.01$^{c, C}$	3.02±0.01$^{c, B}$
D50	1.99±0.02$^{b, B}$	0.2±0.01$^{b, C}$	0.66±0.02$^{a, B}$	2.16±0.02$^{a, B}$
D100	1.95±0.01$^{a, B}$	0.19±0.01$^{a, B}$	0.68±0.01$^{a, B}$	2.11±0.02$^{a, B}$
F200	3.23±0.04$^{e, B}$	0.22±0.01$^{c, C}$	2.04±0.03$^{d, C}$	3.54±0.05$^{d, B}$
F400	2.44±0.02$^{c, A}$	0.18±0.01$^{a, B}$	1.41±0.02$^{b, B}$	2.66±0.01$^{b, A}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

1.12. Soluble sugars

The soluble sugars of the cake are presented on Table 24. As expected, fructose and glucose were not detected at T0, given that only sucrose was added to the batter. Their appearance after fifteen days of storage (T15) is due to the breakdown of sucrose into these monosaccharides, as detailed before (Cordenusi & Lajolo, 1995). For sucrose, the control sample showed the lowest values and all the other samples did not show significant differences among each other, although all of them were higher than the control. After 15 days of storage, the highest amounts of these sugars were detected in the control samples and in D50. For T30, interestingly, there

was an inversion of the contents, for F400 showed the higher amounts and F200 for fructose, while the control sample was lower for all of these compounds, although there were no statistical differences that could tell apart the control from the other samples for glucose. This could be due to Maillard reactions, in which the amide (NH_2) group of aminoacids react with the carboxylic acid (COOH) group of reducing sugars, originating melanoidins, brown pigments that are typical in oven cooked foodstuffs. Thus, antioxidant compounds could avoid this interaction between aminoacids and reducing sugars, avoiding their degradation (Belitz et al., 2009).

Regarding each individual sample across the three storage times, for fructose, D50, F200 and F400 raised during the storage, while C and D100 decreased. For glucose, F200 and F400 rose, the control sample lost this soluble sugar during the storage time, but D50 and D100 did not show differences during the storage. Finally, for sucrose, the content of this sugar rose in the control sample and for all the other it decreased from T0 to T15 and the rose from T15 to T30, probably due to breakdown of starch (Cordenusi & Lajolo, 1995).

Table 24. Soluble sugars profile of the cake samples, presented in g/100 fw, across the storage times

	Fructose	Glucose	Sucrose
		T0	
C	nd	nd	$26.45\pm0.2^{a, A}$
D50	nd	nd	$29.47\pm1^{b, B}$
D100	nd	nd	$28.16\pm0.2^{b, B}$
F200	nd	nd	$26.78\pm1^{a, B}$
F400	nd	nd	$26.3\pm0.5^{a, B}$
		T15	
C	$0.16\pm0.01^{e, B}$	$0.15\pm0.01^{c, B}$	$26.13\pm0.7^{c, A}$
D50	$0.1\pm0.01^{b, A}$	$0.09\pm0.01^{b, A}$	$25.97\pm0.3^{c, A}$
D100	$0.13\pm0.01^{d, B}$	$0.08\pm0.01^{b, A}$	$24.79\pm0.2^{b, A}$
F200	$0.12\pm0.01^{c, A}$	$0.08\pm0.01^{b, A}$	$24.16\pm0.1^{b, A}$
F400	$0.09\pm0.01^{a, A}$	$0.07\pm0.01^{a, A}$	$10\pm0.3^{a, A}$
		T30	
C	$0.1\pm0.01^{a, A}$	$0.09\pm0.01^{a, A}$	$28.84\pm0.2^{a, B}$
D50	$0.13\pm0.01^{b, B}$	$0.09\pm0.01^{a, A}$	$29.24\pm0.3^{a, B}$
D100	$0.12\pm0.01^{b, A}$	$0.09\pm0.01^{a, A}$	$31.9\pm0.2^{c, C}$
F200	$0.14\pm0.01^{c, B}$	$0.09\pm0.01^{a, B}$	$30.8\pm0.5^{b, C}$
F400	$0.14\pm0.01^{c, B}$	$0.13\pm0.01^{b, B}$	$32.89\pm0.3^{d, C}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

1.13. Total polyphenols

Table 25 represents the total polyphenols (reducing agents) in the cakes, carried out by the Folin-Ciocalteu assay. For T0, the highest quantity was detected for F200 and the control samples, while for T15 this was achieved in F400, followed by F200. For T30 the highest samples in phenols content was D100, followed by F200 and F400, being the lowest detected in the control samples and D50.

There were no differences among the control samples across the storage times ($p<0.05$), while D50 reduced their content and F400 increased. D50 and D100 had inverse behaviours regarding rising between two storage times and decreasing among the other. The dried flowers seemed to

be the best option for preserving the antioxidant activity in the cakes, in terms of total phenolic compounds.

Table 25. Quantification of the total polyphenols in the cakes, represented in GAE/g extract

	Total polyphenols
T0	
C	3.16±0.001$^{b, A}$
D50	4.04±0.001$^{d, B}$
D100	3.87±0.001$^{c, B}$
F200	2.29±0.04$^{a, A}$
F400	3.18±0.3$^{b, A}$
T15	
C	3.31±0.2$^{b, A}$
D50	2.16±0.02$^{a, A}$
D100	2.23±0.03$^{a, A}$
F200	5.92±0.1$^{c, C}$
F400	6.16±0.04$^{d, B}$
T30	
C	4.87±0.9$^{a, A}$
D50	4.48±0.5$^{a, A}$
D100	7.09±0.2$^{c, C}$
F200	5.08±0.1$^{a, B}$
F400	6.24±0.05$^{b, B}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

1.14. Antioxidant activity

On Table 26, the antioxidant capacity of the cakes incorporated with the chestnut flowers and their decoctions are presented. The same assays that were carried out for the plant characterization were also applied for the cakes. These assays were applied for the three storage times, to determine the differences in the antioxidant potential of the incorporated cakes. An

initial comparison was made between each assay for all samples, and then a comparison among the storage times for each sample in each assay.

Regarding the T0 storage time, the cakes incorporated with the dried flowers, namely F400 presented the best results for all assays, and the control samples had the worst behaviour, minus for the TBARS assay. For T15, the dried flower incorporated samples proved to present the better results in all performed assays, while F200 presented the better results for DPPH and TBARS assays, and F400 for DPPH and RP. Surprisingly, the control samples had the lowest EC_{50} values for β-C.

It was expected that the antioxidant capacity would decrease along the storage time due to oxidation of the antioxidants. However, after the 30 days of storage time, F400 had the highest antioxidant capacity evaluated by all the assays, followed by F200, that was not the second best for RP and TBARS. Regarding the comparisons for each assay between the samples along the storage time, for DPPH, F200 and F400 increased the EC_{50} value, which resulted in a lower antioxidant capacity, an expected outcome. No significant changes were recorded along the storage time for the control cakes, which had the least antioxidant capacity. D50 and D100 had a higher antioxidant capacity at T30 for the RP assay. In terms of the β-C assay, D100, F200 and F400 had the highest capacity at T30, with F400 not being significantly different from T0. There was a significant loss of antioxidant capacity from T15 to T30 for D50. The TBARS assay, proved no significant differences between T0 and T30 for F400 and the control samples, being only different ($p<0.05$) for T15. F200 decreased its antioxidant capacity along the storage time. The dried flowers incorporations seemed to be the best solution for preserving the antioxidant capacity in the cakes, especially for F400, which was the best solution for nearly all assays in the three storage times, being so also in the tocopherol assays. Furthermore, in terms of total polyphenols, after 15 and 30 days, these samples were also the ones with the highest content in total polyphenols, which could prove their activity as antioxidants. A general tendency could be established with the interpretation along the storage time, the effectiveness of the antioxidants is reduced, as expected in the final storage time, and all the incorporated samples seem to equal their antioxidant capacity. This is also in line with the variations among the fatty acids, with gradual decrease of MUFA and PUFA and increase of SFA by oxidation.

