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FACULTAD DE FARMACIA

DEPARTAMENTO DE QUÍMICA ANALÍTICA, NUTRICIÓN Y BROMATOLOGÍA

Development of new food products of high nutritional and functional value using flowers, fruits and plant stems

DOCTORAL THESIS

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Celestino Santos Buelga, Catedrático de Nutrición y Bromatología de la Universidad de Salamanca, Lillian Bouçada de Barros, Investigadora del Centro de Investigação de Montanha, e Isabel Cristina Fernandes Rodrigues Ferreira, Professora Coordinatora con Agregación del Instituto Politécnico de Bragança (Portugal), directores del trabajo "Development of new food products of high nutritional and functional value using flowers, fruits and plant stems", realizado por Tânia Cristina de São Pedro Pires para optar al Grado de Doctor con Mención Doctorado Internacional, AUTORIZAN la presentación del mismo al considerar que se han alcanzado los objetivos inicialmente previstos.

Salamanca, __de _____ de 2020

Isabel C. F. R. Ferreira

Celestino Santos-Buelga

Lillian Bouçada de Barros

During the development of this PhD Thesis, the following articles derived from the work carried out have been published, copies of wich are included as annexes in the present memory:

[1] Tânia C. S. P. Pires, Maria Inês Dias, Lillian Barros, Isabel C.F.R. Ferreira. Nutritional and chemical characterization of edible flowers and corresponding infusions: Valorization as new food ingredients. Food Chemistry, 2017, 220, 337 – 343 (Annex I)

[2] Tânia C. S. P. Pires, Maria Inês Dias, Ricardo C. Calhelha, Maria José Alves, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Edible flowers as sources of phenolic compounds with bioactive potential, Food Research International, 2018, 105, 580-588 (Annex II)

[3] Tânia C.S.P. Pires, Maria Inês Dias, Lillian Barros, Maria José Alves, M. Beatriz P.P. Oliveira, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Antioxidant and antimicrobial properties of dried Portuguese apple variety (*Malus domestica* Borkh. cv Bravo de Esmolfe). Food Chemistry, 2018, 240, 701-703 (Annex III)

[4] Tânia C. S. P. Pires, Maria Inês Dias, Ricardo C. Calhelha, Maria José Alves, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Phenolic compounds profile, nutritional compounds and bioactive properties of *Lycium barbarum* L.: A comparative study with stems and fruits. Industrial Crops & Products 2018, 122 574–581 (Annex IV)

[5] Tânia C.S.P. Pires, Maria Inês Dias, Lillian Barros, João C.M. Barreira, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Incorporation of natural colorants obtained from edible flowers in yogurts. LWT - Food Science and Technology, 2018, 97 668–675 (Annex V)

The following review article was also directy related to the subject of this PhD Thesis. A copy is also included as an annex:

[6] Tânia C.S. P. Pires, Lillian Barros, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Edible flowers: Emerging components in the diet- review. Trends in Food Science & Technology, 2019, 93, 244-258 (Annex VI)

Articles in process:

[1] Tânia C. S. P. Pires, Cristina Caleja, Celestino Santos-Buelga, Lillian Barros, Isabel C.F.R. Ferreira. *Vaccinium myrtillus* L. fruits as a novel source of phenolic compounds with health benefits and industrial applications - a review.; in review in Current Pharmaceutical Design.

[2] Tânia C.S.P. Pires, Maria Inês Dias, Márcio Carocho, João C.M. Barreira, Celestino Santos-Buelga, Lillian Barros, Isabel C.F.R. Ferreira. Bilberry Fruits as a Source of Natural Colorants: Chemical Characterization and Incorporation in Yogurts; in review in: Food & Function.

[3] Tânia C. S. P. Pires, Maria Inês Dias, Lillian Barros, Ricardo C. Calhelha, Maria José Alves, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Development of new *Vaccinium myrtillus* L. based snacks: nutritional, chemical and bioactive features; in review in: Food Chemistry.

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“I don't know what tomorrow will bring.”

Fernando Pessoa

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“A leader is a seller of hope.”

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Fernando Pessoa

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Raul Minh'alma

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ABBREVIATIONS

a*	Greenness-redness
AI	Adequate Intake
AOPP	Advanced Oxidation Protein Products
aP2	Adipocyte fatty acid-binding protein
arab	Arabinoside
C/EBP	CCAAT- enhancer-binding proteins
CFR	Code of Federal Regulations
CK-MB	Creatine Kinase MB
CO_2	Carbon Dioxide
CPK	Creatine Phosphokinase
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
Cy	Cyanidin
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
Dp	Delphinidin
DPPH	2,2-diphenil-1-picrylhydrazyl
DW	Dry Weight
e.g.	For example
E163	Anthocyanin
EC_{50}	Concentration with 50% of antioxidant activity
EFSA	European Food Safety Authority
EMM	Estimated Marginal Means
ESBL	Spectrum Extended producer of β-lactamases
ESI	Electrospray Ionization
EU	European Union
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FID	Flame ionization detector

FUFOSE	Functional Food Science in Europe
FW	Fresh Weight
gal	Galactoside
glc	Glucoside
HDL	High-Density Lipoprotein
HEK	Human Embryonic Kidney
HPLC	High Performance Liquid Chromatography
IL	Interleukin
IT	Incorporation types
iNOS	Inducible Nitric oxide Synthases
INT	Iodonitrotetrazolium chloride
IS	Internal standard
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid Chromatography
LDA	Linear Discriminant Analysis
LDH	Lactic Dehydrogenase
LPS	Lipopolysaccharides
MAE	Microwave Assisted Extraction
MAP	Modified Atmosphere Packaging
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MS	Mass Spectrometer
MSn	Tandem mass spectrometry
Mv	Malvidin
nd	Not detected
NF-κB	Nuclear Factor κB
NMR	Nuclear magnetic resonance
NY	New York
PAD	Pulsed Amperometric Detection
PDO	Protected Designation of Origin
PLE	Pressurized Liquid Extraction
Pn	Peonidin
PPAR	Peroxisome proliferator-activated receptor

Pt	Petunidin
RI	Refractive index
ROS	Reactive oxygen species
SCF	Scientific Committee for Food
SD	Standard deviation
SFE	Supercritical Fluid Extraction
SLE	Solid-Liquid Extractions
SREBP	Sterol Regulatory Element Binding transcription factor
ST	Storage Time
STAT	Signal Transducer and Activator of Transcription
TBARS	Thiobarbituric acid reactive substances
Th	T helper cell
THP	Human monocytic cell line
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
UAE	Ultrasound Assisted Extraction
UPLC	Ultra Performance Liquid Chromatography
USA	United States of America
YF	Yogurt Formulation

ABSTRACT

Sustainable food options are becoming more prevalent to respond to needs from consumers, which seek to combinations of new ingredients with potential health benefits.

In this work, edible flowers and infusions of *Dahlia mignon*, a mixture of roses (*Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*), *Calendula officinalis* L., *Centaurea cyanus* L., fruits of *Vaccinium myrtillus* L., *Malus domestica* Borkh apples, and *Lycium barbarum* L. fruits and stems were characterized regarding their proximate composition, soluble sugars, organic acids, individual phenolic profile and bioactive potential (antioxidant and antibacterial activities for all samples, and antiproliferative capacity for flowers and their infusions). Furthermore, the flowers, fruits and stems were also characterized in terms of their fatty acid and tocopherol profiles.

Carbohydrates were the most abundant macronutrients in all the studied samples, with fructose, glucose and sucrose being identified in all the flowers and their infusions. Rose flowers and *C. officinalis* infusions showed the highest content of organic acids, with the latter also being the one with the highest quantity of tocopherols. In all samples, polyunsaturated fatty acids predominated over the saturate. The different studied flowers revealed distinct phenolic composition, but all showed high biological potential, in which hidromethanolic extracts of rose flowers revealed the greatest bioactive properties.

Besides the high nutritional value and the diverse chemical composition, the extracts prepared from 'Bravo de Esmolfe' apples, showed a characteristic phenolic profile with presented epicatechin and B-type procyanidins, hydroxycinnamoyl-quinic acids and phloretin derivatives, while also showing antioxidant and antibacterial effects.

Stems of *L. barbarum* presented higher values of energy, monounsaturated fatty acids, tocopherols and flavonols, as well as great antioxidant and antibacterial activities than fruits, while these latter revealed higher contents of sugars, PUFA and hydroxycinnamic acid derivatives, as also greater activity against Gram-positive bacteria.

Since the food industry is always seeking innovative approaches to maintain consumers' interest and fulfil their awareness towards the healthiness of diets, a great interest has been given to natural food additives, namely colourants. In this sense,

aqueous extracts from rose mixture, *C. cyanus* and *D. mignon* and a hydroethanolic extract obtained from *V. myrtillus* were tested as potential substitutes to E163 (anthocyanins). Besides comparing the colouring capacity, the potential occurrence of changes in the chemical composition of yogurts added with the colouring extracts (nutritional parameters, free sugars and fatty acids) was also assessed throughout storage (up to 7 days) and compared with a plain (free of any colourant) yogurt formulation. In general, yogurts prepared with flower and bilberry fruits extracts presented similar nutritional value and free sugars profile to those prepared with E163 and to the plain yogurt. Nevertheless, rose extract turned out to be the most suitable alternative to E163 as these two groups of yogurts presented close scores in colour parameters, besides having similar nutritional, free sugars and fatty acids composition. For their part, bilberry extracts showed lower colouring capacity when compared to E163, but it provided higher stability throughout storage. Another advantage of this natural extract was the potential bioactive properties that it can confer to foods, due to its high content in bioactive compounds.

The sustainable exploitation of fruit and cereal processing by-products is being conducted for the development of new food products, including snacks. The consumption of these type of products has gained a significant importance worldwide and in consumer's health, since nowadays they represent an important source in the daily intake of nutrients for some groups of individuals. The development of *V. myrtillus*-based snacks could, therefore, be an answer for the food industry, due to the nutritional properties of these fruits, but also to their content in high added value compounds and bioactive potential. Having this in mind, three bilbery fruits-based snacks supplemented with edible flowers and fruits were developed and characterized for their nutritional value and contents in fatty acids, sugars, organic acids, tocopherols, and phenolic compounds, as well as their antioxidant, antibacterial and hepatotoxic properties. The incorporation of edible flowers and fruits to the bilbery snacks improved their nutritional, chemical and bioactive inputs, leading to higher content in phenolic acids and anthocyanins, and also higher antioxidant and antibacterial activities.

The results obtained throughout this thesis work are expected to be useful to fulfil the pressing need of the food industry to develop new functional food products.

RESUMEN

El desarrollo de alimentos saludables y sostenibles es cada vez más frecuente en repuesta a las demandas de los consumidores, que buscan combinaciones de nuevos ingredientes a partir de fuentes alternativas a las clásicas y con posibles beneficios para la salud.

En este trabajo, pétalos e infusiones de flores comestibles de *Dahlia mignon*, una mezcla de rosas (*Rosa damascena* 'Alejandría' y *R. gallica* 'Francesa' en *R. canina*), *Calendula officinalis* L. y *Centaurea cyanus* L., frutos de *Vaccinium myrtillus* L., manzanas *Malus domestica* Borkh variedad 'Bravo de Esmolfe', y frutos y tallos de *Lycium barbarum* L. se caracterizaron en cuanto a su composición proximal, contenidos de azúcares solubles, ácidos orgánicos y compuestos fenólicos individuales y potencial bioactivo (actividades antioxidante y antibacteriana para todas las muestras, y capacidad antiproliferativa en flores y sus infusiones). Además, las flores, frutos y tallos también se caracterizaron en términos de sus perfiles de ácidos grasos y tocoferoles.

Los carbohidratos fueron los macronutrientes más abundantes en todos los casos. Fructosa, glucosa y sacarosa fueron identificadas en todas las flores y sus infusiones. Las infusiones de flores de rosa y *C. officinalis* mostraron el mayor contenido de ácidos orgánicos, siendo la última especie la que presentaba mayor cantidad de tocoferoles. En todas las muestras de flores, los ácidos grasos poliinsaturados predominaron sobre los saturados. Las distintas flores estudiadas revelaron perfiles fenólicos diferentes, aunque todas mostraron un alto potencial biológico, siendo los extractos hidrometanólicos de rosas los que presentaron mayor bioactividad.

Además de alto valor nutricional y composición química variada, los extractos preparados a partir de manzanas 'Bravo de Esmolfe' presentaron un perfil fenólico característico, con epicatequina y procianidinas de tipo B, ácidos hidroxicinamoilquínicos y derivados de floretina, así como signficativos efectos antioxidantes y antibacterianos.

Los tallos de *L. barbarum* presentaron valores más altos de energía, ácidos grasos monoinsaturados, tocoferoles y flavonoles que los frutos, así como buena actividad antioxidante y antibacteriana. Por su parte, los frutos revelaron mayores contenidos de azúcares, PUFA y derivados hidroxicinámicos, y una mayor actividad contra bacterias Gram-positivas.

La industria alimentaria está constantemente buscando enfoques innovadores para mantener el interés de los consumidores y satisfacer sus demandas de productos saludables. Un punto de interés en este sentido es el del empleo de aditivos alimentarios naturales, en particular colorantes. Por ello, se exploró la posibilidad de utilizar extractos acuosos de flores (rosas, *C. cyanus* y *D. mignon*) y un extracto hidroetanólico de *V. myrtillus* como fuentes alternativas al aditivo comercial E163 (mezcla de antocianos de origen diverso). Además de comparar la capacidad de coloración, también se evaluaron los posibles cambios que podrían provocar en la composición de los yogures (parámetros nutricionales, azúcares libres y ácidos grasos) y la estabilidad durante el almacenamiento, con respecto a una formulación de yogur natural (sin aditivos). En general, los yogures preparados con extractos de flores y de arándanos presentaron un valor nutricional y un perfil de azúcares libres similares a los incorporados con E163 y al yogur natural. El extracto de rosa resultó ser la alternativa más adecuada al E163, ya que aportaba características cromáticas muy próximas y mantenía valores nutricionales y de composición de azúcares libres y ácidos grasos similares. Por su parte, los extractos de arándano, aunque mostraron menor capacidad de coloración en comparación con el E163, dieron lugar a mayor estabilidad durante el almacenamiento. Otra ventaja de este extracto natural era su contribución a las posibles propiedades funcionales que puede conferir al alimento, debido a su alto contenido en componentes bioactivos.

La explotación sostenible de subproductos del procesado de frutas y cereales es un campo de notable interés en el desarrollo de nuevos productos alimenticios, como puesen ser los aperitivos (*snacks*). El consumo de este tipo de productos está adquiriendo gran importancia en lo hábitos de los consumidores, representando actualmente una fuente importante en la ingesta diaria de nutrientes en algunos sectores de la población. El desarrollo de aperitivos a base de *V. myrtillus* puede ser una respuesta para la industria alimentaria en su búqueda de productos novedosos y saludables, no sólo por sus propiedades nutricionales, sino también por el contenido de este fruto en compuestos con potencial bioactivo y alto valor añadido. Por esta razón, se formularon tres aperitivos a base de frutos de arándano suplementados con flores comestibles y otras frutas (manzana y bayas Goji), que se caracterizaron en cuanto a su valor nutricional, composición química en ácidos grasos, azúcares, ácidos orgánicos, tocoferoles y compuestos fenólicos, y propiedades antioxidantes, antibacterianas y hepatotóxicas. La mezcla de flores y frutas comestibles con frutos de arándano mejoró

las características nutricionales, químicas y bioactivas del producto, conduciendo a mayores contenidos de ácidos fenólicos y antocianos, así como a mayor actividad antioxidante y antibacteriana.

Se espera que los resultados obtenidos en este trabajo de tesis puedan contribuir a satisfacer las crecientes necesidades de la industria alimentaria con relación al desarrollo de nuevos productos alimenticios con características funcionales.

CHAPTER 1

Background

1.1. Functional Foods

The development of new functional products represents a challenge for the scientific community, health authorities and the food industry.

Diet can modulate various body functions, in addition to meeting nutritional needs, and may have beneficial or detrimental effects on some diseases. According to European experts "a food can be considered as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either the state of well-being and health or the reduction of the risk of a disease" (ILSI Europe, 1999). Moreover, a food product can be made functional by using any of these five approaches: i) eliminating a component known to cause or identified as causing a deleterious effect when consumed; ii) increasing the concentration of a component naturally present in food to a point at which it will induce predicted effects or increasing the concentration of a non-nutritive component to a level known to produce a beneficial effect; iii) adding a component that is not normally present in most foods and is not necessarily a macronutrient or a micronutrient, but for which beneficial effects have been shown; iv) replacing a component, normally a macronutrient whose intake is usually excessive and thus a cause of deleterious effects, by a component for which beneficial effects have been shown, and/or v) increasing bioavailability or stability of a component known to produce a functional effect or to reduce the disease-risk potential of the food (Henry, 2010). Given these approaches, this PhD thesis aimed to study the usage and application of edible flowers and the fruits of *Vaccinium myrtillus* L., *Lycium barbarum* L. and *Malus domestica* Borkh. (cv 'Bravo de Esmolfe'), as potential sources of added-value products to be employed as natural ingredients for functional foods with different beneficial effects (antioxidant, antimicrobial and cytotoxic properties), as well as colorants, as an alternative to the massively used artificial additives.

1.2. Edible flowers as foods and sources of functional ingredients

Since ancient times, edible flowers have been used in the human diet (He et al., 2015; Lu, Li, & Yin, 2015; Rop, Mlcek, Jurikova, Neugebauerova, & Vabkova, 2012), being their practice very well documented worldwide, from ancient Greece and Rome, medieval France, Europe, Victorian England, or the Middle Eastern region (He et al., 2015; Lu et al., 2016) to Asian countries like China and Japan, where their consumption has been reported for thousands of years (Rop et al., 2012). Nowadays, globalization and awareness of consumers have contributed in the improvement and comeback of earlier

lifestyles, where edible flowers may have an important role (Liu et al., 2002). This plant part is an abundant natural resource and, in many times, rich in phytochemicals with putative health effects (Lu et al., 2015). Edible flowers have been long used in folk medicine to treat diseases, but recent studies have supported these traditional health benefits, revealing their rich composition in bioactive compounds, which have been correlated to functional properties (He et al., 2015; Lu et al., 2015; Rop et al., 2012; Wetzel et al., 2010). Also, research has been focused on the safety of common edible flowers, so as to ensure their safe use and dosage by different industries and with different purposes (Koike et al., 2015a; Wetzel et al., 2010). There are numerous edible flowers all over the world and only a small part of them have been studied. Therefore, more detailed information regarding this natural matrix is needed, in order to increase their acceptability as food ingredients and to avoid potential risks (Lu et al., 2015). Since not all flowers meet the essential requirements in order to be considered edible, such as being non-toxic and innocuous and having adequate nutritional properties, they cannot be included in the human diet (Lara-Cortés, Osorio-Díaz, Jiménez-Aparicio, & Bautista-Baños, 2013). Some flower species have toxic or antinutritional substances, such as trypsin inhibitors, hemagglutinins, oxalic acid, cyanogenic glycosides or alkaloids. Some of these compounds have been found, for instance, in flowers of *Agave salmiana* Otto ex Salm-Dyck, *Erythrina Americana* Mill., *Erythrina caribaea* Krukoff & Barneby, or *Yucca filifera* Chabaut (Lara-Cortés et al., 2013; Navarro-González, González-Barrio, García-Valverde, Bautista-Ortín, & Periago, 2015; Sotelo, López-García, & Basurto-Peña, 2007). Consequently, those flowers must be considered inedible and hence cannot be included in the human diet.

1.2.1. Production and safety

The appearance of edible flowers in no way differs from the ornamental species and it is crucial to differentiate them by using chemical and biological parameters, so that they could be distinguished for their edibility (Mlcek & Rop, 2011). Purely decorative flowers may have toxic components that can lead to intoxication and be even fatal. In many cases, the cultivation of these plants involves the use of harmful chemicals, whereas edible flowers are usually the result of an organic production, intended for food purposes (Fernandes, Casal, Pereira, Saraiva, & Ramalhosa, 2017).

Bringing innovative products with nutraceutical properties and health benefits to the market is one of the current challenges of producing edible flowers. In the 21st century,

the agro-food industry faces several challenges, namely regarding Food Security (having enough to eat) and Food Safety (safe to eat) (Scotter, 2015). These challenges should be understood not only in an environment of tremendous technological progress and evolution of consumer's life-styles, but also in economic terms, in which the food industry is called to operate under seemingly contradictory market demands (Behe et al., 2010; Chen & Wei, 2017). Consumers preference for natural products, minimally processed foods, packaged without preservatives and free of negative effects (e.g., low in fat, salt and sugar) is gaining increasing attention by the food industry. This is also affected by recently emerged issues, such as climatic change, financial crisis and breakthrough regarding technology information (Chen & Wei, 2017; Scotter, 2015).

Despite all rigor, approval and control by competent organizations in the application of radiation as a safety factor, there are many obstacles related to the cost of this technology and also with the acceptance by consumers, which limit irradiated foods from reaching pioneer levels of commercialization. Specifically, on gamma radiation, there is still the scepticism that the isotopes ^{137}Cs and ^{60}Co could leave radioactive residues (Ornellas et al., 2006; Supriya et al., 2014). Thus, the lack of information available to the consumers about its benefits or harms, particularly with regard to the toxicological risks hypothetically associated, reduces confidence in the use of ionizing radiation in the processing and preservation of food.

1.2.2. Processing and packaging

The marketing of fresh edible plants and their acceptance by the consumers is an important factor regarding their commercialization and post-harvest performance. Although external quality-related attributes, such as appearance, color and odour, are the main criteria for attracting consumers preference and decision-making, there is a great interest in foods with bioactive and nutraceutical components (Behe et al., 2010; Chen & Wei, 2017). The packaging of flowers is usually performed using small and rigid plastic containers, because they are highly perishable (could suffer oxidation) and are easily contaminated by insects, which compromises their nutritional and bioactive characteristics, as also decreases their attractiveness (Fernandes et al., 2018; Villavicencio et al., 2018). Hence, it is essential to develop improved techniques to aid quality retention and extend shelf life of edible flowers. The most common methods used to improve postharvest storage of flowers quality include refrigeration, drying, canning in sugar and preservation in distillates (Fernandes et al., 2017). However, these methods

may cause undesirable biochemical and nutritional changes in the processed product that may affect its overall quality. Food irradiation is an economically viable technology to extend shelf life of perishable commodities, which allows the disinfestation of insects, improves hygiene and helps maintain quality, in addition to preserving the bioactive characteristics and phytochemicals of the irradiated products (Farkas & Mohácsi-Farkas, 2011; Koike et al., 2015a). Koike et al. (2015a) studied the effects of electron-beam and gamma irradiation on the phenolic profile and antioxidant activity of edible flowers of *Viola tricolor* L.; they concluded that irradiation allows increasing the shelf life of these flowers without negatively affecting the levels of phenolic compounds and antioxidant activity, making this technology a suitable commercial alternative. Modified Atmosphere Packaging (MAP) is another technology that has been extensively used to maintain the quality, extend shelf life and decrease microbial growth in perishable products, such as edible flowers, as demonstrated by Kou et al. (2012). These authors investigated the effect of the treatment with the synthetic plant growth regulator 1-methylcyclopropene together with MAP on the shelf life of *Dianthus caryophyllus* L. and *Antirrhinum majus* L. and concluded that this technology significantly extended the storage time of both edible flowers, reducing the weight loss and helping to maintain the visual quality (fresh appearance) (Kou, Turner, & Luo, 2012).

Edible coatings are another methodology used to extend shelf life. The coatings could be derived from proteins, lipids and polysaccharides and can be used to protect perishable food products from deterioration by providing a selective barrier to moisture, oxygen and carbon dioxide. This allows delaying dehydration, suppressing respiration and improving textural quality, while helps to retain volatile flavour compounds and reduces microbial growth, thus making this methodology appropriate to be applied in edible flowers, as demonstrated by Fernandes et al. (2018). Those authors evaluated the effects of alginate coating on the physico-chemical and microbiological quality of *Viola* x *wittrockiana* edible flower under cold storage. The flowers coated with alginate revealed a good appearance until 14 days of storage, 7 days more than the uncoated sample, and also presented a significant reduction of yeasts and moulds counts (Fernandes et al., 2018).

1.2.3. Consumption

Cultural differences and patterns of consumption may determine the acceptance by consumers of a “new food” or “unfamiliar food”. The consumption of edible flowers in the Asian cuisine is already a common practice, and over time they have gained more

common usage in other cultures, including Europe (Rodrigues et al., 2017). Their aroma, taste and appearance turns meals more attractive, which together with their nutritional properties and low fat and energetic content have promoted their consumption worldwide (Rodrigues et al., 2017).

1.2.4. Nutritional and chemical composition

Edible flowers are usually composed by 70 to 95% of water. The composition and levels of other nutrients and phytochemicals depend on the part of the flower. For example, pollen is a source of proteins, carbohydrates, saturated and unsaturated lipids, carotenoids and flavonoids, while the nectar is made up of amino acids, free sugars, proteins, inorganic ions, lipids, organic acids, phenolic compounds, alkaloids and terpenoids, among others. Flowers and other parts of the flowers are richer in vitamins, minerals and antioxidants (Fernandes et al., 2017; Mlcek & Rop, 2011). Carbohydrates are the most abundant macronutrients in edible flowers, with values usuallly above 85% of dry weight. Fiber content is quite variable, ranging from 6.1 to 55.4 g/100 g dry weight, as determined for flowers of *Allium schoenoprasum* L., and *Spilanthes oleracea* L. and *Tagetes erecta* L. (Fernandes et al., 2017). Potassium, phosphorus, calcium and magnesium are the main minerals present, with potassium levels being higher than calcium, bringing greater benefits relative to the prevention of cardiovascular diseases (Fernandes et al., 2017; Rop et al., 2012). The use of edible flowers in the development of children's snacks has a dated application. In Poland, one of the favourite children snacks are developed using flowers of *Trifolium* spp., *Lamium album* and *Robinia pseudacacia* (Łuczaj et al., 2012). The demand for healthier eating habits and low-calorie foods has promoted the development of new functional products obtained from natural sources.

1.2.5. General and medicinal uses of edible flowers by consumers

Edible flowers are usually used to add color (e.g., *C. cyanus* and *V. tricolor*), fragrance or flavour (e.g., *Hibiscus sabdariffa* L.) to food products, such as salads, soups, entrees, desserts and drinks. In addition to these characteristics, different flowers have also been described to possess anti-inflammatory, anti-edematous, anti-HIV, antimicrobial (antibacterial and antifungal), immuno-stimulating and immunomodulatory, spasmolytic, spasmogenic, gastroprotective, insecticidal, genotoxic, antioxidant, or anti-tumour activities (Benvenuti, Bortolotti, & Maggini, 2016; Lara-Cortés et al., 2013). **Table 1** describes some edible flowers and their food and medicinal uses.

The most frequent form to consume edible flowers is in fresh salads, for example *A. majus* and *Bauhinia purpurea* L. are widely employed in this type of meals, but they can also be consumed dried or canned in sugar, as well as incorporated in cocktails in ice cubes (Lai, Lim, & Kim, 2010; Loizzo et al., 2016). Moreover, edible flowers may also be preserved in distillate products or as pickles in vinegar and salt, such as the buds of *Capparis spinosa* L., commonly consumed in Mediterranean countries as seasoning or garnish and that have been related to possess diuretic, antiseptic, and capillary vessels protective properties (Loizzo et al., 2016). *Tropaeolum majus* L. is often consumed as an ingredient in different meals like salads and also in beverages, being its consumption associated to different health benefits, namely antibacterial, antitumor, antithrombotic, diuretic, and hypotensive effects (Benvenuti et al., 2016; Navarro-González et al., 2015). The flowers of *Carthamus tinctorius* L., *Chrysanthemum morifolium* ramat., *Gardenia jasminoides* J. Ellis, *Lonicera japonica* Thunb., and *Rosa chinensis* Jacq are commonly consumed as infusions and also incorporated into cakes; they have been reported to present significant bioactive properties, such as promoting blood circulation, restoring menstrual flow, and heat-clearing and detoxifying activities (Wang et al., 2016).

In general, edible flowers are eaten whole, but there are some flower species where only some parts should be consumed, e.g., flowers of Tulipa, *Chrysanthemum, Rosa spp*. or the flower buds of daisies (*Bellis perenis*) or garden nasturtium (*Tropaeolum majus*) due to their bitterness or other unpleasant characteristics. The acceptability of edible flowers depends on a number of factors, such as the social group, species of flowers, characteristics (taste, texture and appearance), consumers profile (education, gender, annual income), or presentation (composition of flowers, size and price) (Fernandes et al., 2017).

Table 1. Edible and medicinal uses of some flowers.

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Anchusa azurea P. Mill.	Garden anchusa and Italian bugloss	Boraginaceae	Violet light-blue  Source: by Miguel Martínez in www.flickr.com	Soup, boil, fries and salad	Depurative, antitussive, diaphoretic, and diuretic	(Loizzo et al., 2016)
Antirrhinum majus L.	Snapdragon	Plantaginaceae	Red, Rose, White  Source: by naturguker.de in www.flickr.com	Salad	Antiphlogistic, resolvent and stimulant; liver disorders, treatment of scurvy, tumours and as detergent, astringent and diuretic	(Loizzo et al., 2016)
Bauhinia purpurea L.	Orchid Tree, Purple Butterfly Tree, Mountain Ebony, Geranium Tree, Purple Bauhinia	Leguminosae	Purple  Source: by Carlos Beutelspacher in www.flickr.com	Salad	Nephroprotective and thyroid hormone regulating; antibacterial, antidiabetic, analgesic, anti-inflammatory, anti-diarrheal and antitumor activities	(Lai et al., 2010)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Bombax malabaricum L.	Cotton tree	Bombacaceae	Orange, red  Source: by Ilya Borovok in www.flickr.com	Cooked and accompanied with meat and rice	Treatment of chronic inflammation, fever, diarrhoea, hepatitis, and contused wounds	(Zhang et al., 2015)
Calendula officinalis L.	Marigold or Scotch marigold	Asteraceae	Orange  Source: by Marco Ottaviani in www.flickr.com	Salads, omelettes or as an accompaniment cheese	Antioxidant, anti-inflammatory, antitumor, anti-edematous, anti-HIV, antibacterial and antifungal activities; immunomodulatory and immuno-stimulating, spasmolytic, spasmogenic and gastroprotective, insecticidal, heart rate decrease, cardioprotective, genotoxic and antigenotoxic dose-dependent	(Benvenuti, Bortolotti, & Maggini, 2016; Lara-Cortés et al., 2013)
Capparis spinosa L.	Flinders rose	Capparaceae	White-violet  Source: by C. E. Timothy Paine in www.flickr.com	Preserved in vinegar and salt and salad	Antiseptic, diuretic, and protective of capillary vessels	(Loizzo et al., 2016)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Carthamus tinctorius L.	Safflower	Asteraceae	Red  Source: by Färberdistel oder Saflor in www.flickr.com	Infusions and cakes	Restoring menstrual flow and promoting blood circulation	(Wang et al., 2016)
Centaurea cyanus L.	Cornflower and bachelor's button	Asteraceae	Blue  Source: by Atanue D. in www.flickr.com	Infusions, garnish and natural food colorant	Antioxidant activity, soothing, and used in ocular inflammation	(Fernandes et al., 2017)
Chrysanthemum morifolium Ramat	Florist's daisy and hardy garden mum	Asteraceae	Yellow-white  Source: by Inthemind Ofnature in www.flickr.com	Infusions and cakes	Detoxifying and heat-clearing effects	(Wang et al., 2016)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Cichorium intybus L.	Chicory	Astereacea	Light blue  Source: by Hiro. Morison in www.flickr.com	Soup, boil, potage and salad	Depurative, diuretic, laxative, hypoglycaemic, disinfectant of urinary tract and hepatoprotective	(Loizzo et al., 2016)
Gardenia jasminoides J.Ellis	Gardenia, cape jasmine, cape jessamine, danh-danh and jasmin	Rubiaceae	White  Source: by Stefano in www.flickr.com	Infusions and soup	Promoting diuresis and heat-clearing	(Wang et al., 2016)
Hedysarum coronarium L.	Sweetvetch	Fabaceae	Purple  Source: by María García in www.flickr.com	Soups, fries with eggs, and salad	Hypocholesterolemic and laxative effects	(Loizzo et al., 2016)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Hibiscus rosa sinensis L.	Chinese hibiscus, China rose, Hawaiian hibiscus, rose mallow and shoeblackplant	Malvaceae	Rose  Source: by P.L. Tandon in www.flickr.com	Infusions and food supplement	Genito-urinary troubles, bronchial catarrh, fever and cough	(Lu et al., 2015)
Hibiscus sabdariffa L.	Roselle	Malvaceae	Red  Source: by Oleksandr Reva in www.flickr.com	Flavouring agents, beverage (hot and cold), jams preparation of herbal drinks, fermented drinks, wine, ice cream, chocolates, puddings and cakes	Hypertension, abscesses, dysuria, fever and scurvy	(Lu et al., 2015; Xiong et al., 2014)
Jasminum sambac L.	Arabian jasmine and Sambac jasmine	Oleaceae	White  Source: by Robert Sarkisian in www.flickr.com	Infusions and porridge	Skin diseases, cancer, uterine bleeding, ulceration, leprosy and wound healing	(Wang et al., 2016; Xiong et al., 2014)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Lonicera japonica Thunb.	Japanese honeysuckle and golden and silver honeysuckle	Caprifoliaceae	Yellow-green  Source: by Ebroh in www.flickr.com	Infusions and soup	Heat-clearing and detoxifying	(Lu et al., 2015; Wang et al., 2016)
Rosa chinensis Jacq	China rose and Chinese rose	Rosaceae	Red  Source: by Bunga Mawar in www.flickr.com	Flavour extract, jams and infusions	Homeostasis, menstruation disorders, trauma and diarrheal	(Lu et al., 2015)
Tropaeolum majus L.	Garden nasturtium, Indian cress, and monks cress	Tropaeolaceae	Yellow. Orange, Red  Source: by Taylor World in www.flickr.com	Ingredients in meals, salads, foodstuffs and drinks	Antibacterial, antitumor and antithrombotic activities, diuretic and hypotensive effects	(Stefano Benvenuti et al., 2016; Navarro-González et al., 2015)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Viola tricolor L.	Johnny Jump up and heartsease	Violaceae	Yellow, orange, purple, violet  Source: by Nora Caracci and Albert_zsolt in www.flickr.com	Food colorants, sweets, salads, soups, vinegars and drinks	Prevention in Alzheimer, Parkinson, atherosclerosis and various cancers; antiallergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective and vasodilator effects.	(Koike et al., 2015a; Navarro-González et al., 2015)

1.2.6. Toxicity of edible flowers

Currently there are not many studies regarding the toxicity of edible flowers, thus those that are found associate that the toxic effects depends on the plants parts (Egebjerg et al., 2018). For instance, the leaves of *Allium schoenoprasum* present in their composition sulphurous substances that are non-existent in the flowers, which are a non-common plant part consumed (Sobolewska, Podolak, & Makowska-Wąs, 2015). Moreover, the presence of some compounds in plant parts, such as hydrogen cyanide, erucic acid, coumarin and thujone, has to follow a guidance value table set by EFSA or JECFA, which contemplates the Tolerable Daily Intake or Acceptable Daily Intake of these compounds, which will determine their toxicity (Egebjerg et al., 2018). Some of flowers such as *Tropaeolum majus,* when ingested in amounts higher than 39.5 g of fresh flowers will exceed the Tolerable Daily Intake in erucic acid (Egebjerg et al., 2018). Moreover, the ingestion of more than 18 g of *Achillea millefolium* flower would exceed the Acceptable Daily Intake for thujone, and the ingestion of 7 g of *Galium odoratum* flowers the Tolerable Daily Intake for coumarin, for adults (Egebjerg et al., 2018; Kalemba-Drożdż, 2019).

Taking into account all these facts and that these compounds may be present in other food sources, further studies focusing on the recommended doses are needed and should be developed, although most often these doses would not be exceeded if the plant is used as an edible decoration part in food products, such as cakes or desserts (Egebjerg et al., 2018; Kalemba-Drożdż, 2019).

1.2.7. Bioactive compounds present in edible flowers

Phenolic compounds are a group of secondary metabolites found in different plant parts that are considered to have numerous bioactive properties (Kucekova, Mlcek, Humpolicek, 2013). These compounds are classified according to the number of phenol subunits present, in polyphenols and simple phenols (Vuolo, Lima, & Maróstica Junior, 2019), and can be further divided into flavonoids and non-flavonoid compounds. Phenolic acids and derivatives are the main non-flavonoid compounds found in plants, but there are other compounds that are considered in this class, such as stilbenes, lignins, lignans, coumarins, naphtoquinones, xanthones, and anthraquinones, which are also found in natural sources (Huang et al., 2017; Li & Sun, 2017).

Phenolic acids occur naturally in plants and can be divided into hydroxybenzoic benzoic acids (C6-C1) and hydroxycinnamic acids (C6-C3); they may occur as free acids and as derivatives usually combined with sugars or organic acids (e.g., quinic acid). Caffeic, *p*-

coumaric, vanillic, ferulic and protocatechuic acids are present in almost all plants, and their presence in the diet has been linked to the prevention of ageing-related diseases, such as cancer, cardiovascular and neurodegenerative diseases (Butts-Wilmsmeyer et al., 2018; Kucekova, Mlcek, Humpolicek, 2013). Flavonoids are characterized by their C6-C3-C6 skeleton and are constituted by main 6 subgroups: flavan-3-ols, flavonols (e.g. quercetin, kaempferol, myricetin), flavones (e.g. apigenin, luteolin, chrysin), flavanones (e.g. hesperidin, naringenin, eriodictyol), isoflavones (e.g. genistein, daidzein, glycitein) and anthocyanins (e.g. cyanidin, delphinidin, malvidin) (Dias, Carocho, Barros, & Ferreira, 2019; Li & Sun, 2017). Flavan-3-ols may occur in their monomeric forms (catechins) or as polymers (condensed tannins or proanthocyanidins), for which very different bioactive properties have been reported, being related with cardiovascular protection (Dias et al., 2019). Flavones differ from other flavonoids by their double bond between C2 and C3 in the flavonoid skeleton and a keto group at C4 position, with no substitution at the C3 position (Hostetler, Ralston, & Schwartz, 2017). Biological activities, such as anticancer, cytotoxic, hepatoprotective, antidiabetic, anti-inflammatory, antiviral and anti-ageing properties have been described for these compounds (Dias et al., 2019). Flavonols are similar to flavones but they possess a hydroxy group at C3 position; quercetin and kaempferol and their glycosylated and methylated forms are the major representatives of this group. A range of beneficial health effects have been described for flavonols, including anti-inflammatory, genotoxic and antioxidant capacities, as also protective effects against cardiovascular, Parkinson's and Alzheimer's diseases (Dias et al., 2019). Flavanones are mainly made up of three main abundant aglycones (hesperetin, naringenin, and eriodictyol), being mostly present in citrus fruits as glycosylated forms; their presence has been associated with anti-inflammatory, anti-cancer, anti-mutagenic, cardiovascular, anti-proliferative, vasorelaxant, and vasoprotective effects (Dias et al., 2019). Isoflavonoids are mainly found in legumes and have been related to estrogenic, antibacterial, antiviral, anti-inflammatory and anti-ischemic activities (Zheng, Deng, Guo, Chen, & Fu, 2019). Anthocyanins occur in nature mainly in the form of heterosides whose aglycone (or anthocyanidin) consists of a 2-phenylbenzopyrylium (flavylium) skeleton diversely hydroxylated and methoxylated. Currently more than 700 anthocyanins have been described in nature and above 200 have been tentatively identified (Santos-Buelga & González-Paramás, 2019). These compounds are water-soluble pigments highly recognised as colorant molecules, being responsible for the coloration of many fruits and

vegetables and the flowers in most flowers, and they are also acknowledged to have a high bioactive potential.

Flowers may contain a variety of all these phenolic compounds, which are recognised as natural antioxidants, being their presence strongly related to their color, either directly (e.g., anthocyanins and other flavonoid pigments) or indirectly through co-pigmentation processes (Kaisoon, Siriamornpun, Weerapreeyakul, & Meeso, 2011; Skrajda, 2017)

Table 2 presents the main non-anthocyanin phenolic compounds found in some edible flowers. Among them, phenolic acid derivatives, especially hydroxycinnamic acids, are found in relevant amounts, with caffeoylquinic acids being majority phenolic compounds in many species (e.g. *Achillea millefolium* L., *H. sabdariffa* and *Tropaelum majus* L.). Regarding flavonoids, flavonols are within the main phenolics present in edible flowers, in particular quercetin and kaempferol derivatives (**Table 2**). Quercetin was the main phenolic compound quantified in *V. tricolor* and *Hemerocallis fulva* L. (46 mg/g DW and 273 mg/g DW, respectively (Koike et al., 2015a; Wu, Mong, Yang, Wang, & Yin, 2018), while caffeoylquinic acids were the best represented compounds in four of the twelve flowers analysed by Guimarães et al. (2013), being *Matricaria recutita* L., the flower with the highest amount of these derivatives, namely 3,4-*O*-dicaffeolyquinic acid (730 mg/100 g DW).

Moreover, solid-liquid extraction systems (maceration, ultrasound assisted extraction, among others) using different organic solvents and mixtures of organic/water solvents (water, mixture of water and ethanol or methanol and acetone) are the most common methodology applied in the extraction of non-anthocyanin compounds, as it can be visualized in **Table 2.**

Table 2. Content and extraction methodology of the main non-anthocyanin phenolic compounds in edible flowers.

Edible Flowers	Origin	Main phenolic compounds	Identification	Content	Extraction methodology	Reference
Achillea millefolium L.	Bulgaria	Isorhamnetin-3-*O*-(6-*O*-rhamosyl-galactoside)	HPLC-DAD-ESI-QTOF-MS	12.6 mg/g DW	Ultrasound-assisted extraction, with a power of 200W and frequencies of 60 kHz, using ethanol (1:10 plant/solvent ratio), 30 min time, 40 °C	(Villalva et al., 2019)
		Luteolin-7-*O*-glucoside		7.69 mg/g DW		
		Luteolin		4.47 mg/g DW		
		3,5-*O*-Dicaffeoylquinic acid		3.62 mg/g DW		
Hemerocallis fulva L.	China and Taiwan	Quercetin	HPLC-DAD	273 mg/g DW	Solid-liquid extraction with 300 g of sample in 2000 mL boiling distilled water for 1 h.	(Wu et al., 2018)
		Ellagic acid		205 mg/g DW		
		Chlorogenic acid		175 mg/g DW		
Hibiscus sabdariffa L.	Alfândega da Fé, Bragança, Portugal	5-(Hydroxymethyl)furfural	HPLC-DAD-ESI/MSn	5.75 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of ethanol/water (80:20 v/v), 25 °C at 150 rpm for 1 h	(Jabeur et al., 2017)
		3-*O*-Caffeoylquinic acid		2.88 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of water, 25 °C at 150 rpm for 1 h	
		5-*O*-Caffeoylquinic acid		1.53 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of ethanol/water (80:20 v/v), 25 °C at 150 rpm for 1 h	
Lavandula pedunculata Mill.	Ponte de Sôr, Portalegre	Salvianolic acid B	HPLC-DAD-ESI/MSn	582 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of ethanol/water (80:20 v/v), 25 °C at 150 rpm for 1 h	(Lopes et al., 2018)
		Rosmarinic acid		550 mg/g DW		
		Luteolin-7-*O*-glucuronide		84.1 mg/g DW		

Edible Flowers	Origin	Main phenolic compounds	Identification	Content	Extraction methodology	Reference
Lonicera japonica Thunb	China	Chlorogenic acid Rutin Protocatechuic acid	HPLC-DAD	16.0 mg/g DW 0.62 mg/g DW 0.25 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
Matricaria recutita L.	Trás-os-Montes, North-eastern Portugal.	Luteolin-*O*-acylhexoside 3,4-*O*-Dicaffeolyquinic acid Feruloyl hexoside acid dimer	HPLC-DAD–ESI/MS	1290 mg/100g DW 730 mg/100g DW 590 mg/100g DW	Solid-liquid extraction with 1 g in 30 mL of methanol:water (80:20 v/v) for 1 h.	(Guimarães et al., 2013)
Paeonia suffruticosa Andrews	China	Rutin Gallic acid Quercetin	HPLC-DAD	18.1 mg/g DW 7.99 mg/g DW 7.24 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
Prunus persica L.	China	Chlorogenic acid Kaempferol Rutin	HPLC-DAD	6.54 mg/g DW 2.78 mg/g DW 0.70 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
Rosa chinensis Jacq.	China	Gallic acid Chlorogenic acid 3-Hydroxy-4-methoxybenzaldehyde thiosemicarbazone	HPLC-DAD	6.87 mg/g DW 2.66 mg/g DW 1.09 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
Rosa rugosa Thunb.	Poland	Ellagitannin (+)-Catechin Sanguine H-2	UPLC-DAD-Q/TOF-MS	1072 mg/100 g FW 178 mg/100 g FW 166 mg/100 g FW	Solid-liquid extraction with 50 g in 500 mL of ethyl alcohol for 18 days.	(Cendrowski et al., 2017)

Edible Flowers		Origin	Main phenolic compounds	Identification	Content	Extraction methodology	Reference
Trapaeolum majus L.	Yellow	Geneva, NY, USA	*cis* 5-*p*-Coumaroylquinic acid	HPLC-DAD	639 mg/100g DW	Solid-liquid extraction with 0.5 g in 1:1 (w/v) 70% aqueous acetone under a nitrogen atmosphere during 10 min.	(Garzón, Riedl, & Schwartz, 2009; Navarro-González et al., 2015)
			3-*O*-Caffeoylquinic acid		283 mg/100g DW		
			5-*O*-Caffeoylquinic acid		247 mg/100g DW		
	Orange		Kaempferol dihexoside		1199 mg/100g DW		
			5-*O*-Caffeoylquinic acid		233 mg/100g DW		
			3-*O*-Caffeoylquinic acid		182 mg/100g DW		
	Red		Myricetin dihexoside		2265 mg/100g DW		
			Kaempferol dihexoside		268 mg/100g DW		
			5-*O*-Caffeoylquinic acid		138 mg/100g DW		
Viola tricolor L.		São Paulo, Brazil	Quercetin-3-*O*-(6-*O*-rhamnosylglucoside)-7-*O*-rhamnoside	HPLC–DAD–ESI/MS	46 mg/g extract DW	Solid-liquid extraction with 0.5 g in 20 mL of methanol/water 80:20 (v/v), at room temperature, 150 rpm, for 1h	(Koike et al., 2015a)
			Quercetin-3-*O*-rutinoside		28 mg/g extract DW		
			Isorhamnetin-3-*O*-(6-*O*-rhamosyl-galactoside)		12.6 mg/g extract DW		

1.2.8. Anthocyanins composition in edible flowers

The presence of anthocyanins confers the flowers a great diversity of colors, touching practically all the visible spectra, from orange and red to purple and blue hues, making these matrices a potential source of these natural pigments, which can provide new colors and flavours, attracting the attention of consumers. Edible flowers are used to garnish and/or decorate meals, sweets, ice-creams or drinks improving not only the aesthetic effect, but also adding a specific taste and smell to the food dishes. Further, nutritional and bioactive features of edible flowers represent an additional value for their consumption. Many edible flowers have begun to arouse interest in the food industry due to the important amounts of anthocyanins present in their composition. Anthocyanins have been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1982 and by the EU Scientific Committee for Food (SCF) in 1975 and 1997 (Pop, Lupea, Popa, & Gruescu, 2010; Rodriguez-Amaya, 2016), and are authorised as food colorants in the European Union with the common code E163 regardless of their origin, indicating that, at least from a regulatory point of view, they are looked upon as a group of harmless compounds (Santos-Buelga & González-Paramás, 2019).

The growing concern about the substitution of artificial colorants for natural counterparts has promoted the interest in the search of new alternatives, and in this case edible flowers could be interesting sources of these natural molecules. **Table 3** presents some edible flowers and their composition in anthocyanins. It can be seen that the most common anthocyanins present in the majority of the flowers are cyanidin derivatives, namely cyanidin-3-*O*-glucoside. However, other major compounds can also be found, such as malvidin-3-O-glucoside (202.1 mg/kg fresh weight (FW) and delphinidin-3-*O*-glucoside (109 mg/kg FW) in *Nelumbo nucifera* (Gaertn.) (Deng et al., 2013), delphinidin-3,7-*O*-diglucoside (3936 μg/g DW) in *Crocus sativus* L. (Goupy, Vian, Chemat, & Caris-Veyrat, 2013), delphinidin-3-*O*-(4"p-coumaroyl)-rutinoside-5-*O*-glucoside (10.2 mg/g DW) in *V. tricolor* (Koike et al., 2015a), or pelargonidin-3-*O*-sophoroside (591.6 mg/g DW) in *Tropaeolum majus* L. red variety (Garzón, Manns, Riedl, Schwartz, & Padilla-Zakour, 2015).

Anthocyanins have been described to provide a range of health benefits, including antioxidant, anti-inflammatory and anti-proliferative effects. Many fruits and vegetables have been demonstrated to inhibit the initiation, promotion, and progression of several cancers, such as breast, prostate, liver, colorectal, intestinal, blood, or cervical cancers, which has been related to their anthocyanin composition (Hidalgo & Almajano, 2017; Khoo, Azlan, Tang, & Lim, 2017; Li et al., 2017b). Anthocyanin-rich extracts from Hibiscus have shown to be able to significantly suppress rotenone-induced dopaminergic cell death via interference with

microglial activation and amelioration of mitochondrial dysfunction, suggesting their neuroprotective activity and ability to improve cognitive, memory and motor performances, which may have potential application in the prevention of neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases (Li et al., 2017). Cyanidin-3-*O*-glucoside has demonstrated to be able to inhibit carrageenan-induced acute inflammation and peritonitis through downregulation of cyclooxygenase-2 expression and inhibition of prostaglandin E2 production, indicating its anti-inflammatory potential (Li et al., 2017).

Although several in vitro and in vivo studies have been carried out to try to demonstrate the biological activity of anthocyanins, a major drawback for their use is their low bioavailability, as they are considered to be poorly absorbed and largely metabolised, being found in blood under the form of metabolites (Fernandes, Marques et al., 2019). In this respect, the use of nanotechnology can provide promising tools for solving the problems of bioavailability (Sharif, Shah, Butt, & Sharif, 2016). On the other hand, anthocyanin structure may also influence their activity and the molecular mechanisms involved, so that the isolation and purification of specific molecules is required in order to determine their effects (Li, et al., 2017).

Table 3. Anthocyanin content of edible flowers.

Edible flower	Main anthocyanins	Identification	Content	Extraction methodology	Reference
Bauhinia purpurea L.	Cyanidin-3-*O*-glucoside	HPLC-DAD	59.8 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Bombax malabaricum D.C.	Cyanidin-3-*O*-glucoside	HPLC-DAD	63.4 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Brunfelsia acuminate L.	Cyanidin-3-*O*-glucoside	HPLC-DAD	61.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Calliandra haematocephala Hassk.	Cyanidin-3-*O*-glucoside	HPLC-DAD	517 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)

Edible flower	Main anthocyanins	Identification	Content	Extraction methodology	Reference
Crocus sativus L.	Delphinidin-3,7-*O*-diglucoside Petunidin-3,7-*O*-diglucoside Petunidin-3-*O*-glucoside Malvidin-*O*-glucoside	UPLC-DAD-ESI/MS	3936 μg/g DW 380 μg/g DW 475 μg/g DW 13 μg/g DW	Ultrasound-assisted extraction with 2 g for 5 min in 20 mL methanol/water (50:50, v/v) containing hydrochloric acid 1%, followed by a magnetic stirring for 30 min.	(Goupy et al., 2013)
Dendranthema grandiflorum Ramat.	Cyanidin-3-*O*-(3″malonyl)-glucoside Cyanidin-3-*O*-glucoside	HPLC-ESI/MS	11.3 mg/g DW 1.06 mg/g DW	Ultrasound-assisted extraction with 0.1 g of plant material in 2 mL of water-formic acid, 95:5 (v/v) for 5 min	(Park et al., 2015)
Dianthus caryophyllus L.	Cyanidin-3-*O*-glucoside	HPLC-DAD	52.4 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Gerbera jamesonii Bollus ex Hooker F.	Cyanidin-3-*O*-glucoside	HPLC-DAD	60.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)

Edible flower	Main anthocyanins	Identification	Content	Extraction methodology	Reference
Hibiscus rosa-sinensis L.	Cyanidin-3-*O*-glucoside	HPLC-DAD	72.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Hibiscus sabdariffa L.	Delphinidin-3-*O*-sambubioside Delphinidin-3-*O*-glucoside Cyanidin-3-*O*-sambubioside Cyanidin-3-*O*-glucoside	LC–MS	4.11 mg/g DW 0.15 mg/g DW 3.81 mg/g DW 0.46 mg/g DW	Solid-liquid extraction with 0.1 g of plant material in 10 mL of water at 100 ºC for 10 min	(Sindi, Marshall, & Morgan, 2014)
Ipomoea cairica (L.) Sweet	Cyanidin-3-*O*-glucoside	HPLC-DAD	11.0 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Jatropha integerrima Jacq.	Cyanidin-3-*O*-glucoside	HPLC-DAD	641.5 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)

Edible flower		Main anthocyanins	Identification	Content	Extraction methodology	Reference
Lantana camara L.		Cyanidin-3-*O*-glucoside	HPLC-DAD	48.6 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Lilium brownie A. Poit.		Cyanidin-3-*O*-glucoside	HPLC-DAD	10.7 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Nelumbo nucifera (Gaertn.)	Red	Malvidin-3-*O*-glucoside	HPLC–MS	202 mg/kg FW	Solid-liquid extraction with 0.7 g of plant material in 5 mL of 70% methanol aqueous solution containing 2% of formic acid, at 4 °C in dark during 24h	(Deng et al., 2013)
		Delphinidin-3-*O*-glucoside		109 mg/kg FW		
		Petunidin-3-*O*-glucoside		55.6 mg/kg FW		
		Cyanidin-3-*O*-glucoside		30.6 mg/kg FW		
		Peonidin-3-*O*-glucoside		28.2 mg/kg FW		
	Pink	Malvidin-3-*O*-glucoside		81.9 mg/kg FW		
		Delphinidin-3-*O*-glucoside		23.8 mg/kg FW		
		Petunidin-3-*O*-glucoside		13.3 mg/kg FW		
		Cyanidin-3-*O*-glucoside		5.88 mg/kg FW		
		Peonidin-3-*O*-glucoside		8.56 mg/kg FW		
	Yellow	Malvidin-3-*O*-glucoside		2.53 mg/Kg FW		
		Delphinidin-3-*O*-glucoside		0.14 mg/Kg FW		

Edible flower	Main anthocyanins	Identification	Content	Extraction methodology	Reference
Oncidium varicosum	Cyanidin-3-*O*-glucoside	HPLC-DAD	52.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Orostachys fimbriata (Turcz.)	Cyanidin-3-*O*-glucoside	HPLC-DAD	160 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Pelargonium × *hortorum*	Cyanidin-3-*O*-glucoside	HPLC-PAD	497 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Rosa hybrida cv. Noblered	Cyanidin-3,5-di-*O*-glucoside	HPLC-DAD	375 mg /g DW	Solid-liquid extraction with 5 g of plant material in 500 mL of methanol with 1% (v/v) of TFA during 48h at 4 °C, in the dark	(Lee, Lee, & Choung, 2011)

Edible flower		Main anthocyanins	Identification	Content	Extraction methodology	Reference
Rhododendron spp L.		Cyanidin-3-*O*-glucoside	HPLC-DAD	65.9 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Salvia splendens Sellow ex J.A. Schultes		Cyanidin-3-*O*-glucoside	HPLC-DAD	30.6 mg/g W	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 ºC for 30 min in a shaking water bath	(Li et al., 2014)
Tropaeolum majus L.	Yellow	Pelargonidin-3-*O*-sophoroside	HPLC-DAD	126 mg/g DW	Ultrasound-assisted extraction with 0.5 g of plant material with 1:1 (w/v) 70% aqueous acetone under a nitrogen atmosphere for 10 min in a chilled water bath	(Garzón et al., 2015)
		Delphinidin-3-*O*-dihexoside		95.1 mg/g DW		
		Cyanidin-3-*O*-sophoroside		24.8 mg/g DW		
	Orange	Pelargonidin-3-sophoroside		439.6 mg/g DW		
		Delphinidin-3-*O*-dihexoside		35.9 mg/g DW		
		Cyanidin-3-*O*-sophoroside		10 mg/g DW		
	Red	Delphinidin-3-*O*-dihexoside		592 mg/g DW		
		Pelargonidin-3-*O*-sophoroside		213 mg/g DW		
		Cyanidin-3-*O*-sophoroside		76.0 mg/g DW		
Tropaeolum majus L.		Pelargonidin-3-*O*-sophoroside	HPLC-DAD-ESI-MS	5.8 mg/g DW	Solid-liquid extraction with 0.5 g of plant material with 20 mL of methanol, containing 0.5% TFA during 2h.	(Koike et al., 2015b)
		Delphinidin-*O*-dihexoside		3.2 mg/g DW		
		Cyanidin-*O*-dihexoside		0.21 mg/g DW		

Edible flower		Main anthocyanins	Identification	Content	Extraction methodology	Reference
Viola tricolor L.		Delphinidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside	HPLC-DAD-ESI-MS	10.2 mg/g DW	Solid-liquid extraction with 0.5 g of plant material in 20 mL of methanol containing 0.5% trifluoroacetic acid, during 2h	(Koike et al., 2015a)
		Delphinidin-3-*O*-rutinoside		3.30 mg/g DW		
		Cyanidin-3-*O*-rutinoside		0.70 mg/g DW		
Viola x *wittrockiana*	Yellow	Delphinidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside	HPLC-DAD-ESI-MS	4.69 mg/g DW	Solid-liquid extraction with 1.5 g of plant material in 50 mL of ethanol 50% (pH=2)	(Skowyra, Calvo, Gallego, Azman, & Almajano, 2014)
		Petunidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		2.08 mg/g DW		
		Cyanidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		4.39 mg/g DW		
		Malvidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		2.72 mg/g DW		
	Red	Delphinidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		11.40 mg/g DW		
		Petunidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		7.72 mg/g DW		
		Cyanidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		7.25 mg/g DW		
		Malvidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		4.74 mg/g DW		
	Violet	Delphinidin-3-*O*-(4´-*p*-coumaroyl)-rutinoside-5-glucoside		11.6 mg/g DW		
		Petunidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		9.89 mg/g DW		
		Cyanidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		7.74 mg/g DW		
		Malvidin-3-*O*-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside		9.07 mg/g DW		

1.2.9. Flower anthocyanins and extraction methodologies

Anthocyanin contents and composition in edible flowers can present high variability depending on the species (**Table 3**), but also due to edafo-climatic and abiotic factors. Furthermore, the different variables in the extraction process, such as the extraction methodology, employed solvents, solid/liquid ratio, or temperature can also have a great influence in the composition of the obtained extracts.

The polar characteristics of the anthocyanins allow them to be extracted by different polar solvents, such as methanol, acetone, water and ethanol. Methanol and acetone and their aqueous mixtures are among the solvents most commonly used to extract polyphenols, including anthocyanins (Santos-Buelga, Gonzalez-Manzano, Dueñas, & Gonzalez-Paramas, 2012). However, the replacement with greener solvents (water, ethanol or a mixture of both), considering the requirements for food and pharmaceutical industries, in order to obtain "environmentally friendly" products, have been gaining a great consideration (Machado, Pereira, Barbero, & Martínez, 2017). In **Table 3** a summary of the solvents and extraction methods used in edible flowers is shown.

Acidified methanol (Deng et al., 2013; Lee et al., 2011; Skowyra et al., 2014; Koike et al., 2015a), acidified mixtures of methanol/water (Li et al., 2014; Goupy et al., 2013), water (Sindi, et al., 2014), acidified water (Park et al., 2015), and acetone (Garzón et al., 2015) are within the most common extraction solvents. The ability to extract some flavonoids, increases with acidification of the solvent, especially when methanol or ethanol (protic polar solvents) are used, in which the change of the phenol-phenolate equilibrium towards the polar phenyl form, improves the extraction of these molecules (Atta-ur-Rahman, Iqbal Choudhary, & Perry, 2015; Hostettmann, 2014). In the case of the anthocyanins, acidification of the solvents is even necessary, since they are structurally dependent on the pH of the medium, modifying their solubility and stability (Santos-Buelga & González-Paramás, 2014). Nevertheless, as it can be seen in **Table 3**, the solvents applied in anthocyanins extraction from edible flowers include the use of organic solvents with or without acidic agents, such as acetic, hydrochloric, formic and trifluoroacetic acids. The type of the acid used in the extraction solvents also influences the extraction yields of these compounds. For instance, Oancea, Stoia, and Coman (2012) demonstrated that using hydrochloric acid instead of acetic acid improves the extraction yield of anthocyanins. Soft acidic conditions must be used to prevent cleavage of the sugar moieties and labile conjugated residues (Santos-Buelga & González-Paramás, 2014). Anthocyanins are highly susceptible to degradation. In this way it is fundamental

to use methodologies that allow their extraction with the least number of interferers, making a strict control of pH and temperature (Silva et al., 2015). The most common methodology applied in the extraction of anthocyanins is conventional solid-liquid extractions (SLE), due to their simplicity and non-required specific equipment as it can be verified in **Table 3** (Silva et al., 2015). Supercritical Fluid Extraction (SFE), Ultrasound Assisted Extraction (UAE), Pressurized Liquid Extraction (PLE) and Microwave Assisted Extraction (MAE) are alternative methodologies that can also be applied in the extraction of anthocyanins.

Supercritical fluid extraction (SFE) is used to obtain bioactive compounds from natural matrices, applying low viscosity solvents (near the gas), significantly reducing the extraction time and with a higher penetration of the fluids in the solid pores, resulting in a faster and more efficient process. (Otero-Pareja, Casas, Fernández-Ponce, Mantell, & De La Ossa, 2015). Supercritical CO_2 is the usual solvent of choice, although it is only suitable for non-polar or low polarity compounds, so that it is no adequate as such for anthocyanin extraction, for which some percentages of solvent modifiers are required, such as methanol or ethanol (Santos-Buelga & González-Paramás, 2014). This technique has been used by different authors obtaining good yields (Santos-Buelga & González-Paramás, 2014). Ultrasound assisted extraction (UAE) is a key technology for sustainable "green" extraction. This extraction system presents high reproducibility, reducing the consumption of solvent, simplifying the manipulation and the processing, and conferring a greater degree of purity to the final product (Silva et al., 2015). Pressurized Liquid Extraction (PLE) is characterized by the use of liquid solvents, using high temperatures and pressures (Mustafa & Turner, 2011). The solubility, amount of extracted compounds, solid-liquid bonds and the mass transfer rise with increasing temperature, while viscosity and interfacial tension decrease (Machado et al., 2017). Although this methodology presents some advantages, such as low solvent consumption, low extraction time and possibility of process automation, it requires high temperatures that can lead to anthocyanin degradation, therefore, it is not often applied as an alternative extraction method for this type of molecules (Silva et al., 2015). Another system that can be applied to extract anthocyanins is Microwave-assisted extraction (MAE). This technique promotes rapid heating of the solvent and sample, because the microwave energy causes molecular movement and rotation of liquids with a permanent dipole (Yang & Zhai, 2010). It has advantages over conventional extraction methodologies, such as improved

efficiency, reduction of extraction time, low solvent consumption, and high level of automation (Silva et al., 2015).

In general, there is not an ideal extraction system and conditions that can be used for anthocyanin extraction in all situations. A simple or a more technological extraction methodology may be applied, although the lack of data on the direct comparison among technologies, using the same type of samples under the same conditions, limits the selection of an infallible extraction method. Therefore, the choice of the extraction process depends on different factors, among which the final application of the extract obtained and the type of sample used (Ongkowijoyo, Luna-Vital, & Gonzalez de Mejia, 2018; Silva et al., 2015). Some general guidelines can be taken into account for the extraction of anthocyanins in order to select the most adequate extraction process, not forgetting that methodologies involving the use of high temperatures may induce the degradation of these compounds (Sarkis, Jaeschke, Tessaro, & Marczak, 2013). Methodologies with better extraction yields, such as MAE and UAE, which mainly use water as solvent, present economic and ecological advantages, although the costs of production deserve a comparison with the cost of the equipment.

1.3. General description of the studied edible flowers

1.3.1. Rosa canina L.

Rosa canina L. belongs to the *Rosaceae* family, which include about 200 species spread in the temperate and subtropical zones of the Northern hemisphere. *R. canina* is an erect shrub of up to 3.5 m height; its branches are often curved or arched. **Figure 1** presents the flowers of this species, which are white to pale pink, rarely deep pink (Roman, Stănilă, & Stănilă, 2013).

R. canina is known for its fruits, which have constituted an important source of food and medicine for many cultures. Common food preparations have been formulated using the fruits and flowers of this rose species, which include juice, wine, tea, jelly, jam, as well as mixtures with dried salmon and eggs (Roman et al., 2013). Infusions made of the flowers are used with cosmetic effecst and can help heal rashes and abrasions. The infusion of the flowers is good for bringing down fevers, aiding the liver and gallbladder, and treating the symptoms of colds and influenza. In addition, the flowers are also good for diarrhea (Nojavan et al., 2008). The traditional uses of this species are very popular due to their sweet and aromatic flavor (Lara-Cortés et al., 2013), thus these usages have been increasing due to their prophylactic and therapeutic activities against a wide range

of ailments. These activities have been linked to the presence of a variety of bioactive compounds, such as carotenoids and ascorbic acid, along with natural sugars, organic acids, polyunsaturated fatty acids, phenolic compounds, and essential oils. Rose hips are valued as potential candidates for the preparation of functional foods, natural pharmaceuticals, and cosmo-nutraceuticals (Ahmad, Anwar, & Gilani, 2015; Barros, Carvalho, & Ferreira, 2011).

Figure 1. Edible flowers of *Rosa canina* L. Author: Giuliano da Zanche

1.3.2. Rosa damascena Mill.

Rosa damascena Mill. also belongs to the Rosaceae family. It has brilliant colors, rich aroma and high trophic value. It is an important raw material for the production of spices and functional ingredients for food industry (Sengul, Sener, & Ercisli, 2017). The flowers **(Figure 2)** of this rose species are sold in groceries as flavor and laxative agents. Its decoction is traditionally used for treatment of abdominal and chest pains, strengthening the heart, menstrual bleeding, digestive problems and constipation (Mahboubi, 2016).

In its chemical composition, the presence of citronellol, geraniol, nerol, phenyl ethyl alcohol, nonadecane, nonadecene, eicosane, heneicosane, tricosane, α-guaiene, geranyl acetate, and eugenol has been reported (Fathima & Murthy, 2019). The most beneficial effects of *R. damascena* are the ones mentioned above, but they are also recommended

for the reduction of inflammation, especially of the neck, cough remedy for children and as a gentle laxative. Reported pharmacological properties also include anti-HIV, antibacterial, antioxidant, antitussive, hypnotic, antidiabetic, and relaxant effect on tracheal (Boskabady, Shafei, Saberi, & Amini, 2011).

Figure 2. Edible flowers of *Rosa damascena* Mill. Author: Asmad Morad

1.3.3. Rosa gallica Mill.

Rosa gallica, French rose, was bred by the indigenous population of central and south Europe, and the Caucasus region. Its flowers **(Figure 3)** are used for the extraction of oils, or to prepare medicinal herbs with intended antibacterial, astringent, tonic, and antioxidant effects, as well as for mild inflammation of the skin or lining of the mouth and throat (Koczka, Stefanovits-Bányai, & Ombódi, 2018; Lee et al., 2018). As other species of the Rosacea family, it is rich in bioactive compounds, such as terpenes, flavonoids and anthocyanins, and shows strong antioxidant activity and anti-skin aging activity, being recommended for skin whitening and wrinkle suppression (Shin et al., 2019).

Figure 3. Edible flowers of *Rosa gallica* Mill. Author: Charlie.

In the development of this Thesis work, different samples of edible flowers have been used, one of which resulting from the cross of the three species of rose described previously, i.e., *Rosa damascena* 'Alexandria' and *Rosa gallica* draft in *Rosa canina*, as it will be described in **section 2.2**. A picture of this hybrid is shown in **Figure 4**.

Figure 4. Edible flowers of *Rosa damascena* 'Alexandria' and *Rosa gallica* draft in *Rosa canina.* Author: Tânia Pires.

1.3.4. Calendula officinalis L.

Calendula officinalis L. belongs to the family *Asteraceae*, commonly know as calendula or pot marigold. It is native to Central and Southern Europe, Western Asia and the United States.

The flower head consist of an epicalyx of numerous narrow-lanceolate sepals, which are densely covered on both sides with glandular hairs. The inner section of the flower head is made up of orange-yellow tubular florets **(Figure 5)**. The disc florets are pseudohermaphrodites. The zygomorphic ray florets at the edge are sterile female, their stamens are completely absent, and their inferior ovaries are much more developed than those of the tubular florets. The fruit forms only in the female ray flowers and the heterocarp achenes are sickle-shaped, curved and ringed (Muley, Khadabadi, & Banarase, 2009).

Tradicionally *C. officinallis* is used as an ingredient in salads, omelettes or as an accompaniment for cheese, due to its slightly bitter and slightly spicy taste (Lara-Cortés et al., 2013). The presence of several classes of chemical compounds, the main ones being terpenoids, flavonoids, coumarins, quinones, volatile oil, carotenoids and amino acids, have been associated with a broad range of biological effects (Muley, Khadabadi, & Banarase, 2009).

Figure 5. Edible flowers of *Calendula officinalis* L. Author: Tânia Pires

1.3.5. Centaurea cyanus L.

Centaurea cyanus L. is an annual *Asteraceae* species that grows in many countries throughout Europe and Asia (Lockowandt et al., 2019). Its flowers **(Figure 6)**, also called 'blue cornflower' or bachelor's button', have no fragrance, but they have a sweet-to-spicy clove-like flavor. Centaurea flowers are used mixed with other flowers to make dishes more attractive, for sprinkling over salads, or to prepare teas. Dried flowers are richer in tocopherols, organic acids, and apigenin derivatives (mainly apigenin-7-*O*-glucuronide-4-*O*-(6-*O*-malonylglucoside)) that have been related to a long list of medicinal properties, such as antioxidant, antibacterial (Lockowandt et al., 2019), anti-inflammatory and skin cleansing effects, assisting regulating digestion and kidney, gall bladder, liver and menstrual disorders, and increasing immunity (Fernandes, Pereira, Saraiva, Ramalhosa, & Casal, 2019).

Figure 6. Edible flowers of *Centaurea cyanus* L. Author: Tânia Pires.

1.3.6. Dahlia mignon

The genus *Dahlia* belongs to family *Asteraceae* (Compositae), tribe *Heliantheae*. It consists of 36 species, all native to Mexico (Lara-Cortés et al., 2014). *Dahlia mignon* is a mixture of single and low growing flowers with different beautiful and bright colours (yellow, red, pink, purple, white and orange). Dahlia flowers **(Figure 7)** have a bitter flavor and are commonly used in salads and meat sauce decoration or, if they are placed under the crystallization process, they can be used for sweets and cake decoration, especially wedding cakes (Moldovan & Zsolt Szekely-Varga, 2017).

Dahlia is widely used in the pharmaceutical, cosmetic and food industries, and as a raw material for the extraction of dyes (Moldovan & Zsolt Szekely-Varga, 2017). Flowers of this species contain a significant amount of flavonoids, including anthocyanins and flavones, as flowers pigments with antioxidant activity and other reported health benefits, such as increasing the appetite and gastric secretion and modulating the cell wall tone (Lara-Cortés et al., 2013; Moldovan & Zsolt Szekely-Varga, 2017).

Figure 7. Edible flowers of *Dalhia mignon*. Author: Tânia Pires.

1.4. Fruits as a source of bioactive compounds with health benefits

1.4.1. Vaccinium myrtillus L.

Bilberry (*Vaccinium myrtillu*s L.) is a dark blue fruit that belongs to the genus *Vaccinium*, family *Ericaceae,* which comprises around 450 species of trees, shrubs, sub-shrubs and hemiphytes distributed all over the world (Nagulsamy, Ponnusamy, & Thangaraj, 2015). These fruits **(Figure 8)** are usually consumed in the fresh form, however, due to their short shelf life, they are also frozen, dried or processed in the form of jams, juices and wines or liqueurs (Zorenc, Veberic, & Mikulic-Petkovsek, 2018). Bilberries have been conventionally consumed and used in traditional medicine since ancient times, being harvested from wild bushes, although currently the cultivation of these fruits is commonly performed in northern and eastern Europe (Zoratti, Klemettilä, & Jaakola, 2016).

These fruits are described as being an important source of phenolic compounds and carotenoids, also containing moderate levels of other micronutrients such as vitamins. Nevertheless, it is due to their high levels of anthocyanins that these fruits are recognized for their bioactive properties (Colak et. al., 2017). Anthocyanins, besides being responsible for the blue color of bilberries, are the major group of flavonoids in these berries and have been associated to many beneficial health effects, like prevention of cancers, cardiovascular diseases, obesity, diabetes, aging diseases, urinary tract infections and periodontal diseases (Abreu, Barreto, & Prieto, 2014; Crespo & Visioli, 2016). The high content of these flavonoids has also highlighted these fruits as interesting sources of

coloring compounds for food and pharmaceutical applications (Li, Wang, Guo, & Wang, 2011). According to the literature, the anthocyanin profile in bilberry consists of fifteen main compounds, derived from five aglycones (delphinidin, cyanidin, petunidin, peonidin, and malvidin) linked to different sugar moieties (galactose, glucose and arabinose) (Colak et al., 2016; Primetta, Jaakola, Ayaz, Inceer, & Riihinen, 2013).

Consumers are increasingly concerned about choosing foods labeled as healthier and more natural. In this sense, the food industry has been exploring various natural sources in order to enrich different products (Murley & Chambers, 2019). Currently, there are many products in the market with the incorporation of berries, namely bilberry, highlighting their beneficial effects, usually their antioxidant potential (Karam, Petit, Zimmer, Baudelaire Djantou, & Scher, 2016).

Figure 8. Dried fruits of *Vaccinium myrtillus* L. Author: Tânia Pires

1.4.1.1. Nutritional characterization

Due to their reduced environmental adaptability it is extremely difficult to cultivate bilberries. Moreover, there are still few producers of these fruits because of their low productivity, justified by the small size of the fruits when compared to similar blueberries (*Vaccinium corymbosum* L.), which have a larger fruit size. Most of the available bilberries (*V. myrtillus* L.) are mainly obtained from wild plants that grow in northern and southern Europe (Ancillotti et al., 2016; Nin, Petrucci, Del Bubba, Ancillotti, & Giordani, 2017), while highbush blueberries (*V. corymbosum*) are originate from North America

(Može et al., 2011). Sometimes bilberry is also called blueberry because both have similar appearance and are close relatives, but the true blueberry is native to the United States (Chu, Cheung, Lau, & Benzie, 2011). There are several environmental factors, such as climatic conditions, soil type or cultivation conditions, among others, which may affect the plant growth and consequently fruit productivity (Coudun & Gégout, 2007). In the case of bilberries plants, some authors report that light availability influences plant development and consequently fruit yield (Barizza et al., 2013). Additionally, weather conditions can also influence the nutritional and chemical composition of the fruits. This was proven through studies performed from several authors, which demonstrated that bilberries fruits growing in northern latitudes present higher phenolic contents than those from southern latitudes (Åkerström, Jaakola, Bång, & Jäderlund, 2010; Lätti, Jaakola, Riihinen, & Kainulainen, 2010).

There are more than 70 registered ethnomedical and food uses of 36 *Vaccinium* species, being *V. myrtillus* the specie with the highest number of described uses (Abreu et al., 2014). The American Herbal Products Association classified *V. myrtillus* as a Class 1 product, category assigned when considering it as safe to consume (Upton, 2001). The fruits of this plant are usually consumed as food, while the leaves or aerial parts are commonly associated to medicinal use (Abreu et al., 2014; Ferlemi & Lamari, 2016). *V. myrtillus* berries can be consumed fresh, frozen and dried, as well as in processed forms, such as juices, jams or food supplements (Chu, Cheung, Lau, & Benzie, 2011).

The interesting nutritional and functional properties described for bilberries justify the growing interest in these berries. The fruits of *V. myrtillus* are rich sources of micronutrients and phytochemical compounds with health benefits, such as organic acids (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2015; Uleberg et al., 2012; Zorenc, Veberic, Stampar, Koron, & Mikulic-Petkovsek, 2016), sugars (Uleberg et al., 2012; Zorenc et al., 2016), vitamins (Cocetta et al., 2012), fibres (Aura et al., 2015), and phenolic compounds (Ferlemi & Lamari, 2016; Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2012a). Their organoleptic properties are clearly influenced by the different content of the mentioned compounds, namely sugars and organic acids (Silva, Andrade, Mendes, Seabra, & Ferreira, 2002). Since sugars and organic acids are considered the main soluble constituents of berries, some authors describe their content as a direct effect on fruit taste and ripeness and consequently on consumer's acceptability (Bordonaba & Terry, 2010; Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2012a). According to Michalska & Łysiak (2015), beside the taste of the bilberry fruits,

the vitamin C content is important in the consumption of these fruits because 100 g of the fruit provides, on average, 10 mg of ascorbic acid, which is equal to 1/3 of the daily recommended intake. Uleberg et al. (2012) indicated that cool temperatures and genetic factors influence the taste of bilberry fruits, which explains the sweeter taste of fruits cultivated in Northern areas in comparison to those from Southern areas.

Bilberries have different organic acids in their composition (malic, citric, gallic, chlorogenic, ascorbic and quinic acids), being citric, malic and quinic acids the main ones. Fructose and glucose are described as the highest group of free sugars in bilberry, although the presence of sucrose has also been reported as a relevant constituent (Mikulic-Petkovsek et al., 2012a; Uleberg et al., 2012; Zorenc et al., 2016).

The mineral content in fruits and vegetables is very important, contributing to their nutritional value. However, it is necessary to balance these mineral elements in the human diet, because trace elements can be toxic when consumed at higher amount than the recommended intake. Some wild fruits, including bilberry, have been explored and identified as interesting sources of minerals (Damascos, Arribere, Svriz, & Bran, 2008; Desideri, Meli, & Roselli, 2010). Regarding mineral composition, these fruits have three main macroelements (Ca, P, and Mg) and seven main microelements (Fe, Ba, Na, Mn, Cu, Sr, and Zn) (Barizza et al., 2013). Comparative studies between wild and commercial fruits have shown that wild bilberry have higher concentrations of minerals than commercial fruits (Barizza et al., 2013). As mentioned above, these differences may be justified by the cultivation conditions, namely the soil composition (Kabata-Pendias, 2010). Regarding the proximate composition, the fresh fruits contain around 84% of water, 9.7% of carbohydrates, 0.6% of proteins, 0.4% of fats, and 3-3.5 % of fibres, with an estimated energetic value of 192 kJ (Michalska & Łysiak, 2015).

1.4.1.2. Bioactive compounds

Phenolic compounds are a group of secondary plant metabolites recognized for their health-protective activity, namely as antioxidant (Fidaleo, Lavecchia, Maffei, & Zuorro, 2015; Zorenc et al., 2018), anti-inflammatory (Ambriz-Pérez, Leyva-López, Gutierrez-Grijalva, & Heredia, 2016), antihypertensive (Rodrigo, Gil, Miranda-Merchak, & Kalantzidis, 2012), antimicrobial (Bouarab-Chibane et al., 2019) and anticancer agents (Anantharaju, Gowda, Vimalambike, & Madhunapantula, 2016). The fact that *V. myrtillus* has high content of phenolic compounds may account for the growing demand of this fruit.

Flavonoids, such as flavan-3-ols (catechins and proanthocyanidins) and flavonols (e.g., kaempferol, quercetin, myricetin), phenolic acids (mainly hydroxycinnamic acids) and derivatives of stilbenes, are the major non-anthocyanin polyphenols present in *V. myrtillus* fruits (Ferlemi & Lamari, 2016; Michalska & Łysiak, 2015; Tian et al., 2017).

The flavan-3-ols are usually found in varying concentrations in commonly consumed foods such as fruits, legumes, vegetables and nuts. They are natural antioxidants that may contribute to prevent rancidity due to oxidation of unsaturated fats and stabilize food colors, as well as being involved in chemoprevention against a variety of diseases (Rue, Rush, & van Breemen, 2018). The most common flavan-3-ols are procyanidins, consisting of (epi)catechin oligomers (Ge et al., 2016; Rue et al., 2018) and can be classified into A-type and B-type, depending on the stereo configuration and linkage between monomers. B-type procyanidins are the most abundant, with procyanidins B1, B2, B3 and B4 occurring most frequently (Rue et al., 2018). **Table 4** shows the contents of the main phenolic compounds determined in bilberries in recent studies by several authors. Data are expressed in different units and as dry or fresh weight basis, as reported by the authors. Regarding flavan-3-ols, the concentrations of catechin, epicatechin, procyanidin dimers and trimers, and gallocatechin are collected, as well as total flavan-3-ol contents. As it can be seen, only catechin was identified and quantified in all the represented studies.