Table 26. Antioxidant activity of the cakes incorporated with the chestnut flowers presented EC_{50} values (mg/mL of aqueous cake extract)

	DPPH	RP	β-C	TBARS
		T0		
C	226.47±3.7$^{d, B}$	8.56±0.3$^{c, B}$	19.16±0.6$^{d, C}$	2±0.03$^{cb, A}$
D50	131.7±12.1$^{b, B}$	7.59±0.03b, C	14.52±1.5$^{c, B}$	2.7±0.04$^{dc, B}$
D100	153.42±16.6$^{c, B}$	8.36±0.1$^{c, B}$	10±1.2$^{b, B}$	2.9±1.2$^{d, A}$
F200	20.56±2$^{a, A}$	8.36±0.06$^{c, B}$	23.3±0.8$^{e, C}$	0.86±0.2$^{a, A}$
F400	18.33±0.5$^{a, A}$	3.29±0.4$^{a, A}$	7.09±10.4$^{a, A}$	1.56±0.1$^{ba, A}$
		T15		
C	189.56±15.5$^{b, A}$	4.37±0.04$^{c, A}$	7.47±1.3 $^{a, A}$	14.32±2.4$^{c, B}$
D50	85.09±5.8$^{a, A}$	5.57±0.1$^{d, B}$	10.89±1.8$^{b, A}$	4.14±0.3$^{b, C}$
D100	102.62±36.6 $^{a, A}$	9.12±0.1$^{e, C}$	7.5±1.9$^{a, BA}$	4.63±0.05$^{b, C}$
F200	95.36±2.9$^{a, B}$	4.04±0.1$^{b, A}$	11.76±0.1$^{b, B}$	2.08±0.3 $^{a, B}$
F400	99.73±2.9 $^{a, B}$	3.18±0.3 $^{a, A}$	10.35±0.4$^{b, B}$	4.75±0.2$^{b, B}$
		T30		
C	229.07±12.2$^{bc, A}$	5.09±0.3 $^{a, B}$	11.42±0.8$^{b, B}$	1.55±0.1$^{b, A}$
D50	237.46±9.1$^{c, C}$	5.26±0.1$^{a, A}$	23.15±0.3$^{c, C}$	1.19±0.2 $^{a, A}$
D100	219.39±9.1$^{c, C}$	5.02±0.1$^{a, A}$	6.36±2.9$^{a, A}$	3.75±0.2$^{c, B}$
F200	212.38±4.5 $^{a, C}$	9.39±0.2$^{b, C}$	8.32±1.4 $^{a, A}$	4.73±0.2$^{d, C}$
F400	210.14±8 $^{a, C}$	5.26±0.2 $^{a, B}$	7.67±0.9 $^{a, A}$	1.4±0.1$^{ba, A}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time, while majuscules refer to differences between both storage times between each individual cake type (both achieved through a Tukey's HSD test). Significance level was set at $p<0.05$.

"Serra da Estrela" Cheese

The "Serra da Estrela" cheeses were incorporated with decoctions of the chestnut flowers, lemon balm and basil plants, as well as with their dried counterparts. They were then analysed at two time periods, immediately after the incorporation (T0) and after 6 months (T6), in order to detect changes in their nutritional properties and bioactive compounds. The parameters analysed were the nutritional profile, mineral composition, fatty acids, the antioxidant capacity (only for T0) and external colour (only for T0), in line with articles 6, 7 and 8 of Part V.

1.15. Nutritional profile

On Table 27, it is clear that the predominant parameter is moisture regardless of the incorporation of the plants. Furthermore, very little differences are found in the analysed compounds, for the samples are not very statically different among each other. At T0, the cheese with the lowest moisture was the one incorporated with lemon balm plant, while the one with the highest amount was the one incorporated with chestnut flowers decoctions. Regarding crude fat content, the least fat cheeses were the ones incorporated with chestnut flowers, regardless of the extraction type, while the fattest cheese was the ones incorporated with basil plants. Furthermore, no significant differences ($p<0.05$) were found between lemon balm incorporation types. In terms of protein content, the least amount was found for basil decoction incorporated cheese, while the highest content was found for both the chestnut flowers and lemon balm, regardless of the extraction type. The ash content, as expected, was the least abundant parameter, therefore the differences were even grimmer, being the cheeses incorporated with both types of basil and the chestnut flowers decoctions the most abundant in ash. While the least abundant were the lemon balm incorporations and the dried chestnut incorporated samples. Regarding energy value, the lowest energetic cheese were the ones incorporated with chestnut flowers, while the highest were the control samples. No differences ($p<0.05$) were also found between the incorporations of lemon balm.

With regard to the T6 cheeses, a very clear decrease in moisture can be observed, whilst the other components rose. In terms of moisture, the lowest registered was for the basil incorporated cheese, with the decoctions showing even less than the dried plant. The sample that most retained water was the control sample, followed by the one with decoctions of lemon balm. Among the dried plant samples and the decocted samples of chestnut flowers, no statistical differences ($p<0.05$) could be found. In terms of the fat fraction, the lowest recorded values were observed for the control cheese and the one incorporated with decoctions of lemon balm, whilst the highest was found in the decoctions of basil, being this plant, in both forms of incorporations, the one with the highest fat quantities. Inversely, the proteins decreased in a higher manner for the basil incorporated plants, with the least recorded for the dried basil, having the chestnut and the lemon balm dried plants the highest quantities. Furthermore, the cheeses with the decoctions of lemon balm showed no statistical differences ($p<0.05$) from the control samples. In terms of ash, the highest amounts were detected in the control and the dried chestnut flowers incorporated samples, whilst the least were recorded for the decoctions of the

flowers the basil, and the dried lemon balm. Once again no differences could be pointed out between the decoctions of lemon balm and the basil plants. Finally regarding the energy values, the decoctions of basil showed the highest amounts, whilst the cheeses with the decoctions of lemon balm had the least. The second most energetic cheeses were the plants of lemon balm, followed by the chestnuts flowers. The majuscule letters refer to comparisons between the two storage times for each individual cheese type, and the classification was based on a student's t test, with $p<0.05$. It is clear that significant statistical differences were found for all parameters except ash content for C and CP, implying a change in the nutritional profile over maturation time.

Table 27. Proximate composition of cheeses represented in g/100g of fresh weight for T0 and T6