The individual and total contents of flavonols in fruits of *V. myrtillus* are also presented in the **Table 4**. All the reported studies identified kaempferol, quercetin and myricetin glycosides as the main compounds, although Zorenc et al. (2018) also reported laricitrin, syringetin and isorhamnetin glycosides.

Table 4. Contents of flavan-3-ols, flavonols and phenolic acids in fruits of *Vaccinium myrtillus* L.

Phenolic compounds	(Zorenc et al., 2018)	(Tumbas et al., 2015)	(Xiao et al., 2017)	(Stanoeva et al., 2017)	(Može et al., 2011)	(Değirmencioğlu et al., 2017)	(Ancillotti et al., 2016)
Catechin	29.67 mg/100 g (dw)	15.04 μg/100 g (dw)	91.64 mg/g (dw)	96 mg/100 g (dw)	0.2 mg/100g (fw)	2.72 mg/kg (fw)	13.9 mg/kg (dw)
Epicatechin	59.14 mg/100 g (dw)	nd	nd	7.9 mg/100 g (dw)	2 mg/100g (fw)	28.54 mg/kg (fw)	255 mg/kg (dw)
Procyanidin dimers	72.10 mg/100 g (dw)	nd	nd	117 mg/100 g (dw)	nd	nd	nd
Procyanidin trimers	59.33 mg/100 g (dw)	nd	nd	109 mg/100 g (dw)	nd	nd	nd
Gallocatechin	35.72 mg/100 g (dw)	nd	nd	nd	nd	6.66 mg/kg (fw)	nd
Total flavan-3-ols	**244.09 mg/100 g (dw)**	**15.04 μg/100 g (dw)**	**91.64 mg/g (dw)**	**329.9 mg/100 g (dw)**	**2.2 mg/100g (fw)**	**37.92 mg/kg (fw)**	**268.9 mg/kg (dw)**
Myricetin hexoside	29.61 mg/100 g (dw)	nd	28.60 mg/g (dw)	nd	nd	nd	nd
Myricetin pentoside	3.34 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Myricetin-3-glucuronide	2.23 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Myricetin-3-rhamnoside	2.12 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Laricitrin-3-galactoside	0.10 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Laricitrin-3-glucoside	4.33 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Laricitrin-3-glucuronide	0.34 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Myricetin	6.20 mg/100 g (dw)	40.66 μg/100 g (dw)	nd	nd	0.4 mg/100g (fw)	nd	36.9 mg/kg (dw)
Syringetin-3-galactoside	8.69 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Syringetin-3-glucoside	1.01 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Isorhamnetin-3-arabinoside	nd	nd	nd	6 mg/100 g (dw)	nd	nd	nd
Isorhamnetin-3-galactoside	5.27 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Isorhamnetin-3-glucoside	36.60 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Quercetin-3-arabinoside	nd	nd	38.14 mg/g (dw)	nd	nd	nd	nd

Quercetin-3-rhamnoside	67.36 mg/100 g (dw)	51.80 μg/100 g (dw)	nd	6 mg/100 g (dw)	nd	nd	nd
Quercetin-7-rhamnoside	nd	nd	nd	48 mg/100 g (dw)	nd	nd	nd
Quercetin-3-galactoside	4.03 mg/100 g (dw)	nd	45.78 mg/g (dw)	nd	nd	nd	nd
Quercetin-3-glucuronide	2.82 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Quercetin-3-glucoside	4.03 mg/100 g (dw)	nd	nd	236 mg/100 g (dw)	nd	nd	nd
Quercetin-3-(6”-acetyl) glucoside	nd	nd	nd	97 mg/100 g (dw)	nd	nd	nd
Quercetin-3-pentoside	nd	nd	29.29 mg/g (dw)	64 mg/100 g (dw)	nd	nd	nd
Quercetin-3-rutinoside	nd	51.80 μg/100 g (dw)	27.03 mg/g (dw)	196 mg/100 g (dw)	0.2 mg/100g (fw)	3.84 mg/kg (fw)	nd
Quercetin	nd	243.30 μg/100 g (dw)	4.40 mg/g (dw)	146 mg/100 g (dw)	0.8 mg/100g (fw)	1.62 mg/kg (fw)	2.2 mg/kg (dw)
Kaempferol	nd	15.64 μg/100 g (dw)	nd	30 mg/100 g (dw)	nd	1.72	nd
Kaempferol-3-glucoside	nd	nd	nd	nd	nd	nd	nd
Kaempferol-3-glucuronide	2.82 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Kaempferol-3-(6”-acetylglucoside)	nd	nd	nd	8 mg/100 g (dw)	nd	nd	nd
Kaempferol-3-rhamnoside	nd	nd	nd	10.3 mg/100 g (dw)	nd	nd	nd
Total flavonols	**173.03 mg/100 g (dw)**	**403.2 μg/100 g (dw)**	**173.24 mg/g (dw)**	**847.3 mg/100g (dw)**	**1.4 mg/100g (fw)**	**57.82 mg/kg (fw)**	**39.1 mg/kg (dw)**
Dicaffeoylquinic acid	nd	nd	nd	31.6 mg/100g (dw)	nd	nd	nd
4-*O*-Caffeoylquinic acid	nd	nd	nd	37 mg/100g (dw)	nd	nd	nd
trans 5-*O*-Caffeoylquinic acid	818.39 mg/100 g (dw)	nd	nd	649 mg/100g (dw)	nd	nd	nd
cis 5-*O*-Caffeoylquinic acid	31.84 mg/100 g (dw)	nd	nd	nd	nd	nd	nd
Caffeic acid derivatives	66.83 mg/100 g (dw)	nd	nd	nd	nd	nd	nd

Caffeic acid	4.24 mg/100 g (dw)	15.33 mg/100 g (dw)	6.26 mg/g (dw)	nd	0.3 mg/100g (fw)	5.46 mg/kg (fw)	3.1 mg/kg (dw)
Chlorogenic acid	nd	21.0 mg/100 g (dw)	6.40 mg/g (dw)	nd	23.1 mg/100g (fw)	1.25 mg/kg (fw)	1320 mg/kg (dw)
3-*p*-Coumaroylquinic acid	nd	nd	nd	7.3 mg/100g (dw)	nd	nd	nd
4-*p*-Coumaroylquinic acid	nd	nd	nd	3.5 mg/100g (dw)	nd	nd	nd
5-*p*-Coumaroylquinic acid	3.04 mg/100 g (dw)	57.87 mg/100 g (dw)	nd	29.2 mg/100g (dw)	nd	nd	nd
p-Coumaric acid hexoside	3.99 mg/100 g (dw)	nd	nd	nd	0.3 mg/100g (fw)	7.63 mg/kg (fw)	1.20 mg/kg (dw)
Coumaroyl iridoid isomers	31.68 mg/100 g (dw)	nd	nd	82.5 mg/100g (dw)	nd	nd	nd
Coumaric acid derivatives	3.18 mg/100 g (dw)	nd	nd	243.7 mg/100g (dw)	nd	nd	nd
Ellagic acid	nd	9.99 mg/100 g (dw)	nd	nd	1.2 mg/100g (fw)	nd	nd
Ferulic acid	4.84 mg/100 g (dw)	22.76 mg/100 g (dw)	nd	7.3 mg/100g (dw)	0.4 mg/100g (fw)	10.60 mg/kg (fw)	0.44 mg/kg (dw)
Gallic acid	nd	7.24 mg/100 g (dw)	nd	nd	6.2 mg/100g (fw)	19.19 mg/kg (fw)	33.3 mg/kg (dw)
Gallic acid derivatives	nd	nd	nd	36 mg/100g (dw)	nd	nd	nd
Protocatechuic acid	nd	19.41 mg/100 g (dw)	7.66 mg/g (dw)	nd	nd	nd	nd
Syringic acid	nd	27.43 mg/100 g (dw)	nd	nd	nd	637.43 mg/kg (fw)	nd
Vanillic acid	nd	nd	nd	nd	nd	532.97 mg/kg (fw)	nd
Total phenolic acids	**968.02 mg/100 g (dw)**	**181.03 mg/100 g (dw)**	**20.32 mg/g (dw)**	**1127.1 mg/100 g (dw)**	**31.5 mg/100 g (fw)**	**1214.53 mg/kg (fw)**	**1358.04 mg/kg (dw)**

Abbreviations used: nd- not detected, fw- fresh weight, dw- dry weight

Phenolic acids are phenols that possess one carboxylic acid function and include two main groups: the hydroxycinnamic and hydroxybenzoic acids (Abo et al., 2013). Caffeic, *p*-coumaric, vanillic, ferulic, and protocatechuic acids are widely present in many plants (Değirmencioğlu et al., 2017; Tumbas Šaponjac, Čanadanović-Brunet, Ćetković, Djilas, & Četojević-Simin, 2015), whereas gentisic and syringic acids have a more restrictive distribution, being reported in *V. myrtillus* by Tumbas et al. (2015) and Değirmencioğlu et al. (2017) **(Table 4)**.

Regarding stilbenes, there are few studies that reveal their presence in bilberry fruits, however, Ehala, Vaher, & Kaljurand (2005) and Može et al. (2011) identified and quantified *trans*-resveratrol in concentrations ranging 97.8 % and 0.3 mg/100 g fw, relating their presence to the antioxidant activity of the fruit (Ehala et al., 2005; Može et al., 2011).

Anthocyanins are pigments commonly present in plants, where they are responsible for characteristic blue, red or purple colors. Actually, anthocyanins are considered the most important water-soluble pigments in plants, being particularly relevant in flowers and berries, namely bilberry, cherry or blackcurrant (Silva et al., 2015). Many studies describe anthocyanins as the most abundant polyphenol class in *V. myrtillus* fruits, being responsible for the characteristic dark blue color of bilberry. Glucosides, galactosides and arabinosides of delphinidin, cyanidin, petunidin, peonidin and malvidin characterize the anthocyanin profile of bilberry (Ancillotti et al., 2016). Owing to their interest and relevance in the sensory and health properties of fruits, a more profound discussion regarding this group of compounds is made the following section.

1.4.1.3. Natural pigments

Pigments are colored chemical compounds capable of reflecting selective wavelengths of the visible light. Textile, food, painting, cosmetic, and pharmaceutical industries are some of the sectors where color plays a very important role. Research on natural pigments is of enormous importance as it has a direct impact on environmental, economic and human health safety (Lu et al., 2009). Natural pigments generally extracted from different parts of plants, namely seeds, fruits, vegetables or roots have played an important role since ancient times and in particularly in textile dyeing (Parmar & Singh, 2018). For its part, the food industry routinely applies pigments to foods to restore color losses or make them more attractive to consumers. Food colorants can be classified as artificial or natural. It is estimated that around 31% of the colorants used by the food industry are obtained from natural sources, such as plants, animals and microorganisms (Mapari, Thrane, & Meyer, 2010).

Currently, due to several published scientific studies that associate the consumption of certain artificial additives with potential adverse health effects, an aversion has been created to this type of colorants. This has put pressure on the industry that is looking for natural alternatives capable of performing the same functions as artificial colorants and additionally offering bioactive properties (Carocho, Barreiro, Morales, & Ferreira, 2014).

Several studies have shown that extracts rich in anthocyanins could be used not only as colorants but also as potential functional food ingredients and/or dietary supplements due to their biological properties (Uleberg et al., 2012). Anthocyanins are classified in the USA as natural food colorings in the fruit (21 CFR 73,250) and vegetable (21 CFR 73,260) category, and in the EU they are included as additives under code E163 (Loypimai, Moongngarm, Chottanom, & Moontree, 2015).

Anthocyanins are the most abundant and widely studied class of bioactive compounds in the fruits of *V. myrtillus.* The anthocyanin profile in bilberry is characterized by fifteen main compounds, among which delphinidin glycosides constitute the best represented ones, as it is shown in the Table 5, where the concentrations determined by different authors are collected.

The type and quantity of anthocyanins is affected by several internal and external factors, such as genetic factors (varietal and regional), environmental variables (light intensity, temperature, humidity, the use of fertilisers), fruit size, ripening stage, pre-harvest environmental conditions and storage. The influence of regional factors on the anthocyanin content in bilberry fruits has been shown by Uleberg et al. (2012), which demonstrated differences between anthocyanin content in samples collected in northern and southern Finland, with higher anthocyanin and total polyphenol contents in the northern region.

The use of extracts obtained from natural sources rich in anthocyanins has gained prominence, in view to their use by the food and pharmaceutical industries, with different purposes. The low stability of these compounds requires the utmost care. In most cases water and alcoholic solutions are used as extraction solvents under acidic conditions, so as to maintain the stability of flavylium ion and increase the intensity of the red hue of the anthocyanin. Anthocyanins, in addition to making products more attractive, play a key role as bioactive compounds with putative healthy effects. Studies developed by Prior et al. (2010) and ggest the potential of anthocyanins to be used as nutraceuticals and functional food ingredients to fight obesity and type 2 diabetes. Fernandes, Marques et al. (2019), in a study carried out with mice, showed the hypoglicemic effects of cyanidin-3-glucoside, reducing blood glucose levels and enhancing insulin sensitivity by downregulation of the retinol binding protein-4 expression. Prior et al. (2010) found that purified anthocyanins from *V. myrtillus* as well as bilberry juice could prevent

dyslipidemia and obesity in the same animal model. Also, Yamaura et al. (2012) showed that cyanidin-3-glucoside and a quercetin fraction from bilberry have beneficial dermatological efects and inhibit inflammation of injured skin, correcting the Th1/Th2 balance and reducing IL-17.

Not only individual anthocyanins from *V. myrtillus* present beneficial effects, but Cooke et al., (2006) demonstrated that one extract with 40% anthocyanins, containing the main fifteen bilberry compounds, possesed chemopreventive effects for colorectal cancer in rats.

Table 5. Contents of individual anthocyanins in *Vaccininium myrtillus* fruits.

	Anthocyanins															
Origin	**Cy-3-arab**	**Cy-3-gal**	**Cy-3-glu**	**Dp-3-arab**	**Dp-3-gal**	**Dp-3-glu**	**Mv-3-arab**	**Mv-3-gal**	**Mv-3-glu**	**Pn-3-arab**	**Pn-3-gal**	**Pn-3-glu**	**Pt-3-arab**	**Pt-3-gal**	**Pt-3-glu**	**References**
Finland Southern (mg/100 g fw)	37.0	34.0	41.0	57.5	45.7	54.6	2.6	13.3	16.6	nd	2.1	9.3	nd	10.0	25.9	(Uleberg et al., 2012)
Finland Northern (mg/100 g fw)	44.0	59.5	50.9	87.8	98.9	76.4	9.6	34.2	46.8	nd	4.8	17.7	nd	26.3	45.3	(Uleberg et al., 2012)
Slovenia (mg/kg fw)	161.92	542.45	194.47	640.31	700.46	381.11	42.45	109.97	149.63	9.66	19.42	108.09	78.31	213.63	416.06	(Zorenc et al., 2016)
Poland (mg/g dw)	936	375	396	741	1060	1247	295	127	506	15	107	198	340	581	541	(Müller et al., 2012)
Italy Northern (mg/100 g fw)	37.1	56.3	55.0	97.4	127.1	119.0	nd	19.7	56.1	nd	nd	24.8	20.1	33.1	65.9	(Benvenuti et al., 2018)
Slovenia (mg/100g fw)	110.6	122.6	13.04	152.3	167.1	169.1	28.24	21.66	24.89	22.99	17.82	20.75	19.98	14.76	16.72	(Može et al., 2011)
Finland (mg/kg fw)	450	493	488	632	629	562	91	124	350	20	34	187	137	167	359	(Kähkönen et al., 2003)
Mustasaari, Finland (mg/kg fw)	220	300	220	340	360	300	53	100	150	18	36	75	84	120	170	(Laaksonen, et al., 2010)
Milan, Italy (mg/g extract)	32.18	40.03	41.81	49.06	49.07	54.21	8.75	13.23	40.15	1.75	4.34	15.90	13.44	17.92	38.49	(Luo et al., 2014)

	Anthocyanins															
Origin	**Cy-3-arab**	**Cy-3-gal**	**Cy-3-glu**	**Dp-3-arab**	**Dp-3-gal**	**Dp-3-glu**	**Mv-3-arab**	**Mv-3-gal**	**Mv-3-glu**	**Pn-3-arab**	**Pn-3-gal**	**Pn-3-glu**	**Pt-3-arab**	**Pt-3-gal**	**Pt-3-glu**	**References**
Bulgaria (mg/100 g dw)	78.13	244.81	563.38	101.57	91.54	733.01	27.65	17.80	46.65	15.42	14.39	57.61	38.67	27.74	17.53	(Ivayla et al., 2016)

Abbreviations used: dp- delphinidin; cy- cyanidin; pt- petunidin; pn- peonidin; mv- malvidin; gal- galactoside; glc- glucoside; arab- arabinoside; nd- not detected; fw- fresh weight; dw- dry weight.

The use of *V. myrtillus* fruits as a source of natural pigments has also been evaluated by some authors. Thus Camire et al. (2002) compared breakfast cereals colored with an extract rich in anthocyanin from *V. myrtillus* and an extract obtained from grape juice. The results concluded that bilberry concentrate possessed much higher anthocyanin content (125.4 mg/100 g, fresh weight) than the grape juice extract (28.3 mg/100 g, fresh weight) and storage over a period of three months did not change the anthocyanin content in bilberry extrudates.

Although the external appearance of food is the organoleptic characteristic that arouses the first attention of consumers, it is necessary to assess acceptability. Pasqualone, Bianco, & Paradiso (2013) evaluated the acceptability of consumers to biscuits added with anthocyanin extracts obtained from bilberry fruits. This study revealed that most consumers, while detecting differences in color when compared to biscuits without anthocyanin incorporation, accepted the anthocyanin-enriched product satisfactorily. Another study revealed that the incorporation of *V. myrtillus* in ice-cream increased the levels of some major and minor essential elements, such as K, Se, Mn and Zn, and presented a good score by the panellists. Therefore, the addition of *V. myrtillus* extracts to ice cream was recommended as a natural source to increase the nutritional value and improve physicochemical properties, thus increasing the added value of the product (Eekaya Kotan, 2018).

1.4.1.4. Health benefits

The berries of *V. myrtillus* are popular worldwide and consumed since ancient times constituting an important part of the usual diet, as well as used in several popular medicines (Colak et al., 2016). These fruits are recognized as rich natural sources of polyphenols and others bioactive compounds with health benefits. Among others, antioxidant, anti-obesity, anti-proliferative, cardioprotective, anti-inflammatory, ocular, hypoglycemic and antibacterial effects have been associated with consuming bilberry fruits (de Mello et al., 2017; Karcheva-Bahchevanska, Lukova, Nikolova, Mladenov, & Iliev, 2017; Schink et al., 2018). The numerous scientific studies that support the beneficial effects associated with these fruits explain the increase in production and consumption of novel products and dietary supplements containing bilberry (Prokop et al., 2019). However, some studies have warned that high dose consumption of this berry may cause some unwanted effects, including possible interactions with concurrently and subsequently administered medicinal products (Prakash et al., 2015). In this section, the

principal health benefits associated to the consumption of berries or products development from *V. myrtillus* are reviewed.

Antioxidant properties. Antioxidants are thought to prevent chronic complications in part through their interactions with reactive oxygen species (ROS) and their ability to scavenge free radicals (Tian et al., 2017). The antioxidant properties of polyphenols have been strongly related to their ability to act as reducing agents (Lima, Vianello, Corrêa, A, & Borguini, 2014; Tumbas, Čanadanović-Brunet, Gille, Dilas, & Ćetković, 2010). Dróżdż, Šėžienė, & Pyrzynska (2017) demonstrated that fruits of *V. myrtillus* exhibited high antioxidant activity as good electron donors, and their extracts were able to reduce copper (II)-neocuproine chelate, as well as to quench 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH$^{\bullet}$). Veljković et al. (2017), in studies in rats, found relevant EC_{50} values for the DPPH radical scavenging activity and inhibition of lipid peroxidation in liposomes (0.202 ± 0.008 mg/mL and 0.33 ± 0.01 mg/mL respectively) for bilberry fruits. The same authors, in assays with Wistar albino rats, showed significantly decreased lipid peroxidation (MDA) and protein oxidation (AOPP) levels ($p < 0.001$) in the groups were *V. myrtillus* was supplemented than in the control group (Veljković et al., 2017).

Anti-obesity effects. The anti-obesity mechanisms for berries may include a reduction in decrease in lipogenesis, an increase in lipolysis, and an inhibition of pro-inflammatory adipokine secretion (Kowalska, Olejnik, Rychlik, & Grajek, 2015; Kowalska, Olejnik, Szwajgier, & Olkowicz, 2017). Studies carried by Kowalska et al. (2017), demonstrated the capability of *V. myrtillus* to diminish lipid accumulation with a concomitant down-regulation of peroxisome proliferator-activated receptor gamma (*PPARγ*), enhancer-binding protein alpha (*C/EBPα*) and sterol regulatory element binding transcription factor 1 (*SREBP*1c) in mouse embryo 3T3-L1 adipocytes, as well as to suppress the expression of adipocyte fatty acid-binding protein (a*P*2) and resistin (Kowalska et al., 2017).

Anti-proliferative properties. Polyphenols, which are plentiful in *V. myrtillus*, are among the most promising anti-carcinogenic agents in plants. Demirel Sezer et al. (2009) found that quercetin and kaempferol, the most abundant non-anthocyanin polyphenols present in *V. myrtillus* extracts, showed strong cytotoxic, antioxidant and apoptotic effects. *V. myrtillus* anthocyanins have also demonstrated ability to upregulate tumor suppressor genes, induce apoptosis in cancer cells, repair and protect genomic DNA

integrity and improve neuronal and cognitive brain function (Thibado, Thornthwaite, Ballard, & Goodman, 2017). Nguyen et al. (2010) concluded that extracts from *V. myrtillus* were able to inhibit proliferation and induce apoptosis in breast cancer cells through a mechanism that did not involve action in the microtubules or mitosis, although when the concentration of the extracts increases the organization of the microtubules was affected, leading to accumulation of cells at mitosis by a direct action on microtubules.

Cardioprotective effects. Cardiovascular diseases remain one of the leading causes of death and they are, therefore, a primary focus of research and treatment (Brader, Overgaard, Christensen, Jeppesen, & Hermansen, 2013). Several studies have shown that the intake of berry fruits was associated with a reduced risk of cardiovascular diseases. Ashour et al. (2011) demonstrated the ability of *V. myrtillus* to protect against DOX-induced cardiotoxicity in rats, which could be attributed, at least in part, to its antioxidant activity. *V. myrtillus* significantly inhibited DOX-induced elevations of LDH, CPK and CK-MB activity in serum, as well as troponin I levels; furthermore, in the histopathological examination the severity of the histological changes was much lower in sections from rats pre-treated with *V. myrtillus* (Ashour et al., 2011).
Erlund et al. (2008) studied the effects of *V. myrtillus* berries consumption on platelet function, blood pressure and HDL-cholesterol, and concluded that daily consumption of moderate amounts (100 mg) during two months could contribute to explain the cardiovascular disease protective role of a diet rich in fruit and vegetables. Žiberna et al. (2009) observed that the administration of a *V. myrtillus* extract (0.01-5 mg/L) to rats increased coronary flow up to 2.5-fold, and decreased lactate dehydrogenase (LDH) release rate during reperfusion by 3.7-fold at 0.1 mg/L and by 6.7-fold at 1 mg/L, compared to controls. It was also effective in the prevention of arrhythmias, whose duration was maximally shortened at 0.1 mg/L to 3.2 ± 0.2%, and at 1 mg/L to 4.4 ± 0.3%, in relation to the untreated group (Žiberna et al., 2009).

Anti-inflammatory effects. The progression and development of several diseases, such as autoimmune diseases, organ fibrosis, diabetes, obesity, allergies and dysfunction, are influenced by acute and chronic inflammation. Schink et al. (2018), in a screening on 99 ethanolic plant extracts, found that *V. myrtillus* displayed strong anti-inflammatory activity combined with high cell viability in THP-1, HeLa-TLR4, and HEK-TLR2/HEK-TLR4 cell lines. Anthocyanin-rich extracts from bilberry also showed anti-inflammatory

effects against liver inflammation in mice, leading to suppression of LPS-induced inducible nitric oxide synthase (iNOS), TNF-α, IL-1β and IL-6 transcripts, and iNOS, TNF-α and NF-κB protein levels (Luo et al., 2014). Furthermore, bilberry demonstrated ability to reduce serum C-reactive protein (CRP), IL-6, IL-12 and LPS levels, and downregulate genes associated with the TLR pathway in individuals with metabolic syndrome (Kolehmainen et al., 2012; Schink et al., 2018). All in all, these studies reveal not only the anti-inflammatory potential of *V. myrtillus*, but also their oral effectiveness in humans.

Hypoglycemic effects. Postprandial hyperglycemia is a condition that can be improved through lifestyle and diet, preventing the development of cardiovascular diseases and diabetes type 2 (Ceriello & Genovese, 2016; Granfeldt & Björck, 2011). Xu et al. (2018) showed that the consumption of fruits, like *V. myrtillus* rich in phenolic compounds, can attenuate postprandial glycemic and insulin responses in young adults, with bilberry fruits having the most insulin lowering effect at 30 min postprandial; this effect was maintained throughout the early postprandial period and was related with the consumed amount of phenolic compounds. Different authors also reported inhibitory effects on α-glucosidase and amyloglucosidase activities of polyphenol-rich extracts from *V. myrtillus* fruits; in particular, phenolic acid-enriched fractions were able to inhibit α-glucosidase *in vitro*, which is considered one of the most effective ways to control type 2 diabetes (de Mello et al., 2017; de Sales, de Souza, Simeoni, Magalhães, & Silveira, 2012; McDougall & Stewart, 2005; Xiao et al., 2017).

Ocular effects. Eye fatigue, pain, dry eye sensation, excess of tears, blurry vision, glaucoma and cataracts are the most common changes that can impair the quality of vision, especially in individuals with daily work that require more eye strain (Ozawa et al., 2015). Thus, the development of products which could improve eye health has gained prominence from researchers. Riva et al. (2017) studied the bioavailability of a standardized *V. myrtillus* extract and its ability to alleviate dry eye symptoms and concluded that it could improve tear secretion in subjects suffering from dry eye symptoms. Other studies described beneficial ocular effects of *V. myrtillus* extracts, namely night vision improvement (Canter & Ernst, 2004), cataract and glaucoma prevention (Head, 2001).

Antimicrobial effects. Some studies suggest that bilberry may protect against human pathogenic bacteria, due to its composition in phenolic compounds and organic acids (Puupponen-Pimiä, Nohynek, Ammann, Oksman-Caldentey, & Buchert, 2008). Toivanen et al. (2011) demonstrated that juices produced from *V. myrtillus* showed potential against pneumococcal infections caused by *Neisseria meningitidis* with a 63% growth inhibition at a concentration of 10 mg/mL. Huttunen et al. (2011) studied the inhibitory activity of wild berry juice fractions composed mostly of sugars and some amounts of small size phenolics against *Streptococcus pneumoniae* binding to human bronchial cells and concluded that the highest concentration used in their antimicrobial tests (~86 mg/g) was extremely effective and the growth of *S. pneumonia* was totally inhibited by *V. myrtillus* extract.

Overall, bilberry has potential to be used in vision improvement, and in the treatment or prevention of conditions associated with dyslipidemia, inflammation, hyperglycemia, increased oxidative stress, cardiovascular disease (CVD), cancer, diabetes, and other age-related diseases, besides their antimicrobial activity.

1.4.1.5. Industrial applications

Appearance is a recurrent concern of the food and pharmaceutical industries where color plays a key role. The demand for the use of natural pigments has increased not only because of the concern with the use of artificial colorants, which is increasingly evident, but also for their nutraceutical properties (Murley & Chambers, 2019). The discovery of compounds able to meet the coloring and healthy requirements, with minimal toxicity, has led many researchers to focus on the use of extracts rich in anthocyanins obtained from fruits, including the fruits of *V. myrtillus*, in the development of innovative products. Daily anthocyanin consumption can range from milligrams to hundreds of milligrams per person depending on the diet and the sources they are ingested from. The use of anthocyanins as food colorings, especially in more acidic foods, which may favour their stability, has exponentially raised. The exploitation of *V. myrtillus* fruits for medicine and human diet purposes has received great attention, being the economically most important wild berries of Northern Europe, widely used by the food industry (Trivedi et al., 2019). This fruit is consumed not only in a fresh manner, but also in processed products (press cake) and derivatives (juice, jam and liqueur) (Benvenuti, Brighenti, & Pellati, 2018; Zhou et al., 2018). Fruits drying and its transformation into powder represent a suitable

alternative widely used by consumers and the food industry that allow to have them available throughout the year for subsequent use as an ingredient in based foods (extruded products, bakeries, sauces, beverages, ice creams, yogurts, and confectionary) (Karam, Petit, Zimmer, Baudelaire Djantou, & Scher, 2016; Oliveira et al., 2019). The by-products resulting from the production of bilberries, as well as the fruits that due to their exterior appearance or size do not meet commercialization standards, may also be used for the preparation of polyphenols extracts and the production of novel foods, conferring them added value and reducing the environmental impact. Some authors showed that press cake of *V. myrtillus*, a by-product from juice production, can be a suitable and green approach to be used in the preparation of value-added products by the food industry (Bobinaitė et al., 2015; Pataro et al., 2017; Zhou et al., 2018). For instance, Fidaleo et al. (2015) studied the suitability of phenolic extracts obtained from *V. myrtillus* residues as an ingredient in drinking yogurt and condensed milk with high antioxidant capacity. There are already in the market several companies that have incorporated bilberry in products from several sectors claiming benefits to the consumer health. Lusoberry® (http://lusoberry.com/), a Portuguese company based in Oliveira do Hospital, is a bilberry producer that began to add value to the fruits using them in the production of oil and wine. The company expounds that bilberry oil does not have as strong taste or acidity as olive oil and that has higher concentration of magnesium and potassium. For its part, the bilberry wine, not being a novelty in the world, emerges for the first time in the Iberian Peninsula.

Yogurts are an important element of the human diet and often consumed by a large part of the population of all age groups. This may justify the diversity of dairy companies interested in developing bilberry-enriched yogurt. PIÁ®, a Brazilian company, has developed a yogurt with bilberry preparations, containing 25% less sugar content than usual (http://www.pia.com.br/produto/tipo/iogurtes/). Also, Biedermann® company from Switzerland has developed BioSkyr®, a protein-rich fermented milk containing 9.2% bilberry fruit (https://biomolkerei.ch). Both companies have promoted marketing campaigns around these products highlighting the health benefits. A Brazilian company dedicated to the preparation of more natural ice cream has launched a claimed healthy product based on bilberry fruit (http://www.santofruto.eco.br/).

Another sector that is of great importance in the human diet and which has also been influenced by consumer demand for healthier foods is the bakery industry. Mirtiflor® (https://www.mirtiflor.pt/), a family Portuguese company producer of wild fruits, has invested in the development of several products aiming in the full use of these fruits. In

the particular case of bilberry, the company, in addition to preparing traditional jams and liqueurs, has launched in the market bilberry cookies. Also, the well-known BioGerminal® (https://www.germinalbio.it/), that is dedicated to the production of healthy food, has developed an integral spelled pie with bilberry. Moreover, Little Bellies® (https://bellies.com.au/) is a well-known children's food brand that has been focusing on launching products labeled as healthier and more natural. In its diversity of choices, there is a snack for children from nine months of age based on puffed corn with organic bilberry. In addition to being advertised as a natural snack with antioxidant properties, this snack is also gluten free.

Similarly, the big chocolate companies have also been testing new recipes using powerful fruits to somehow make unhealthy products more attractive to consumers. So, Schogetten® (https://www.schogetten.com) features chocolate with dehydrated bilberry and muesli, and the well-known Guylian® Belgian chocolate (https://www.guylian.com/) features a variety of fruit berries topped with its famous chocolate, among them bilberry berries.

In addition to the various sectors of the food industry, the cosmetic industry has also been focusing on enriching different products with bilberry. Panvel Vert® (https://www.panvel.com) has an extensive experience in cosmetic products and have developed a body spray with soothing and relaxing properties with bilberry extract, enriching the product with vitamins, minerals and other antioxidants. Also, the hair cosmetics industry has been betting on innovation, and brand Loweel® (http://www.lowell.com.br/) has developed a hair care kit for repair and nutrition incorporating bilberry antioxidant active agents, claiming the protection from hair aging.

In conclusion, *V. myrtillus* is a widely consumed fruit, with interesting nutritional and therapeutic properties, rich in phytochemical compounds, which can be used in various industrial sectors, not only as such or as derived products, but also taking advantage of the resulting by-products, without losing its claimed beneficial effects.

1.4.2. Lycium barbarum L.

Lycium barbarum L. belongs to *Solanaceae* family, it is widely distributed in Mediterranean area and Southwest and Central Asia, and has different vernacular names, but the most commom names are "goji" and "wolfberry" (Donno, Beccaro, Mellano, Cerutti, & Bounous, 2015). Since the beginning of the 21st century, goji products have been introduced in Europe and North America and their consumption has increased rapidly due to their claimed beneficial properties for wellbeing and longevity (D'Amato, Esteve, Fasoli, Citterio, & Righetti, 2013). Goji berries have been associated with a wide range of health benefits, including the treatment of diseases related to liver, kidney, eyesight, immune system, circulation and longevity, as also with sexual activity (Tang et al., 2012).

The berries (**Figure 9**) are commonly consumed in soups, as porridge with rice and added to numerous meat and vegetable dishes, eaten raw, as a juice, wine or in tea preparations, as also processed as tinctures, powders, and tablets (Potterat & Food, 2010). Goji is also found in conventional food products, such as yogurt (Donno et al., 2015), and as food supplements, in particular, the concentrated juice or extracts from this fruit are added to some beverages with the aim to improve the hepatic function and lower the oxidative stress (Masci et al., 2018). Leaves and stems (**Figure 10**) are used in Taiwan as tea and vegetables, and claimed to provide beneficial effects such as antioxidant, immuno-stimulating, anti-obesity and anti-tumor activities (Jabbar, Abid, & Zeng, 2014) , which have been associated to their content in polyphenols.

Over the last decade, more than 200 different components, comprising carotenoids, phenylpropanoids, flavonoids and other polyphenols, and polysaccharides, have been identified and characterised. All of them with some interesting biological properties (Masci et al., 2018).

Polysaccharides consist of a complex mixture in most cases, with 90–95% of arabinose, glucose, galactose, mannose, rhamnose, xylose, and/or galacturonic acid representing the most important group of substances in the fruit of *L. barbarum*; they are followed by carotenoids, which are responsible for the red coloration of these fruits (Potterat & Food, 2010). Zeaxanthin represents the major compound of this class and plays an important protective role against utraviolet radiation. Different types of polyphenols, including phenylpropanoids, coumarins, lignans, flavonoids (quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside), isoflavonoids, chlorogenic acid derivatives and

hydroxybenzaldehyde and hydroxybenzoic acid derivatives have been identified and related with their antioxidant activity (Masci et al., 2018; Zheng et al., 2017).

Nutritionally goji berries contain carbohydrates as a major macronutrient (51%), low concentrations of free amino acids (proline and taurine), vitamins (thiamine and riboflavin) and small amounts of fatty acids (palmitic, linoleic and myristic) (Masci et al., 2018; Potterat & Food, 2010). Moreover, goji berries also have been attributed multiple effects, including anti-aging, neuroprotection, anti-fatigue, hypoglycemic, antiproliferative, cytoprotection, immunomodulation and antioxidant properties (Jabbar et al., 2014; Mocan, Vlase, Raita, et al., 2015a) .

Figure 9. Fruits of *Lycium barbarum* L. Author: Tânia Pires

Figure 10. Stems of *Lycium barbarum* L. Author: Tânia Pires

1.4.3. Malus domestica Borkh. cv Bravo de Esmolfe

'Bravo de Esmolfe' (**Figure 11**) is a Portuguese apple variety with an intense aroma, highly appreciated by consumers. This apple has been recognised as a product with Protected Designation of Origin (PDO; N^{o}1107/96, 2001), being therefore a high added value product with impact in the local and national economy (Reis, Rocha, Barros, Delgadillo, & Coimbra, 2009). In the last few years the 'Bravo de Esmolfe' apple has doubled its price compared to exotic varieties, such as Golden and Starking (Feliciano et al., 2010). Its production is carried out in a restricted and small inland region in northern Portugal, corresponding to a production of 200,000 kg per year, but commercial demand is now increasing, due to its appealing sensory properties, namely sweetness and flavour Apple fruits have a wide variety and well-balanced composition, being moderately energetic and well-proportioned in sugar and acid contents, giving it a pleasant taste. They are mostly constituted by water (84%), minerals, complex B vitamins (Feliciano et al., 2010), monosaccharides, dietary fibre, and various biologically active compounds, such as vitamin C, and certain phenolic compounds (Róth et al., 2007; Wu et al., 2007)

An apple hardly contains 0.16%-0.18% fat, mostly characterized by the presence of linoleic acid (18:2n6) and oleic acid (18:1n9). Linoleic acid is an essential fatty acid, with some studies suggesting that it improves impaired glucose tolerance and reduces the risk of atherosclerosis and body fat deposition (Skinner, Gigliotti, Ku, & Tou, 2018). Apples contain a large percentage of total carbohydrates and free sugars, such as frutose and glucose, with higher concentration of the first (Skinner et al., 2018). They also present complex carbohydrates such as polysaccharides, responsible for improvement in the levels of serum triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), insulin, and adiponectin, as described by Skinner et al. (2018) and Raphaelli et al. (2019). Soluble fiber accounts for 13.5%-14.6% of total fiber, responsible to promote gastrointestinal health and to reduce the risk for diverticular diseases and certain cancers, particularly colorectal cancer (Skinner et al., 2018).

Various authors (Malec et al., 2014; Mayr, Treutter, Santos-Buelga, Bauer, & Feucht, 1995; Scafuri et al., 2016; Shoji, Masumoto, Moriichi, Kanda, & Ohtake, 2006; Shoji et al., 2003; Verdu et al., 2013; Wojdyło, Oszmiański, & Laskowski, 2008) analysed the phenolic profile of different apple cultivars, but none of the previously mentioned authors have studied the bioactive properties and compounds from the cultivar 'Bravo de Esmolfe'. Flavonols (quercetin, kaempferol, and rutin), dihydrochalcones (phloretin and

phloridzin), flavan-3-ols (epicatechin and procyanidins) and phenolic acids (caffeic acid and coumaric acid) constitute the main classes of polyphenols present in apples (Rana & Bhushan, 2016), with hydroxycinnamic acids, flavan-3-ols, flavonols and dihydrochalcones being the prevailing polyphenols.

The phenolic compounds are responsible for several reported bioactive properties, such as antioxidant, antiproliferative, anticancer, anti-inflammatory, anti-diabetic and cardiprotective (Rana & Bhushan, 2016). Epicatechin showed protective effect against neuronal cell death provoked by oxidative stress, good antioxidant activity and was found to reduce lipid peroxidation and also inhibit human blood platelet aggregation (Neiva, Morais, Polack, Simões, & D'Amico, 1999). Quercetin and quercetin-3-*O*-glucoside have been reported to have strong antiproliferative activity against HepG2 and MCF-7 cells, and the dihydrochalcone phloretin reported to inhibit the proliferation of SMMC-7721 hepatoma cells (He & Liu, 2008). Procyanidins were indicated to play a significant role in tumor cell apoptosis, with good results for the inhibit growth of human breast cancer cells (e.g., MDA MB-231, 436, 468, SKBR-3, and MCF-7) (Ramljak et al., 2005). Kaempferol and quercetin have been found to inhibit the activation of STAT-1 and NF-κB transcription factors of nitric oxide synthase (iNOS) in macrophages that were exposed to an inflammatory lipopolysaccharide, thus controlling nitric oxide production (Hämäläinen, Nieminen, Vuorela, Heinonen, & Moilanen, 2007).

Figure 11. Lyophilized slices of the Portuguese apple variety "Bravo de Esmolfe"
Author: Tânia Pires

1.5. Objectives and working plan

Consumption habits are increasingly diversified and oriented towards more sustainable food options with improved functional value. The range of species and parts of plants used for food is also increasingly varied, seeking not only to provide ingredients with potential health benefits for consumers, but also new colors, textures and flavors. Thus, in the development of new food products it is increasingly common to incorporate non-traditional elements such as edible flowers, exotic berries or stems, along with other products more common to the consumer, such as red fruits or apples.

The motivation for the improvement of health and quality of life is increasingly present in the general population, so that there is a growing concern for adopting healthier lifestyles, among which diet plays a preponderant role. This has led to increase the interest in foods with optimized functional characteristics and nutritional supplements, usually based on fruits and other parts of plants, as well as the development of new combinations of products and ingredients more attractive to the consumer.

The general objective of this work is the development of food products of high nutritional and bioactive value based on bilberries mixed with flowers and other fruits (**Figure 12**). The products developed are intended to be transferred into the market by RBRfoods company (Castro Daire, Portugal), which provided the plant materials.

With this aim, the following **specific objectives** were proposed:

1) To study the chemical and nutritional composition of different matrices to be further incorporated as ingredients in yogurts and used in the preparation of snack mixtures, in particular bilberry (fruits of *Vaccinum myrtillus* L.), apple (*Malus domestica* Borkh, cv Bravo-de-Esmolfe), Goji berries and stems (*Lycium barbarum* L.), and edible flowers, specifically *Dalia mignon*, *Rosa damascena* 'Alexandria' and *Rosa gallica* 'Francesa' mixed on *R. canina*, *Calendula officinalis* L., and *Centaurea cyanus* L..

2) To analyse the phenolic composition and evaluate the bioactive properties of the selected plant matrices.

3) To assess the suitability of the incorporation of natural ingredients obtained from the studied matrices into a dairy product (yogurt).

4) To characterize the physico-chemical, nutritional and bioactive properties of different snacks prepared based on mixtures of bilberry with the other considered fruits and edible flowers (**Figure 12**).

Sample	Composition (total of 50 g)
	100% Bilberry fruit (50 g)
	98% Bilberry fruit (49 g) 2% Rose flowers (1 g)
	99.98% of Bilberry (49.97 g) 0.02% Calendula flowers (0.03 g)
	60% Bilberry fruit (30 g) 36% Apple fruit (18 g) 4% Goji fruit (2 g)

Figure 12. Snacks prepared with bilberries mixed with flowers and other fruits. Author: Tânia Pires

A scheme of the studies to perform for the development of the proposed objectives is presented in **Figure 13.**

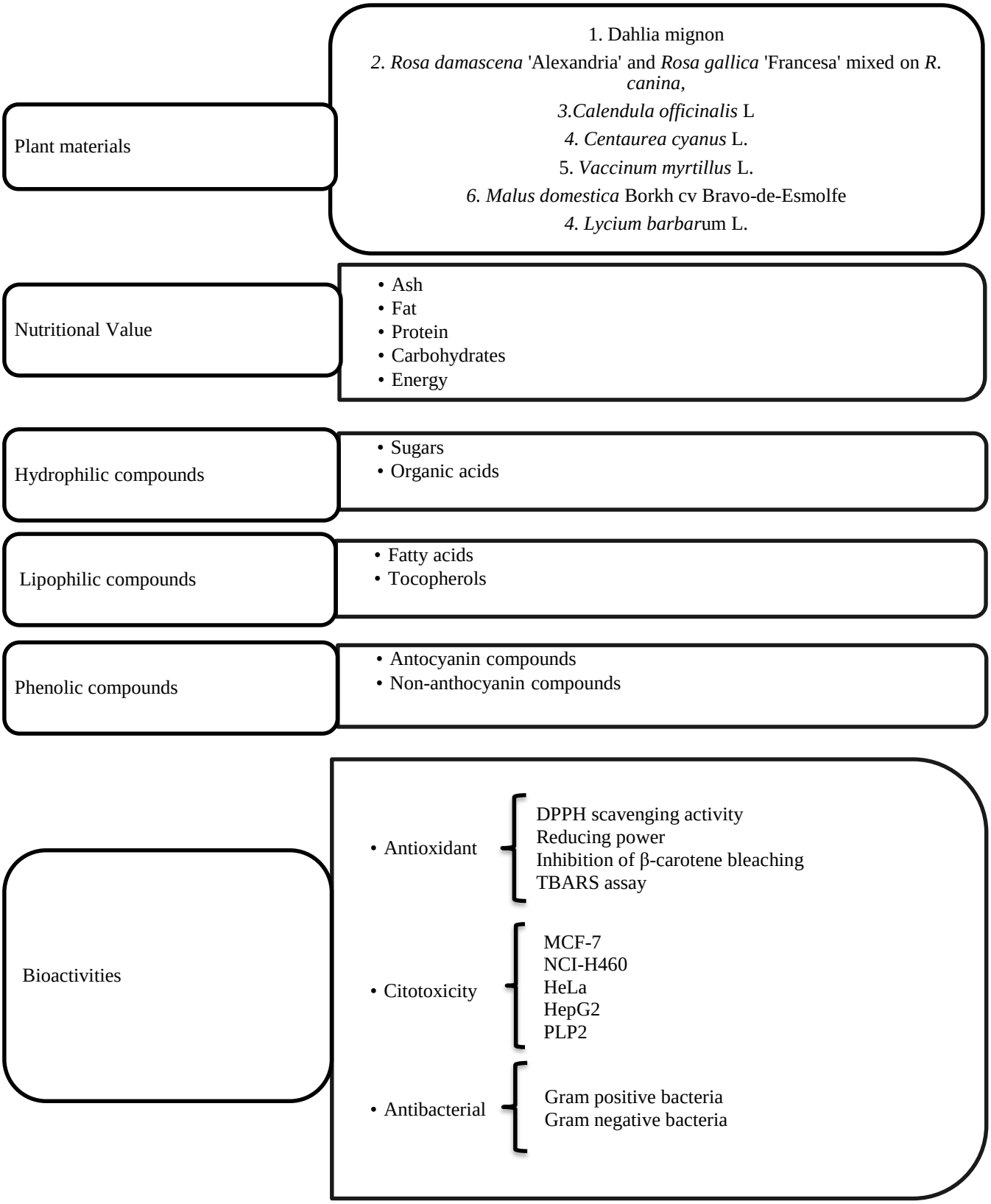


Figure 13. Scheme of the proposed studies.

CHAPTER 2

Materials and Methods

2.1. Standards and reagents

For chemical analisis: Acetonitrile, n-hexane and ethyl acetate were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (FAME) reference standard mixture (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, MO, USA), as well α- and δ-tocopherols, sugar and organic acid standards (oxalic acid, quinic acid, malic acid, shikimic acid, citric acid, succinic acid, and fumaric acid). Racemic tocol, 50 mg/mL, and β- and γ-tocopherols were purchased from Matreya (Pleasant Gap, PA, USA). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain).

Non-anthocyanin phenolic standards (apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, hesperetin, isoliquiritigenin, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, naringenin, *p*-coumaric acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, catechin, ferulic acid, sinapic acid, syringic acid and myricetin) were from Extrasynthèse (Genay, France). Anthocyanin standards (cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside; pelargonidin-3-*O*-glucoside, peonidin 3-*O*-glucoside, malvidin-3-*O*-glucoside) were from Polyphenols (Sandnes, Norway).

Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

For antioxidant activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, tris(hydroxymethyl) aminomethane (Tris), formic acid, β-carotene and linoleic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tween 80 was acquired from Panreac Applichem (Barcelona, Spain)

For antiproliferative activity and hepatotoxicity: Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma-Aldrich (St. Louis, MO, USA).

The Griess Reagent System Kit was purchased from Promega (Madison, WI, USA). Human tumor cell lines tested: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) from DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).

For antimicrobial activity*: p*-Iodonitrotetrazolium chloride (INT) was from Panreac Applichem (Barcelona, Spain), Tryptic Soy Broth (TSB) and Mueller- Hinton (MH) were from Biolab® (Hungary).

2.2. Samples

The flower samples (*Dahlia mignon* (commercial seeds mixture), *Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*, *Calendula officinalis* L., and *Centaurea cyanus* L.), fruits (*Vaccinium myrtillus* L., *Lycium barbarum* L. and *Malus domestica* Borkh cv *Bravo de Esmolfe*) and stems (*Lycium barbarum* L.) were graciously supplied by the company RBR Foods (Castro Daire, Portugal) as dry material. After reception in the laboratory all the samples were reduced to a fine powder (20 mesh) that was mixed to obtain homogenate samples.

2.2.1. Hidromethanolic extracts

1 g of each sample was submitted to extraction with a methanol:water mixture (80:20, v/v; 30 mL) at 25 ºC and 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. Afterwards, the residue was extracted with one additional portion of the hydromethanolic mixture and the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland). All preparations were frozen and then lyophilized for their storage till performing the assays.

2.2.2. Preparation of infusions

Only edible flowers were used to prepare infusions**.** Boiling distilled water (100 mL, pH 6.6) at 100 ºC was added to each sample (1 g) and left to stand at room temperature for 5 min, Afterwards, the infusions were filtered (0.22 μm), frozen and then lyophilized.

2.2.3. Preparation of aqueous extracts

In order to prepare the aqueous extracts for anthocyanin analyses and incorporation into yogurts, the powdered samples (20 mesh) were extracted by maceration (25 ºC, 150 rpm, 1 h) using a stirring plate (VELP scientific, Keyland Court, NY, USA) by adding 1 g of dry material to 50 mL of distilled water. Afterwards, the mixture was filtered through Whatman filter paper No. 4, frozen and lyophilized.

2.2.4. Incorporation in yogurts

For flowers: The base formulation natural yogurts (fat 3.8 %; protein 5.0% and carbohydrates 4.7%) were purchased at a local market. Five groups (three samples/group) of yogurts (70 g each) were prepared: i) plain samples (BY); ii) yogurts with commercial colorant, E163 (AY); iii) yogurts with rose flowers extract (RY); iv) yogurts with *Centaurea cyanus* L. flowers extract (CY), and v) yogurts with *Dahlia mignon* flowers extract (DY). All colorants were added to a portion of 70 g of yogurt and were prepared in duplicate. The E163 colorant was added at a 0.02% concentration; in the case of yogurts added with flowers extracts, higher concentrations of each extract (0.05% for dahlia extract; 0.15% for rose extract; 0.10% for centaurea extract) were added (the quantity was added until an evident change in color was obtained).

For fruits: The same base yogurt formulation described in the previous seccion was used for incorporating a dried aqueous extract obtained from fruits of *Vaccinium myrtillus L.* Yogurts were then divided into three groups with three samples (70 g each) per group. Group A consisted of the plain yogurt group, without any incorporation, while Group B was incorporated with 0.02% of E163 (0.014 g; CHR Hansen, Denmark, prepared from grapes from the Mediterranean region), and Group C was incorporated with 0.42% of bilberry extract (0.294 g). All samples were stored for 7 days (5 ºC) and the analyses were conducted in day 0 and day 7 to evaluate their stability.

2.3. Nutritional value and chemical composition

2.3.1. Proximate composition and energetic value

Protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC, 2005, 2016). Crude protein content (N×6.25) was estimated by the macro-Kjeldahl method. This method is based on the amount of nitrogen present in a sample

and relies on the destruction of all organic matter by addition of a strong acid (sulphuric acid) that retains nitrogen under the form of $(NH_4)_2SO_4$. Further addition of NaOH releases the nitrogen as NH_3 that is collected by steam distillation on a solution of 0.1N H_2SO_4; afterwards a titration with 0.1N NaOH using methyl red as an indicator is made to calculate the amount of nitrogen. Crude fat was determined using a Soxhlet apparatus by extracting a known weight of sample with petroleum ether. The ash content was determined by incineration at 600 ± 15 °C.

Total carbohydrates were calculated by difference, and total energy was calculated according to the following equation:

Energy (kcal) = (protein mass (g)+ carbohydrates mass (g)) ×4 + fat mass (g) ×9

Equation 1. Equation for energy determination.

2.3.2. Hydrophilic compounds

Soluble sugars. Dried sample powder (1.0 g) was spiked with melezitose as internal standard (IS, 5 mg/mL), and extracted with 40 mL of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000 *g* for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm Whatman nylon filters (Pinela et al., 2011). The HPLC equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD, USA) and an RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw) (Barros et al., 2013).

Organic acids. Organic acids were determined using ultra-fast liquid chromatography coupled to a photodiode array detector (UFLC–DAD). Samples (approximately 2 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25 ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample was filtered through 0.2 μm nylon filters. The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 mm· 4.6 mm i.d.) thermostatted at 35 ºC. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out in the DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm (245 nm for ascorbic acid) with calibration curves obtained from commercial standards of each compound: oxalic acid (y= 9x106x + 45973, R^2=0.9901); quinic acid (y = 610607x + 46061, R^2=0.9995); malic acid (y= 912441x + 92665, R^2= 0.999); shikimic acid (y= 7x107x + 175156, R^2=0.9999); citric acid (y= 1x106x + 45682, R^2=0.9997); succinic acid (y= 592888x + 50689, R^2=0.9996) and fumaric acid (y= 154862x + 1x106, R^2=0.9977). The results were expressed in g per 100 g of dry weight (Barros et al., 2013).

2.3.3. Lipophilic compounds

Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC–FID)/capillary column, following trans-esterification. Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v:v), during at least 12 h in a bath at 50 ºC and 160 rpm; then 3 mL of deionized water were added, to obtain phase separation; the FAME were recovered with 3 mLof diethyl ether by shaking in vortex, and the upper phase was passed through a micro-column of sodium sulphate anhydrous in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 μm nylon filter from Whatman (Pinela et al., 2012). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 °C) and a Macherey-Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm df). The oven temperature

programme was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid (Barros, Pereira, Calhelha, et al., 2013).

Tocopherols. Tocopherols were determined by HPLC, coupled to a fluorescence detector (FP-2020; Jasco). BHT solution in hexane (10 mg/mL; 100 μL) and IS solution in hexane (tocol; 50 μg/mL; 400 μL) were added to the sample prior to the extraction procedure. The samples (approximately 500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000 *g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of n-hexane, dehydrated with anhydrous sodium sulphate, filtered through 0.2 μm nylon filters from Whatman, and transferred into a dark injection vial for the analysis (Pinela et al., 2011). The fluorescence detector was programmed for excitation (λ_{ex}) at 290 nm and emission (λ_{em}) at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm x 4.6 mm i.d.) normal-phase column from YMC Waters operating at 30 ºC. 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, and the injection volume was 20 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on calibration curves obtained from commercial standards of each compound using the IS methodology. The results were expressed in mg/100 g of dry weight (dw) (Barros et al., 2013).

2.4. Physico-chemical parameters

External color was analyzed using a portable colorimeter CR400 (Konica Minolta, Chiyoda, Tokyo, Japan) using the C65 illuminant, which represents the midday light in

Europe, and a standard from the International Commission of Illumination (CIE). The CIE L*, a*, and b* color space coordinates were used, where L* represents lightness, a* represents redness (red-green), and b* yellowness (yellow-blue), with a 10º observer angle and 8 mm of aperture. The variation in total color difference (ΔE*) between yogurt samples was calculated by the equation:

$$\Delta E^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

Equation 2. Equation for determining the variation in total color difference.

The pH values of the samples were measured directly with a HI 99161 pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA).

2.5. Phenolic compounds analyses

2.5.1. Non-anthocyanin compounds

The non anthocyanic compounds were determined in the previously obtained hydromethanolic extracts dissolved in 20% aqueous ethanol at 5 mg/mL and filtered through a 0.22-µm disposable LC filter disk. Chromatographic analysis were performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to an electrospray ionization mass detector (HPLC-DAD-ESI/MSn), a quaternary pump, an auto-sampler (kept at 5 °C), a degasser and an automated thermostatted column compartment. Chromatographic separation was performed using a Waters Spherisorb S3 ODS-2 C18 (3 µm, 4.6 × 150 mm). Double online detection was carried out in a DAD (using 280, 330 and 370 nm as preferred wavelengths) and a mass spectrometer operated in negative mode (Linear Ion Trap LTQ XL mass spectrometer equipped with an ESI source, ThermoFinnigan, San Jose, CA, USA) connected to the HPLC system via the DAD cell outlet. The identification was performed using standard compounds, when available, and based on their retention behaviour, UV-vis and mass spectra compared with our database library and literature data. For quantitative analysis, calibration curves with known concentration (200-5 µg/mL) for each available phenolic standard: apigenin-7-*O*-glucoside ($y = 10683x - 45{,}794$, $R^2 = 0.9906$), caffeic acid ($y = 388345x + 406369$, $R^2 = 0.9949$); chlorogenic acid ($y = 168823x - 161172$, $R^2 = 0.9999$), hesperetin (y =

34156x + 268,027, R^2 = 0.9999), isoliquiritigenin (y = 42820x + 184,902, R^2 = 0.999), isorhamnetin-3-*O*-glucoside (y = 11117x + 30,861, R^2 = 0.9999), kaempferol-3-*O*-rutinoside (y = 11117x + 30861, R^2 = 0.9998), naringenin (y = 18433x + 78,903, R^2 = 0.9998), *p*-coumaric acid (y = 301950x + 6966.7, R^2 = 0.9999), quercetin-3-*O*-glucoside (y = 34843x – 160173, R^2 = 0.9998), catechin (y = 84950x – 23200, R^2 = 0.9999), ferulic acid (y = 633126x – 185462, R^2 = 0.9999), quercetin-3-*O*-rutinoside (y = 13343x + 76751, R^2 = 0.9998), sinapic acid (y = 197337x + 30036, R^2 = 0.9997), syringic acid (y = 376056x + 141329, R^2 = 0.9995), myricetin (y = 23287x – 581708, R^2 = 0.9988) were constructed based on the UV signal at 280 nm. Quantification was made from the areas of the peaks recorded at 280 nm by comparison with the calibration curve obtained from a standard of the same family (Bessada, Barreira, Barros, Ferreira, & Oliveira, 2016).

2.5.2. Anthocyanins

In the lyophilized extracts obtained from edible flowers and fruits: Anthocyanins were analysed in the lyophilized aqueous extracts of the different samples dissolved in water at 5 mg/mL. Chromatographic separation was achieved in a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm x 150 mm, Waters, Milford, MA, USA) column working at 35 °C. The gradient elution was the following: 10% B for 3 min, from10 to 15% B for 12 min, 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min, from 30 to 35% B for 5 min, and from 35 to 10% B for 10 min, followed by column reconditioning of 10 min, using a flow rate of 0.5 mL/min. Double online detection was carried out in a DAD using 520 nm as the preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in positive mode, using a Linear IonTrap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. Nitrogen served as the sheath gas (50 psi); the system was operated with a spray voltage of 4.8 kV, a source temperature of 320 °C, and a capillary voltage of 14V. The tube lens offset was kept at a voltage of 75 V. The full scan covered the mass range from *m/z* 100 to 1500. The collision energy used was 20 (arbitrary units). Data acquisition was carried out with Xcalibur data system (Thermo Finnigan, San Jose, CA, USA) (Gonçalves et al., 2017). Retention times, UV-Vis and mass spectra were compared with available standards and with literature data to identify the anthocyanins. Calibration curves of the

available anthocyanin standards: cyanidin-3-*O*-glucoside (y = 243287x - 1E+06, R^2 = 0.995), pelargonidin-3-*O*-glucoside (y = 276117x - 480418; R^2 = 0.9979), malvidin-3-*O*-glucoside (y = 477014.9x + 38.376, R^2 = 0.999), delphinidin-3-*O*-glucoside (y = 557274x + 126.24, R^2 = 0.997) and peonidin 3-*O*-glucoside (y = 537017x - 71.469, R2=0.999) were constructed based on the UV signal at 520 nm to perform quantitative analysis. In case of unavailable commercial standards, the compounds were quantified via the calibration curves of the most similar available standards.

In yogurt samples incorporated with anthocyanin rich extracts: 3 g of dry yogurt were dispersed in water at 25 ºC, 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. The remaining residue was re-extracted with an additional portion of water mixture, stored at -20 ºC and lyophilized for analysis, using the HPLC-DAD-ESI/MS system mentioned above in the previous section**.**

2.6. Evaluation of bioactive properties

2.6.1. Antioxidant activity

The lyophilized plant extracts were re-dissolved in methanol:water (80:20, v/v) to obtain stock solutions of 2.5 mg/mL, which were further diluted to obtain a range of concentrations (2.5 mg/mL to 0.07 mg/mL) for antioxidant activity evaluation by DPPH radical-scavenging, reducing power, inhibition of β-carotene bleaching, and TBARS inhibition assays (Barros, Pereira, Calhelha, et al., 2013). The final results were expressed as EC_{50} values (mg/mL), which means sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as a positive control.

DPPH scavenging activity. This method is based on the reduction of the DPPH radical by hydrogen donation from an antioxidant. The DPPH radical is a nitrogen compound that is stable due to the relocation of the free electron and that possesses a purple color. It reacts easily with molecules that can donate hydrogen atoms, changing to yellow when it accepts them (**Figure 14**). This assay is widely used as a preliminary antioxidant study (Antolovich et al., 2002; Amarowicz et al., 2004; Moon and Shibamoto, 2009).

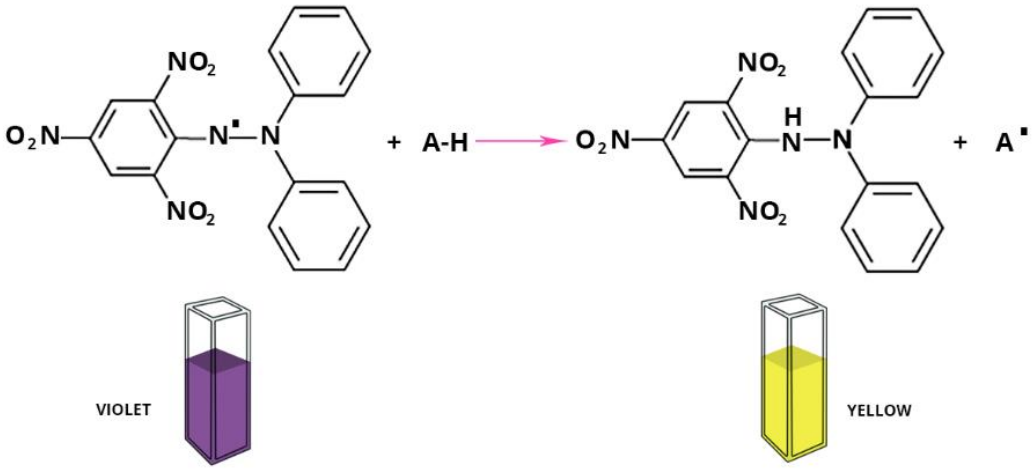


Figure 14. Reduction of the DPPH radical. Source: http://chimactiv.agroparistech.fr

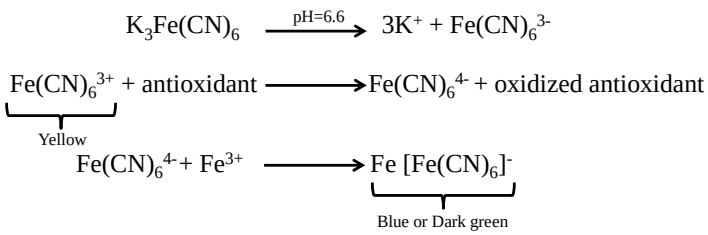
The assay was performed using an ELX800 microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA). The reaction mixture on a 96 well plate consisted of sample solutions (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm.

The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the **equation 3**, where 'A_S' is the absorbance of the solution containing the sample, and 'A_{DPPH}' is the absorbance of the DPPH solution.

$$\% \text{ RSA} = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100$$

Equation 3. Equation for calculation of the radical scavenging activity (RSA) in the DPPH method

Reducing power. This method measures the ability of antioxidants to reduce Fe(III) to Fe(II). The chemistry of the assay can be summarized in the following reactions:



The sample solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured into a well of a 48 wells plate, deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL) were then incorporated, and the absorbance was measured at 690 nm in the Microplate Reader mentioned above.

Inhibition of β-carotene bleaching. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing sample solutions (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero-time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was measured by the **Equation 4**, where 'A_{β}' corresponds to absorbance of β-carotene in zero time or after 2 hours.

$$\%\ \beta\text{-Carotene bleaching inhibition} = \frac{A_{\beta\text{-Carotene after 2 h}}}{A_{\beta\text{-Carotene}}} \times 100$$

Equation 4. Equation for the calculation of β-carotene bleaching inhibition.

TBARS assay. Porcine brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate that was centrifuged at 3000 *g* for 10 min. An aliquot (100 µL) of the supernatant was incubated with the sample solutions (200 µL) in the presence of $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL at 37 °C for 1h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 µL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 µL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000 *g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the **Equation 5**, where '$A_{Control}$' and 'A_{Sample}' were the absorbance of the control and the sample solution, respectively:

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Equation 5. Equation for calculation of the inhibition ratio in the TBARS assay.

2.6.2. Antibacterial activity

The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal. Seven Gram-negative bacteria (*Escherichia coli*, *E. coli* ESBL (extended spectrum of beta-lactamase), *Klebsiella pneumoniae*, *K. pneumoniae* ESBL, *Morganella morganii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, isolated from urine and expectoration) and five Gram-positive bacteria (*MRSA*-methicillin-resistant *Staphylococcus aureus*, *MSSA*- methicillin-susceptible *Staphylococcus aureus*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis*) were used to screen the antibacterial activity. Minimum Inhibitory Concentrations (MIC) determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay.

The extracts were re-dissolved in water in order to obtain stock solutions of 100 mg/mL, and then submitted to further dilutions. A 190-µL aliquot of this concentration was added in the first well (96-well microplate) in duplicate. In the remaining wells place

90 μL of medium MHB or TSB. Then the samples were serially diluted obtain the concentration ranges (20 at 0.15 mg/mL). To finish, 10 μL of inoculum (standardized at 1.5×10^8 Colony Forming Unit (CFU)/mL) was added at all well. Three negative controls were prepared (one with (MHB)/(TSB), another one with the extract, and the third with medium, antibiotic and bacteria). One positive control was prepared with MHB and each inoculum. For the Gram-negative bacteria, antibiotics, such as amikacin, tobramycin, amoxicillin/clavulanic acid, and gentamicin were used. For the Gram-positive bacteria, ampicillin and vancomycin were selected. The microplates were covered, and incubated at 37 °C for 24 h. The MIC of samples was detected following addition (40 μL) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37 °C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determinated by change the coloration from yellow to pink if the microorganisms are viable. The **Figure 15 s**hows an example of results obtained in an assay of antibacterial activity.

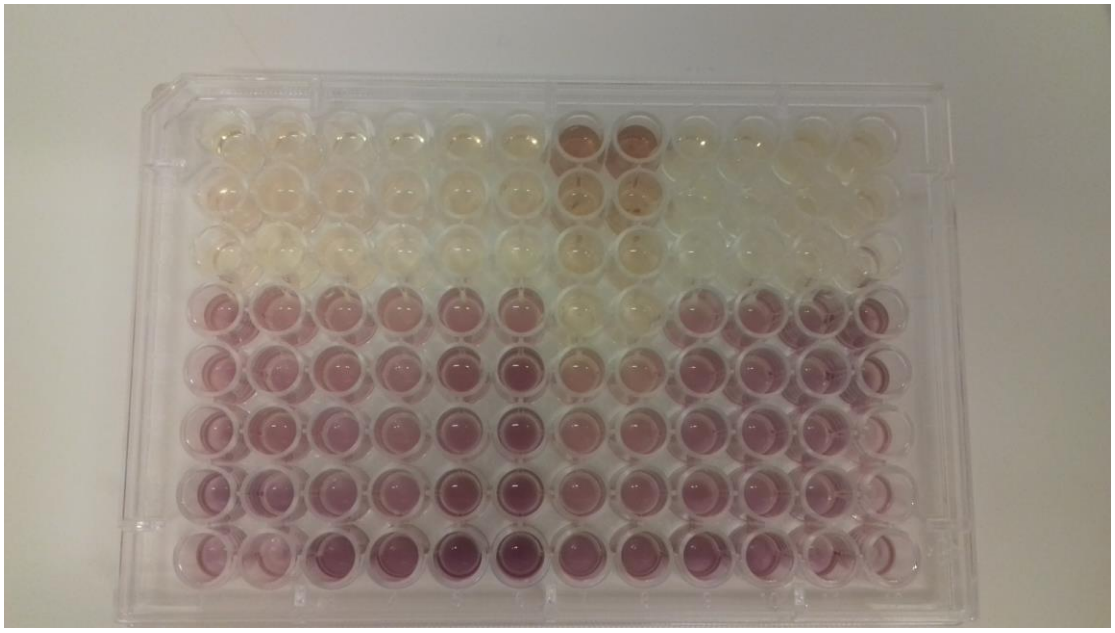


Figure 15. Example of results obtained in an assay of antibacterial activity. Author: Tânia Pires

2.6.3. Antiproliferative activity

The lyophilized extracts were re-dissolved in water to obtain stock solutions of 4 mg/mL, and then submitted to further dilutions. Four human tumor cell lines were tested: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma). Sulforhodamine B assay

was performed according to a procedure previously described by the authors (Barros et al., 2013).

Each of the cell lines were plated in a 96-well plate, at an appropriate density (1.0 × 104 cells/well) and allowed to attach for 24 h. The cells were then incubated in the presence of different extract concentrations during 48 h. Afterwards, cold trichloroacetic acid (TCA 10%, 100 μL) was added in order to bind the adherent cells and further incubated for 60 min at 4 ºC. After the incubation period, the plates were washed with deionized water, dried, sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 μL) was incorporated to each plate well, and incubated for 30 min at room temperature. The plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200 μL) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) (Guimarães et al., 2013).

For evaluation of the hepatotoxicity in non-tumour cells, a cell culture (named as PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by the authors (Abreu et al., 2011). The liver tissues were rinsed in Hank's balanced salt solution containing penicillin (100 U/mL) and streptomycin (100 μg/mL), and divided into 1×1 mm^3 explants. A few of these explants were transferred to tissue flasks (25 cm^2) containing DMEM medium supplemented with fetal bovine serum (FBS, 10%), nonessential amino acids (2 mM), penicillin (100 U/mL), and streptomycin (100 mg/mL) and incubated at 37 ºC with a humidified atmosphere (5% CO_2). The medium was changed every two days and the cell cultivation was continuously monitored using a phase contrast microscope. When confluence was reached, the cells were sub-cultured and plated in a 96-well plate (density of 1.0×10^4 cells/well) containing DMEM medium supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). Ellipticine was used as positive control and the results were expressed in GI_{50} values (concentration that inhibited 50% of the net cell growth). The **Figure 16** shows an example of the results obtained in an antoproliferative assay.

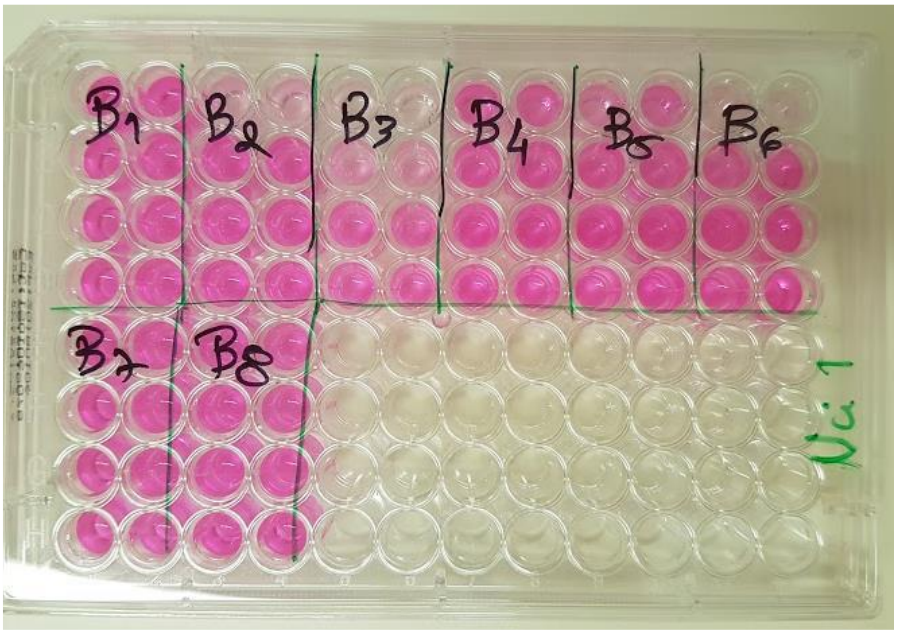


Figure 16. Example of results obtained in an antoproliferative assay. Author: Ricardo Calhelha

2.6.4. Hepatotoxicity

The extracts were re-dissolved in water to obtain stock solutions of 4 mg/mL, and then submitted to further dilutions. For hepatotoxicity evaluation, a porcine liver cells primary culture (PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by the authors (Abreu et al., 2011). Ellipticine was used as positive control and the results were expressed in GI_{50} values, concentration that inhibited 50% of the net cell growth.

2.7. Statistical analysis

In general: For all the experiments three samples (n=3) were analysed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD).

During the development of this thesis some common comparative methodologies were used, such as one-way ANOVA, 2-way ANOVA (specifically the generalized linear model), Student's *t*-test, Tukey's HSD Test and Tamhane's T2, advanced classification tools such as principal components analysis (PCA) and linear discriminant analysis (LDA). Also, Pearson correlation coefficient was calculated. All these methodologies were performed using IBM SPSS Statistics for Windows, Version 22.0., Version 23.0. and Version 24.0 (IBM Corp., Armonk, New York, USA).

2.7.1. One-way ANOVA

This test is used in this PhD Thesis to check differences among at least three groups, comparing means of two or more samples followed by simple Student's T test or a Tukey's multiple comparison test when the means were homoscedastic.

2.7.2. Two-way ANOVA

This test is used to examine the influence of two different categorical independent variables on one continuous dependent variable. The two-way ANOVA not only aims at assessing the main effect of each independent variable but also if there is any interaction between them. An analysis of variance with type III sums of squares was performed using the GLM (General Linear Model) procedure. When a statistically significant interaction among factors was detected, the two factors were evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistically significant interaction was verified, means within each factor were compared using appropriate tests.

This analysis was performed in the evaluation of the results obtained in the following studies:

Incorporation to yogurt of natural colorants obtained from edible flowers: To compare the variables evaluated in the prepared yogurts with the factors "yogurt formulation" (YF) and "storage" (SE).

Bilberry fruits as a source of natural colorants: pigment characterization and incorporation in yogurts: To assess of the effect of each individual factor, storage time (ST) and incorporation type (IT).

2.7.3. Linear discriminant analysis (LDA)

A linear discriminant analysis (LDA) was used to have a better understanding about the overall effect on yogurt formulations (YF). A stepwise technique was applied, considering the Wilks' ʎ test with the usual probabilities of F (3.84 to enter and 2.71 to be removed) for variable selection. Only variables with a statistically significant classification performance ($p < 0.050$) were maintained by the statistical model. The significant independent variables were selected following the stepwise method of LDA.

This procedure is based on sequential forward selection and backward elimination steps, where the inclusion of a new variable requires verifying the significance of all previously selected variables (Zielinski et al., 2014).

This analysis was performed in the evaluation of the results obtained in the studies of **incorporation of natural colorants obtained from edible flowers in yogurts**, in order to determine which independent variables contributed more to the differences in the average score profiles of different YF.

2.7.4. Principal Components Analysis (PCA)

Principal components analysis was applied as pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha variable (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the number of components selected. The number of plotted dimensions was chosen in order to allow meaningful interpretations.

This analysis was performed in the evaluation of the results obtained in the studies on **Bilberry fruits as a source of natural colorants: pigment characterization and incorporation in yogurts**, in order to evaluate the affinity (correlation) of each studied variable (nutritional parameters, individual compounds, color parameters and pH) with different mathematical functions (principal components).

2.7.5. Post-hoc tests

During the statistical analysis when no significant interaction (>0.05) was observed, each variable was evaluated independently using a simple Student's T test or a Tukey's multiple comparison test when the means were homoscedastic, and a Tamhane's T2 for heteroscedastic samples. Homoscedasticity was evaluated using a Levene's test.

2.7.5.1 Student's t-test

This test was used when necessary to determine significant differences between two different samples, with $p = 0.05$.

This analysis was performed in the evaluation of the results obtained in the following studies:

Edible flowers as sources of phenolic compounds with bioactive potential: To evaluate significant differences in the phenolic profiles among the hidromethanolic extracts of different flowers and infusions.

Phenolic compounds profile, nutritional compounds and bioactive properties of *Lycium barbarum* L.: A comparative study with stems and fruits: To determine differences in the proximate composition, soluble sugars, organic acids, fatty acids and tocopherols in fruits and stems of *Lycium barbarum* L., and for the determination of significant differences in the phenolic profiles between fruits and stems of *Lycium barbarum* L.

Bilberry fruits as a source of natural colorants: pigment characterization and incorporation in yogurts: To evaluate nutritional profile, individual fatty acids and external colour profile as a function of storage time.

2.7.5.2. Tukey's HSD Test

Tukey's multiple comparison test was used when necessary to determine significant differences between multiple samples, with $p = 0.05$.

This analysis was performed in the evaluation of the results obtained in the following studies:

Nutritional and chemical characterization of edible flowers and corresponding infusions: Valorization as new food ingredients: To determine differences in the proximate composition, energy, composition in soluble sugars, organic acids, fatty acids and tocopherols of dried flowers and corresponding infusions.

Edible flowers as sources of phenolic compounds with bioactive potential: In the evaluation of antioxidant, antiproliferative, hepatotoxic and antibacterial activities of hydromethanolic extracts and infusions of the studied flowers.

Bilberry fruits as a source of natural colorants: pigment characterization and incorporation in yogurts: In the evaluation of the nutritional profile, individual fatty acids and external colour profile as a function of the type of incorporation (plain sample, E163 or bilberry).

Development of new *Vaccinium myrtillus* L. based snacks: nutritional, chemical and bioactive features: 1) In the evaluation of the nutritional values, fatty acids content, soluble sugars, organic acids and tocopherols in bilbery fruits (P0), fruits with rose flowers (P1), calendula flowers (P2) and apple and goji berries (P3); 2) determination of significant differences in anthocyanin phenolic compounds and non

anthocyanin phenolic compounds in the hydromethanolic extracts of the four different mixtures of bilbery fruits (P0, P1, P2, and P3), and 3) determination of significant differences in antioxidant, antibacterial and hepatotoxicity activity in bilbery fruits (P0), fruits with rose flowers (P1), calendula flowers (P2) and apple and goji berries (P3).

2.7.5.3. Tamhane`s T2

This test is a pair-wise procedure based on Student t-distribution. It is a more conservative post hoc comparison for data with unequal variances, appropriate when variances are unequal and/or when the sample sizes are different.

This analysis was performed in the evaluation of the results obtained in the studies on **Bilberry fruits as a source of natural colorants: pigment characterization and incorporation in yogurts**, for evaluation of nutritional profile for the different incorporation types when no interaction was found and the distribution was heteroscedastic.

2.7.6. Pearson Correlation Coefficient

This test is a measure of the linear correlation between two variables. It was performed in the evaluation of the results obtained in the studies on **Phenolic compounds profile, nutritional compounds and bioactive properties of *Lycium barbarum* L.: A comparative study with stems and fruits**, for determination of the correlations between antioxidant and antimicrobial activities and diferent groups of phenolic compounds.

CHAPTER 3

Results and Discussion

3.1. Chemical composition, bioactive compounds and development of a novel product from edible flowers

3.1.1. Proximate and chemical composition of dried edible flowers and corresponding infusions

The results obtained in the analyses of the nutritional composition and energetic value of edible flowers from four different species- *Dahlia mignon* (dahlia), mixture of roses (rose) *Calendula officinalis* L. (calendula) and *Centaurea cyanus* L. (centaurea), and of the corresponding infusions are shown in **Table 6.**

Carbohydrates were the most abundant macronutrients in all the dried flowers, followed by proteins (5.93 to 7.58 g/100 g dw) and ashes (4.29 to 6.93 g/100 g dw). Rop et al. (2012) presented lower values of crude protein in *C. officinalis* flowers (0.673 g/100 g) originated from Czech Republic. Calendula flowers presented higher amount of fat (5.33 g/100 g dw) when compared to the other samples, and also higher energetic contribution (421.58 kcal/100 g). These results are in accordance with the ones described by Miguel et al. (2016) who reported similar values of fat and energy in calendula flowers. Regarding the infusions, rose and dahlia samples presented the highest contribution in carbohydrates (0.19 mg/100 mL), and also the highest energetic value (0.80 and 0.76 kcal/100 mL, respectively).

Table 6. Proximate composition of dried flowers and corresponding infusions (mean ± SD).