	Moisture	Fat	Protein	Ash	Energy value (kcal/100g FW)
			T0		
C	$49.97\pm0.01^{cb, B}$	$24.68\pm0.03^{c, A}$	$29.13\pm0.5^{c, A}$	$3.92\pm0.1^{cb, A}$	$338.7\pm5.5^{d, A}$
CP	$49.48\pm0.06^{cb, B}$	$21.91\pm0.09^{a, A}$	$29.7\pm0.1^{dc, A}$	$4.19\pm0.05^{d, A}$	$316\pm1.3^{a, A}$
CD	$50.11\pm0.04^{c, B}$	$21.52\pm0.2^{a, A}$	$30.175\pm0.2^{d, A}$	$3.76\pm0.1^{ba, B}$	$314.4\pm1.4^{a, A}$
MP	$47.99\pm0.03^{a, B}$	$22.850.07^{b, A}$	$30.32\pm0.2^{d, A}$	$4.09\pm0.01^{c, B}$	$327.2\pm4.1^{c, A}$
MD	$48.63\pm0.08^{ba, B}$	$22.9\pm0.2^{b, A}$	$30.28\pm0.2^{d, A}$	$4.19\pm0.05^{d, B}$	$327.2\pm3.3^{c, A}$
OP	$49.03\pm0.05^{cba, B}$	$25.75\pm0.1^{d, A}$	$26.81\pm0.2^{b, A}$	$3.55\pm0.04^{a, B}$	$339.9\pm6.1^{d, A}$
OD	$49.91\pm0.06^{cb, B}$	$24.85\pm0.01^{c, A}$	$24.25\pm0.02^{a, A}$	$3.74\pm0.1^{ba, B}$	$320.6\pm4.4^{b, A}$
			T6		
C T6	$36.40\pm0.8^{e, A}$	$27.84\pm0.02^{ba, B}$	$36.43\pm0.1^{b, B}$	$3.98\pm0.05^{a, A}$	$396\pm3.1^{4b, B}$
CP T6	$27.72\pm0.5^{c, A}$	$31.04\pm1^{d, B}$	$39.97\pm0.3^{c, B}$	$4.17\pm0.02^{a, A}$	$439.3\pm2.3^{e, B}$
CD T6	$29.25\pm0.6^{d, A}$	$28.67\pm0.4^{cb, B}$	$41.88\pm0.2^{d, B}$	$5.94\pm0.01^{c, A}$	$425.6\pm3.2^{d, B}$
MP T6	$27.84\pm0.7^{c, A}$	$29.77\pm0.02^{c, B}$	$38.87\pm0.04^{c, B}$	$6.1\pm0.4^{c, A}$	$423.4\pm2.1^{c, B}$
MD T6	$36.87\pm0.8^{e, A}$	$26.94\pm0.2^{a, B}$	$36.16\pm0.06^{b, B}$	$5.37\pm0.02^{b, A}$	$387.1\pm4.4^{a, B}$
OP T6	$24.45\pm1^{b, A}$	$37.21\pm0.5^{e, B}$	$33.03\pm0.8^{a, B}$	$5.23\pm0.1^{b, A}$	$467.1\pm4.1^{f, B}$
OD T6	$21.93\pm0.9^{a, A}$	$38.74\pm0.05^{f, B}$	$33.23\pm0.8^{a, B}$	$5.94\pm0.1^{c, A}$	$481.5\pm6.2^{g, B}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time (achieved through a Tukey's HSD test), while majuscules refer to differences between both storage times between each individual cheese type (achieved through a student T-test). Significance level was set at $p<0.05$.

1.16. Mineral elements composition

By analysing Table 28, it is clear that the most abundant minerals are the macroelements Ca and Na, with very slight variations among samples in both storage times, but an increase in nearly all minerals were visible from T0 to T6. Still, for T0, regarding Ca, the highest was detected in the cheeses with the dried chestnut flowers. No statistical differences ($p<0.05$) were found among both basil incorporations and the lemon balm dried plants. In terms of Mg, the sample with the highest amount was detected in two dried plant incorporations (basil and chestnut) and the decoction of basil. Regarding Na, the control cheeses and the chestnut flower decoction ones showed the least amounts, while the highest was detected in the basil, with the decoction incorporated samples showing statistical differences ($p<0.05$) from the dried plant incorporated ones. The highest K amounts were observed in the basil plant incorporated cheese. No statistical differences ($p<0.05$) could be noticed in the lemon balm extractions.

Regarding microelements, all samples except the control and the basil decoctions showed the lowest Fe amounts, while the highest was found for many samples, namely the decoctions of chestnut flowers and lemon balm, and the dried chestnuts and basil. Interestingly, Zn showed no statistical differences among samples. Finally, Cu was the highest for the cheese with chestnut decoctions. No differences could be established between the controls and the chestnut flowers and lemon balm dried plant samples.

After 6 months of maturation (T6 samples), and with regard to Ca, the highest detected amounts were found in the basil and chestnuts, with no statistical differences among the incorporation methods. Mg and Na showed the highest amounts for the same samples, control and lemon balm decoction (although Na had no statistical differences for the chestnut flowers), differing on the least abundant, which for Mg was the basil decoction and for Na was the lemon balm dried plant. In both these minerals, the chestnut dried flower incorporated cheeses did not show statistical differences from the decocted flower incorporated cheese, although this also occurred for basil in terms of the Na content. For K, the lowest amount was found in the control cheeses and the decoctions of chestnut and lemon balm. For Fe, the cheese with basil decoction and chestnut dried flower showed the highest amounts. Zn was most abundant in many samples, namely the ones incorporated with the decoctions of lemon balm, and both types of incorporation of basil, but also for the control. Finally, Cu was detected in a higher amount in the cheeses decocted with chestnut flower and lemon balm plants. Regarding the comparisons

between each individual cheese for the two storage times, statistical differences ($p<0.05$) were sought for all minerals.

In the case of Na, the cheese with the lowest amount of this mineral element was CD, with only 785 mg/100g. Consumption of 100 g of the cheese with 6 months of maturation (higher amounts of minerals per 100g) represents, in terms of percentage of the admissible daily intake 179.67% of Ca, 37.85% of Mg, 155.66% of Na, 10.49% of K, and 0.02% of Fe. As previously mentioned, the admissible quantity of Na varies between 1.5 and 2.0 g/day, but the EFSA (2005) estimates the daily intake of sodium between 3 to 5 g daily. As with all other cheeses, in the "Serra da Estrela" the amounts of Ca and Na exceed these allowances, still, it is not likely that someone would consume 100 g of cheese at once. Still, an excessive consumption of cheese is not advisable for people with hypertension problems. All the other mineral elements are under the daily allowances.

Table 28. Mineral composition of the cheeses, represented in mg/100g of fresh weight for T0 and T6

	Ca	Mg	Na	K	Fe	Zn	Cu
				T0			
C	832.2±13.1$^{a, A}$	50.03±1.4$^{a, A}$	819.22±5.3$^{a, A}$	120.2±4$^{b, A}$	0.88±0.01$^{ba, A}$	5.15±0.01$^{a, A}$	0.50±0.01$^{dc, A}$
CP	1129±33$^{c, A}$	69.21±7.4$^{cb, A}$	869.24±11.3$^{b, A}$	133.62±3$^{c, A}$	0.91±0.01$^{b, A}$	5.92±0.1$^{a, A}$	0.49±0.01$^{dc, A}$
CD	858.2±12.1$^{a, A}$	53.75±2$^{a, A}$	785.75±13.2$^{a, A}$	98.4±5.2$^{a, A}$	0.99±0.01$^{cb, A}$	5.5±0.05$^{a, A}$	0.55±0.01$^{d, A}$
MP	1053±1.5$^{b, A}$	59.37±3.7$^{ba, A}$	1048.63±21.1$^{d, A}$	137.2±2.4$^{c, A}$	1.07±0.2$^{cb, A}$	5.03±0.3$^{a, A}$	0.45±0.05$^{c, A}$
MD	877.3±1.2$^{a, A}$	55.98±0.9$^{a, A}$	922.82±3$^{c, A}$	127.2±1.3$^{cb, A}$	0.98±0.1$^{cb, A}$	3.83±2.6$^{a, A}$	0.35±0.02$^{b, A}$
OP	1026±12.4$^{b, A}$	71.6±4.8$^{c, A}$	1014.61±27.4$^{e, A}$	186.3±6.5$^{d, A}$	1.23±0.2$^{c, A}$	4.86±0.3$^{a, A}$	0.21±0.01$^{a, A}$
OD	1026.2±33$^{b, A}$	67.98±3.5$^{cb, A}$	1144.25±7.3$^{d, A}$	201.92±4.3$^{e, A}$	0.61±0.102$^{a, A}$	4.45±0.1$^{a, A}$	0.26±0.03$^{a, A}$
				T6			
C T6	1137.3±18$^{a, B}$	73.1±7$^{a, B}$	1569.08±52.7$^{ba, B}$	233.10±6.5$^{a, B}$	0.31±0.1$^{a, B}$	5.78±0.2$^{a, B}$	0.34±0.04$^{cb, B}$
CP T6	1432±37$^{cb, B}$	92.74±5.9$^{b, B}$	1587.71±91.7$^{ba, B}$	279.81±9.5$^{c, B}$	1.64±0.04$^{ed, B}$	7.04±0.3$^{dc, B}$	0.28±0.01$^{b, B}$
CD T6	1430±24$^{cb, B}$	87.44±2.6$^{b, B}$	1847.6±241.4$^{bc, B}$	247.11±7.7$^{ba, B}$	1.12±0.1$^{cb, B}$	7.73±0.5$^{d, B}$	0.1±0.01$^{a, B}$
MP T6	1359.3±61$^{b, B}$	83±7.8$^{ba, B}$	2335.58±28.8$^{d, B}$	281.67±12.7$^{c, B}$	1.94±0.2$^{e, B}$	6.77±0.4$^{cb, B}$	0.04±0.01$^{a, B}$
MD T6	1027±155$^{a, B}$	73.39±1.1$^{a, B}$	1479.1±40.5^{a}	227.46±0.1$^{a, B}$	1.35±0.04$^{dc, B}$	6.53±0.3$^{cba, B}$	0.25±0.02$^{b, B}$
OP T6	1617±49.2$^{c, B}$	151.42±2.5$^{d, B}$	1881.23±31.6$^{c, B}$	259.85±1.5$^{cb, B}$	0.82±0.09$^{b, B}$	6.33±0.1$^{cba, B}$	0.41±0.05$^{dc, B}$
OD T6	1583.5±31$^{c, B}$	132.47±1.4^{B}	1962.41±34.4$^{c, B}$	325.2±15.1$^{d, B}$	1.69±0.3$^{ed, B}$	6.12±0.3$^{ba, B}$	0.52±0.03$^{d, B}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time (achieved through a Tukey's HSD test), while majuscules refer to differences between both storage times between each individual cheese type (achieved through a student T-test). Significance level was set at $p<0.05$.