	Dried fowers (g/100 g dw)				Infusions (g/100 mL infusion)			
	Dahlia	Rose	Calendula	Centaurea	Dahlia	Rose	Calendula	Centaurea
Nutritional value								
Fat	2.23 ± 0.05b	2.01 ± 0.04b	5.33 ± 0.45a	0.140 ± 0.001	nd	nd	nd	nd
Protein	5.93 ± 0.2bc	7.58 ± 0.84a	6.43 ± 0.68b	5.79 ± 0.1c	nd	nd	nd	nd
Ash	5.83 ± 0.04b	4.29 ± 0.1d	6.93 ± 0.14a	5.68 ± 0.13c	np	np	np	np
Total available carbohydrates	86.02 ± 0.2b	86.12 ± 0.8b	81.32 ± 0.75c	88.39 ± 0.13a	0.19 ± 0.02a	0.19 ± 0.01a	0.17 ± 0.01b	0.14 ± 0.01c
	Dried flowers (kcal/100 g dw)				Infusions (kcal/100 mL infusion)			
Energy	387.83 ± 0.37c	392.87 ± 0.58b	421.58 ± 3.54a	377.99 ± 0.50d	0.76 ± 0.08a	0.80 ± 0.08a	0.68 ± 0.02b	0.56 ± 0.04c

dw- dry weight basis; np-not performed; nd-not detected. In each row and within dry flowers or infusions different letters mean significant differences between samples (p<0.05), where “a” and “d” correspond to the highest and lowest values, respectively.

Soluble sugars and organic acids composition of the studied dried flowers and corresponding infusions are presented in **Table 7**.

Dahlia and rose dried flowers (10.24 and 10.75 g/100 g dw) and infusions (0.19 g/100 mL of infusion) showed the highest total sugars amount, while centaurea dried flowers (1.5 g/100 g dw) and infusion (0.14 mg/100 mL) presented the lowest levels of total sugars. Fructose, glucose and sucrose were detected in all the studied flowers and infusions, being fructose the main sugar present with the exception of calendula samples and centaurea infusion, where sucrose was predominant. This is in accordance with the results reported by Barros et al. (2011) in *R. canina*. flowers, in which fructose was also the main sugar. In *C. officinalis* samples analysed by Miguel et al. (2016), fructose was also the main sugar detected, but followed by sucrose and xylose. Currently, the EFSA does not have a recommended daily dose for sugars intake, since data on the matter are insufficient to set an upper limit of consumption for these compounds (EFSA, 2010). Nevertheless, the World Health Organization recommends reducing the intake of free sugars to less than 10% of total energy intake, in both adults and children (WHO, 2015). Taking into account the sugar contents in the studied flowers and their normal way of consumption, it should not be expected that they can contribute in a relevant manner to exceed this level, so that they can be used "in" and "as" foods without any concern in this respect.

Regarding organic acids, the studied samples presented very distinct profiles (**Table 7**). The highest content was found in rose dried flowers mainly due to the presence of quinic and malic acids (1.53 and 1.23 g/100 g dw, respectively). The dried flowers of calendula also presented high levels of organic acids, with particular contribution of malic and succinic acids (1.14 and 1.77 g/100 g dw, respectively). The existence of relevant amounts of malic acid in *C. officinalis* flowers was also reported by Miguel et al. (2016), although with citric acid as the main organic acid, whereas they did not detect the presence of succinic acid. Centaurea revealed the lowest content of organic acids, presenting only minor amounts of oxalic and shikimic acids. Fumaric acid was found in trace amounts in all the studied flowers. Among the infusions, calendula presented the highest concentrations, mostly by the presence of quinic (14.5 mg/100 mL) and succinic acids (11.2 mg/100 mL). Curiously, centaurea was the second infusion with the highest organic acis content, mostly due to the presence of citric (15.5 mg/100 mL) and quinic acids (7.4 mg/100 mL), which were not detected in the flower.

Table 7. Soluble sugars and organic acids composition in dried flowers and corresponding infusions (mean ± SD).

	Dried flowers (g/100 g dw)				Infusions (mg/100 mL)			
	Dahlia	**Rose**	**Calendula**	**Centaurea**	**Dahlia**	**Rose**	**Calendula**	**Centaurea**
Soluble sugars								
Fructose	3.87 ± 0.23^{b}	5.14 ± 0.48^{a}	1.47 ± 0.12^{c}	0.65 ± 0.04^{d}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	0.066 ± 0.001^{b}	0.07 ± 0.004^{b}
Glucose	3.23 ± 0.25^{a}	3.23 ± 0.41^{a}	0.61 ± 0.07^{b}	0.47 ± 0.02^{b}	0.079 ± 0.02^{a}	0.064 ± 0.004^{b}	0.021 ± 0.001^{c}	0.04 ± 0.001^{d}
Sucrose	3.14 ± 0.15^{a}	2.39 ± 0.17^{b}	1.53 ± 0.18^{c}	0.38 ± 0.01^{d}	0.016 ± 0.001^{c}	0.035 ± 0.001^{b}	0.078 ± 0.001^{a}	0.03 ± 0.01^{b}
Sum	10.24 ± 0.62^{a}	10.75 ± 1.05^{a}	3.61 ± 0.37^{b}	1.5 ± 0.1^{c}	0.19 ± 0.02^{a}	0.19 ± 0.01^{a}	0.17 ± 0.01^{b}	0.14 ± 0.01^{c}
Organic acids								
Oxalic acid	0.23 ± 0.01^{c}	0.26 ± 0.01^{b}	0.702 ± 0.002^{a}	0.18 ± 0.01^{d}	tr	1.31 ± 0.01	tr	tr
Quinic acid	0.466 ± 0.003^{b}	1.52 ± 0.01^{a}	0.35 ± 0.01^{b}	nd	nd	9.33 ± 0.41^{b}	14.5 ± 0.3^{a}	7.4 ± 0.3^{c}
Malic acid	0.74 ± 0.01^{c}	1.23 ± 0.02^{a}	1.14 ± 0.02^{b}	nd	nd	4.1 ± 0.4^{a}	1.16 ± 0.15^{b}	tr
Shikimic acid	0.0497 ± 0.0003^{c}	0.062 ± 0.001^{b}	nd	0.108 ± 0.001^{a}	tr	0.368 ± 0.001^{b}	tr	1.05 ± 0.003^{a}
Citric acid	nd	1.2 ± 0.1	nd	nd	nd	nd	nd	15.5 ± 0.5
Succinic acid	nd	nd	1.77 ± 0.03	nd	nd	nd	11.2 ± 0.5	nd
Fumaric acid	tr	0.011 ± 0.001	tr	tr	nd	tr	tr	tr
Sum	1.49 ± 0.01c	4.26 ± 0.13a	3.98 ± 0.02b	0.29 ± 0.01d	tr	15.01 ± 0.1c	26.9 ± 0.3a	23.9 ± 0.8b

dw- dry weight basis; nd- not detected; tr- traces (LOD (μg/mL) and LOQ (μg/mL) for oxalic acid (12.6 and 42, respectively), quinic acid (24 and 81, respectively), malic acid (36 and 1.2×10^{2}, respectively), shikimic acid (6 and 19, respectively), citric acid (10 and 35, respectively), succinic acid (19 and 64, respectively) and fumaric acid (0.080 and 0.26, respectively). In each row and within dry flowers or infusions different letters mean significant differences between samples ($p<0.05$), where “a” and “d” correspond to the highest and lowest values, respectively.

Table 8 shows the results on the contents of lipophilic compounds, namely fatty acids and tocopherols, determined in the studied flowers.

Twenty-four fatty acids were identified, being polyunsaturated fatty acids (PUFA) predominant in all the samples, with the exception of dahlia that showed slightly higher concentration of saturated fatty acids (SFA). Linoleic acid (C18:2n6) was the major fatty acid found in dahlia and rose samples (36.54 and 31.87%, respectively), followed by palmitic acid (C16:0) and linolenic acid (C18:3n3), respectively. Calendula presented linolenic acid (36.90%) as the main fatty acid, followed by palmitic acid (21.70%), while centaurea presented eicosapentaenoic acid (C20:5n3, 26.93%) as the main fatty acid, followed by linolenic acid (18.75%). The results found for *C. officinalis* are in accordance with the ones described by Dulf et al. (2013), in which PUFA content was around 60 to 64% of total fatty acids, and the saturated fraction mainly consisted of palmitic acid. This tendency was not found by Miguel et al. (2016) in calendula samples, which presented a SFA fraction much higher than the PUFA fraction (78% and 21%, respectively). According EFSA (2010) recommendations, the daily intake of SFA should be as low as possible, whereas an adequate intake (AI) of 4% of the total dietary energy was set for linoleic acid and an AI of 250 mg for the intake eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) in adults. In this respect the flowers of centaurea would be the more balanced among those analysed, owing to their lower SFA percentage and the presence of EPA, only present in this sample.

Regarding tocopherols, *C. officinalis* was the sample that revealed the highest content (60.88 mg/100 g dw), mainly due to the presence of α-tocopherol isoform (56.78 mg/100 g dw). Miguel et al. (2016) also described α-tocopherol as the main isoform in calendula flowers, however, those authors found lower values of total tocopherols. In all the samples, that isoform occurred in higher amounts than the remaining isoforms. The lowest contents of tocopherols were determined in centaurea flowers, where β- and δ-tocopherol were not detected; this latter form was not present in calendula, either. From the determined tocopherol levels, calendula flowers could be considered a suitable source of α-tocopherol, for which an AI of 9 mg/day has been established by EFSA (2015).

Table 8. Fatty acids and tocopherols composition in dried flowers (mean ± SD).

	Dahlia	Rose	Calendula	Centaurea
Fatty acids (relative percentage, %)				
C6:0	0.89 ± 0.07	0.18 ± 0.01	0.27 ± 0.01	0.17 ± 0.01
C8:0	0.90 ± 0.09	0.23 ± 0.02	0.28 ± 0.06	0.07 ± 0.00
C10:0	0.99 ± 0.04	0.33 ± 0.05	0.18 ± 0.08	0.12 ± 0.00
C11:0	nd	nd	0.13 ± 0.03	nd
C12:0	0.74 ± 0.03	1.22 ± 0.05	1.65 ± 0.18	nd
C13:0	nd	0.03 ± 0.00	nd	nd
C14:0	3.11 ± 0.20	2.55 ± 0.14	9.92 ± 0.39	0.89 ± 0.05
C14:1	0.59 ± 0.03	0.31 ± 0.00	nd	0.21 ± 0.02
C15:0	0.66 ± 0.00	0.31 ± 0.01	0.18 ± 0.01	0.37 ± 0.01
C16:0	24.61 ± 0.77	17.10 ± 1.06	21.70 ± 0.10	15.40 ± 0.10
C16:1	0.87 ± 0.00	0.22 ± 0.00	0.23 ± 0.03	0.28 ± 0.02
C17:0	0.91 ± 0.09	0.53 ± 0.04	0.19 ± 0.04	0.82 ± 0.02
C18:0	7.60 ± 0.28	16.80 ± 0.27	3.95 ± 0.08	9.67 ± 0.08
C18:1n9	5.75 ± 0.08	1.95 ± 0.19	1.56 ± 0.06	4.41 ± 0.04
C18:2n6	36.54 ± 0.85	31.87 ± 0.33	20.35 ± 0.14	6.72 ± 0.08
C18:3n3	8.60 ± 0.56	19.54 ±0.79	36.90 ± 0.55	18.75 ± 0.14
C20:0	1.57 ± 0.08	3.62 ± 0.03	0.63 ± 0.02	5.34 ± 0.05
C20:2	0.40 ± 0.03	nd	nd	nd
C20:3n3	0.63 ± 0.10	0.33 ± 0.00	0.26 ± 0.01	0.51 ± 0.08
C20:5n3	nd	nd	nd	26.93 ± 0.29
C22:0	2.15 ± 0.19	1.81 ± 0.13	0.56 ± 0.04	2.04 ± 0.00
C22:1n9	nd	nd	nd	6.01 ± 0.12
C23:0	0.21 ± 0.02	0.08 ± 0.01	0.13 ± 0.03	0.15 ± 0.00
C24:0	2.31 ± 0.01	1.01 ± 0.07	0.93 ± 0.09	1.14 ± 0.10
SFA	46.64 ± 1.46a	45.79 ± 1.30b	40.70 ± 0.70c	36.18 ± 0.28d
MUFA	7.20 ± 0.11b	2.47 ± 0.19c	1.79 ± 0.02d	10.91 ± 0.13a
PUFA	46.16 ± 1.35d	51.74 ± 1.11c	57.51 ± 0.68a	52.91 ± 0.15b
Tocopherols (mg/100 g dw)				
α-Tocopherol	4.36 ± 0.07c	8.16 ± 0.08b	56.78 ± 1.06a	0.55 ± 0.02d
β-Tocopherol	1.77 ± 0.01a	0.18 ± 0.01c	1.16 ± 0.06b	nd
γ-Tocopherol	0.72 ± 0.02b	0.77 ± 0.01b	2.94 ± 0.08a	0.29 ± 0.02c
δ-Tocopherol	0.43 ± 0.01a	0.14 ± 0.01b	nd	nd
Sum	7.28 ± 0.04c	9.25 ± 0.04b	60.88 ± 0.92a	0.84 ± 0.04d

dw- dry weight basis; nd- not detected. C6:0 - Caproic acid; C8:0 - Caprylic acid; C10:0- Capric acid; C11:0 - Undecylic acid; C12:0- Lauric acid; C13:0 - Tridecanoic acid; C14:0- Myristic acid; C14:1 - Myristoleic acid; C15:0- Pentadecanoic acid; C16:0- Palmitic acid; C16:1 - Palmitoleic acid; C17:0 -

Heptadecanoic acid; C18:0 - Stearic acid; C18:1n9- Oleic acid; C18:2n6- Linoleic acid; C18:3n3- Linolenic acid; C20:0- Arachidic acid; C20:2- *cis*-11,14 - Eicosadienoic acid; C20:3n3 - Eicosatrienoic acid; C20:5n3 - Eicosapentaenoic acid; C22:0 - Behenic acid; C22:1n9- Erucic acid; C23:0 - Tricosanoic acid; C24:0 - Lignoceric acid. SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids. In each row different letters mean significant differences between samples ($p<0.05$), where "a" and "d" correspond to the highest and lowest values, respectively.

3.1.2. Charaterization of bioactive compounds in hydromethanolic extracts and infusions of edible flowers

The extraction yields obtained for the hydromethanolic extracts were, 47% for dahlia, 39% for rose, 25% for calendula, and 24% for centaurea, while the infusions presented the following extraction yields 37% for dahlia, 34% for rose, 27% for calendula, and 22% for centaurea. Dahlia hydromethanolic extract and infusions showed the most promising yields.

The results obtained in the HPLC analyses of the phenolic composition of the flowers extracts and infusions are presented in **Tables 9-12** and some examples of the HPLC chromatograms obtained are shown in **Figure 17**.

Table 9. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds detected in the extracts of dahlia flowers.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydrometha nolic extracts	Infusions
1d	6.1	287	653	287(100)	Eriodictyol-acetyldihexoside[C]	DAD/MS	tr	tr
2d	7.4	320	353	191(100),179(79),173(20),161(5),135 (12)	5-*O*-Caffeolquinic acid[B]	DAD/MS; comercial standard	1.82 ± 0.06*	0.53 ± 0.03*
3d	8.8	282	611	449(100),287(48)	Eriodictyol-dihexoside[C]	(Pereira et al., 2013)	0.12 ± 0.02*	tr
4d	10.9	283	611	449(100),287(57)	Eriodictyol-dihexoside[C]	(Pereira et al., 2013)	tr	tr
5d	11.4	268	627	465(100),447(87),345(9),285(6)	Pentahydroxyflavanone-dihexoside[G(	(Lin et al., 2007)	0.93 ± 0.04*	0.59 ± 0.03*
6d	11.9	274	653	449(100),287(59)	Eriodictyol-acetyldihexoside[C]	DAD/MS	tr	tr
7d	13.6	269	669	465(38),447(100),285(11)	Pentahydroxyflavanone-acetylhexoside-hexoside[G]	(Lin et al., 2007)	0.76 ± 0.05*	0.47 ± 0.00*
8d	15.3	285	595	287(100)	Eriodictyol-deoxyhexosyl-hexoside[C]	DAD/MS	1.14 ± 0.06*	0.12 ± 0.07*
9d	16.2	347	771	285(100)	Kaempferol-pentosyl-rhamnosyl-hexoside[E]	(Harbaum et al., 2007)	2.23 ± 0.02*	0.11 ± 0.01*
10d	16.3	285	449	287(100)	Eriodictyol-hexoside[C]	(Guimarães et al., 2013)	1.56 ± 0.07*	0.18 ± 0.03*
11d	16.7	361	579	417(100),255(57)	Isoliquiritigenin-dihexoside[D]	DAD/MS	1.57 ± 0.01	tr
12d	17.7	354	609	301(100)	Quercetin-3-*O*-rutinoside[H]	DAD/MS; comercial standard	0.89 ± 0.03*	0.29 ± 0.03*
13d	18.1	350	431	269(100)	Apigenin-hexoside[A]	DAD/MS; comercial standard	2.08 ± 0.05*	0.83 ± 0.04*
14d	20.7	287	637	475(100),271(52)	Naringenin-hexoside-acetylhexoside[F]	DAD/MS	0.82 ± 0.01*	0.64 ± 0.00*
15d	21.0	284	433	271(100)	Naringenin-3-*O*-glucoside[F]	DAD/MS; comercial standard	2.92 ± 0.03*	0.79 ± 0.01*
16d	21.3	364	621	459(100),255(48)	Isoliquiritigenin-hexoside-acetylhexoside[D]	DAD/MS	0.10 ± 0.01	tr
17d	21.6	285	579	301(100)	Hesperetin-pentosyl-rhamnoside[C]	DAD/MS	0.24 ± 0.01	tr
18d	23.3	380	433	271(100)	Butein-4'-glucoside (Coreopsin)[C]	(Chen et al., 2016; Yang et al., 2016)	0.81 ± 0.05*	0.01 ± 0.00*

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydrometha nolic extracts	Infusions
19d	26.4	348	563	285(100)	Kaempferol-pentosyl-rhamnoside[E]	(Barros et al., 2013)	0.17 ± 0.03*	tr
20d	28.2	381	475	271(100)	Acetylcoreopsin[C]	(Chen et al., 2016; Yang et al., 2016)	tr	tr
21d	28.7	377	577	433(100),271(32)	Coreopsin derivative	DAD/MS	0.70 ± 0.02*	0.17 ± 0.00*
					Sum of phenolic acid derivatives		1.817 ± 0.061*	0.53 ± 0.03*
					Sum of flavonoids		17.040 ± 0.007*	4.20 ± 0.13*
					Sum of phenolic compounds		18.857 ± 0.068*	4.73 ± 0.01 *

Standard calibration curves: A- apigenin-7-*O*-glucoside ($y = 10683x - 45794$, $R^2 = 0.9906$); B- chlorogenic acid ($y = 168823x - 161172$, $R^2 = 0.9999$); C- hesperetin ($y = 34156x + 268027$, $R^2 = 0.9999$); D- isoliquiritigenin ($y = 42820x + 184902$, $R^2 = 0.999$); E- kaempferol-3-*O*-rutioside ($y = 41843x + 220192$, $R^2 = 0.9998$); F- naringenin ($y = 18433x + 78903$, $R^2 = 0.9998$); G- quercetin-3-*O*-glucoside ($y = 34843x - 160173$; $R^2 = 0.9998$); H- quercetin-3-*O*-rutinoside ($y = 13343x + 76751$, $R^2 = 0.9998$). tr- traces; nq- not quantified; * - *t*-Students test *p*-value <0.001.

Table 10. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds detected in the extracts of rose flowers.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydromethanolic extracts	Infusions
1r	18.2	348	477	301(100)	Quercetin-glucuronide[B]	(Guimarães et al.,2013)	0.79 ± 0.03*	0.49 ± 0.01*
2r	18.6	355	463	301(100)	Quercetin-hexoside[B]	(Guimarães et al.,2013)	1.37 ± 0.04	0.73 ± 0.01
3r	19.0	355	463	301(100)	Quercetin-3-*O*-glucoside[B]	DAD/MS; comercial standard	2.87 ± 0.07*	1.31 ± 0.01*
4r	21.1	348	593	285(100)	Kaempferol-3-*O*-rutinoside[A]	DAD/MS; comercial standard	tr	tr
5r	21.6	353	433	301(100)	Quercetin-pentoside[B]	(Guimarães et al., 2013)	0.66 ± 0.01*	0.47 ± 0.01*
6r	22.0	348	461	285(100)	Kaempferol-glucuronide[A]	(Guimarães et al., 2013)	tr	tr
7r	22.5	348	447	285(100)	Kaempferol-3-*O*-glucoside[A]	DAD/MS; comercial standard	2.74 ± 0.06*	0.88 ± 0.01*
8r	25.1	348	417	285(100)	Kaempferol-pentoside[A]	(Barros et al., 2011)	tr	tr
9r	25.9	347	417	285(100)	Kaempferol-pentoside[A]	(Barros et al., 2011)	tr	nd
10r	27.2	348	431	285(100)	Kaempferol-rhamnoside[A]	(Barros et al., 2011)	0.29 ± 0.02	tr
11r	30.1	314	609	463(100),301(12)	Quercetin-(*p*-coumaroyl)hexoside[B]	(Guimarães et al., 2013)	0.46 ± 0.01*	0.37 ± 0.00*
12r	33.1	314	593	447(9),285(100)	Kaempferol-(*p*-coumaroyl)hexoside[A]	(Guimarães et al., 2013)	tr	tr
					Sum of phenolic compounds (flavonoids)		9.18 ± 0.23*	4.24 ± 0.01*

Standard calibration curves: A- kaempferol-3-*O*-rutinoside (y =41843x+220192, R^2=0.998); B- quercetin-3-*O*-glucoside (y =34843x-160173, R^2=0.998). nq- not quantified; tr-traces; * *t*-Students test *p*-value <0.001.

Table 11. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds detected in the extracts of calendula flowers.

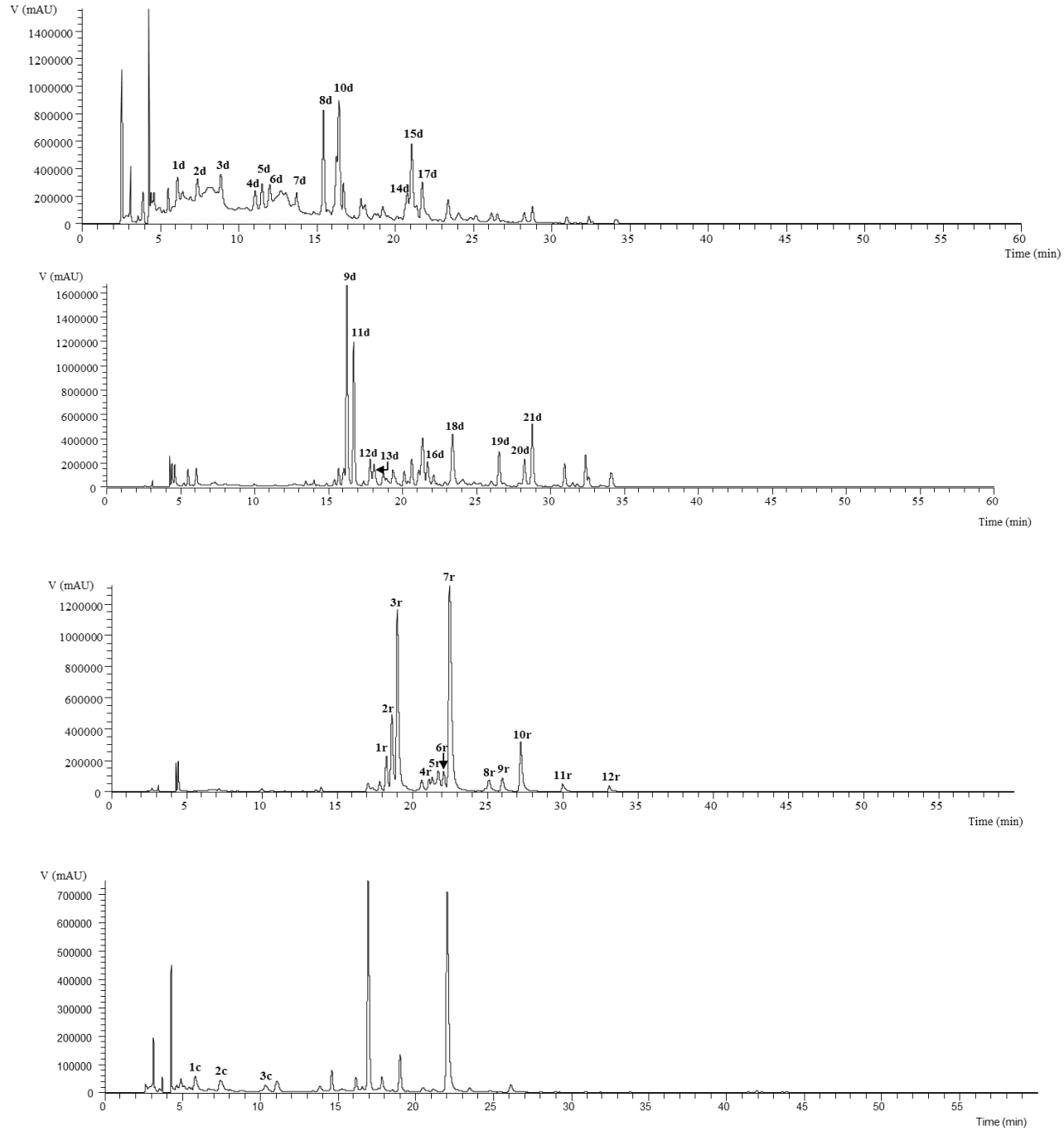
Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydromethanolic extracts	Infusions
1c	5.8	320	341	179(100)	Caffeic acid hexoside[A]	(Miguel et al., 2016)	0.03 ± 0.01	tr
2c	7.4	326	353	191(100),179(79),173(20), 161(5),135(8)	5-*O*-Caffeoylquinic acid[B]	(Miguel et al., 2016)	0.12 ± 0.01*	0.10 ± 0.00*
3c	10.4	324	179	161(5),135(100)	Caffeic acid[A]	DAD/MS; comercial standard	0.01 ± 0.00	tr
4c	14.7	354	755	301(100)	Quercetin-3-*O*-rhamnosylrutinoside[E]	(Miguel et al., 2016)	0.31 ± 0.00*	0.40 ± 0.00*
5c	16.3	354	609	301(100)	Quercetin-deoxyhexosylhexoside[E]	(Miguel et al., 2016)	0.33 ± 0.00	tr
6c	16.7	348	739	285(100)	Kaempferol-rhamnosylrutinoside[D]	(Miguel et al., 2016)	tr	tr
7c	17.1	355	769	315(100)	Isorhamnetin-3-*O*-rhamnosylrutinoside[C]	(Miguel et al., 2016)	3.99 ± 0.04*	2.71 ± 0.01*
8c	18.0	354	609	301(100)	Quercetin-3-*O*-rutinoside[E]	DAD/MS; comercial standard	0.30 ± 0.00*	0.38 ± 0.00*
9c	19.1	354	623	315(100)	Isorhamnetin-3-*O*-neohesperidoside[C]	(Miguel et al., 2016)	0.69 ± 0.00*	0.35 ± 0.00*
10c	20.7	352	505	301(100)	Quercetin-3-*O*-(6"-acetyl)-glucoside[E]	(Miguel et al., 2016)	0.23 ± 0.00*	0.33 ± 0.00*
11c	20.2	355	623	315(100)	Isorhamnetin-3-*O*-rutinoside[C]	(Miguel et al., 2016)	5.40 ± 0.04*	3.20 ± 0.00*
12c	23.7	354	477	315(100)	Isorhamnetin-3-*O*-glucoside[C]	(Miguel et al., 2016)	0.04 ± 0.01	tr
13c	26.3	355	519	315(100)	Isorhamnetin-3-*O*-(6"-acetyl)-glucoside[C]	(Miguel et al., 2016)	0.16 ± 0.00	tr
					Sum of phenolic acid derivatives		0.16 ± 0.03*	0.10 ± 0.00*
					Sum of flavonoids		11.15 ± 0.09*	7.37 ± 0.01*
					Sum of phenolic compounds		11.31 ± 0.07*	7.47 ± 0.02*

Standard calibration curves: A - caffeic acid (y =388345x+406369, R^2=0.994); B - chlorogenic acid (y = 168823x – 161172, R^2= 0.9999); C - isorhamnetin-3-*O*-glucoside (y=11117x+30861, R^2=0.9999); D - kaempferol-3-*O*-rutinoside (y =41843x+220192, R^2=0.998); E - quercetin-3-*O*-glucoside (y =34843x-160173, R^2=0.998). nq- not quantified; tr-traces; * *t*-Students test *p*-value <0.001.

Table 12. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds detected in the extracts of centaurea flowers.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydromethanolic extracts	Infusions
1ce	5.2	294,320sh	627	465(100),303(3),285(3)	Taxifolin derivatives[E]	DAD/MS	0.93 ± 0.02*	0.31 ± 0.00*
2ce	5.7	263	341	179(100),161(1),135(1)	Caffeic acid hexoside[B]	(Miguel et al., 2016)	0.25 ± 0.01	tr
3ce	7.3	326	353	191(100),179(80),173(24),161(5),135(10)	*cis*-5-*O*-Caffeoylquinic acid[C]	DAD/MS; (Barros et al., 2012)	1.50 ± 0.18*	0.17 ± 0.01*
4ce	7.4	326	353	191(100),179(80),173(42),161(5),135(12)	*trans*-5-*O*-Caffeoylquinic acid[C]	DAD/MS; commercial standard	1.40 ± 0.06*	0.24 ± 0.02*
5ce	7.8	346	325	163(100)	*p*-Coumaric hexoside[F]	(Barros et al., 2012)	0.93 ± 0.01*	0.09 ± 0.01*
6ce	8.2	312	325	163(100)	*p*-Coumaric hexoside[F]	(Barros et al., 2012)	0.44 ± 0.06*	0.04 ± 0.00*
7ce	13.0	350	667	505(100),463(43),301(14)	Quercetin-hexoside-acetylhexoside[E]	(Barros et al., 2012)	0.78 ± 0.00*	0.26 ± 0.00*
8ce	13.4	274,317sh	627	465(100),303(3),285(4)	Taxifolin derivatives[E]	DAD/MS	1.08 ± 0.04*	0.28 ± 0.01*
9ce	13.7	276,316sh	627	465(100),303(1),285(3)	Taxifolin derivatives[E]	DAD/MS	1.11 ± 0.12*	0.25 ± 0.01*
10ce	17.8	320	649	473(100),269(8)	Apigenin-glucuronide-acetylhexoside[A]	DAD/MS	1.25 ± 0.00*	0.46 ± 0.01*
11ce	18.9	346	461	285(100)	Luteolin-glucuronide[E]	(Miguel et al., 2016)	0.83 ± 0.01*	0.26 ± 0.00*
12ce	20.4	353	505	463(23),301(100)	Quercetin-3-*O*-(6"-acetyl)-glucoside[E]	(Barros et al., 2012)	0.83 ± 0.01*	0.25 ± 0.00*
13ce	23.9	337	445	269(100)	Apigenin-glucuronide[A]	(Guimarães et al., 2013)	12.22 ± 0.09*	1.52 ± 0.13*
14ce	24.8	330	489	285(100)	Kaempferol-acetylhexoside[D]	(Barros et al., 2012)	tr	tr
					Sum of phenolic acid derivatives		4.52 ± 0.17*	0.55 ± 0.02*
					Sum of flavonoids		19.03 ± 0.06*	3.59 ± 0.03*
					Sum of phenolic compounds		23.55 ± 0.11*	4.14 ± 0.05*

Standard calibration curves: A- apigenin-7-*O*-glucoside (y =10683x-45794, R^2=0.991); B - caffeic acid (y =388345x+406369, R^2=0.994); chlorogenic acid (y = 168823x – 161172, R^2= 0.9999); D - kaempferol-3-*O*-rutinoside (y =41843x+220192, R^2=0.998); E - quercetin-3-*O*-glucoside (y =34843x-160173, R^2=0.998); F - *p*-coumaric acid (y =301950x+6966.7, R^2=0.9999). nq- not quantified; tr-traces; * *t*-Students test *p*-value <0.001.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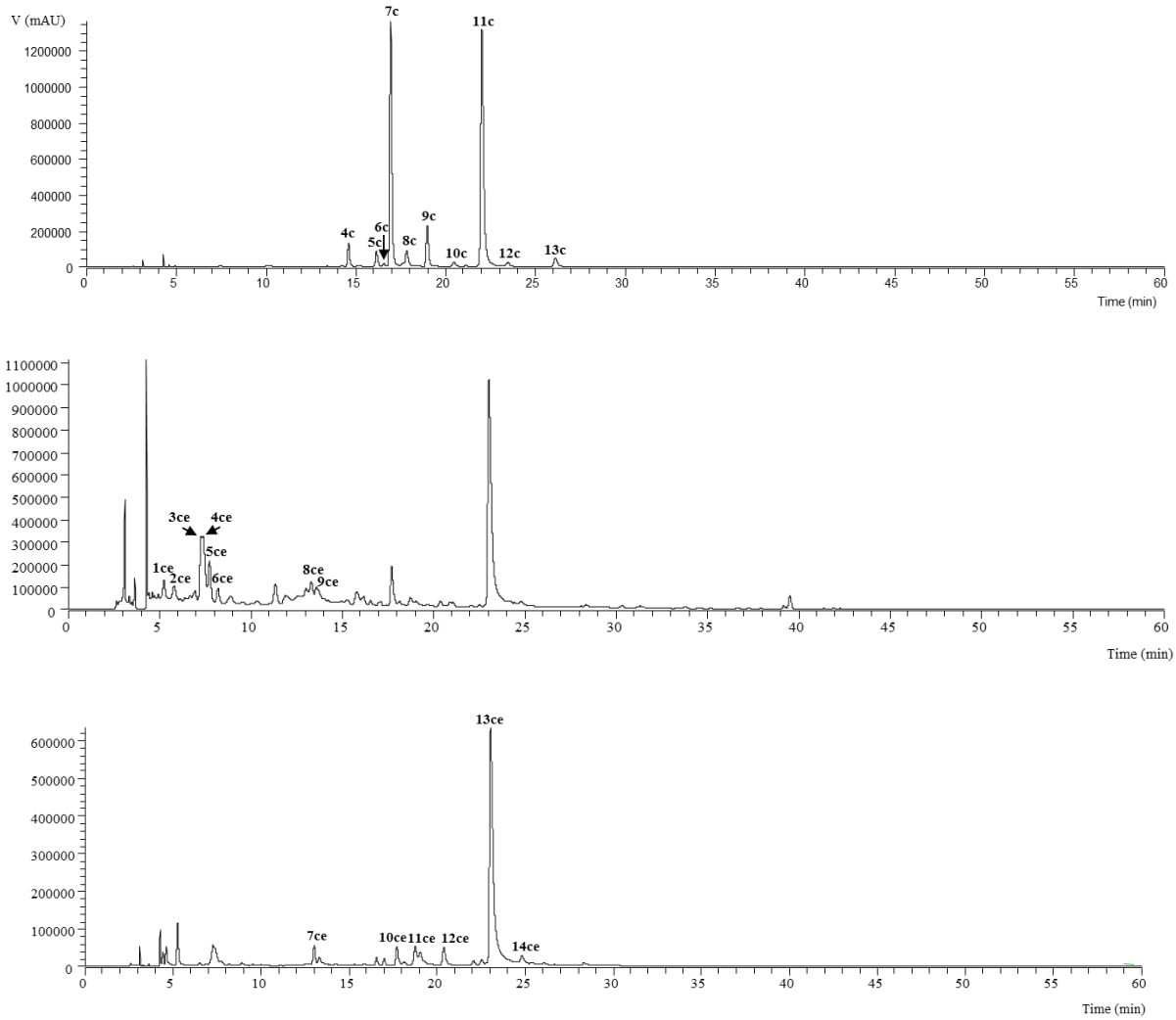
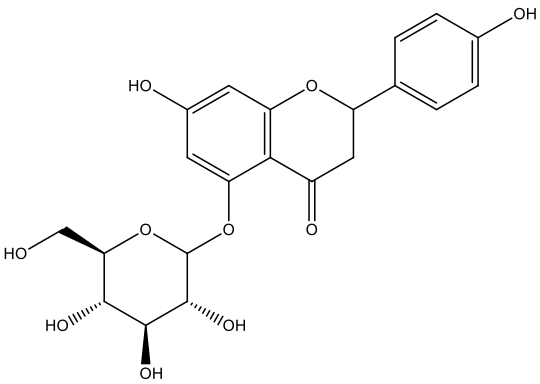
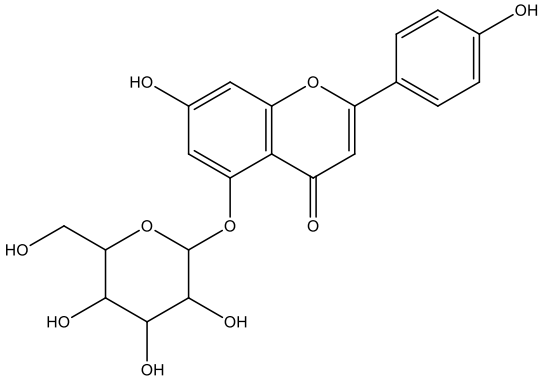
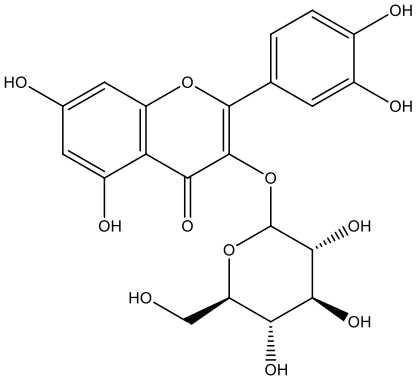


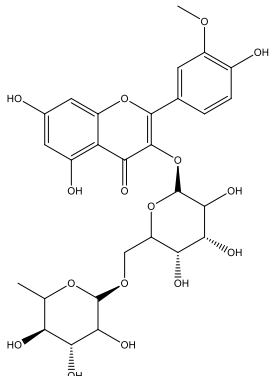
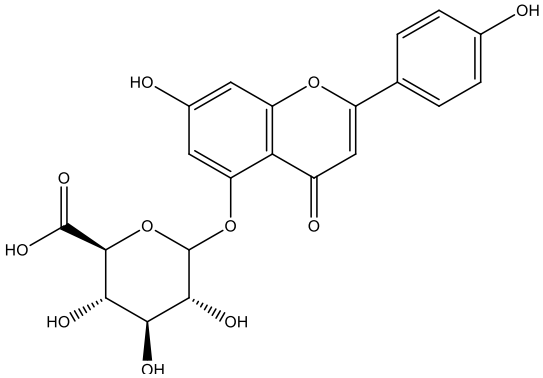
Figure 17. Chromatograms of the phenolic profiles obtained for the hydromethanolic extracts of the studied flowers: dahlia (A and B recorded at 280 and 370 nm, respectively), rose (C recorded at 370 nm), calendula (D and E recorded at 280 and 370 nm, respectively), and centaurea (F and G recorded at 280 and 370 nm, respectively).

One phenolic acid derivative (chlorogenic acid), twenty flavonoids (eryodictiol, kaempferol, quercetin, apigenin, naringenin, hesperetin, butein, and isoliquiritigenin glycoside derivatives) and two unknown compounds were detected in dahlia samples. Rose flowers showed twelve flavonoids, all of them derived from kaempferol and quercetin, and did not reveal any phenolic acid derivative. Three caffeic acid derivatives and ten flavonoids (kaempferol, quercetin, and isorhamnetin glycoside derivatives) were identified in calendula samples. Finally, centaurea samples presented five

phenolic acid derivatives (derived from caffeic and *p*-coumaric acids), nine flavonoids (quercetin, kaempferol, apigenin, luteolin, and taxifolin glycoside derivatives) and one unknown compound. Due to the complexity of the discussion, letters were attributed to the peak numbers to identify the plant in which they were found: dahlia (d), rose (r), calendula (c), and centaurea (ce). **Table 13** shows the main compounds identified in each sample and type of extract.

Table 13. Main phenolic compounds identified in the four flower samples studied in their respective hydromethanolic extracts and infusions.

	Hydromethanolic extract	**Infusion**
Dahlia		
	Naringenin-3-*O*-glucoside (Peak 15d)	Apigenin-hexoside (Peak 13d)
	Hydromethanolic extract and infusion	
Rose		
	Quercetin-3-*O*-glucoside (Peak 3r)	

Calendula	
	Isorhamnetin-3-*O*-rutinoside (Peak 11c)
Centaurea	
	Apigenin-glucuronide (11ce)

3.1.2.1. Flavonoids

Several classes of flavonoids were found in the composition of the edible flowers, namely flavonols, flavones, flavanones, dihydroflavonols and chalcones, as described below.