1.17. Fatty acids

By analysing Table 29, it is easy to gather that the saturated fatty acids are more abundant than the monounsaturated ones, being the most abundant fatty acids C16:0 and C18:1. Fatty acids with relative quantities under 1% in all samples were not recorded in the table, given their negligible contribution. Regarding T0, no statistical differences ($p<0.05$) were found between the cheese samples for C14:0, C16:1 and C18:0. Overall, the incorporations did not show very clear effects in terms of addition of fatty acids to the cheese.

In terms of T6, it was expected that the fatty acids could suffer changes due to the preserving actions of the plants against lipid peroxidation, among others. For SFA, and particularly C17:0 the control samples showed the highest values, and the percentage of the fatty acids was overall maintained. Between the unsaturated fatty acids, the ones that were not maintained by the incorporations were C16:1 and C18:2, in which the control sample cheeses showed higher values. But for all the other unsaturated ones, the incorporated samples avoided their breakdown. The best plant extraction for fatty acids preservation seemed to be basil, for both its incorporation preserved C18:2 (predominant fatty acid) and C18:3. Furthermore, its decoctions proved to better preserve C20:4. In terms of the SFA, the lowest was also detected for both the basil incorporations, and the highest amount of MUFA for the cheeses with the decoctions of this plant. Finally, the chestnut dried chestnut flowers better protected the PUFA.

Regarding the comparisons of each cheese formulation over time, by using a student's t-test, some interesting findings were achieved. As expected, most of the saturated fatty acids increased over time, due to their degradation into SFA. For the control samples, most of the unsaturated fatty acids decreased, including MUFA and PUFA. SFA increased over the course of the six months. For the cheeses with the dried chestnut flowers, C4:0, C6:0 and C17:0 did not show statistical differences ($p<0.05$) during the six months. All but C16:1 unsaturated fatty acids decreased, while the SFA also increased over time. For the cheese with the decoctions of the chestnut flowers, the behaviour was similar to the dried flowers incorporated ones, being the most striking conclusion the maintenance of PUFA. For the lemon balm plants incorporated cheese, C4:0, C8:0, C17:0 and C18:0 were maintained, with no statistical differences ($p<0.05$) being registered over time. Furthermore, all other unsaturated fatty acids except C16:1 decreased, including MUFA and PUFA. The same conclusions could be drawn for the decoctions of lemon balm, although less fatty acids had no statistical differences (only C16:1

and C17:0). Regarding basil incorporated cheese in both forms, these seemed to be the most preserving incorporations, for more unsaturated fatty acids were maintained. In the plant incorporated cheese, C10:0, C16:1 and SFA were maintained, while C18:1, C18:3, C20:4 and MUFA increased over time. The only notorious decrease was registered for PUFA and particularly C18:2. Finally, for the decocted samples, SFA and particularly C12:0, C20:0 were maintained. The same fatty acids increased in these samples as the ones in the basil incorporated ones, with exception to C20:5, which maintained. Decreases were only found for C16:1, C18:2 and PUFA.

Basil, in its both forms is the most suitable incorporation for fatty acid preservation, although it was not the plant with the highest polyphenols and/or antioxidant activity. Other compounds could be responsible from this preserving effects, namely organic acids, which are in higher quantities in basil, when compared to the other plant samples.

Table 29. Fatty acid profile of the cheeses, represented in percentage for T0 and T6