Flavonols. Quercetin derivatives were detected in the four flower samples. Quercetin-3-*O*-glucoside (peak **3r**) and quercetin-3-*O*-rutinoside (peaks **8c** and **12d**) were identified according to their UV spectra, elution order, and fragmentation pattern in comparison with commercial standards. Compounds **3r** and **8c** were previously identified in rose fruits by Guimarães et al. (2013), and in calendula flowers by Miguel et al. (2016), respectively. Peaks **4c** and **10c** were identified as quercetin-3-*O*-rhamnosylrutinoside and quercetin-3-*O*-(6''-acetyl)-glucoside, respectively, which were also previously reported in *C. officinalis* (Miguel et al., 2016). Peak **12ce** corresponded to the same compound as peak **10c.** Peak **7ce** ([M-H]$^-$ at *m/z* 667) with

three MS^2 fragments at *m/z* 505 (-162 u, loss of a hexosyl moiety), *m/z* 463 (-42 u, loss of an acetyl moiety), and *m/z* 301 (-162 u, loss of a hexosyl moiety) was tentatively identified as quercetin-hexoside-acetylhexoside. Peaks **1r** and **5r** were tentatively assigned as quercetin-glucuronide and quercetin-pentoside, respectively, being also previously reported by Guimarães et al. (2013) in *R. canina* fruits. Peak **2r** presented the same pseudomolecular ion and fragmentation pattern as peak **3r** (quercetin-3-*O*-glucoside), but a lower retention time, therefore it was tentatively assigned as a quercetin-hexoside. Similarly, peak **5c** showed the same spectral characteristics as peak **8c** (quercetin-3-*O*-rutinoside), but different retention time, being tentatively identified as a quercetin-deoxyhexosylhexoside. Peak **11r (**$[M-H]^-$ at *m/z* 609) also presented the same pseudomolecular ion as **8c**, but different UV spectra, fragmentation pattern and retention time. The observation in its MS^2 spectrum of a product ion at *m/z* 463, from the loss of 146 u and the UV maximum at 314 nm, as well as its late elution, were coherent with the presence of a coumaroyl residue instead of rhamnose. Therefore, it was tentatively assigned as quercetin-(*p*-coumaroyl) hexoside.

Kaempferol derivatives were also observed in the four studied flowers, being especially relevant in the rose sample. Peaks **4r** and **7r** were identified according to their UV spectra, elution order, fragmentation pattern, and comparison with commercial standards, as kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside, respectively. The remaining compounds detected in rose flowers (i.e., peaks **6r**, **8r**, **9r**, **10r**, and **12r**) were assigned following similar reasoning as for quercetin derivatives. Dahlia samples presented two kaempferol derivatives (peaks **9d** and **19d**) with pseudomolecular ions $[M-H]^-$ at *m/z* 771 and *m/z* 563, both releasing a unique MS^2 fragment at *m/z* 285, being associated to kaempferol-pentosyl-rhamnosyl-hexoside and kaempferol-pentosyl-rhamnoside, respectively. Centaurea and calendula samples presented one kaempferol derivative each (peaks **14ce** and **6c**) that were tentatively identified according to their mass spectral characteristics as kaempferol-acetylhexoside and kaempferol-rhamnosyl-rutinoside, respectively. This latter compound has already been reported in *C. officinalis* flowers by our group (Miguel et al., 2016).

Isorhamnetin derivatives were only detected in the calendula samples (peaks **7c**, **9c**, **11c**, **12c**, and **13c**), being identified as isorhametin-3-*O*-rhamnosylrutinoside ($[M-H]^-$ at *m/z* 769), isorhametin-3-*O*-neohesperidoside ($[M-H]^-$ at *m/z* 623), isorhametin-3-*O*-rutinoside ($[M-H]^-$ at *m/z* 623), isorhametin-3-*O*-glucoside ($[M-H]^-$ at *m/z* 477) and

isorhametin-3-*O*-(6"-acetyl)-glucoside ([M-H]$^-$ at *m/z* 519), respectively, all of them previously reported in *C. officinalis* flowers by Miguel et al. (2016).

Flavones. Peaks **13d** ([M-H]$^-$ at *m/z* 431) and **13ce** ([M-H]$^-$ at *m/z* 445) presented a unique MS^2 fragment at *m/z* 269 (associated to apigenin), corresponding to the loss of hexosyl (-162 u) and glucuronyl (-176 u) moieties, so that they were tentatively identified as apigenin-hexoside and apigenin-glucuronide, respectively. An identity of peak **13d** as apigenin-7-*O*-glucoside was discarded by comparison with a standard. Peak **10ce** ([M-H]$^-$ at *m/z* 649) released two MS^2 fragments at *m/z* 473 (176 u, loss of a glucuronyl moiety) and *m/z* 269 (162+42 u, further loss of an acetylhexosyl residue), being tentatively identified as an apigenin-glucuronide-acetylhexoside. Peak **11ce**, with a pseudomolecular ion [M-H]$^-$ at *m/z* 461 releasing a unique MS^2 fragment at *m/z* 285 (loss of 176 u, corresponding to a glucuronyl moiety), was tentatively identified as a luteolin-glucuronide.

Flavanones. They were only detected in dahlia samples. Peaks **1d** ([M-H]$^-$ at *m/z* 653), **3d** and **4d** ([M-H]$^-$ at *m/z* 611), **6d** ([M-H]$^-$ at *m/z* 653), **8d** ([M-H]$^-$ at *m/z* 595), and **10d** ([M-H]$^-$ at *m/z* 449]), presenting characteristic UV spectra with λ_{max} around 274 nm and an MS^2 product ion at *m/z* 287, were associated as eriodictyol derivatives. According to their pseudomolecular ions they were tentatively identified as eriodictyol-acetyldihexoside (**1d** and **6d**), eriodictyol-dihexoside (**4d**), eriodictyol-deoxyhexosyl-hexoside (**8d**), and eriodictyol-hexoside (1**0d**). Peak **15d** ([M-H]$^-$ at *m/z* 433) was identified as naringenin-3-*O*-glucoside based on its LC-MS characteristics in comparison with data available in our compound library. Peak**14d** ([M-H]$^-$ at *m/z* 637) showing an MS^2 fragmentation pattern at *m/z* 475 ([M-H-162]$^-$) and 271 (further loss of 162+42 u) was tentatively assigned as naringenin-hexoside-acetylhexoside. Peak **17d** ([M-H]$^-$ at *m/z* 579) presented a unique MS^2 fragment at *m/z* 301 (-132-146 u, loss of pentosyl rhamnosyl moieties) and was tentatively identified as hesperetin-pentosyl-rhamnoside.

Peaks **5d** and **7d** showed a pseudomolecular ion [M-H]$^-$ at *m/z* 465 that is coherent with a pentahydroxyflavanone structure, also the UV spectra presented by this compounds at λ_{max} 270 nm with a shoulder at 320 nm is also characteristic of dihydroflavonoids, so

that they were tentatively assigned as pentahydroxyflavanone-dihexoside (**5d**) and pentahydroxyflavanone-acetylhexoside-hexoside (**7d**).

Dihydroflavonols. Peaks **1ce**, **8ce**, and **9ce**, all of them showing the same pseudomolecular ion $[M-H]^-$ at *m/z* 627, detected in centaurea samples, were associated to taxifolin (i.e., dihydroquercetin; *m/z* at 303) derivatives bearing hexose and caffeic acid residues (both with 162 mu). The presence of caffeoyl moieties is also supported by the characteristic UV spectra shape with a shoulder over 320 nm.

Chalcones. Seven compounds detected in dahlia samples were identified as chalcones. Peaks **11d** and **16d** presented an aglycone with *m/z* at 255 that fits both the flavanone liquiritigenin and its corresponding chalcone isoliquiritigenin. However, the flavanone nature was discarded based on their UV spectra showing λ_{max} around 360 nm, characteristic of chalcones, as also checked by comparison with a commercial standard of isoliquiritigenin. Based on this observation and their pseudomolecular ions and MS^2 fragmentation patterns, these peaks were tentatively identified as isoliquiritigenin-dihexoside and isoliquiritigenin-hexoside-acetylhexoside.

Peaks **18d**, **20d**, and **21d** showed similar spectra shapes as the previous ones with λ_{max} around 370-380 nm, and a common MS^2 product ion at *m/z* at 271 that matched the chalcone butein. The pseudomolecular ion ($[M-H]^-$ at *m/z* 433) of peak **18d** was coherent with butein hexosides, being the first of them (the majority one) assigned as coreopsin (butein-4'-*O*-glucoside), described as a main flavonoid in other species of the Asteraceae family, such as *Coreopsis tinctoria* (Chen et al., 2016; Yang et al., 2016). Peak **20d**, with a molecular weight 42 u higher that peak **18d**, was tentatively assigned as acetylcoreopsin, also reported in *Coreopsis tinctoria* (Yang et al., 2016). Peak **23d** should also correspond to a coreopsin derivative, although no definite identity could be assigned to it.

The most abundant flavonoids in dahlia (except dahlia's infusions), rose, calendula, and centaurea were naringenin-3-*O*-glucoside, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, and apigenin-glucuronide, respectively. Calendula hydromethanolic extracts and infusions presented the highest concentration of flavonoids (11.15 and 7.37 mg/g, respectively) and total phenolic compounds (11.31 and 7.47 mg/g, respectively) from all the studied samples. This flower sample showed higher concentration of

phenolic compounds when compared to the results reported by Miguel et al. (2016) in *C. officinalis* samples. This difference could be related to the type of treatment given to the flowers (e.g. drying process), as well as the geographic origin of the samples.

3.1.2.2. Phenolic acids

Phenolic acid derivatives represent the second major class of polyphenols found in the studied flower samples, except in rose flowers where this kind of compounds were not detected. Caffeic acid (peaks **3c**) and 5-*O*-caffeoylquinic acid (chlorogenic acid; peaks **2d, 2c**, and **4ce**) were positively identified according to their retention, mass, and UV-vis characteristics by comparison with commercial standards. Peak **3ce** was assigned as the *cis* form of 5-*O*-caffeoylquinic acid, since the *cis* hydroxycinnamoyl derivatives would be expected to elute before the corresponding *trans* ones (Barros et al., 2012). Peaks **1c** and **2ce** ($[M-H]^-$ at *m/z* 341) were tentatively identified as caffeic acid hexoside. The presence of caffeic acid hexoside and 5-*O*-caffeoylquinic acid was already reported in *C. officinalis* by our group (Miguel et al., 2016). Finally, peaks **5ce** and **6ce** were tentatively identified as *p*-coumaric hexoside based on their pseudomolecular ion ($[M-H]^-$ at *m/z* 325) and the MS^2 fragment at *m/z* 163 ($[\text{coumaric acid-H}]^-$, -162 u, loss of a hexosyl moiety). The observation of two compounds could be explained by a different location of the sugar residue on the coumaric acid or the existence of *cis/trans* isomers.

The hydromethanolic extract from centaurea samples presented the highest concentration in phenolic acids; while for the infusions the highest concentration of phenolic acids was found in centaurea sample. Chlorogenic acid was the most abundant phenolic acid present in all samples, which may be interesting since this compound has been correlated with various biological effects, including antioxidant, antiobesity, antiapoptosis, and antitumor activities (Kamiyama et al., 2015; Rakshit et al., 2010).

3.1.2.3. Characterization of anthocyanin profiles in the edible flowers

Owing to the powerful coloring capacity of anthocyanins, these compounds were thoroughly characterized in the extracts obtained from the flowers of each selected species. The extraction yields (mg of anthocyanin per 100 g of flowers) obtained for

each sample extract were: ~53% for dahlia; ~46% for rose; and ~23% for centaurea samples.

Nine anthocyanin compounds were detected in dahlia, two in rose and eight in centaurea extracts. Peak characteristics, tentative identification and compound quantification are presented in **Table 14.**

Table 14. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification, and quantification of anthocyanins in dahlia, rose, and centaurea extracts. Results are presented as mean±standard deviation.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g extract)
Rose						
1	11.5	514	611	449(10),287(100)	Cyanidin 3,5-di-*O*-glucoside [A]	13.19 ± 0.01
2	18.5	516	449	287 (100)	Cyanidin-3-*O*-glucoside[A]	0.131 ± 0.004
					Total Anthocyanins	**13.326 ± 0.002**
Centaurea						
1	11.7	512	611	449(5),287(100)	Cyanidin 3,5-di-*O*-glucoside [A]	5.5 ± 0.2
3	18.03	516	697	535(62),449(8),287(46)	Cyanidin 3-*O*-(6"-malonylglucoside)-5-*O*-glucoside [A]	6.2 ± 0.3
4	20.38	516	711	549(3),449(48),287(100)	Cyanidin 3-*O*-(6’’-succinylglucoside)-5-*O*-glucoside [A]	11.2 ± 0.5
5	29.6	518	465	303(100)	Delphinidin-hexoside[C]	1.5 ± 0.2
6	31.5	518	463	287(100)	Cyanidin-glucuronide[A]	0.85 ± 0.06
7	32.6	518	561	303(100)	Delphinidin-malonylhexoside[C]	tr
8	38.1	501	695	609(9),433(2),271(82)	Pelargonidin 3-*O*-(6’’-succinylglucoside)-5-*O*-glucoside [B]	0.18 ± 0.01
9	39.2	502	519	271(100)	Pelargonidin-malonylhexoside[B]	0.17 ± 0.01
					Total Anthocyanins	**26 ± 1**
Dahlia						
10	11.6	516	449	287(100)	Cyanidin-hexoside[A]	2.98 ± 0.01
11	13.4	504	449	287(100)	Cyanidin-hexoside[A]	2.654 ± 0.001
12	15.1	514	579	271(100)	Pelargonidin-rutinoside[B]	1.4 ± 0.1

Peak	Rt (min)	λ_{max} (nm)	Molecular ion (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g extract)
13	17.2	514	491	287(100)	Cyanidin-acetylhexoside[A]	5.36 ± 0.01
14	19.4	501	431	269(100)	Methylapigeninidin-hexoside[A]	4.1 ± 0.1
15	20.8	518	595	287(100)	Cyanidin-rutinoside[A]	0.8 ± 0.1
16	28.5	504	595	271(100)	Pelargonidin 3,5-di-*O*-glucoside [B]	0.8 ± 0.1
17	31.5	518	491	287(100)	Cyanidin-acetylhexoside[A]	0.33 ± 0.02
18	32.7	516	433	271(100)	Pelargonidin-hexoside[B]	0.450 ± 0.001
					Total Anthocyanins	**18.8 ± 0.2**

tr-trace amounts; Standard calibration curves: A – cyanidin-3-*O*-glucoside (y = 243287x - 1E+06; R^2= 0.995); B – pelargonidin-3-*O*-glucoside (y = 276117x – 480418; R^2= 0.9979); C- delphinidin-3-*O*-glucoside (y = *557274*x+126.24; R^2= 0.9979).

Cyanidin (Cy; peaks **1**, **2**, **3**, **4**, **6**, **10**, **11**, **13**, **15**, and **17**), pelargonidin (Pg; peaks **8**, **9**, **12**, **16**, and **18**), and delphinidin (Dp; peaks **5** and **7**) were identified as main aglycones, based on the observation of their characteristic fragments in MS^2 spectra. As reviewed by Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal (2009), these non-methylated anthocyanidins are the most commonly found in flowers, being cyanidin derivatives the most abundant in the analysed samples. Cyanidin is a reddish-purple (magenta) anthocyanidin that is the major pigment in berries (Seeram, Momin, Nair, & Bourquin, 2001) and edible flowers such as *R.canina* (Hvattum, 2002). Anthocyanins are the glycosylated forms of anthocyanidins. Several foods, like yogurt, are considered healthy but they lack phenolic compounds, therefore, a possible incorporation of plant extracts rich in anthocyanins in such fermented products beyond imparting a desirable red color can also enhance their health status due to the presence of phenolic compounds, which are recognized for their multi-functional positive role in human health (Mourtzinos et al., 2018).

Peak **1**, detected in rose and centaurea samples, was positively identified as cyanidin 3,5-di-*O*-glucoside based on the HPLC-DAD-MS results and comparison with our database library. This compound was already described as the main anthocyanin in flowers of *R. damascena* (Velioglu & Mazza, 1991) and *R. hybrida* (Lee, Lee, & Choung, 2011a), as well as in flowers from different *Centaurea* species (Mishio, Takeda, & Iwashina, 2015). Peak **2**, found in rose samples, was also positively identified as Cy-3-*O*-glucoside according with its retention time and mass spectral data by comparison with a standard. The presence of this anthocyanin in rose hips (*R. canina*) was also previously reported by Hvattum (2002). This compound, also known as chrysanthemin, shows red-purple colour and is the most abundant anthocyanin in nature.

Peak **4** ($[M]^+$ at *m/z* 711) was the majority anthocyanin in Centaurea samples. Its MS^2 spectra yielded fragments at *m/z* 549 (-162 mu, loss of a hexose), 449 (-262 mu, loss of succinylhexose) and 287 (cyanidin), coherent with an identity as Cy-3-*O*-(6''-succinylglucoside)-5-*O*-glucoside, a compound consistently identified in centaurea flowers also referred to as centaurocyanin (Mishio et al., 2015; Takeda & Tominaga, 1983), and whose combination with a flavone glycoside and metal ions give rise to protocyanin, a stable complex pigment considered to be the main responsible for the blue color of *Centaurea cyanus* flowers (Takeda et al., 2005). This compound has

interesting properties regarding color and stability, making it suitable to be used as a natural additive in food products. Similarly, mass spectral characteristics of peak **3**, with a molecular ion $[M]^+$ at *m/z* 697 and MS^2 fragments at *m/z* 535 (-162 mu, loss of a hexose), 449 (-248 mu, loss of malonylhexose) and 287 (cyanidin), allowed tentatively assigning it as Cy-3-*O*-(6''-malonylglucoside)-5-*O*-glucoside, also previously identified in flowers from different *Centaurea* species (Mishio et al., 2015). Peak **6** ($[M]^+$ at *m/z* 463) was another cyanidin derivative, tentatively identified as Cy-*O*-glucuronide based on the loss of 176 mu (a glucuronyl moiety) to yield the unique MS^2 product ion at *m/z* 287.

Peaks **8** and **9** in centaurea samples were associated as pelargonidin derivatives based on their characteristic absorption spectra, which differs from the other usual anthocyanidins, showing λ_{max} at 501 nm, and the fragment ion observed at *m/z* 271 (Pg). Pelargonidin appears as an orange-red pigment in nature, giving rise to an orange hue to some flowers and red to some fruits and berries, such as strawberry (Jaakola, 2013; Khoo et al., 2017). It has been indicated to possess notable anti-inflamatory (Duarte et al., 2018). Peak **8** ($[M]^+$ at *m/z* 695), with similar fragmentation behaviour as peak **4**, was identified as Pg-3-*O*-(6''-succinylglucoside)-5-*O*-glucoside, previously described in *Centaurea cyanus* flowers by Takeda, Kumegawa, Harborne, & Self (1988). Peak **9** ($[M]^+$ at *m/z* 519) was tentatively assigned as a Pg-*O*-malonylhexoside based on the loss of 248 mu (malonylhexoside) to yield the aglycone ion at *m/z* 271. In a similar way, peak **7** ($[M]^+$ at *m/z* 561), yielding a unique MS^2 fragment at *m/z* 303 (-248 mu; delphinidin), was associated to Dp-*O*-malonylhexoside, whereas **p**eak **5** ($[M]^+$ at *m/z* 465) was assigned as a Dp-*O*-hexoside; a possible identity as Dp-3-*O*-glucoside was discarded by comparison with our database library. Delphinidin appears as a purple pigment in the nature. The blue hue of some flowers is related to the presence of delphinidin glycosides, making them interesting for the development of processed foods and beverages (Khoo et al., 2017). Dp has also associated to anti-inflammatory, antioxidant, and anti-tumorigenic activities (Ko et al., 2015).

Similar reasoning was applied to identify anthocyanins in Dahlia samples as cyanidin (peaks **10**, **11**, **13**, **15** and **17**) and pelargonidin derivatives (peaks **12**, **16** and **18),** types of aglycones previously reported in Dahlia flowers (Deguchi, Ohno, Hosokawa, Tatsuzawa, & Doi, 2013; Takeda, Harborne, & Self, 1986; Yamaguchi et al., 1999). The presence of Pg-3,5-*O*-diglucoside in flowers of *Dahlia variabilis* was identified by

Yamaguchi et al. (1999) and Deguchi et al. (2013), which could correspond to peak **16** ($[M]^+$ at *m/z* 595) in our samples. For the remaining anthocyanins (peaks **10**, **11**, **12**, **13**, **15**, **17, and 18**) the substituent moieties were assigned based on the mass losses observed in their MS^2 spectra, as hexosides (-162 mu), acetylhexosides (-204 mu) or deoxyhexosylhexosides (-308 mu), although no conclusions about the precise identity of the sugar nature and location could be obtained. Curiously, none of the observed peak losses indicates the presence of malonylglucosides, a type of derivatives usually reported in Dahlia flowers (Deguchi et al., 2013; Takeda et al., 1986; Yamaguchi et al., 1999). Cy-acetylhexoside (peak **13**) was the most abundant pigment present in dahlia, responsible by the coloration of its edible flowers. In addition to improving the sensory characteristics of yogurt, that anthocyanin was proposed as a promising antiglycation agent for preventing or ameliorating AGEs-mediated diabetic complications (Suantawee et al., 2016). Finally, peak **14** presented a molecular ion $[M]^+$ at *m/z* 431 and a unique MS^2 fragment at *m/z* 269, which could match the mass of methylapigeninidin, so that it might be associated to a methylapigeninidin-hexoside, a 3-deoxyanthocyanin pigment reported in red sorghum (Wu & Prior, 2005). Nevertheless, the absorption spectrum of peak **14** would not be coherent with such an identity, as maximum absorption in the visible region for that compound should be expected around 470 nm (Awika, 2008; Yang, Dykes, & Awika, 2014). Thus, the identity of peak **14** remains uncertain, although in case it is confirmed as a methylapigeninidin-hexoside it would be the first description of this type of pigments in Dahlia flowers.

Among the analyzed flowers, centaurea presented the highest anthocyanins concentration, followed by dahlia and rose samples. All of them present a potential to be used as natural colorants and in the development of innovative products with new sensory characteristics and possible healthy properties, including their recognized antioxidant activity.

3.1.2.4. Bioactivities of the hydromethanolic extracts and infusions of the flower samples

Data regarding antioxidant, antiproliferative, and antibacterial activities of the hydromethanolic extracts and infusions of the four studied flowers are presented in **Table 15.**

According to the results obtained in the distinct antioxidant assays, all the studied samples showed acceptable capacity to inhibit lipid peroxidation and to prevent oxidative damage, as well as to promote free radicals scavenging, when compared to the Trolox control. As reviewed by Lu et al. (2015), phytochemicals present in edible flowers, such as anthocyanins, flavonoids, phenolic acids, alkaloids, and glycosides exert high antioxidant activities.

Among the studied samples, the hydromethanolic extracts and the infusions of rose flowers were those with the highest antioxidant activity (lower EC_{50} values) in the different assays, i.e., DPPH radical scavenging (0.18 and 0.82 mg/mL, for extracts and the infusions respectively), reducing power (1.29 and 0.64 mg/mL) and β-carotene bleaching inhibition (0.38 and 1.12 mg/mL) These results were in accordance with those obtained by Barros et al. (2011) for flowers of *Rosa canina* L.

As for the other samples, the hydromethanolic extracts of calendula showed higher antioxidant activity in the DPPH and β-carotene bleaching inhibition assays than the ones reported by Miguel et al. (2016).

Table 15. Antioxidant, antiproliferative, hepatotoxic and antibacterial activities of hydromethanolic extracts and infusions of the studied flowers (mean ± SD).

	Hydromethanolic extracts				Infusions			
	Dahlia	**Rose**	**Calendula**	**Centaurea**	**Dahlia**	**Rose**	**Calendula**	**Centaurea**
Antioxidant activity EC_{50} values (mg/mL)[A]								
DPPH scavenging activity	0.63 ± 0.01^{c}	0.18 ± 0.02^{d}	1.37 ± 0.08^{a}	0.83 ± 0.03^{b}	1.17 ± 0.05^{c}	0.82 ± 0.01^{d}	16.71 ± 0.29^{a}	10.78 ± 0.26^{b}
Reducing power	1.33 ± 0.07^{b}	1.29 ± 0.09^{b}	7.96 ± 0.08^{a}	8.14 ± 0.18^{a}	0.799 ± 0.001^{d}	0.64 ± 0.01^{c}	10.19 ± 0.12^{a}	5.10 ± 0.03^{b}
β-carotene bleaching inhibition	0.48 ± 0.02^{bc}	0.38 ± 0.03^{c}	0.66 ± 0.08^{b}	1.17 ± 0.01^{a}	2.01 ± 0.07^{c}	1.12 ± 0.04^{d}	8.50 ± 0.08^{a}	8.06 ± 0.30 ^{b}
Antiproliferative activity GI_{50} values (µg/mL)[B]								
MCF-7 (breast carcinoma)	361.99 ± 28.83	>400	>400	>400	303.27± 26.13^{b}	377.09 ± 32.09^{a}	>400	>400
NCI-H460 (non-small cell lung carcinoma)	>400	>400	>400	>400	>400	>400	>400	>400
HeLa (cervical carcinoma)	223.65 ± 2.78^{b}	308.45 ± 17.13^{a}	>400	>400	>400	>400	>400	>400
HepG2 (hepatocellular carcinoma)	339.15± 15.14^{a}	296.82 ± 23.71^{b}	>400	>400	>400	315.33 ± 19.28	>400	>400
Hepatotoxicity GI_{50} values (µg/mL)[B]								
PLP2	>400	>400	>400	>400	>400	>400	>400	>400
Antibacterial activity MIC values (mg/mL)								
Gram-negative bacteria								
Acinetobacter baumannii	5	2.5	10	5	10	2.5	>20	>20
Escherichia coli ESBL 1	5	5	10	5	10	10	10	20
Escherichia coli ESBL 2	5	1.25	5	5	-	-	-	-
Escherichia coli	2.5	5	20	20	10	5	20	20
Klebsiella pneumoniae	5	2.5	5	10	>20	10	>20	>20
Klebsiella pneumoniae ESBL	5	2.5	10	5	>20	10	>20	>20

	Hydromethanolic extracts				Infusions			
Antibacterial activity MIC values (mg/mL)	**Dahlia**	**Rose**	**Calendula**	**Centaurea**	**Dahlia**	**Rose**	**Calendula**	**Centaurea**
Morganella morganii	2.5	1.25	20	10	2.5	1.25	20	20
Pseudomonas aeruginosa	>20	2.5	5	10	20	2.5	>20	20
Gram-positive bacteria								
Enterococcus faecalis	2.5	2.5	5	20	5	>20	20	>20
Listeria monocytogenes	5	10	5	20	5	>20	20	>20
Staphylococcus aureus	2.5	1.25	10	5	1.25	0.625	20	5
MRSA	5	1.25	5	5	2.5	0.625	>20	20
MSSA	5	1.25	10	10	2.5	1.25	20	10

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. A - Trolox EC_{50} values: 62.98 μg/mL (DDPH), 45.71 μg/mL (reducing power), 10.25 μg/mL (β-carotene bleaching inhibition); B- Ellipticine GI_{50} values: 1.21 mg/mL (MCF-7), 1.03 mg/mL (NCI-H460), 0.91 mg/mL (HeLa), 1.10 mg/mL (HepG2) and 2.29 mg/mL (PLP2). MIC values correspond to the minimal sample concentration that inhibited the bacterial growth. In each row and for the different extraction procedures, different letters mean significant differences ($p < 0.05$).

Regarding antiproliferative activity, not all the samples studied were able to inhibit the growth of the studied tumor cell lines. Dahlia hydromethanolic extracts and infusion gave the lowest GI_{50} values against HeLa (223.65 μg/mL) and MCF-7 (361.99 μg/mL, 303.27 μg/mL respectively) cell lines. The presence of flavanones, only detected in dahlia samples, may have contributed to their antiproliferative activity against cell lines (Manthey et al., 2002). Rose hydromethanolic extract also presented the capacity to inhibit the growth of some tumor cell lines, such as those from cervical and hepatocellular carcinoma. Similar results were also described by Nadpal et al. (2016) for rose samples in a cervical carcinoma cell line (308.5 μg/mL). Centaurea and calendula hydromethanolic extracts and infusions showed hardly or no antiproliferative activity, with GI_{50} values higher than 400 μg/mL in all the tested cells. None of the extracts or infusions presented hepatoxicity toward the non-tumor liver primary culture (PLP2).

As for antimicrobial activity, the obtained results showed that samples were active against all the microorganisms used, although in most cases, they were found to be more active against Gram-positive bacteria with MICs ranging from 0.625 to 10 mg/mL. Similar observations were made by Nowak et al. (2014).

For Gram-positive bacteria, the infusions of rose samples showed the best results for *Staphylococcus aureus* (0.625 mg/mL), MRSA (0.625 mg/mL), and MSSA (1.25 mg/mL). The hydromethanolic extracts of rose sample also showed the best antibacterial activity against Gram-negative bacteria, *E. coli* and *Morganella morganii* (1.25 mg/mL, for both). Considering the determined MICs, these antibacterial effects might be obtained with the consumption of a portion of 3.9 mg of rose plant/mL (e.g. 0.78 g per cup of infusion of 200 mL). For the remaining plants the necessary portions would be 2.4, 5 and 4.8 mg of dahlia, calendula and centaurea, respectively per mL. Extended-spectrum beta-lactamase-producing (ESBL 2) *Escherichia coli* was not affected by any of the studied infusions.

3.1.3. Incoporation of an anthocyanin-rich extract obtained from edible flowers in yogurts

The trend to incorporate natural additives instead of artificial compounds in food products is evident, mainly due to their expected lack of harmful effects on consumers' health (Carocho et al., 2014). Nevertheless, the acceptability of these products is highly dependent on their appearance and rheological properties (Caleja et al., 2016; Santillán-Urquiza, Méndez-Rojas, & Vélez-Ruiz, 2017). In what concerns yogurt, similarly to several other food products, color is a determining factor for consumers' acceptance. Bearing this in mind, different plant species were selected as potential sources of coloring agents to be incorporated in yogurt. To have a better idea about the coloring capacity of each plant extract they were compared with a set of yogurts added with a commercial anthocyanin extract (E163, authorized by EFSA). Likewise, "white" yogurts (free of any coloring agent) were also used as a "blank" for comparison of the nutritional and stability characteristics.

For the discussion, the following codes were used: i) plain yogurt samples (BY); ii) yogurts with commercial colorant, E 163 (AY); iii) yogurts with rose flowers extract (RY); iv) yogurts with *Centaurea cyanus* L. flowers extract (CY), and v) yogurts with *Dahlia mignon* flowers extract (DY). In addition, the stability of the different yogurt formulations (YF) during storage was also evaluated, specifically by performing the same evaluation assays on the preparation day and after 7 days of storage at 8 ºC (SE). Since the effect of each factor (YF or SE) might be affected by the second factor level (*i.e.*, different storage effects according on each YF, or *vice versa*), the interaction (YF×SE) was also evaluated. In the case where a significant interaction was found (p<0.050), multiple comparisons could not be performed. In those cases, overall conclusions were obtained from the corresponding estimated marginal means (EMM) plots.

3.1.3.1. Proximate and chemical composition in the different yogurt formulations

The results obtained for nutritional parameters in the different yogurt formulations (YF) at the two storage times (SE) are presented in **Table 16.**

A significant interaction between YF and SE (YF×SE) was found in all cases, thereby indicating that each YF reacted differently to storage. Considering each factor individually, YF-related differences were significant in most cases, except water and

energy, while SE had no significant effects in any case. Nevertheless, despite the statistically significant differences among values presented by each YF, the nutritional profile was very similar in all tested samples, with water as the main component (≈85 g/100 g), followed by carbohydrates (slightly higher in RY and CY and lower in DY) and proteins (a bit higher in CY and lower in RY), both with concentrations of ≈5.5 g/100 g, fat (from 3.2 g/100 g in CY to 3.4 g/100 g DY and AY) and ash (<0.9 g/100 g in all yogurts). This profile resulted in energy values around 74 kcal/100 g in all cases. Actually, considering the low quantity of added coloring agent, it was not expectable to have great differences among the different yogurt formulations, particularly in what concerns fat amounts, as the flower extracts were prepared in water. Nevertheless, some minute changes could be expected taking into account the different nutritional composition of distinct plant species used (see section 3.1.1). In either case, these obtained results validate the maintenance of the nutritional quality that typifies natural yogurt (herein identified as BY).

In what concerns individual sugars, lactose was, as expected, the main compound (≈4.8 g/100 g, with slightly higher values in AY). Minor levels of galactose were also quantified, varying from the highest values in CY (0.76 g/100 g) to the lowest in BY (0.69 g/100 g).

More significant differences were, as observable in **Table 17**, found in the case of color parameters, which is in line with the main purpose of this work.

Table 16. Nutritional composition (g/100 g fresh weight) and energy values (kcal/100 g fresh weight) in the different yogurt formulations (YF) and storage times (SE). Results are presented as mean±standard deviation.[1]

		Water	Fat	Protein	Ash	Carbohydrates	Galactose	Lactose	Energy
YF	**BY**	85.0 ± 0.4	3.3 ± 0.1	5.3 ± 0.3	0.79 ± 0.03	5.6 ± 0.1	0.69 ± 0.01^{c}	4.7 ± 0.1	73 ± 2
	RY	84.8 ± 0.4	3.3 ± 0.2	5.3 ± 0.2	0.85 ± 0.01	5.8 ± 0.2	0.71 ± 0.04^{bc}	4.7 ± 0.2	74 ± 3
	DY	85.0 ± 0.1	3.4 ± 0.1	5.4 ± 0.1	0.86 ± 0.02	5.4 ± 0.1	0.71 ± 0.01^{b}	4.8 ± 0.1	73 ± 1
	CY	84.8 ± 0.1	3.2 ± 0.1	5.4 ± 0.1	0.86 ± 0.02	5.7 ± 0.1	0.76 ± 0.02^{a}	4.8 ± 0.1	74 ± 1
	AY	84.9 ± 0.2	3.4 ± 0.1	5.3 ± 0.1	0.82 ± 0.02	5.5 ± 0.1	0.72 ± 0.02^{b}	4.9 ± 0.1	74 ± 1
	ANOVA *p*-value (n = 18)[2]	0.083	0.001	0.039	<0.001	<0.001	<0.001	<0.001	0.632
SE	**0 days**	85.0 ± 0.2	3.3 ± 0.1	5.3 ± 0.2	0.84 ± 0.04	5.6 ± 0.2	0.73 ± 0.03	4.8 ± 0.1	73 ± 1
	7 days	84.8 ± 0.3	3.4 ± 0.1	5.4 ± 0.1	0.84 ± 0.03	5.6 ± 0.2	0.71 ± 0.03	4.8 ± 0.1	74 ± 2
	ANOVA *p*-value (n = 45)[3]	0.056	0.061	0.119	0.763	0.258	0.100	0.408	0.081
YF×SE	*p*-value (n = 90)[4]	<0.001	<0.001	<0.001	<0.001	<0.001	0.272	<0.001	<0.001

[1]Results are reported as mean values of each YF, aggregating results from 0 and 7 days, and mean values of SE, combining all YF. [2]If $p<0.05$, the corresponding parameter presented a significantly different value for at least one YF. [3]If $p<0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts. [4]In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

Table 17. Chromatic parameters (CIE *L, *a** and *b**) and pH values in the different yogurt formulations (YF) and storage times (SE). Results are presented as mean±standard deviation.[1]**

		*L**	*a**	*b**	pH
YF	**BY**	93 ± 1	-3.5 ± 0.1	9.8 ± 0.4	4.3 ± 0.1
	RY	88 ± 1	2.2 ± 0.1	9.0 ± 0.3	4.3 ± 0.1
	DY	84 ± 1	2.1 ± 0.3	17.7 ± 0.4	4.4 ± 0.1
	CY	90 ± 1	-1.1 ± 0.2	9.5 ± 0.5	4.2 ± 0.1
	AY	89 ± 1	3.1 ± 0.5	6.5 ± 0.5	4.8 ± 0.1
	ANOVA *p*-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001
SE	**0 days**	88 ± 3	1 ± 3	10 ± 3	4.4 ± 0.2
	7 days	89 ± 3	0 ± 2	11 ± 3	4.4 ± 0.2
	ANOVA *p*-value (n = 45)[3]	0.056	0.250	0.312	0.946
IF×ST	*p*-value (n = 90)[4]	<0.001	<0.001	<0.001	0.867

[1]Results are reported as mean values of each YF, aggregating results from 0 and 7 days, and mean values of SE, combining all YF. [2]If $p<0.05$, the corresponding parameter presented a significantly different value for at least one YF. [3]If $p<0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts. [4]In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

Yogurts free of any additive (BY) showed the highest *L** values, followed by CY, AY, RY and DY. On the contrary, BY yogurts presented the lowest *a** values, followed by CY, while AY, RY and DY reached the highest (without significantly different values among them), highlighting the cornflower extract as the less effective coloring agent. On the other hand, the absence of significant differences for *a** values among AY, RY and DY indicate that rose and dahlia extracts might be potential alternatives to E163.

Considering their potential usefulness as indicators of suitable conservation conditions, fatty acids profiles were also characterized Pereira et al., 2016). Fatty acids present in relative percentages above 1% are showed in **Table 18**, but the complete profiles included also other fatty acids (C11:0, C13:0, C14:1, C17:0, C17:1, C18:3n6, C20:0, C20:1, C20:4n6, C20:5n3, C22:0, C23:0, C24:0), which were also included in the Linear Discriminant Analysis.

Table 18. Fatty acids profile (relative percentage) in the different yogurt formulations (YF) and storage times (SE). Results are presented as mean ± standard deviation.[1]

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n3	SFA	MUFA	PUFA
YF	**BY**	1.3 ± 0.2	1.7 ± 0.1	1.3 ± 0.1	2.9 ± 0.2	3.6 ± 0.1	11.9 ± 0.4	1.4 ± 0.1	35 ± 1	1.4 ± 0.1	11.0 ± 0.2	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	71 ± 1	23 ± 2	5.0 ± 0.1
	RY	0.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.6 ± 0.1	12.2 ± 0.2	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.1	20 ± 1	2.2 ± 0.1	1.3 ± 0.1	72 ± 1	23 ± 1	4.6 ± 0.2
	DY	1.0 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.5 ± 0.1	12.1 ± 0.1	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.2	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	72 ± 1	23 ± 1	5.1 ± 0.1
	CY	1.1 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.6 ± 0.1	12.0 ± 0.1	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.1	20 ± 1	2.5 ± 0.2	1.5 ± 0.1	72 ± 1	23 ± 1	5.3 ± 0.2
	AY	1.2 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.7 ± 0.1	12.0 ± 0.3	1.5 ± 0.1	35 ± 1	1.4 ± 0.1	11.3 ± 0.1	21 ± 1	2.3 ± 0.1	1.4 ± 0.1	72 ± 1	23 ± 1	4.9 ± 0.2
	ANOVA *p*-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	0.133	<0.001	<0.001	0.180	0.125	<0.001
SE	**0 days**	1.1 ± 0.2	1.4 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.5 ± 0.1	11.9 ± 0.3	1.5 ± 0.1	35 ± 1	1.5 ± 0.1	11.3 ± 0.3	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	71 ± 1	23 ± 1	5.0 ± 0.2
	7 days	1.0 ± 0.3	1.6 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.6 ± 0.1	12.2 ± 0.2	1.5 ± 0.1	36 ± 2	1.5 ± 0.1	11.4 ± 0.1	20 ± 1	2.3 ± 0.1	1.4 ± 0.1	72 ± 1	22 ± 1	5.0 ± 0.1
	t-student *p*-value (n = 45)[3]	0.018	<0.001	<0.001	<0.001	<0.001	<0.001	0.737	0.001	0.984	0.213	<0.001	0.063	<0.001	<0.001	<0.001	0.532
IF×ST	*p*-value (n = 90)[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Results are reported as mean values of each YF, aggregating results from 0 and 7 days, and mean values of SE, combining all YF. [2]If $p<0.05$, the corresponding parameter presented a significantly different value for at least one YF. [3]If $p<0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts. [4]In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

Since milk and cream were the main sources of fatty acids and bearing in mind that the added extracts were obtained using water, the high similarity among different YF was expectable. Nevertheless, C18:1n9 (p = 0.133), SFA (p = 0.180) and MUFA (p = 0.125) were the only cases not showing significant differences among tested YF, most likely because the results presented for each YF correspond to the average values obtained in the preparation day and after 7 days of storage, and the added extracts might behave differently in preventing the oxidation of specific fatty acids. Actually, the effect induced by this factor was associated with SE, as validated by the p values of their interaction (YF×SE), precluding, therefore, the possibility of presenting the statistical classification of the corresponding results. However, the analysis of the EMM plots (data not shown) allowed obtaining some general conclusions: BY presented higher percentages C4:0 (1.3%), C6:0 (1.7%), C8:0 (1.3%), C10:0 (2.9%), while C15:0 (1.5%), C16:1 (1.5%), C18:0 (11.5%), C18:2n6 (2.5%) and PUFA (5.3%) were maximized in CY; RY on the other hand, had the highest percentages of C14:0 (12.2%) and C16:0 (36%), whilst C12:0 was slightly higher in AY (3.7%).

In what concerns the SE effect, almost all considered fatty acids showed significant differences, except in the cases of C15:0 (p = 0.737), C16:1 (p = 0.984), C18:0 (p = 0.213), C18:2n6 (p = 0.063) and PUFA (p = 0.532). In stored samples, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0 and SFA were quantified in higher percentages, while C4:0, C18:1n9, C19:3n3 and MUFA tended to present higher values in non-stored samples, thereby generally corroborating the higher resistance to storage of the saturated forms.

3.1.3.2. Linear Discriminant Analysis in different yogurt formulations

After illustrating specific individual differences among different YF, it was intended to verify the overall differences that better characterize each tested YF, by evaluating changes in all parameters and variables simultaneously.

The first three discriminant functions included 97.7% (first function: 61.4%; second function: 30.0%; third function: 6.3%) of the observed variance (**Figure 18**).

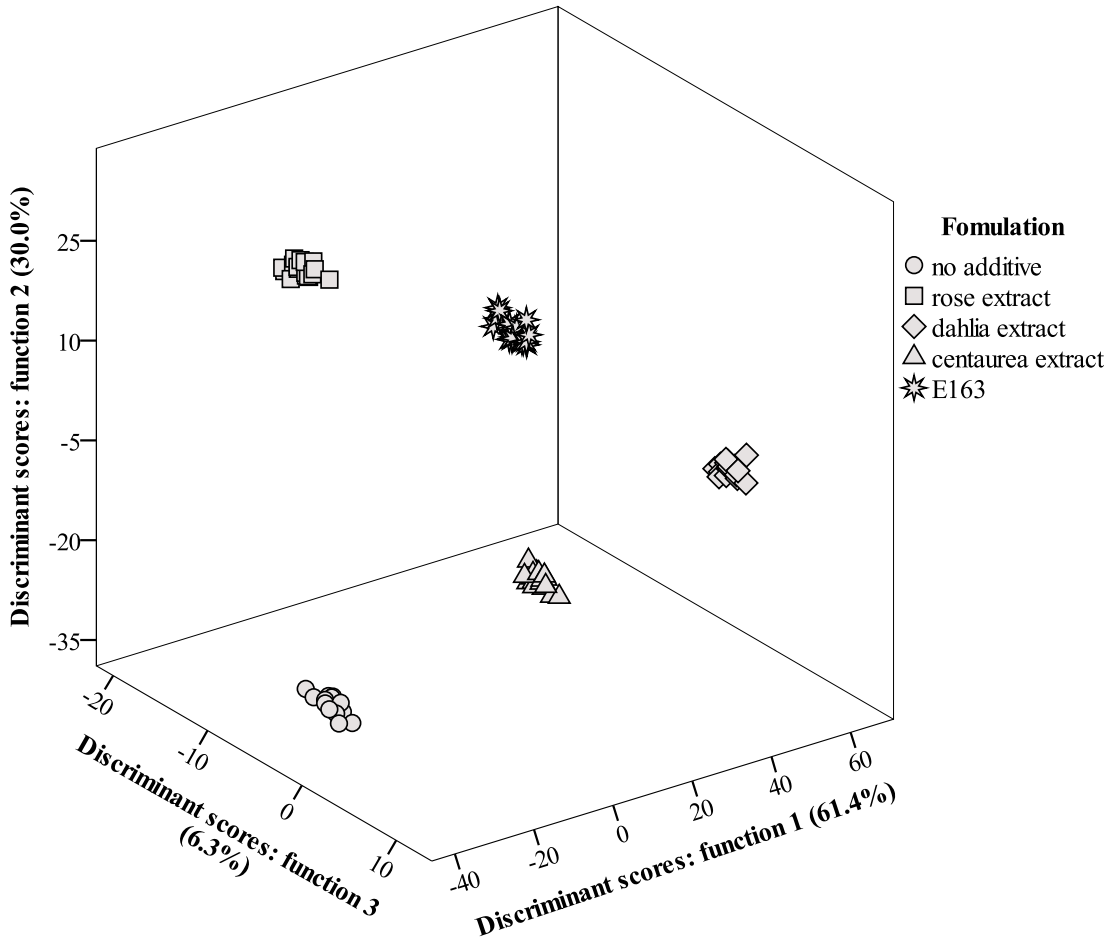


Figure 18. Three-dimensional distribution of YF markers according to the canonical discriminant functions coefficients defined from different yogurt variables.

From the 41 variables under analysis, the discriminant model selected *b**, *a**, *L**, pH, C4:0, C8:0, C13:0, C16:1, C17:1, C18:3n3, C18:3n6, C20:1, C20:4n6, C20:5n3, C23:0, C24:0 and PUFA as those having discriminant ability, reinforcing color parameters and fatty acids as the variables with most significant changes.

In what concerns the correlations among functions and variables, function 1 was highly correlated with *b** and *L** placing markers corresponding to DY and BY in the farthest positions, indicating that these yogurts had the most dissimilar values of blueness (highest in DY) and lightness (highest in BY). Function 2, in turn, was mostly correlated with *a**, and its discriminating effect was particularly noticeable in separating markers corresponding to AY and RY (positive end of the axis) from BY

(negative end of the axis), which represents a good indicator of RY as the most suitable alternative to E163, considering the main purpose of confering color in the yellow-orange range. According to function 3, on the other hand, the most similar yogurts were CY and AY, which together with the highest correlations of function with C20:4n6 and C20:5n3, indicates DY as having the most similar antioxidant performance with AY among all tested YF.

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

3.2. Chemical characterization, bioactive compounds and bioactivities of dried apples (*Malus domestica* Borkh. cv Bravo de Esmolfe)

3.2.1. Proximate composition and energetic value of 'Bravo de Esmolfe'

The results obtained regarding the proximate composition and energetic value of 'Bravo de Esmolfe' dried apples are shown in **Table 19.**

Carbohydrates were the most abundant macronutrients, followed by fat, proteins and ash. The USDA (United States Department of Agriculture) reported similar values for carbohydrates (95.72 g/100 g dw) and energetic value (360 kcal/100 g dw) in apple raw samples without skin, but lower fat content (0.97 g/100 g dw) (USDA, 2016) than those obtained in the present study. Feliciano et al. (2010), in a screening on different apple varieties from Portugal, reported lower protein contents for "Bravo de Esmolfe" (0.08 g/100 g of edible portion, which would be equivalent to 1.23 g/100 g dw) than those determined in our samples (2.61 g/100 g dw).

Sixteen fatty acids were identified with the predominance of saturated fatty acids, mostly palmitic acid, followed by stearic and linoleic acids (28.94%, 16.4% and 15.8%, respectively). Interestingly, previous studies carried out by Wu et al. (2007) using other apple varieties (Delicious, Golden Delicious, Ralls, Fuji, QinGuan, Granny Smith, Jonagold, Orin and Average), reported linoleic acid as the most abundant fatty acid. α-Tocopherol (0.52 mg/100 g dw) was the only tocopherol isoform found in this sample, which is in agreement with Feliciano et al. (2010) for 'Bravo de Esmolfe' (0,65-0,7 mg/100 g dw). Fructose, glucose and sucrose were the sugars detected in the analyzed sample (19.0, 8.4 and 1.38 g/100 g dw, respectively), being fructose the most abundant one, as also reported by Feliciano et al. (2010), although those authors found sucrose as the the second important sugar.

Regarding organic acids, malic acid was the main molecule present, followed by quinic, oxalic and shikimic acids (1.36, 0.15, 0.101 and 0.0002 g/100 g dw, respectively). Malic acid was also the main organic acid described by Feliciano et al. (2010) in 'Bravo de Esmolfe' apples (~ 0.9 g/100 g dw), together with citric acid (~ 0.016 g/100 g dw). The presence of other organic acids, such as succinic and fumaric was also reported in different apple varieties, such as Delicious, Golden Delicious, Ralls, Fuji, QinGuan, Granny Smith, Jonagold, Orin and Average (Wu et al., 2007) and apple juices (Chinnici, Spinabelli, Riponi, & Amati, 2005).

The differences in the chemical composition of apples could depend on the cultivar, production region and horticultural practices (Róth et al., 2007).

Table 19. Nutritional and chemical composition of 'Bravo de Esmolfe' apples (mean ± SD)

Nutritional value (g/100 g dw)		**Soluble sugars (g/100 g dw)**	
Fat	5.9 ± 0.3	Fructose	19.0 ± 0.2
Proteins	2.61 ± 0.02	Glucose	8.4 ± 0.3
Ash	1.84 ± 0.04	Sucrose	1.38 ± 0.02
Total carbohydrates	89.68 ± 0.03	Sum	28.8 ± 0.1
Energy (kcal/100 g dw)	492 ± 1		
Fatty acids (relative percentage, %)		**Organic acids (g/100 g dw)**	
C10:0	0.63 ± 0.01	Oxalic acid	0.101 ± 0.004
C12:0	0.81 ± 0.01	Quinic acid	0.15 ± 0.01
C14:0	1.92 ± 0.07	Malic acid	1.36 ± 0.01
C14:1	2.30 ± 0.05	Shikimic acid	0.0002 ± 0.0001
C15:0	1.04 ± 0.08	Sum	1.6 ± 0.1
C16:0	28.94 ± 0.07	**Tocopherols (mg/100 g dw)**	
C17:0	1.8 ± 0.1	α-Tocopherol	0.52 ± 0.02
C18:0	16.4 ± 0.1		
C18:1n9	5.89 ± 0.04		
C18:2n6	15.8 ± 0.4		
C18:3n3	7.6 ± 0.3		
C20:0	1.52 ± 0.09		
C20:3n3	0.98 ± 0.04		
C22:0	3.76 ± 0.01		
C23:0	1.02 ± 0.05		
C24:0	9.6 ± 0.1		
SFA	67.4 ± 0.2		
MUFA	8.19 ± 0.08		
PUFA	24.4 ± 0.1		

dw- dry weight basis. C10:0- Capric acid; C12:0- Lauric acid; C14:0- Myristic acid; C14:1 - Myristoleic acid; C15:0- Pentadecanoic acid; C16:0- Palmitic acid; C17:0 - Heptadecanoic acid; C18:0 - Stearic acid; C18:1n9- Oleic acid; C18:2n6- Linoleic acid; C18:3n3- Linolenic acid; C20:0- Arachidic acid; C20:3n3 - Eicosatrienoic acid; C22:0 - Behenic acid; C23:0 - Tricosanoic acid; C24:0 - Lignoceric acid. SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids dw- dry weight basis; Calibration curves for organic acids: oxalic acid (y= 9x106x + 45973, R^2=0.9901); quinic acid (y= 610607x + 46061, R^2=0.9995); malic acid (y= 912441x + 92665, R^2= 0.999) and shikimic acid (y= 7x107x + 175156, R^2=0.9999); (<LOD: 12.6, 24, 36 and 0.01 µg/mL for oxalic, quinic, malic and shikimic acids, respectively); (<LOQ: 42, 81, 1.2x10^2 and 0.02, µg/mL for oxalic, quinic, malic and shikimic acids, respectively).

3.2.2. Phenolic profile of the hidromethanolic extract from 'Bravo de Esmolfe'

The phenolic profile of the hydromethanolic extract prepared from 'Bravo de Esmolfe' dried apples, recorded at 280 nm is shown in **Figure 19**. UV and mass spectral were obtained for the fifteen phenolic compounds marked in the chromatogram (**Table 20**).

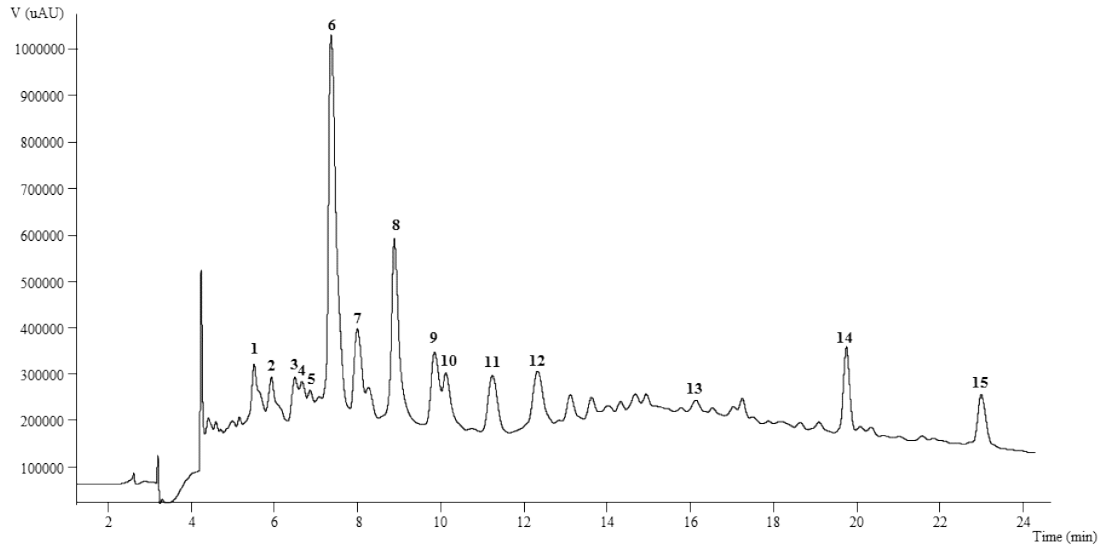


Figure 19. HPLC chromatogram recorded at 280 nm showing the phenolic profile of the 'Bravo de Esmolfe' hydromethanolic extract (numbers correspond to the compounds collected in Table 20).

Table 20. Retention time (Rt), wavelengths of maximum absorption (λ_{max}) mass spectral data, tentative identification and quantification of phenolic compounds in the 'Bravo de Esmolfe' hydromethanolic extracts.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/100 g dw)	Reference used for identification
1	5.56	281	577	451(24),425(100),407(21),289(12)	Procyanidin B1[1]	11.38 ± 0.04	(Mayr et al., 1995; Shoji et al., 2006, 2003; Verdu et al., 2013; Wojdyło et al., 2008)
2	5.98	271	373	327(17),165(100),121(12),93(14)	Unknown	-	
3	6.54	280	865	739(74),713(44),695(100),577(64),575(37),425(10), 407(9),289(8),287(7)	B-type epicatechin trimer[1]	11.3 ± 0.1	(Shoji et al., 2006, 2003)
4	6.7	281	865	739(69),713(43),695(100),577(68),575(36),425(11), 407(7),289(6),287(8)	B-type epicatechin trimer[1]	9.6 ± 0.2	(Shoji et al., 2006, 2003)
5	6.96	322	353	191(12),179(1),173(100),161(1),135(2)	4-*O*-Caffeoylquinic acid[2]	5.8 ± 0.1	(Dias et al., 2016)
6	7.4	327	353	191(100),179(6),173(2),161(1),135(1)	5-*O*-Caffeoylquinic acid[2]	51.5 ± 0.5	(Dias et al., 2016)
7	8.09	280	577	451(17),425(100),407(19),289(7)	Procyanidin B2[1]	34.5 ± 0.3	(Mayr et al., 1995; Shoji et al., 2006, 2003; Verdu et al., 2013; Wojdyło et al., 2008)
8	8.95	311	337	191(3),173(95),163(8),145(4),119(3)	4-*p*-Coumaroylquinic acid[3]	14.1 ± 0.6	(Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
9	9.96	281	289	245(100),203(5),187(1),161(2),137(2)	Epicatechin[1]	18.2 ± 0.4	DAD/MS
10	10.19	312	337	191(2),173(100),163(7),119(2)	5-*p*-Coumaroylquinic acid[3]	6.93 ± 0.04	(Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
11	11.34	280	865	739(83),713(53),695(100),577(82),575(43),425(14), 407(9),289(8),287(12)	Procyanidin C1[1]	19.56 ± 0.01	(Mayr et al., 1995; Shoji et al., 2006, 2003; Verdu et al., 2013; Wojdyło et al., 2008)
12	12.42	280	1153	865(19),863(18),577(6),575(11),289(3),287(4)	(C4,C8)-epicatechin tetramer[1]	24.77 ± 0.01	(Santos-Buelga, C.; García-Viguera, C.; Tomás-Barberán, 2003; Shoji et al., 2006, 2003)
13	16.2	280	579	289(56),245(100),203(9)	Unknown biflavonoid[1]	16.7 ± 0.4	
14	19.85	285	567	273(100),167(5),123(5)	Phloretin-2'-*O*-xyloglucoside[4]	7.87 ± 0.02	(Malec et al., 2014; Mayr et al., 1995; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
15	23.09	285	435	273(100),167(4),123(5)	Phlorizin (phloretin-2'-*O*-glucoside)[4]	4.84 ± 0.01	(Malec et al., 2014; Mayr et al., 1995; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
					Total phenolic compounds	237 ± 1	

Standard calibration curves recorded at 280 nm: (1) catechin (y=158.42x +11.38, R^2=0.999); (2) chlorogenic acid (y= 168823x – 161172; R^2 = 0.9999); (3) *p*-coumaric acid (y =706.09x +1228.1, R^2=0.9989); (4) isoliquiritigenin (y= 42820x + 184902, R^2 = 0.999).

The main family of compounds were flavan-3-ols, as also reported for other apple varieties (Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008). Epicatechin (peak **9**) was positively identified by comparison with a commercial standard. Two B-type procyanidin dimers ([M-H]$^-$ at *m/z* 577, peaks **1** and **7**), three trimers ([M-H]$^-$ at *m/z* 865, peaks **3**, **4** and **11**) and one tetramer ([M-H]$^-$ at *m/z* 1153, peak **12**) were detected. Peaks **1**, **7** and **11** were identified as procyanidins B1 [epicatechin-(4,8)-catechin], B2 [epicatechin-(4,8)-epicatechin], and C1 (5) [epicatechin-(4,8)-epicatechin-(4,8)-epicatechin] by comparison with our database library; these compounds have been consistently reported as majority procyanidins in apple (Mayr et al., 1995; Shoji et al., 2006, 2003; Verdu et al., 2013; Wojdyło et al., 2008). Peak **12** could be assigned as the (4,8)-linked epicatechin tetramer, in coherence with its elution order (Santos-Buelga, García-Viguera, Tomás-Barberán, 2003) and previous identification in apple (Shoji et al., 2006, 2003), whereas peaks **3** and **4** might correspond to the trimers epicatechin-(4,8)-epicatechin-(4,8)-catechin and epicatechin-(4,6)-epicatechin-(4,8)-catechin, also described in apple (Shoji et al., 2003, 2006). Apple procyanidins are known to be mostly based on epicatechin extension units (Mayr et al., 1995; Shoji et al., 2003, 2006).

Peaks **14** and **15** were identified as phloretin-2'-*O*-xyloglucoside and phlorizin (phloretin-2'-*O*-glucoside), respectively, chalcones that are also commonly present in apple (Malec et al., 2014; Mayr et al., 1995; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008). Peak **13**, presenting a pseudomolecular ion [M-H]$^-$ at *m/z* 579, might correspond to a biflavonoid containing an (epi)catechin unit, owing to the characteristic MS^2 fragments at *m/z* 289, 245 and 203, although definite identity could be established. Similarly, no identity could be concluded for peak **2**.

Peaks **5** and **6** were tentatively identified as 4-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid, respectively, based on their fragmentation patterns and elution order (Dias et al., 2016). Similarly, peaks **8** and **10** were tentatively identified as 4-*p*-coumaroylquinic and 5-*p*-coumaroylquinic acids. This kind of hydroxycinnamoyl derivatives have already been cited in apple (Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008).

The most abundant compound present in 'Bravo de Esmolfe' variety was 5-*O*-caffeoylquinic acid (peak **6**, 51.5 mg/100 g dw), followed by procyanidin B2 (peak **7**, 34.5 mg/100 g dw).

3.2.3. Antioxidant and antibacterial activity of the hydromethanolic extract from 'Bravo de Esmolfe'

Data regarding the antioxidant and antibacterial activities of the hydromethanolic extract prepared from 'Bravo de Esmolfe' dehydrated apples are presented in **Table 21.**

Table 21. Antioxidant and antibacterial activities of 'Bravo de Esmolfe' hydromethanolic extracts (mean ± SD).

Antioxidant activity EC_{50} values (mg/mL)*	
DPPH scavenging activity	0.71 ± 0.05
Reducing power	1.38 ± 0.01
β-carotene bleaching inhibition	7.19 ± 0.04
TBARS inhibition	0.45 ± 0.005
Antibacterial activity MIC values (mg/mL)	
Gram negative bacteria	
Acinetobacter baumannii	>20
Escherichia coli	5
Escherichia coli ESBL	5
Klebsiella pneumoniae	>20
Klebsiella pneumoniae ESBL	>20
Morganella morganii	5
Pseudomonas aeruginosa	>20
Gram positive bacteria	
Enterococcus faecalis	5
Listeria monocytogenes	5
MRSA	5
MSSA	2.5

*EC_{50} values correspond to the extract concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 43.03 ± 1.71 μg/mL (DDPH), 29.62 ± 3.15 μg/mL (reducing power), 2.63 ± 0.14 μg/mL (β-carotene bleaching inhibition) and 3.73 ± 1.9 μg/mL (TBARS inhibition). MIC values correspond to the minimal extract concentration that inhibited the bacterial growth. ESBL - extended spectrum β-lactamases. MRSA - Methicillin-resistant *Staphylococcus aureus*. MSSA - Methicillin-susceptible *Staphylococcus aureus*.

The lowest EC_{50} values (highest antioxidant activity) were observed in the TBARS inhibition and DPPH scavenging activity assays (0.45 and 0.71 mg/mL, respectively). These values of antioxidant activity were superior to those determined in apple extracts by other authors. Thus, Hamauzu, Yasui, Inno, Kume, & Omanyuda (2005) reported an EC_{50} of 8.4 mg/100 mL using the DPPH assay in *Malus domestica* Mill. *var*. Fuji, and

Luo, Zhang, Li, & Shah (2016) found EC_{50} values ranging from 0.26 to 9.30 mg/mL (DPPH assay) and from 0.36 to 1.82 mg/mL (ABTS assay) in different parts of the same apple variety. These differences might be related with the distinct studied varieties and also the way of preparation of the extracts (e.g., solvents).

Regarding antibacterial actvity, the hydromethanolic extracts showed the lowest MIC values against Gram-positive bacteria, namely methicillin-susceptible *Staphylococcus aureus* (MSSA) (MIC=2.5 mg/mL). The methicillin-resistant *Staphylococcus aureus* (MRSA) and other Gram-positive bacteria such as *Listeria monocytogenes* and *Enterococcus faecalis* had higher MIC values (MIC=5 mg/mL). Regarding Gram-negative bacteria, *Escherichia coli*, *E. coli* producing extended spectrum β-lactamases (ESBL) and *Morganella morganii* presented the lowest MIC values (MIC=5 mg/mL). Luo et al. (2016) also reported antibacterial activity of polyphenolic apple extracts from the Fuji variety finding lower MIC values, which might be expected, since those authors used ATCC reference standard microorganisms, while the bacteria used in the present study were obtained from clinical isolates with multiresistant profiles (Dias et al., 2016). Furthermore, the extract studied herein uses different solvents, which can lead to different results.

3.3. Chemical characterization, bioactive compounds and bioactivities of *Lycium barbarum* L.: A comparative study with stems and fruits

3.3.1. Proximate composition and energetic value of L. barbarum L.

Data on the proximate composition and energetic value of *L. barbarum* fruits and stems are shown in **Table 22.**

Table 22. Proximate composition, soluble sugars and organic acids in fruits and stems of *Lycium barbarum* L. (mean ± SD).

	Fruits	Stems	*t*-Students test *p*-value
Nutritional value (g/100 g dw)			
Fat	4.1 ± 0.3	4.6 ± 0.3	0.040
Proteins	5.3 ± 0.2	7.4 ± 0.2	<0.001
Ash	3.21 ± 0.02	9.9 ± 0.1	<0.001
Total carbohydrates	87± 6	78.1 ± 0.4	<0.001
Energy contribution (kcal/100 g dw)	408 ± 1	383 ± 2	<0.001
Soluble sugars (g/100 g dw)			
Fructose	12.7 ± 0.4	0.45 ± 0.01	<0.001
Glucose	14.4 ± 0.4	0.42 ± 0.01	<0.001
Sucrose	0.8 ± 0.1	0.21 ± 0.02	<0.001
Sum	27.9 ± 0.9	1.08 ± 0.05	<0.001
Organic acids (g/100 g dw)			
Oxalic acid	0.010 ± 0.001	0.65 ± 0.001	<0.001
Quinic acid	nd	0.53 ± 0.03	-
Malic acid	nd	0.899 ± 0.004	-
Citric acid	1.29 ± 0.02	nd	-
Succinic acid	0.77 ± 0.07	nd	-
Sum	2.07 ± 0.01	2.08 ± 0.03	0.677

dw- dry weight basis; nd- not detected.

Carbohydrates were the most abundant macronutrients in fruits and stems (87 and 78.1 g/100 g dw, respectively). Stems presented the highest contents of ash, proteins and fat (9.9, 7.4, and 4.6 g/100 g dw, respectively), while fruits presented proteins as the second major macronutrient (5.3 g/100 g dw), followed by fat and ash (4.1 and 3.21 g/100 g dw, respectively). Yan et al. (2014) reported different results for goji fruits from China, describing higher contents of proteins and fat (12.1 and 6.89 g/100 g dw, respectively) and lower ash content (0.95 g/100 g dw). These differences might be explained by the

cultivar and/or different edaphoclimatic conditions that can lead to variable nutritional contents.

Fructose, glucose and sucrose were the only forms of monosaccharides detected in fruits and stems, being glucose the most abundant one, followed by fructose and sucrose. As expected, fruits presented much higher content in soluble sugars (27.9 g/100 g dw) than stems (1.08 g/100 g dw). These results are in agreement with those obtained by Mikulic-Petkovsek et al. (2012a) in goji fruits from Slovenia, where glucose and fructose were also the prevailing sugars (23.1 g/kg fw, each) with much lower levels of sucrose (2.73 g/kg fw), concentrations that are higher than those determined herein, especially taking into account that they were expressed by fresh weight.

Regarding organic acids, fruits and stems presented very different profiles, however, no statistically significant differences were found in the sum of the organic acids between both samples. Citric, succinic and oxalic (1.29, 0.77, and 0.010 g/100 g dw, respectively) acids were detected in the fruit; while malic, oxalic and quinic (0.899, 0.65, and 0.53 g/100 g dw, respectively) acids were found in the stems. Oxalic acid was the only organic acid common in both samples. Donno et al. (2015), in goji fruits from Italy, reported the presence of several organic acids, including malic, quinic and tartaric acids that were not detected in samples of this study. These differences might be due to the different origin of the samples, physical state of the samples and/or the extraction method, as Donno et al. (2015) analysed the organic acids in fresh or semi-fresh samples (stored at 4 ºC for a few days) and using ethanol as extraction solvent.

Fatty acids were also determined in fruits and stems of goji fruits and the results are shown in **Table 23.**

Table 23. Fatty acids and tocopherols in fruits and stems of *Lycium barbarum* L. (mean ± SD)

	Fruits	Stems	*t*-Students test *p*-value
Fatty acids (relative percentage, %)			
C8:0	0.65 ± 0.04	0.60 ± 0.04	0.020
C10:0	0.10 ± 0.01	0.15 ± 0.01	<0.001
C12:0	0.19 ± 0.02	0.19 ± 0.02	0.442
C14:0	0.38 ± 0.02	1.7 ± 0.1	<0.001
C14:1	0.37 ± 0.03	0.35 ± 0.02	0.015
C15:0	0.21 ± 0.02	0.29 ± 0.01	<0.001
C16:0	12.77 ± 0.07	15.94 ± 0.08	<0.001
C16:1	0.29 ± 0.02	nd	-
C17:0	0.48 ± 0.05	0.90 ± 0.04	<0.001
C18:0	7.50 ± 0.06	9.1 ± 0.2	<0.001
C18:1n9	16.5 ± 0.5	5.12 ± 0.06	<0.001
C18:2n6	53.4 ± 0.5	9.7 ± 0.2	<0.001
C18:3n3	1.68 ± 0.02	14.8± 0.3	<0.001
C20:0	1.30 ± 0.07	12.84 ± 0.01	<0.001
C20:2	nd	1.3 ± 0.2	-
C20:3n3	0.35 ± 0.04	0.73 ± 0.04	0.000
C22:0	2.75 ± 0.08	10.4 ± 0.1	<0.001
C23:0	nd	0.69 ± 0.01	-
C24:0	nd	15.3 ± 0.3	-
SFA	26.1 ± 0.1	68.0 ± 0.5	<0.001
MUFA	17.2 ± 0.6	5.46 ± 0.04	<0.001
PUFA	56.8 ± 0.5	26.6 ± 0.4	<0.001
Tocopherols (mg/100 g dw)			
α-Tocopherol	0.23 ± 0.02	3.37 ± 0.01	<0.001
β-Tocopherol	nd	0.22 ± 0.04	-
δ-Tocopherol	0.09 ± 0.01	nd	-
Sum	0.33 ± 0.03	3.59 ± 0.05	<0.001

dw- dry weight basis; nd- not detected. C8:0 - Caprylic acid; C10:0- Capric acid; C12:0- Lauric acid; C14:0- Myristic acid; C14:1 - Myristoleic acid; C15:0- Pentadecanoic acid; C16:0- Palmitic acid; C16:1 - Palmitoleic acid; C17:0 - Heptadecanoic acid; C18:0 - Stearic acid; C18:1n9- Oleic acid; C18:2n6- Linoleic acid; C18:3n3- Linolenic acid; C20:0- Arachidic acid; C20:2- *cis*-11,14 - Eicosadienoic acid; C20:3n3 - Eicosatrienoic acid; C22:0 - Behenic acid; C23:0 - Tricosanoic acid; C24:0 - Lignoceric acid. SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids

Sixteen fatty acids were detected in the fruits, being polyunsaturated fatty acids (PUFA) the predominant group, mainly due to the presence of linoleic acid (C18:2n6, 53.4%), followed by oleic acid (C18:1n9, 16.5%) and palmitic acid (C16:0, 12.77%). Similar results were obtained by Yan et al. (2014) in goji fruits from China, that described linoleic acid (66.81%) and oleic acid (17.13%) as the major fatty acids. In stem samples, eighteen fatty acids were identified, but quite a different profile, being saturated fatty acids (SFA) predominant, especially palmitic (C16:0, 15.94%) and lignoceric acids (C24:0, 15.3%), followed by linolenic acid (C18:3n3, 14.8%).

Regarding tocopherols (**Table 23**), both samples presented only two vitamers, although different in each case. The highest content of tocopherols (3.59 mg/100 g dw) was determined in the stems, mostly due to the presence of α-tocopherol (3.37 mg/100 g dw), with minor levels of β-tocopherol (0.22 mg/100 g dw). Significant lower concentrations of tocopherols were found in the fruits, also containing α-tocopherol, but with δ-tocopherol as the second vitamer (0.23 and 0.09 mg/100 g dw, respectively). To the authors' best knowledge, there are no previous studies of tocopherols composition in goji fruits and stems.

3.3.2. Individual phenolic profile of L. barbarum fruits and stems

The peak characteristics (retention time, wavelength of maximum absorption and mass spectral data), tentative identification and quantification of phenolic compounds present in the hydromethanolic extracts of *L. barbarum* fruits and stems are shown in **Table 24**, and HPLC chromatograms, recorded at 280 nm, are shown in **Figure 20.**

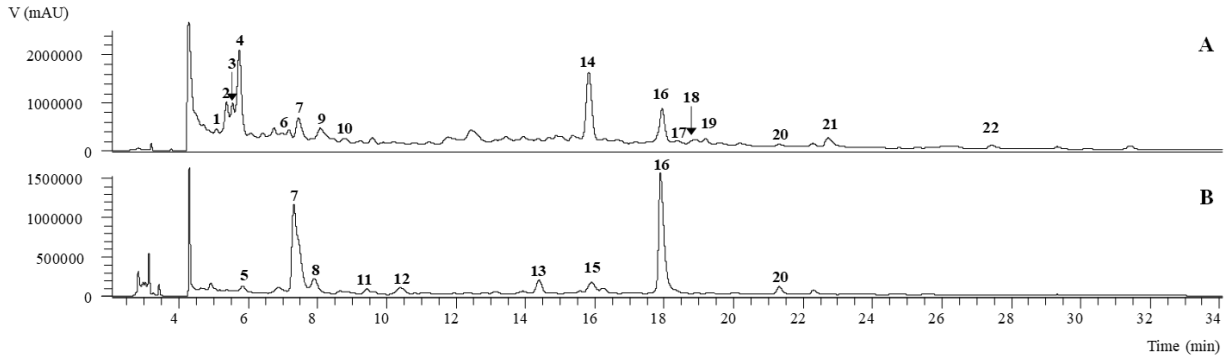


Figure 20. HPLC chromatograms recorded at 280 nm with the phenolic profiles of the hydromethanolic extracts of fruits (A) and stems (B) of *L. barbarum*.

Table 24. Retention time (Rt), wavelengths of maximum absorption in the UV-visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds detected in fruits and stems of *Lycium barbarum* L.

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Fruits	Stems	*t*-Students test *p*-value
1	5.08	262	311	179(100),135(5)	Caftaric acid[A]	(Mocan et al., 2015a; Mocan et al., 2015b)	0.86 ± 0.04	nd	-
2	5.37	313	353	191(100),179(7),161(3)	*cis* 3-*O*-Caffeoylquinic acid[A]	(Clifford et al., 2003, 2005)	2.9 ± 0.1	0.36 ±0.02	<0.001
3	5.54	296	487	163(100),119(40)	*p*-Coumaroyl acid dihexoside[B]	(Bondia-Pons et al., 2014; Zhou et al., 2017)	3.6 ± 0.2	nd	-
4	5.74	304	353	191(100),179(7),161(3)	*trans* 3-*O*-Caffeoylquinic acid[C]	(Clifford et al., 2003, 2005)	8.87 ± 0.01	0.59 ± 0.02	<0.001
5	5.82	264	343	191(3),169(100),125(3)	Galloylquinic acid[A]	(Guimarães et al., 2013)	nd	1.59 ± 0.01	-
6	7.16	324	933	609(100),301(5)	Quercetin-dihexoside-rutinoside[D]	(Bondia-Pons et al., 2014)	3.73 ± 0.03	nd	-
7	7.47	315	353	191(100),179(3),161(3)	*trans* 5-*O*-Caffeoylquinic acid[A]	DAD/MS, standard	3.3 ± 0.1	8.03 ± 0.01	<0.001
8	7.92	284	385	223(100),207(50),179(40),163(14),149(3)	Sinapic acid hexoside[E]	(Chahdoura et al., 2014)	nd	2.8 ± 0.1	-
9	8.07	315	289	245(2),20(13),137(20)	Catechin[F]	DAD/MS, standard	10.4 ± 0.4	nd	-
10	8.75	318	517	193(100), 179(5),149(20)	Ferulic acid dihexoside[G]	(Dias et al., 2016)	0.9 ± 0.1	nd	-
11	9.36	284	385	223(100),207(40),179(2),161(19),153(36),149(2)	Sinapic acid hexoside[E]	(Chahdoura et al., 2014)	nd	0.9 ± 0.1	-
12	10.36	322	179	161(5),159(4),135(100)	Caffeic acid [A]	DAD/MS, standard	nd	0.52 ± 0.01	-
13	14.31	272	787	635(12),617(14),483(3),465(4),447(5),423(20),313(2),271(10)	Tetragalloyl-glucose[C]	(Rached et al., 2016)	nd	2.4 ± 0.1	-
14	15.79	310	163	119(100)	*p*-Coumaric acid[B]	DAD/MS, standard	12.3 ± 0.4	nd	-
15	15.84	290	577	289(76),245(14),203(18)	Procyanidin dimer[F]	DAD/MS	nd	6.2 ± 0.1	-
16	17.71	352	609	301(100)	Quercetin-3-*O*-rutinoside (rutin)[D]	DAD/MS, standard	16.6 ± 0.1	48 ± 1	-
17	18.42	nd	447	301(100)	Quercetin-3-*O*-rhamnoside (quercitrin)[H]	(Mocan et al., 2014; Protti et al., 2017; Zhou et al., 2017)	tr	nd	-
18	18.84	355	463	301(100)	Quercetin-3-*O*-galactoside (hyperoside)[H]	(Qian et al., 2004; Donno et al.; 2016)	0.70 ± 0.01	nd	-
19	19.11	353	463	301(100)	Quercetin-3-*O*-glucoside (isoquercitrin)[H]	DAD/MS, standard	2.42 ± 0.04	nd	-
20	21.12	348	593	285(100)	Kaempferol-3-*O*-rutinoside[G]	DAD/MS, standard	tr	0.83 ± 0.01	-

Peak	Rt (min)	λmax (nm)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Fruits	Stems	*t*-Students test *p*-value
21	22.27	343	447	285(100)	Kaempferol-3-*O*-glucoside[G]	DAD/MS, standard	4.21 ± 0.04	nd	-
22	27.41	nd	431	285(100)	Kaempferol-rhamnoside[G]	MS	tr	nd	-
					Sum of phenolic acid		32.7 ± 0.8	17.2 ± 0.2	<0.001
					Sum of flavan-3-ols		10.4 ± 0.4	6.2 ± 0.1	<0.001
					Sum of flavonols		27.6 ± 0.1	48.5 ± 0.6	<0.001
					Sum of phenolic compounds		71 ± 1	71.9 ± 0.9	0.113

tr-trace amounts; nd- not detected. Standard calibration curves: A - caffeic acid ($y = 388345x + 406369$, R^2=0.9949); B - *p*-coumaric acid ($y = 301950x + 6966.7$, R^2=0.9999); C - chlorogenic acid ($y = 168823x – 161172$, R^2=0.9999); D - quercetin-3-*O*-rutinoside ($y = 13343x + 76751$, R^2=0.9998); E - sinapic acid ($y = 197337x + 30036$, R^2=0.9997); F - catechin ($y = 84950x – 23200$, R^2=0.9999); G - ferulic acid ($y = 633126x – 185462$, R^2=0.9999); H - quercetin-3-*O*-glucoside ($y = 34843x – 160173$, R^2=0.9998); G - kaempferol-3-*O*-rutinoside ($y = 11117x + 30861$; R^2=0.9998).

Benzoic (galloyl derivatives) and hydroxycinnamic acid derivatives (caffeic, *p*-coumaric, ferulic and sinapic acid derivatives), flavan-3-ols and flavonols (quercetin and kaempferol derivatives) were detected in the samples, but with different phenolic profiles in fruits and stems.

Sixteen compounds were identified in fruit samples: eight flavonols (peaks **6**, **16**, **17**, **18**, **19**, **20**, **21**, and **22**), seven phenolic acid derivatives (peaks **1**, **2**, **3**, **4**, **7**, **10**, and **14**), and one flavan-3-ol (peak **9**), while eleven compounds were detected in the stems, most of which were phenolic acid derivatives (peaks **2**, **4**, **5**, **7**, **8**, **11**, **12**, and **13**), together with two flavonols (peaks **16** and **20**) and one flavan-3-ol (peak **15**). Only three chlorogenic acids (peaks **2**, **4** and **7**) were common to both samples. Peaks **7**, **9**, **12**, **14**, **16**, **19**, and **20** (5-*O*-caffeoylquinic acid, catechin, caffeic acid, *p*-coumaric acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside, respectively) were identified by their UV and mass spectra, and retention characteristics in comparison with commercial standards. Compounds **19** and **20** had been previously reported by other authors in goji leaves (Mocan et al., 2017) and fruits (Bondia-Pons et al., 2014; Inbaraj, Lu, Kao, & Chen, 2010).