	T0						
	C	CP	CD	MP	MD	OP	OD
C4:0	2.31±0.2$^{ba, A}$	2.13±0.2$^{a, A}$	2.37±0.1$^{cba, A}$	2.11±0.1$^{a, A}$	2±0.04$^{a, A}$	2.75±0.1$^{c, A}$	2.63±0.2$^{bc, A}$
C6:0	2.57±0.1$^{cb, A}$	2.31±0.2$^{ba, A}$	2.69±0.1$^{c, A}$	2.44±0.08$^{cba, B}$	2.28±0.03$^{ba, A}$	2.34±0.01$^{ba, A}$	2.22±0.1$^{a, A}$
C8:0	2.42±0.06$^{dc, A}$	2.23±0.2$^{cba, A}$	2.66±0.01$^{d, A}$	2.31±0.1$^{cb, A}$	2.21±0.04$^{cba, A}$	2.05±0.07$^{ba, A}$	1.94±0.1$^{a, A}$
C10:0	6.88±0.1$^{dc, A}$	6.4±0.3$^{c, A}$	7.560.2$^{d, A}$	6.49±0.4$^{c, A}$	6.34±0.08$^{cb, A}$	5.51±0.3$^{ba, A}$	5.21±0.4$^{a, A}$
C12:0	4.29±0.1$^{dc, A}$	4.06±0.1$^{dcb, A}$	4.46±0.1$^{d, A}$	4.11±0.2$^{dc, A}$	4±0.02$^{cb, A}$	3.66±0.2$^{ba, B}$	3.54±0.2$^{a, A}$
C14:0	10.65±0.2$^{a, A}$	10.37±0.1$^{a, A}$	10.72±0.3$^{a, A}$	10.54±0.06$^{a, A}$	10.2±0.05$^{a, A}$	10.77±0.3$^{a, B}$	10.64±0.3$^{a, B}$
C15:0	1.34±0.01$^{ba, A}$	1.32±0.01$^{a, A}$	1.33±0.02$^{ba, A}$	1.36±0.01$^{ba, A}$	1.32±0.01$^{a, A}$	1.38±0.01$^{b, B}$	1.38±0.01$^{b, B}$
C16:0	23.35±0.3$^{a, A}$	24.1±0.2$^{b, A}$	23.43±0.06$^{a, A}$	23.74±0.1$^{ba, A}$	23.81±0.1$^{ba, A}$	27.04±0.2$^{c, B}$	26.82±0.3$^{c, B}$
C16:1	0.89±0.02$^{a, A}$	0.89±0.01$^{a, A}$	0.88±0.02$^{a, A}$	0.92±0.03$^{a, A}$	0.85±0.01$^{a, A}$	1.11±0.4$^{a, A}$	0.81±0.8$^{a, B}$
C17:0	0.94±0.07$^{b, A}$	1.05±0.02$^{c, A}$	0.95±0.02$^{b, A}$	0.99±0.03$^{cb, A}$	1.02±0.01$^{cb, A}$	0.66±0.01$^{a, A}$	0.66±0.01$^{a, A}$
C18:0	11.48±0.3$^{a, B}$	11.77±0.4$^{a, B}$	10.59±0.3$^{a, B}$	10.69±0.5$^{a, A}$	11.48±0.03$^{a, B}$	10.78±0.5$^{a, A}$	13.34±0.8$^{b, A}$
C18:1	26.4±0.2$^{cba, B}$	26.89±0.5$^{cb, B}$	28.1±0.5$^{cba, B}$	27.79±1.3$^{c, B}$	28.1±0.5$^{c, B}$	25.94±0.5$^{ba, A}$	24.89±0.8$^{a, A}$
C18:2	2.6±0.05$^{b, B}$	2.62±0.07$^{b, B}$	2.38±0.02$^{a, B}$	2.59±0.1$^{b, B}$	2.54±0.03$^{ba, B}$	2.56±0.1$^{ba, B}$	2.57±0.01$^{ba, B}$
C18:3	1.95±0.01$^{b, B}$	1.92±0.4$^{b, B}$	1.85±0.01$^{b, B}$	1.99±0.08$^{b, B}$	1.93±0.01$^{b, B}$	1.69±0.09$^{a, A}$	1.56±0.06$^{a, A}$
C20:4	0.25±0.03$^{a, B}$	0.26±0.01$^{ba, B}$	0.24±0.01$^{a, B}$	0.24±0.01$^{a, B}$	0.2±0.01$^{a, B}$	0.27±0.02$^{ba, A}$	0.29±0.01$^{b, A}$
SFA	67.04±0.05$^{cba, A}$	66.51±0.54$^{cba, A}$	67.39±0.1$^{dcb, A}$	65.54±1.52$^{ba, A}$	65.41±0.4$^{a, A}$	67.67±0.2$^{dc, A}$	69.15±0.7$^{d, A}$
MUFA	27.55±0.2$^{cba, B}$	28.03±0.4$^{cb, B}$	27.6±0.08$^{cba, B}$	28.97±1.3$^{cb, B}$	29.23±0.5$^{c, B}$	27.28±0.1$^{ba, A}$	25.89±0.7$^{a, B}$
PUFA	5.41±0.2$^{ba, B}$	5.45±0.1$^{b, B}$	4.85±0.4$^{a, A}$	5.49±0.2$^{b, B}$	5.36±0.06$^{ba, B}$	5.05±0.3$^{ba, B}$	4.97±0.02$^{ba, B}$
	T6						
	C	CP	CD	MP	MD	OP	OD
C4:0	2.29±0.04$^{b, A}$	1.97±0.01$^{a, A}$	2.6±0.1$^{c, B}$	2±0.2$^{a, A}$	3.1±0.03$^{c, B}$	3.43±0.1$^{d, B}$	3.71±0.02$^{e, B}$
C6:0	2.56±0.02$^{b, A}$	2.19±0.01$^{a, A}$	3.01±0.1$^{d, B}$	2.13±0.2$^{a, A}$	3.14±0.1$^{d, B}$	2.72±0.1$^{cb, B}$	2.92±0.01$^{dc, B}$
C8:0	2.84±0.02$^{c, B}$	2.52±0.01$^{b, B}$	3.22±0.1$^{d, B}$	2.46±0.1$^{ba, A}$	3.32±0.08$^{d, B}$	2.28±0.02$^{a, B}$	2.47±0.06$^{ba, B}$
C10:0	8.61±0.1$^{c, B}$	8.1±0.02$^{cb, B}$	9.4±0.2$^{d, B}$	7.97±0.3$^{b, B}$	9.44±0.01$^{d, B}$	5.65±0.1$^{a, A}$	6.09±0.3$^{a, B}$
C12:0	5.62±0.1$^{dcb, B}$	5.54±0.03$^{cb, B}$	5.83±0.09$^{d, B}$	5.5±0.05$^{b, B}$	5.8±0.06$^{dc, B}$	3.22±0.08$^{a, A}$	3.4±0.2$^{a, A}$
C14:0	12.55±0.1$^{b, B}$	12.65±0.04$^{b, B}$	12.29±0.04$^{b, B}$	12.56±0.08$^{b, B}$	12.33±0.1$^{b, B}$	9.15±0.2$^{a, A}$	9.40±0.3$^{a, A}$
C15:0	1.57±0.02$^{cb, B}$	1.57±0.01$^{cb, B}$	1.56±0.01$^{cb, B}$	1.61±0.06$^{c, B}$	1.53±0.02$^{b, B}$	1.08±0.01$^{a, A}$	1.05±0.01$^{a, A}$
C16:0	26.56±0.2$^{b, B}$	27.7±0.2$^{c, B}$	21.21±0.1$^{b, B}$	27.63±0.3$^{c, B}$	26.12±0.08$^{b, B}$	23.09±0.1$^{a, A}$	23.04±0.07$^{a, A}$
C16:1	1.07±0.01$^{f, B}$	0.99±0.01$^{ed, B}$	0.95±0.03$^{dc, B}$	1.06±0.01$^{fe, B}$	0.9±0.06$^{cb, A}$	0.84±0.01$^{b, A}$	0.24±0.01$^{a, A}$
C17:0	1.11±0.01$^{ba, B}$	1.06±0.01$^{ba, A}$	1.14±0.04$^{ba, B}$	1.26±0.2$^{b, A}$	1.04±0.04$^{ba, A}$	0.97±0.01$^{a, B}$	0.94±0.01$^{a, B}$
C18:0	10.33±0.2$^{b, A}$	10.4±0.3$^{b, A}$	9.32±0.03$^{a, A}$	10.49±0.2$^{b, A}$	9.49±0.2$^{a, A}$	15.2±0.1$^{c, B}$	14.92±0.3$^{c, B}$
C18:1	19.74±0.4$^{b, A}$	19.81±0.2$^{b, A}$	18.93±0.4$^{a, A}$	20.11±0.02$^{b, A}$	18.68±0.01$^{a, A}$	27.29±0.01$^{c, B}$	27±0.3$^{c, B}$
C18:2	1.81±0.01$^{cb, A}$	1.84±0.04$^{c, A}$	2.04±0.01$^{d, A}$	1.74±0.01$^{b, A}$	2.06±0.05$^{d, A}$	0.57±0.01$^{a, A}$	0.55±0.01$^{a, A}$
C18:3	1.74±0.01$^{b, A}$	1.73±0.01$^{b, A}$	1.57±0.01$^{a, A}$	1.7±0.01$^{b, A}$	1.59±0.03$^{a, A}$	2.07±0.01$^{c, B}$	2.05±0.03$^{c, B}$
C20:4	0.15±0.01$^{b, A}$	0.16±0.01b$^{, A}$	0.12±0.01$^{a, A}$	0.14±0.01$^{ba, A}$	0.12±0.01$^{a, A}$	0.38±0.01$^{d, B}$	0.33±0.01$^{c, B}$
SFA	74.96±1$^{dc, B}$	74.46±0.01$^{cb, B}$	75.72±0.5$^{dc, B}$	74.39±0.02$^{b, B}$	76±0.15$^{d, B}$	67.68±0.08$^{a, A}$	68.84±0.08$^{a, A}$
MUFA	21.22±0.5$^{b, A}$	21.06±0.2$^{b, A}$	20.12±0.4$^{a, A}$	21.49±0.03$^{b, A}$	19.79±0.05$^{a, A}$	28.38±0.02$^{d, B}$	27.49±0.4$^{c, B}$
PUFA	3.82±0.5b$^{a, A}$	4.32±0.07$^{b, A}$	2.65±1.5$^{a, A}$	4.11±0.05$^{ba, A}$	4.21±1$^{ba, A}$	3.94±0.1$^{ba, A}$	3.67±0.1$^{ba, A}$

Minuscule letters mean statistical significant differences among the samples for one individual storage time (achieved through a Tukey's HSD test), while majuscules refer to differences between both storage times between each individual cheese type (achieved through a student T-test). Significance level was set as $p<0.05$.

1.18. Antioxidant activity

Regarding the antioxidant capacity of the cheeses, by interpreting Table 30, it is clear that the plant and plants extracts incorporation as functional ingredients was a success, for the control samples showed higher EC_{50} values for all the assays (meaning a higher amount is needed to display the same antioxidant activity). Individually, basil showed lower EC_{50} for two assays (RP and β-C), while two plants incorporations presented the better antioxidant activity (basil dried plant for RP assay and lemon balm dried plant for TBARS), and two decoction extracts incorporation presented better results than the rest (basil and lemon balm for β-C). Individually, and regarding RP, the least effective incorporation was the MD, and no statistical difference ($p<0.05$) could be found for the chestnut incorporation methods. In terms of lipid peroxidation inhibition, for β-C assay, the incorporation with the lower antioxidant activity was MP, and once again no statistical differences ($p<0.05$) were found for the chestnut flower incorporation. Finally, in terms of TBARS assay, the lest effective incorporation in terms of lipid peroxidation inhibition methods was the dried basil plants, and no differences could be detected among the chestnut and lemon balm incorporations.

Table 30. EC_{50} values of the cheese samples, represented in mg/mL of aqueous cheese extract

	RP	β-C	TBARS
C	37.58 ± 0.1^{e}	142.74 ± 9.9^{e}	33.66 ± 1.4^{e}
CP	26.19 ± 0.4^{c}	11.64 ± 1.4^{cba}	4.2 ± 0.6^{ba}
CD	25.92 ± 2.1^{c}	13.82 ± 1.3^{c}	5.61 ± 0.07^{cb}
MP	26.49 ± 1^{c}	45.27 ± 2.9^{d}	3.92 ± 0.2^{a}
MD	29.65 ± 1.8^{d}	7.56 ± 0.8^{ba}	5.57 ± 0.4^{cba}
OP	13.19 ± 0.2^{a}	12.79 ± 0.5^{cb}	21.20 ± 0.1^{d}
OD	19.98 ± 0.2^{b}	6.5 ± 0.2^{a}	6.29 ± 2.4^{c}

Minuscule letters mean statistical differences between each parameter. Significance level was set at $p<0.05$.

1.19. External color

The external color of the cheeses incorporated with the plants, as well as their decoctions are depicted on Table 31. The color for these samples was only analysed at T0. The highest values

reported for the *L** parameter belonged to the control sample, although it was only statistically different from OD ($p<0.05$). This parameter varies between black (*L**=0) and white (*L**=100), and all samples varied between 62 and 67, showing a very slight overall difference in lightness. The samples with the lowest value (darker samples) were those functionalised with the decoction of basil. The *a** value parameter measures the greenness-redness tendency, and all samples showed values close to 0, which indicated the absence of intense red or green colours. No statistical differences ($p<0.05$) were found among the samples for this parameter. When positive, the *b** value indicates a yellow tone, while if negative, it indicates the presence of blue tones. All samples had positive and similar values, ranging from 22 to 27. The highest values were recorded for OD and the least for CP and OP. Overall, in terms of lightness the brighter cheeses were the OD incorporated ones, for greenness-redness no statistical differences ($p<0.05$) were found among the samples, and finally for yellowness-blueness the OD showed brighter yellow colours, although not statistically different ($p<0.05$) from the control, CD, MP and MD. The fact that the OP had lower yellow colors is due to the presence of the leaves of basil in the cheese. The same occurred for the CP and MP, although not statistically different ($p<0.05$) from the rest of the samples. This visual alteration could prove to be appealing to consumers for being different from the traditional colors of cheese, which is a yellow tone without any other colours.

Table 31. External colour for the cheeses with chestnut flowers and lemon balm. T0 storage time

	*L**	*a**	*b**
Control	67 ± 2^{b}	-4 ± 1^{a}	24 ± 1^{ba}
CP	62 ± 3^{ba}	0 ± 1^{a}	22 ± 1^{a}
CD	65 ± 2^{b}	-2 ± 1^{a}	23 ± 1^{ba}
MP	63 ± 3^{ba}	-4 ± 1^{a}	23 ± 1^{ba}
MD	65 ± 3^{b}	-3 ± 1^{a}	24 ± 1^{ba}
OP	62 ± 1^{ba}	-1 ± 3^{a}	22 ± 2^{a}
OD	56 ± 3^{a}	-1 ± 0.5^{a}	27 ± 2^{b}

In each column, different letters mean statistical significant differences among the samples. Significance level was set at $p<0.05$

Part VII. **CONCLUSIONS // CONCLUSIONES**

The main objective of this work was to screen three plants traditionally consumed in Portugal to act as possible functional and preserving ingredients in two different foodstuffs, which are very typical in Portugal, the "económicos" cakes and the "Serra da Estrela" cheese. This work aids the worldwide pursue of new sources of food additives, which, apart from being renewable are also natural, and do not pose a threat to the health of consumers. The chosen plants, lemon balm and basil leaves, as well as the flowers of chestnut trees are all known to have beneficial properties to health, and are all included in the Portuguese diet, in one form or another. The "económicos" cakes are a highly consumed foodstuff in the North-east of Portugal, while the "Serra da Estrela" cheese is one of Portugal's most appreciated dairy delicacies, as well as being one of the most recognizable Portuguese foodstuffs.

Partial conclusions

1.1. Plants and their decoctions as functional and preserving ingredients

After analysing the selected plants, which are traditionally consumed in Portugal, different conclusions could be drawn in terms of the bioactive compounds, as well as, their bioactivities.

a) Most of the major phenolic compounds detected in the samples have antioxidant and/or antimicrobial activities. Trigalloyl-HHDP-glucoside was the most representative for the chestnut flowers, *trans*-rosmarinic acid for the lemon balm leaves and rosmarinic acid for the basil leaves.
b) Chestnut flower decoctions showed the highest amounts of soluble sugars, while lemon balm and basil leaves were highlighted due to their organic acid content.
c) The lemon balm proved to have the highest antioxidant capacity, and the "Judia" cultivar of the chestnut flower were the extract with the highest antifungal activity.
d) Lemon balm showed the best results as an antibacterial agent, as well as, had the lowest GI_{50} against the various tumor cell lines.

1.2. **"Económicos" cakes**

The functional and preserving effects of chestnut flowers and extracts were evaluated in the "económicos" cakes, which were incorporated with the chestnut flowers, in two different forms, direct incorporation of the dried flower and their decoctions, and in two different concentrations.

a) Overall, the functionalization of the cakes was greatly achieved without compromising the nutritional and organoleptic profile of the cakes.
b) The incorporation did not interfere in the nutritional composition of the cakes.
c) The dried flowers seemed to be better in conferring antioxidant capacity than the decoctions extracts.
d) A significant decrease in moisture, organic acids and sucrose was observed in all lots along the storage time.
e) Monosaccharides like fructose and glucose only appeared after 15 days, probably due to sucrose degradation.
f) The ambiguous variation of mineral elements composition could be due to an uneven distribution in the batter, allied to their relative low quantity in the cakes.
g) For all the nutrients, the interaction of influence between the storage time and the incorporation types was significant, which led to the extraction of conclusions through the EMM.
h) The interaction among storage time and incorporation type was also significant for the fatty acids profile.

1.3. **“Serra da Estrela” cheese**

In “Serra da Estrela” cheese, the functional and preserving effects of three selected plants and their decoctions were evaluated, using the same principal for the cake incorporations, although no two-fold concentrations were added.

a) The proteins were not deeply affected by the plant incorporation, regardless of the incorporation type.
b) For the antioxidant capacity (only T0), the decoction of basil proved to be the most antioxidant among the three plants.
c) The chestnut flowers and lemon balm dried plant incorporated cheese seemed to have a higher preserving effect than the decoctions, but for the basil incorporations, the decoctions had a higher effect. One control cheese displayed interior contamination after the six months, while none of the incorporated samples had any occurrence of the sort.
d) The moisture saw a reduction along the storage time (6 months), although it was higher for the plants incorporated cheese, especially the dried flowers, which could contribute to cheese maturation and preservation.
e) Contrarily to the cakes, the incorporation of the plants in their various forms had a higher influence than the storage time.
f) Regarding fatty acid profile, SFA was the predominant, being scarcely after plant incorporation and along stored time. Also no significant variation was observed in MUFA fraction, specifically, for basil cheese incorporations. The incorporations of dried basil in cheeses tended to preserve C18:1 in a higher manner than the all the other incorporations. Being demonstrated the preservative effect of the selected plants against lipid peroxidation process.
g) Dried leaves incorporated cheeses displayed differences in the external appearance (due to the visible leave and flower parts), although this did not very deep color changes.

Concluding remarks and future perspectives

The use of plants as multifunctional food additives is possible, recommendable, cheaper, renewable, healthy and viable in the demanding food industry. The general acceptance of the public, the proven benefits of their consumption and the simple manner these ingredients can be manipulated are characteristics that must be taken into consideration for future foodstuffs. In the case of the cakes, bioactivities were conferred to them, for one, they were functionalized and can carry beneficial compounds for the consumer, while also constituting added value to them. This functionalization was done without altering the nutritional profile or their appearance. Although minimal preserving effects were achieved, this was partially expected due to the low moisture and the heating process that could inactivate some compounds. For the cheese, functionalization was achieved, as well as preserving effects, which could constitute healthier foodstuffs, with no chemical additives as well as conferring added value to the cheese. By reducing the maturation time, production costs could be lowered. Finally, in terms of consumer acceptance, the appearance of the cheese, with the green leaves as basil incorporation in cheese, could prove to be a desirable aspect for consumers seeking new and thrilling experiences. For sceptic consumers, the preservation and bioactivity could be guaranteed by the decoctions that do not alter the appearance.

Chestnuts flowers, lemon balm and basil, apart from being wonderful plants, also proved their worthiness as source of natural food additives for the future!

El objetivo principal del presente trabajo fue la caracterización de tres plantas, tradicionalmente consumidas en Portugal, con el fin de incluirlas como posibles ingredientes funcionales y conservantes naturales en dos alimentos tradicionales de Portugal, como son los bollos denominados "económicos" y el queso "Serra da Estrela". Este trabajo se fundamentó en la necesidad mundial de buscar nuevas fuentes de ingredientes y aditivos naturales, que no presenten riesgo para la salud de los consumidores. Las plantas seleccionadas en el presente estudio fueron, las flores de castaño, así como las hojas de melisa y albahaca, todas ellas conocidas por sus propiedades beneficiosas para la salud y que forman parte, de un modo o de otro, de la dieta portuguesa. Los "económicos" son un producto de bollería ampliamente consumido en el Norte de Portugal, así como el queso "Serra da Estrela" es uno de los quesos más apreciados en todo el país y típicamente reconocido a nivel mundial como alimento tradicional portugués.

Conclusiones parciales

1.4. Plantas y sus decocciones como ingredientes funcionales

Tras el análisis de las plantas seleccionadas, en relación a su contenido en compuestos bioactivos y actividad biológica, podemos concluir que:

a) La mayoría de los compuestos fenólicos caracterizados en las especies seleccionadas presentaron actividad antimicrobiana y/o antioxidante. Destacando el glucósido de HHDP-trigaloil en las flores de castaño, el ácido *trans*-rosmarínico en las hojas melisa, y el ácido rosmarínico en las hojas albahaca.
b) Las mayores concentraciones de azúcares solubles se encontraron en las decocciones de flores de castaño, mientras que las hojas de melisa y de albahaca destacaron por su contenido en ácidos orgánicos.
c) Las hojas de melisa se destacaron por presentar los mejores resultados de capacidad antioxidante, así como las decocciones de la variedad de castaño "Judia" presentaron los mejores resultados de actividad antifúngica.
d) Las hojas de melisa presentaron los mejores resultados de actividad antimicrobiana, así como los valores más bajos de GI_{50} frente a diversas líneas de células tumorales.

1.5. **Bollos “económicos”**

En los bollos "económicos" se evaluó el efecto de la incorporación de las flores castaños y sus extractos acuosos (decocciones) como posibles ingredientes funcionales y conservantes.

a) En general, la incorporación de la flor de castaño y sus decocciones no afectaron negativamente al perfil nutricional ni a las características organolépticas de los bollos.
b) Los bollos a los que se les adicionó las flores de castaño presentaron mejores resultados de actividad antioxidantes que los bollos a los que se les adicionó los extractos acuosos.
c) Un descenso en la humedad, sacarosa y ácidos orgánicos se observó en todos los lotes tras el periodo de almacenamiento.
d) Los monosacáridos fructosa y glucosa aparecieron tras 15 días de almacenamiento, probablemente debido a la degradación de la sacarosa.
e) Respecto al contenido en elementos minerales, las variaciones observadas podrían deberse a una distribución heterogénea de la masa, así como a la baja concentración de estos compuestos en los bollos.
f) Para todos los nutrientes y compuestos bioactivos evaluados, la interacción entre el tiempo de almacenamiento y los tipos de incorporación fue significativa, así como se demuestra en las conclusiones se extraerán del EMM.
g) La interacción entre el tiempo de almacenamiento y el tipo de incorporación también fue significativa para el perfil de ácidos grasos.

1.6. **Queso "Serra da Estrela"**

En el queso "Serra da Estrela" se evaluó el efecto de la incorporación de las plantas seleccionadas, utilizándose el mismo principio que las incorporaciones de los bollos, aunque no se añadieron concentraciones diferentes.

a) No se observaron variaciones significativas en el contenido en proteínas tras la adición de las plantas estudiadas, independientemente del tipo de incorporación.
b) Los quesos adicionados con las decocciones de albahaca presentaron los mejores resultados de capacidad antioxidante (T0) en comparación con el resto de plantas y extractos.
c) La incorporación de flores de castaño y las hojas de melisa presentaron mejores resultados como conservantes, en comparación con los extractos acuoso, a excepción de la incorporación con las decocciones de las hojas de albahaca. Durante el proceso de almacenamiento uno de los quesos control presentó contaminación microbiana en su interior, mientras que los lotes de queso incorporados no se vieron alterados.
d) A diferencia de los resultados obtenidos para los bollos, se observó una variación significativa a lo largo del tiempo de almacenamiento (6 meses) debido a la incorporación de las plantas y sus extractos.
e) La humedad disminuyó a lo largo del periodo de almacenamiento, viéndose acentuada dicha disminución en los lotes de quesos adicionados respecto al lote control, lo que puede contribuir positivamente al proceso de maduración y conservación de los quesos.
f) En relación al perfil de ácidos grasos, los AGS fueron la fracción predominante, viéndose apenas modificados por la adición de plantas y durante el periodo de almacenamiento. Así mismo, no se observaron diferencias significativas en los AGMI, especialmente en los quesos adicionados con las hojas de albahaca. La adición de las plantas a los quesos preservó mejor el contenido en ácido oleico (C18:1), en comparación con las decocciones. Demostrándose así el posible efecto conservante frente al proceso de peroxidación lipídica
g) Los quesos incorporados con plantas secas mostraron diferencias en su apariencia externa, no modificándose sustancialmente el color de los mismo.

Observaciones finales y perspectivas de futuro

El uso de plantas como posibles ingredientes y aditivos alimentarios naturales, hoy en día es una opción factible, recomendable, más barata, renovable, saludable y viable en la industria alimentaria. La aceptación general del público, los beneficios probados de su consumo y la facilidad de manipulación de estos ingredientes, son características que deben tenerse en cuenta para el desarrollo de futuros productos alimenticios. En el caso de los bollos, la incorporación con la flor de castaño y sus extractos permitió la funcionalización de los mimos, incorporándose así compuestos beneficiosos para la salud del consumidor, y aportando un valor añadido para los mismos.

En estos bollos no se observaron diferencias en el perfil nutricional ni en su apariencia. El efecto "conservante" no fue tan acentuad como cabría esperar, dado el potencial antioxidante y antimicrobiano de la flor de castaño, probablemente debido a que el tratamiento térmico durante el horneado de los bollos pudo degradar en gran medida los compuestos bioactivos presentes en los mismos. En el caso del queso, la incorporación de las plantas y sus extractos permitió obtener un alimento más funcional, así como conservarlo sin la adición de aditivos químicos, reduciendo el tiempo de maduración y costes de producción. Además, con la adición de hojas de albahaca obtenemos quesos con apariencia diferente a la tradicional, permitiendo la obtención de nuevos productos alimenticios más "deseables" para aquellos consumidores que buscan nuevas experiencias culinarias. En cambio, para los consumidores más tradicionales, la adición de los extractos acuosos permite garantizar la conservación y funcionalización de los quesos sin modificarse su apariencia.

El consumo de las flores de castaño, y las hojas de melisa y albahaca, además de ser muy interesantes para la salud, han demostrado su potencial utilidad como fuente de aditivos naturales.

Part VIII. **REFERENCES**

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Carocho, M., Barros, L., Bento, A., Santos-Buelga, C., Morales, P., Ferreira, I.C.F.R. (2014). *Castanea sativa* Mill. flowers amongst the most powerful antioxidant matrices: A phytochemical approach in decoctions and infusions. *BioMed Research International*, Article ID 232956, 7 pages. (DOI: 10.1155/2014/232956)

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Carocho, M., Morales, P., Ferreira, I.C.F.R. (2015). Natural Food additives: *Quo vadis? Trends in Food Science & Technology*, 45, 284-295. (DOI: 10.1016/j.tifs.2015.06.007)

Carocho, M., Barreira, J.C.M., Bento, A., Fernández-Ruiz, V., Morales, P., Ferreira, I.C.F.R. (2016). Chestnut and lemon balm based ingredients as natural preserving agents of the nutritional profile in matured "Serra da Estrela" cheese. *Food Chemistry*, 204, 185-193. (DOI: 10.1016/j.foodchem.2016.02.136)

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Book Chapters

Pinela, J., Carocho, M., Dias, M.I., Caleja, C., Barros, L., Ferreira, I.C.F.R. Chapter 9: Wild Plant-based functional foods, nutraceutical or drugs. Book: Wild Plants, Mushrooms and Nuts: Functional Food Properties and Applications. Wiley-Blackwell Publishing, 2016.

Carocho, M., Morales, P., Ferreira, I.C.F.R. Chapter 6: Aditivos antioxidantes. Book: Aditivos Alimentários. Dextra Editorial, 2016.

Carocho, M., Morales, P., Ferreira, I.C.F.R. Chapter 13: Aditivos edulcorantes. Book: Aditivos Alimentários. Dextra Editorial, 2016.

Barros, L., Barreira, J.C.M., Heleno, S.A., Fernandes, Â., Dias, M.I., Pinela, J., Caleja, C., Pereira, E., Carocho, M., Ferreira, I.C.F.R. Valorização de produtos de montanha do ponto de vista nutricional e bioativo: Estudo de caso em plantas, cogumelos e frutos secos. Book: Sustentabilidade da Montanha Portuguesa: Realidade e Desafios. Centro de Investigação de Montanha, 2016.

Conference Attendance

Carocho, M., Barros, L., Morales, P., Ferreira, I.C.F.R. (December 2013). Chromatographic analysis of fatty acids and sugars in traditional pastry from Trás-os-Montes. 8º Encontro Nacional de Cromatografia. Covilhã, Portugal.

Carocho, M., Bento, A., Morales, P., Ferreira, I.C.F.R. (April 2014). Atividade antioxidante de decocções e infusões de flores de castanheiro, uma árvore de grande interesse apícola. III Congresso Ibérico de Apicultura. Mirandela, Portugal.

Carocho, M., Barros, L., Santos-Buelga, C., Bento, A., Ferreira, I.C.F.R. (April 2014). Perfil em ácidos orgânicos e açúcares livres de decocções e infusões de flores de castanheiro. Mirandela, Portugal.

Carocho, M. (May 2014). A biotecnologia como ciência transversal:Do processamento industrial à criação de novos alimentos. XV Semana das Ciências Agrárias. Bragança, Portugal

Carocho, M., Barros, L., Bento, A., Santos-Buelga, C., Morales, P., Ferreira, I.C.F.R. (June 2014). *Castanea sativa* Mill. Flowers as a source of bioactive phenolic compounds. 8th World Congress on Polyphenols Applications. Lisbon, Portugal.

Carocho, M., Antonio, A., Morales, P., Ferreira, I.C.F.R. (October 2014). New foods: A case study of Portuguese "Serra da Estrela" cheese incorporated with chestnut flowers. 4th International Congress on Food Studies. Prato, Italy.

Carocho, M., Antonio, A., Morales, P., Ferreira, I.C.F.R. (November 2014). Chestnut flowers and natural antioxidants in "Serra da Estrela" cheese. Encontro de Jovens Investigadores, Bragança, Portugal.

Carocho, M., Morales, P., Ferreira, I.C.F.R. (May 2015). Alimentos tradicionales de Portugal y su relación con la salud. II Jornadas de Agrodiversidad, Cultura y Salud. Madrid, Spain.

Morales, P., Carocho, M., Barros, L., Santos-Buelga, C., Cámara, M., Ferreira, I.C.F.R. (June 2015). Decocciones de *Melissa officinalis* L.: Su composición y actividad antioxidante. XIV Congreso Nacional de Ciencias Hortícolas. Orihuela, Spain. (ISBN: 978-84-606-8280-6).

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Carocho, M., Barros, L., Calhelha, R.C., Ciric, A., Soković, M., Santos-Buelga, C., Morales, P., Ferreira, I.C.F.R. (September 2015). Potential of basil (*Ocimum basilicum* L.) as a bioactive ingredient and natural preserver. 5th MoniQA International Conference. Porto, Portugal.

Carocho, M., Morales, P., Ferreira, I.C.F.R. (November 2015). Manjericão como ingrediente bioativo em queijo da Serra da Estrela. 2º Simpósio Nacional "Promoção de uma Alimentação Saudável e Segura – Qualidade Nutricional & Processamento Alimentar (SPASS 2015). Lisbon, Portugal.

Carocho, M., Bento, A., Morales, P., Ferreira, I.C.F.R. Utilização de plantas como ingredientes bioativos e aditivos naturais em queijo da Serra da Estrela. I Congresso Nacional das Escolas Superiores Agrárias. Bragança, Portugal.

Awards

Awarded a 200$ "Graduate Scholar Award" at the 4th International Congress on Food Studies in Prato, Italy. October 2014.

Awarded the prize for best Poster at the XIV Congreso Nacional de Ciencias Hortícolas in Orihuela, Spain. June 2015.

ANNEX 2. FIGURE CAPTIONS

ANNEX 3. TABLE CAPTIONS

ANNEX 4. EQUATION CAPTIONS