Flavonols were the most abundant phenolic compounds in goji stems, although mostly due to the presence of quercetin-3-*O*-rutinose (rutin, peak **16**), with minor levels of kaempferol-3-*O*-rutinose (peak **20**). The presence of rutin as a major flavonol in different parts of goji plants has been consistently reported by several authors (Affes et al., 2017; Bondia-Pons et al., 2014; Mocan et al., 2014, 2015a, 2015b, 2017; Protti et al., 2017; Qian, Liu, & Huang, 2004; Zhang, Chen, Zhao, & Xi, 2016). Flavonols were less abundant in the fruits, despite they presented greater variety of these compounds. Quercetin-3-*O*-glucoside (isoquercitrin, peak **19**) was positively identified by comparison with a standard. Peak **18** presented the same UV and mass spectral characteristics as compound **19 (**$[M-H]^-$ at *m/z* 463), thus corresponding to a quercetin hexoside, which was tentatively assigned as hyperoside (i.e., quercetin-3-*O*-galactoside), owing to the previous identification of both isoquercitrin and hyperoside in goji fruits (*Lycium* spp) by Qian et al. (2004) and Donno et al. (2015). This identity is also coherent with their chromatographic behaviour, as galactosides are expected to elute before their corresponding glucosides (Santos-Buelga et al., 2013). Peak **17** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 447 releasing an MS^2 fragment at *m/z* 301, allowing their identification as a quercetin-deoxyhexoside, tentatively associated to quercitrin (quercetin-3-*O*-rhamnoside), previously reported in goji samples (Mocan et al., 2014,

2015a; Protti et al., 2017; Zhou et al., 2017). Peak **6** showed a UV spectra characteristic of a quercetin derivative, and a pseudomolecular ion $[M-H]^-$ at *m/z* 933, yielding fragments at *m/z* 609 ($[M-H-324]^-$, loss of two hexosyl units) and *m/z* 301 ($[M-H-308]^-$, loss of a rutinosyl unit), being tentatively identified as quercetin-dihexoside-rutinoside. A compound with the same characteristics (rutin di-hexose) was reported in hydromethanolic extracts of goji fruits from Finland (Bondia-Pons et al., 2014). Other two flavonols derived from kaempferol were also detected in the fruits. As above indicated, peak **21** was identified as kaempferol-3-*O*-glucoside, a compound previously reported in goji fruits and leaves by Affes et al. (2017) and Mocan et al. (2017). Peak **22** was tentatively assigned as kaempferol-rhamnoside based on its pseudomolecular ion ($[M-H]^-$ at *m/z* 431) releasing a unique fragment at *m/z* 285, by analogy with the identifications made for quercetin glycosides.

Two flavan-3-ol derivatives were detected in the analysed samples and stems. Catechin (peak **9**) was positively identified in the fruit by comparison with a commercial standard, whereas peak **15**, found in the stems, was associated to a procyanidin dimer according to its UV spectrum, pseudomolecular ion ($[M-H]^-$ at *m/z* 577) and MS^2 fragments at *m/z* 289, 245 and 203.

The remaining compounds detected in goji samples corresponded to phenolic acid derivatives, most of them derivatives of hydroxycinnamic acids, which were the most abundant compounds in the fruits. Three chlorogenic acids, peaks **2**, **4** and **7** showing a pseudomolecular ion $[M-H]^-$ at *m/z* 353 yielding a main product ion at *m/z* 191 (deprotonated quinic acid), were identified as *cis* and *trans* 3-*O*-caffeoylquinic acids and *trans* 5-*O*-caffeoylquinic acid, respectively, based on the hierarchical keys previously described by Clifford et al. (2003, 2005). This type of compounds is among the most common phenolic compounds usually reported in goji samples, although authors generally do not indicate the particular derivative, but just refer to them as chlorogenic acid or isomers (Affes et al., 2017; Bondia-Pons et al., 2014; Donno et al., 2015; Mocan et al., 2014, 2015a, 2015b; Qian et al., 2004; Zhang et al., 2016; Zhou et al., 2017). Only Mocan et al. (2017) described the presence of different caffeoylquinic acids in the leaves of cultivated *L. barbarum* from Romania, with particular high contents of 3-*O*-caffeoylquinic acid. Inbaraj et al. (2010) also reported 3-*O*-caffeoylquinic acid in the fruits of *L. barbarum*, although in lower amounts than the ones reported in this paper.

Peak **14**, identified as *p*-coumaric acid by comparison with a standard, was the majority phenolic acid derivative in the fruits, whereas lower levels of caffeic acid (peak **12**) were

present in the stems. Other hydroxycinnamoyl derivatives detected in the samples were caftaric acid (peak **1**), previously described in the leaves of *L. barbarum* (Mocan et al., 2015a, 2015b), and different glycosides (peaks **3**, **8, 10** and **11**). Peak **3** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 487 releasing fragments at *m/z* 163 (-324 mu, loss of two hexosyl moieties) and 119, which is coherent with a *p*-coumaroyl acid dihexoside, as reported in goji fruits from Finland and Spain (Bondia-Pons et al., 2014); 6-*O*-trans-p-coumaroyl-2-*O*-glucopyranosyl-a-D-glucopyranoside was also recently identified by Zhou et al. (2017) in wolfberries from China. Similarly, peak **10**, with a pseudomolecular ion $[M-H]^-$ at *m/z* 517 and a main MS^2 product ion at *m/z* 193 from the loss of 324 mu, could be tentatively identified as a ferulic acid dihexoside. Peaks **8** and **11** presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 385 and an MS^2 fragment at *m/z* 223 (sinapic acid aglycone), corresponding to the loss of an hexosyl unit, so that they were tentatively identified as sinapic acid hexosides.

Finally, peaks **5** and **13** were associated to galloyl derivatives. The first one was identified as galloylquinic acid based on its pseudomolecular ion ($[M-H]^-$ at *m/z* 343) and the major MS^2 fragment at *m/z* 169 $[gallic\ acid-H]^-$, from the loss of quinic acid moiety (-152 mu). Peak **13** was assigned as tetragalloyl-glucose from its pseudomolecular ion $[M-H]^-$ at *m/z* 787 and fragment ions at *m/z* 635, 483, and 313 from the consecutive loss of three gallic acid units. The identification of both compounds was supported by their comparison with previously obtained data available in our compound library (Guimarães et al., 2013; Rached et al., 2016; Ferreira et al., 2013). To the authors' best knowledge, these compounds have not been previously cited in goji samples.

The total content of phenolic compounds did not show any statistically significant difference between fruits and stems goji samples. However, significant differences were found between samples when considering each family of phenolic compounds, being phenolic acid derivatives the majority compounds in the fruits (32.7 mg/g *vs* 17.2 mg/g in the stems) and flavonols in the stems (48.5 mg/g *vs* 27.6 mg/g in the fruits).

Quercetin-3-*O*-rutinoside was by far the major phenolic compound in stems (48 mg/g extract) and fruits (16.6 mg/g extract), followed in these latter by *p*-coumaric acid (12.3 mg/g extract). The differences between fruits and stems are exed by the clear difference in plant tissues. Although a greater amount of flavonols might be expected in the fruits, the obtained results could be exed by the edafoclimatic characteristics, degree of ripening and storage conditions (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012).

3.3.3. Bioactivities of fruit and stem hydromethanolic extracts

Data regarding the antioxidant, hepatotoxic and antibacterial activities are presented in **Table 25**.

The hydromethanolic extracts of *L. barbarum* stems showed the highest antioxidant activity in all assays: DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS inhibition (EC_{50} = 0.28, 0.23, 0.26, and 0.07 mg/mL, respectively). Similar results were reported by Liu et al. (2017) in ethanolic extracts of *L. barbarum* stems from Taiwan, namely DPPH scavenging activity and reducing power (0.102 and 0.167 mg/mL, respectively). On the other hand, Jabbar et al. (2014) reported lower EC_{50} values in methanolic extracts of goji fruits from China, regarding DPPH scavenging activity and reducing power (0.042 and 0.121 mg/mL, respectively), in comparison with the herein analysed hydromethanolic extract.

The antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule that would be strengthened by steric hindrance. The electron-withdrawing properties of the carboxylate group in benzoic acids has a negative influence on the H-donating abilities of the hydroxy benzoates. Hydroxylated cinnamates are more effective than benzoate counterpart (Rice-Evans, Miller, & Paganga, 1996). The presence of multiple hydroxyl groups in flavonoids and other phenolics structures gives a reducing character. In fact, it has been shown in in vitro assays that many of these compounds possess a strong antioxidant activity. This activity is particularly high, three to fourfold higher than in other flavonoids, in ortho-dihydroxy flavonoids – those containing a catechol group in their aromatic rings, such as flavonols or flavanol (Vicente & Boscaiu, 2018). In this way, the difference in the families of phenolic compounds present in the samples, namely phenolic acids for fruits and flavonols in the stems, could explain the greater antioxidant capacity of the stems.

As it can also be seen in **Table 25** good correlations were obtained between the different families of analysed phenolic compounds and the antioxidant activity ($r^2 < 0.8$).

Table 25. Antioxidant, hepatotoxic and antimicrobial activity of fruits and stems of *Lycium barbarum* L. (mean ± SD).

	Fruits	Stems	*t*-Students test *p*-value	Correlation factor r^2			
				Phenolic acids	Flavan-3-ols	Flavonols	Phenolic compounds
Antioxidant activity EC_{50} values (mg/mL)[A]							
DPPH scavenging activity	6.25 ± 0.2	0.28 ± 0.02	<0.001	0.998	0.996	0.999	0.880
Reducing power	1.09 ± 0.02	0.23 ± 0.01	<0.001	0.999	0.997	0.999	0.880
β-carotene bleaching inhibition	1.9 ± 0.3	0.26 ± 0.02	<0.001	0.973	0.971	0.974	0.857
TBARS inhibition	3.9 ± 0.2	0.07 ± 0.02	<0.001	0.995	0.993	0.996	0.877
Hepatotoxicity GI_{50} values (µg/mL)[B]							
PLP2	>400	>400	-	-	-	-	-
Antimicrobial activity MIC values (mg/mL)							
Gram-negative bacteria							
Acinetobacter baumannii	>20	20	-	-	-	-	-
Escherichia coli ESBL 1	5	5	-	-	-	-	-
Escherichia coli ESBL 2	5	10	-	0.999	0.997	0.999	0.880
Escherichia coli	2.5	2.5	-	-	-	-	-
Klebsiella pneumoniae	20	10	-	0.999	0.997	0.999	0.880
Klebsiella pneumoniae ESBL	20	20	-	-	-	-	-
Morganella morganii	5	5	-	-	-	-	-
Pseudomonas aeruginosa	20	10	-	0.999	0.997	0.999	0.880

	Fruits	**Stems**	***t*-Students test *p*-value**	**Correlation factor r^2**			
Gram-positive bacteria				**Phenolic acids**	**Flavan-3-ols**	**Flavonols**	**Phenolic compounds**
Enterococcus faecalis	2.5	10	-	0.999	0.997	0.999	0.880
Listeria monocytogenes	5	5	-	-	-	-	-
Staphylococcus aureus	2.5	2.5	-	-	-	-	-
MRSA	2.5	5	-	0.999	0.997	0.999	0.880
MSSA	2.5	10	-	0.999	0.997	0.999	0.880

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. A- Trolox EC_{50} values: 43.03 ± 1.71 μg/mL (DDPH), 29.62 ± 3.15 μg/mL (reducing power), 2.63 ± 0.14 μg/mL (β-carotene bleaching inhibition) and 3.73 ± 1.9 μg/mL (TBARS inhibition); B - Ellipticine GI_{50} values: 2.29 mg/mL (PLP2). MIC values correspond to the minimal extract concentration that inhibited the bacterial growth. ESBL - extended spectrum β-lactamases. MRSA - Methicillin-resistant *Staphylococcus aureus*. MSSA - Methicillin-susceptible *Staphylococcus aureus*.

Regarding antibacterial activity (**Table 25)**, both samples showed better results against Gram-positive than against Gram-negative bacteria, with MIC values ranging between 2.5 and 10 mg/mL. The lowest MIC values were determined for *E. faecalis* (2.5 mg/mL), *L. monocytogenes* (5 mg/mL), *S. aureus* (2.5 mg/mL), MRSA (2.5 mg/mL), and MSSA (2.5 mg/mL). As for Gram-negative bacteria, the stems presented higher activity against *A. baumannii* (20 mg/mL), *K. pneumonia* (10 mg/mL), and *P. aeruginosa* (10 mg/mL). Mocan et al. (2015b; 2017) reported lower MIC values in goji leaves and flowers against both Gram-negative and positive bacteria. A possible explanation could be that the bacteria used by those authors were ATCC (with no resistance profile), so that lower concentrations of extracts could be needed to inhibit the growth of the bacterial strains.

Good correlation coefficients between phenolic compounds, either flavonoids or chlorogenic acids, and antibacterial activities were obtained for *Escherichia coli* ESBL 2, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*.

The antimicrobial activity of polyphenols has been attributed to their structural features, as well as pH and sodium chloride concentration, resulting in physiological changes in the microorganisms and eventual cell death (Kabir et al., 2014). Studies carried out by Kabir et al. (2014) showed that chlorogenic acid and related compounds exhibit potent antimicrobial activities, and a synergistic effect between compounds. Flavonoids are also known to be synthesized by plants in response to microbial infection. Their activity is probably due to their ability to complex with extracellular and soluble proteins; lipophilic flavonoids may also disrupt the microbial membrane (Cushnie & Lamb, 2005; Kabir et al., 2014).

Neither fruits nor stems revealed toxicity towards a porcine liver primary culture (PLP2).

3.4. Bilberry fruits as a source of natural colorants: pigment characterization and incorporation in yogurts

3.4.1. Identification and quantification of anthocyanins in a bilberry extract

The anthocyanin profile of the extract obtained from bilberry fruits is presented in **Table 26**. Eleven anthocyanin glycosides were detected derived from delphinidin (Dp; peaks **1**, **2** and **4**), cyanidin (Cy; peaks **3** and **6**), petunidin (Pt; peaks **5** and **8**), peonidin (Pn; peaks **7** and **10**), and malvidin (Mv; peaks **9** and **11**). Compounds were identified based on their chromatographic behaviour and absorption and mass spectra. Peaks presented MS^2 fragments corresponding to distinct losses of hexosyl (-162 u) and pentosyl (-132 u) moieties, identified as galactosides, glucosides and arabinoside, as previously described for bilberry anthocyanins (Aaby, Grimmer, & Holtung, 2013; Colak et al., 2017; Paes, Dotta, Barbero, & Martínez, 2014). The nature of the sugar moieties in the different anthocyanins was assigned taking into account their elution behaviour according to the expected polarity (Santos-Buelga et al., 2003). Compounds identities were further supported by comparison with data in the literature (Aaby, Grimmer, & Holtung, 2013; Colak et al., 2017; Paes, Dotta, Barbero, & Martínez, 2014), as well as by comparison with available commercial standards and data from our compound library.

Malvidin glycosides, especially Mv-3-*O*-galactoside and Mv-3-*O*-arabinoside, were the main compounds present, comprising 48% of the total anthocyanin content, whereas Dp derivatives (Dp-galactoside, Dp-glucoside and Dp-arabinoside) represented 22%, and Pt 20% (Pt-galactoside and Pt-arabinoside).

Table 26. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of the anthocyanins present in the hydromethanolic extracts of bilberry fruits.

Peak	Rt (min)	λmax (nm)	$[M]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g of extract)
1	14.4	524	465	303(100)	Delphinidin-3-*O*-galactoside[A]	2.65 ± 0.01
2	15.9	526	465	303(100)	Delphinidin-3-*O*-glucoside[A]	0.162 ± 0.001
3	16.9	518	449	287(100)	Cyanidin-3-*O*-galactoside[B]	0.62 ± 0.02
4	17.7	524	435	303(100)	Delphinidin-3-*O*-arabinoside[A]	1.91 ± 0.02
5	19.2	528	479	317(100)	Petunidin-3-*O*-galactoside[C]	2.50 ± 0.02
6	20.2	514	419	287(100)	Cyanidin-3-*O*-arabinoside[B]	0.464 ± 0.004
7	21.7	518	463	301(100)	Peonidin-3-*O*-galactoside[D]	0.45 ± 0.01
8	22.6	516	449	317(100)	Petunidin-3-*O*-arabinoside[C]	1.67 ± 0.03
9	22.8	528	493	331(100)	Malvidin-3-*O*-galactoside[C]	5.64 ± 0.05
10	26.1	522	433	301(100)	Peonidin-3-*O*-arabinoside[D]	0.550 ± 0.004
11	28.9	528	463	331(100)	Malvidin-3-*O*-arabinoside[C]	4.45 ± 0.02
					Total anthocyanins	**21.1 ± 0.2**

Standard calibration curves used for quantification: A- delphinidin-3-*O*-glucoside (y=557274x+126.24, R^2=0.999); B- cyanidin-3-*O*-glucoside (y=630276x–153.83, R^2=0.999); C- malvidin-3-*O*-glucoside (y=477014.9x+38.376, R^2=0.999); and D - peonidin 3-*O*-glucoside (y=537017x–71.469, R^2=0.999).

3.4.2. Nutritional profile of yogurt formulations

The main objective of analysing the nutritional profile was to verify if any change took place along the seven days storage at 5 ºC in the different yogurt formulations. Since two different factors were contributing for results variability, a 2-way ANOVA was used, allowing to evaluate the effect of each factor, independently of the others. **Table 27** shows the nutritional profile of each yogurt formulation (plain, E163, and bilberry extract) at the two times of storage (0 and 7 days). The nutrient values indicated in each part of the table correspond respectively to the means of each storage time (ST) including the three incorporation types (IT), and *vice-versa*, therefore the standard deviation values should not be regarded as an accuracy measure. Besides the effect of each individual factor, the significance of their interaction (ST×IT) was also evaluated. If a significant interaction was found, the classification obtained for multiple comparisons could not be observed, since the effect of each factor was not equal for all levels of the other. In those cases, the presented general tendencies were obtained from the Estimated Marginal Means (EMM) plots. Inversely, if no interaction was found ($p > 0.050$), the factor was classified individually using either Tukey's or Tamhane T2 tests (depending on the homoscedasticity of the distribution), for IT, and a Student's t-test for ST.

Yogurts presented a moisture content above 85 g/100 g and approximately the same levels (~5 g/100 g) of proteins and carbohydrates, with slightly lower (~3.3 g/100 g) fat content, corresponding to energy values around 70 kcal/100 g, validating the labelled information, and showing to be consistent with the typical nutritional composition of yogurt (Van Nieuwenhove et al., 2019). Storage time (ST) and incorporation types (IT) did not show to exert a cooperative effect over fat content, which was significantly higher in yogurts containing E163 and in stored samples. In turn, the interaction among factors (ST×IT) was significant ($p<0.050$) for all other parameters in **Table 27**.

Nonetheless, some conclusions could be obtained from the estimated marginal means corresponding to the combined results of these parameters, particularly the higher energy values in samples at 7 days (73±2 kcal/100 g fw), as it seems logical owing to the moisture loss during storage. The ash content was significantly higher in yogurts containing E163 (1.0±0.2 g/100 g fw) and the bilberry extract, indicating that minerals were incorporated as a part of the composition of those additives. Despite the mathematical significance of these differences, all yogurt samples showed very slight nutritional differences. This fact should be expected as food additives are added to food for a technological goal and should

not change in any way the nutritional and chemical aspects of food, except the cases it is intended for.

3.4.3. Individual compound analysis of yogurt formulations

Table 28 shows the results obtained for some individual compounds. The fatty acids present in highest percentages were palmitic acid: C16:0 (~35%), oleic acid: C18:1n9 (~21%), myristic acid: C14:0 (~12%), and stearic acid: C18:0 (~11%), which is agreement with the results reported in a similar work (Van Nieuwenhove et al., 2019). As expected, lactose constitutes the main sugar.

In line with the results obtained in the nutritional analysis, the differences obtained for each of the studied individual compounds reflected the significant interaction of both factors (ST and IT), except in the case of lactose, which showed statistically higher contents (despite its low magnitude) in yogurts added with bilberry. Accordingly, the few overall tendencies had to be obtained from the EMM plots, which indicated lower C18:0 percentages and galactose contents in plain yogurts.

Table 27. Nutritional profile of the yogurt samples as a function of the added colorant and the storage time expressed in g/100 g of fresh weight, and energy in kcal/100 g of fresh weight.

		Moisture	Fat	Proteins	Ash	Carbohydrates	Energy
Storage time (ST)	0 days	85.8±0.3	3.2±0.2	5.2±0.2	0.8±0.1	5.0±0.3	70±2
	7 days	85.3±0.1	3.4±0.2	5.3±0.2	0.7±0.4	5.3±0.4	73±2
***p*-value (n = 27)**	Student's t-test	<0.001	<0.001	0.139	0.569	0.033	<0.001
Incorporation Type (IT)	Plain yogurt	85.7±0.5	3.3±0.1[b]	5.3±0.3	0.5±0.2	5.2±0.5	72±4
	E163	85.3±0.1	3.5±0.1[a]	5.3±0.3	1.0±0.2	4.9±0.1	72±1
	Bilberry	85.5±0.3	3.1±0.2[c]	5.1±0.1	0.9±0.2	5.4±0.3	70±1
***p*-value (n = 18)**	Tukey's HSD test	0.010	<0.001	0.003	<0.001	<0.001	0.069
ST×IT (n = 54)	*p*-value	<0.001	0.166	<0.001	<0.001	<0.001	<0.001

In each row and within each storage period, different letters mean significant statistical differences between plain yogurts, yogurts incorporated with E163 and yogurts incorporated with bilberry extract ($p<0.05$)

Table 28. Individual fatty acids, expressed as relative percentages, and sugars contents (g/100 g fw) in the yogurt samples as a function of the added colorant and the storage time

		C14:0	C16:0	C18:0	C18:1n9	SFA	MUFA	PUFA	Galactose	Lactose
Storage time (ST)	0 days	12.0±0.5	35.0±0.5	11.3±0.4	21±1	71±1	24±1	4.8±0.5	0.6±0.1	4.1±0.2
	7 days	12.2±0.2	35.8±0.2	11.3±0.1	20±1	73±1	23±1	4.8±0.2	0.6±0.1	4.1±0.3
***p*-value (n = 27)**	Student's t-test	0.190	<0.001	0.451	0.001	<0.001	<0.001	0.916	0.465	0.985
Incorporation Type (IT)	Plain yogurt	11.9±0.4	35.2±0.5	11.0±0.2	21±1	72±1	23±1	5.0±0.1	0.5±0.1	3.9±0.1[b]
	E163	12.0±0.3	35.2±0.5	11.3±0.1	21±1	72±1	23±1	5.2±0.5	0.7±0.1	4.0±0.1[b]
	Bilberry	12.5±0.5	35.8±0.4	11.6±0.3	20±1	73±1	23±1	4.1±0.4	0.6±0.1	4.4±0.3[a]
***p*-value (n = 18)**	Tukey's HSD test	<0.001	0.004	<0.001	0.240	0.119	0.244	<0.001	<0.001	<0.001
ST×IT (n = 54)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.748

In each row and within each storage period, different letters mean significant statistical differences between plain yogurts, yogurts incorporated with E163 and yogurts incorporated with bilberry extract ($p<0.05$).

3.4.4. External color and pH of yogurt formulations

Owing to the incorporation of the colouring materials, the main differences were expected to be observed for colour parameters. Besides the Cartesian coordinates (L*, a* and b*), the cylindrical coordinates (C* and h) were also obtained; C*, which stands for chroma (related to saturation), was calculated as $\sqrt{a^{*2} + b^{*2}}$, while the formula used to calculate the hue angle (h) depended on the values of a* and b*, *e.g.*, if a* and b* were negative, the formula was $180+\left(\arctan\frac{\frac{b^*}{a^*}}{2\pi}\right)*360$.

As depicted in **Table 29**, all parameters showed significant differences among different yogurt formulations, while the storage time had hardly effect, but for L* values, which were slightly lower in samples at day 0. Despite the significant effect of IT, the statistical classification results could not be presented, since the interaction among factors was statistically significant in all cases. Nonetheless, the EMM plots allowed concluding that L*, b*, and C* were higher in plain yogurt samples, which in turn showed lower h and, in particular, a* values, as it is logical taking into account that they lacked the red color existing in the samples added the colorants.

Figure 21. Variation of the yogurt colors during the storage time.

A visual representation of the yogurt color is shown in **Figure 21**, where it is clear that both colorants provided color to the yogurts, although there was a lower color intensity for the E163 yogurts, particularly at day 7 and mostly due to a decrease in the values of the a* component (**Figure 22**).

This is corroborated by the calculated ΔE (total color difference), which after 7 days reached a value of 3.86 for the E163 yogurts compared to day 0, while the difference for bilberry colored yogurts was 0.91, very similar to the plain yogurt sample variation of 0.73. This proves the higher stability of the bilberry extract and its viability as a food colorant for yogurt. Similar results of enhanced stability of colorants from natural sources were reported by Nontasan et al. (2012) who found stable L* and C* values in yogurts added with black rice bran for 21 days under 4 ºC storage, and by Mouhammadi-Gouraji et al. (2019), following addition of phycocyanin extracted from *Spirulina platensis* in similar storage conditions (Mohammadi-Gouraji, Soleimanian-Zad, & Ghiaci, 2019; Nontasan, Moongngarm, & Deeseenthum, 2012). This trend of incorporation of natural colorants in yogurts, not only with pigments from fruits but also flowers and other plant parts, seems to have gained traction, having good consumer acceptance due to a higher correlation in the color-flavor perception. (Freitas-Sá et al., 2018; Gomes, Petito, Costa, Falcão, & De Lima Araújo, 2014)

In what concerns the pH values, they were statistically different for each IT, with yogurts containing E163 presenting the less acidic values. On the other hand, ST had no effect at all in pH values, independently of IT.

Table 29. pH values and external color profile of the yogurts as a function of the added colorant and along the storage time expressed as L*, a*, b*, C* and h.

		L*	a*	b*	C*	h	pH
Storage time (ST)	0 days	89±3	1±3	7±2	7±2	199±65	4.6±0.2
	7 days	91±2	0±2	8±1	8±1	208±70	4.6±0.2
***p*-value (n = 27)**	Student's t-test	0.030	0.325	0.179	0.602	0.636	0.365
Incorporation Type (IT)	Plain yogurt	93±1	-3.5±0.1	9.9±0.4	10.5±0.4	110±1	4.4±0.1
	E163	89±1	3.1±0.5	6.4±0.5	7.3±0.3	244±12	4.9±0.1
	Bilberry	88±1	1.5±0.3	6.2±0.5	6.4±0.5	256±2	4.6±0.1
***p*-value (n = 18)**	Tukey's HSD test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×IT (n = 54)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.006

In each row and within each storage period, different letters mean significant statistical differences between plain yogurts, yogurts incorporated with E163 and yogurts incorporated with bilberry extract ($p<0.05$)

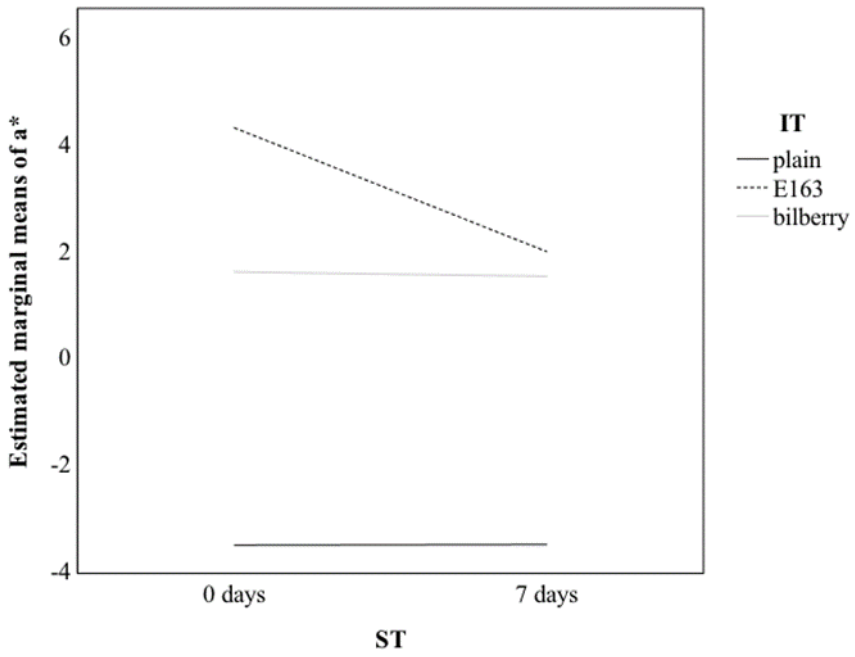


Figure 22. Estimated marginal mean plots of a* in the yogurt formulations along storage time.

3.4.5. Principal component analysis

This analysis was performed to verify the variables with the highest differences among each assayed IT. The first two defined dimensions (first: Cronbach's α = 0.920, eigenvalue = 8.232, explained variance = 47.4%; second: Cronbach's α = 0.871, eigenvalue = 5.940, explained variance = 37.0%) are plotted in **Figure 23**.

As it can be observed, three main groups were formed, two groups corresponding to plain yogurt markers and a big third group containing markers corresponding to yogurts added with E163 or added with bilberry extracts. In an initial analytical approach, it is easily to observe that markers corresponding to plain yogurts and those added with E163 or bilberry extracts were basically separated by dimension 1. Considering the variables placement, it is also straightforward that color parameters were the ones with highest contribution for this separation. Specifically, plain yogurts were mainly characterized by their high L*, b* and C* values, while yogurts containing coloring agents present high a* and h as the most distinguishable features.

In turn, the second dimension separated two distinct groups of markers, both belonging to plain yogurts. According to the SPSS output, these two groups correspond exactly to the two assayed periods: 0 days and seven days, which indicates that this type of yogurts did not maintain their characteristics throughout time. The bottom left group includes the 0 days markers (characteristically containing high moisture, C18:1n9 and MUFA

contents), while the upper left group contains the markers corresponding to plain yogurts stored for 7 days (high fat, protein and energy contents).

Nonetheless, in what concerns yogurts added with E163 or bilberry extracts, there was no separation among non-stored and stored samples, which is a solid evidence of the stability provided by both additives. Despite the resemblance among yogurts added with E163 or bilberry extract, the markers corresponding to this last IT were generally placed on the right of those added with E163, which is an overall indicator of a higher coloring effectiveness in the case of bilberry extracts (closest location to the a* variable).

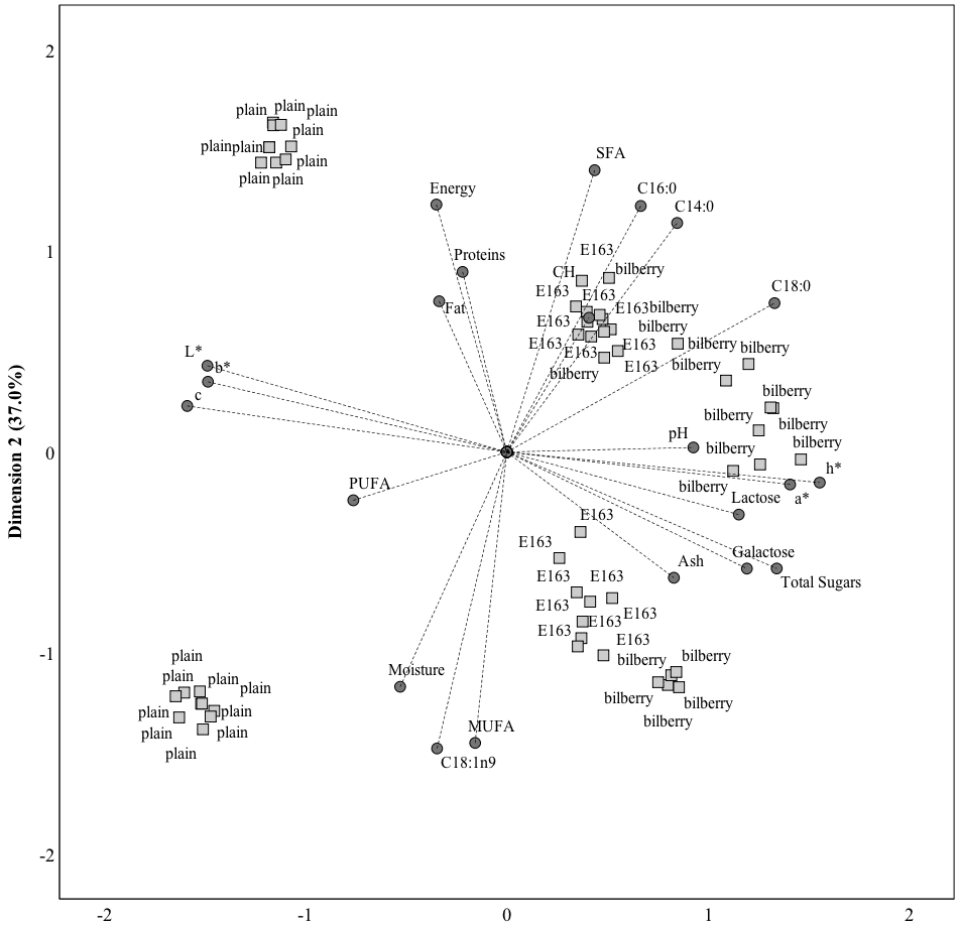


Figure 23. Canonical discriminant functions coefficients defined from the evaluated parameters and plotted to highlight differences among incorporation types.

3.5. Nutritional, chemical and bioactive features of new *Vaccinium myrtillus* L. based snacks

3.5.1. Proximate composition and chemical characterization

The main objective of this study was to fully characterize three bilberry-based mixtures combined with rose flowers (P1), calendula flowers (P2), dehydrated apple and goji fruits (P3), comparing all the results with a plain sample containing only bilberry fruits (P0). Rose and calendula flowers, dehydrated apple and goji berries samples have already been studied individually in previous sections of this Thesis.

Data regarding the nutritional composition, fatty acids, soluble sugars, organic acids and tocopherols contents are shown in **Table 30**.

Overall, despite the existence of some significant statistical differences between the four samples, the composition profiles were very similar, except for tocopherols content. As expected, carbohydrates were the most abundant macronutrient in all samples, ranging from 94.32 to 94.80 g/100 g dw, followed by protein (2.6 ± 0.5 to 3.7 ± 0.6 g/100 g dw), ash (1.3 ± 0.4 to 1.6 ± 0.1 g/100 g dw) and fat (0.7 ± 0.04 to 1.1 ± 0.1 g/100 g dw).

Regarding sugars, fructose, glucose and sucrose were detected in all samples, being fructose the most abundant one (29.2 ± 0.4 to 36.4 ± 0.7 g/100 g dw). Similar values were reported by Mikulic-Petkovsek et al. (2015) in fruits of *V. myrtillus*, where fructose was the most abundant sugar. The addition of the different components did not induce significant differences in sugar contents in mixtures P1 and P2 when compared to P0, but significant lower contents were found in P3 (added with apple and goji fruits), which could be attributed to the lower proportion of bilberry in P3 sample.

Table 30. Nutritional values, fatty acids profile, soluble sugars, organic acids and tocopherols of the four prepared snacks: bilberry fruits (P0), combined with rose flowers (P1), calendula flowers (P2) and apple and goji berries (P3) (mean ± SD).

	P0	P1	P2	P3
Nutritional value (g/100 g dw)				
Fat	1.1 ± 0.3^{a}	1.1 ± 0.2^{a}	0.8 ± 0.1^{b}	0.70 ± 0.04^{c}
Proteins	3.0 ± 0.4^{c}	3.7 ± 0.6^{a}	3.0 ± 0.2^{b}	2.6 ± 0.5^{d}
Ash	1.6 ± 0.1^{a}	1.3 ± 0.4^{c}	1.5 ± 0.1^{a}	1.5 ± 0.2^{a}
Total available carbohydrates	94.6 ± 0.3^{c}	94.3 ± 0.4^{c}	94.57 ± 0.04^{b}	94.8 ± 0.2^{a}
Energy (kcal/100g)	399 ± 1^{a}	399.9 ± 0.4^{a}	398.1 ± 0.6^{b}	397.5 ± 0.4^{b}
Fatty acids (relative percentage, %)				
C16:0	4.7 ± 0.1^{c}	5.4 ± 0.2^{b}	5.3 ± 0.6^{b}	7 ± 1^{a}
C18:0	2.14 ± 0.01^{b}	2.4 ± 0.2^{a}	2.1 ± 0.3^{b}	2.3 ± 0.1^{a}
C18:1n9	15.71 ± 0.01^{a}	15.2 ± 0.2^{bc}	15.3 ± 0.3^{b}	15.0 ± 0.2^{c}
C18:2n6	42.1 ± 0.2^{a}	40.8 ± 0.3^{b}	40.8 ± 0.8^{b}	41.0 ± 0.3^{b}
C18:3n3	32.9 ± 0.2^{a}	32.0 ± 0.8^{b}	32.1 ± 0.6^{b}	30.9 ± 0.4^{c}
SFA	8.8 ± 0.1^{c}	10.2 ± 0.7^{b}	10 ± 1^{b}	11.4 ± 0.7^{a}
MUFA	16.00 ± 0.01^{a}	15.7 ± 0.1^{b}	15.9 ± 0.3^{a}	15.7 ± 0.2^{b}
PUFA	75.3 ± 0.1^{a}	74.1 ± 0.9^{b}	74 ± 1^{b}	72.9 ± 0.9^{c}
Soluble sugars (g/100 g dw)				
Fructose	36.4 ± 0.7^{a}	35.8 ± 0.7^{a}	36.4 ± 0.7^{a}	29.2 ± 0.4^{b}
Glucose	29.6 ± 0.6^{a}	29.1 ± 0.6^{a}	29.6 ± 0.6^{a}	21.4 ± 0.5^{b}
Sucrose	2.52 ± 0.01^{b}	2.52 ± 0.003^{b}	2.53 ± 0.01^{a}	2.04 ± 0.002^{c}
Sum	69 ± 1^{a}	67 ± 1^{a}	69 ± 1^{a}	52.6 ± 0.9^{b}
Organic acids (g/100 g dw)				
Oxalic acid	0.080 ± 0.002^{a}	0.075 ± 0.001^{b}	0.067 ± 0.001^{c}	0.04 ± 0.01^{d}
Quinic acid	0.31 ± 0.01^{a}	0.21 ± 0.03^{c}	0.31 ± 0.02^{a}	0.28 ± 0.01^{b}
Malic acid	nd	0.07 ± 0.02^{b}	0.057 ± 0.003^{c}	0.44 ± 0.02^{a}
Shikimic acid	0.003 ± 0.001^{b}	0.002 ± 0.001^{c}	0.003 ± 0.001^{a}	tr
Citric acid	2.8 ± 0.1^{b}	2.945 ± 0.001^{a}	2.94 ± 0.01^{c}	1.90 ± 0.04^{c}
Fumaric acid	tr	tr	tr	tr
Sum	3.15 ± 0.04^{c}	3.30 ± 0.05^{b}	3.37 ± 0.01^{a}	2.67 ± 0.01^{d}
Tocopherols (mg/100 g dw)				
α-Tocopherol	1.8 ± 0.1^{c}	2.0 ± 0.1^{b}	4.74 ± 0.05^{a}	1.31 ± 0.08^{d}
β-Tocopherol	nd	0.004 ± 0.001^{b}	0.070± 0.003^{a}	nd
γ-Tocopherol	1.185 ± 0.004^{b}	1.176 ± 0.004^{b}	1.49 ± 0.05^{a}	0.715 ± 0.003^{c}
δ-Tocopherol	nd	0.0030 ± 0.0001	nd	nd
Sum	3.04 ± 0.12^{c}	3.2 ± 0.1^{b}	6.301 ± 0.003^{a}	2.02 ± 0.08^{d}

The results are expressed on fresh weight basis, dw- dry weight basis; nd- not detected C10:0- Capric acid; C12:0- Lauric acid; C14:0- Myristic acid; C14:1 - Myristoleic acid; C15:0- Pentadecanoic acid; C16:0- Palmitic acid; C16:1 - Palmitoleic acid; C17:0 - Heptadecanoic acid; C18:0 - Stearic acid; C18:1n9- Oleic acid; C18:2n6- Linoleic acid; C18:3n3- Linolenic acid; C20:0- Arachidic acid; C20:1-Eicosenoic acid; C20:2- Eicosadienoic acid; C20:3n6 - Eicosatrienoic acid; C20:4n6- Arachidonic acid; C20:3n3- Eicosatrienoic acid; C20:5n3- Eicosapentaenoic acid; C22:0 - Behenic acid; C22:1n9- Erucic acid; C22:2- Docosadienoic acid; C23:0 - Tricosanoic acid; C24:0 - Lignoceric acid. SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids. In each row different letters mean significant differences between samples ($p<0.05$), where "a" and "d" correspond to the highest and lowest values, respectively.

The profile in organic acids was similar in all samples, being identified six main compounds. The main organic acid found was citric, followed by quinic acid; fumaric acid was in trace amounts in all four samples. Malic acid was not found in P0 sample, contrary to what happens in the other three samples. This was an expectable result, since malic acid was previously found in rose and calendula flowers (P1 and P2, respectively, and apple (P3) samples. As for sugars, significant lower organic acid content was found in P3 compared to P0 and the other mixtures.

Twenty-four fatty acids were identified, being polyunsaturated fatty acids (PUFA) predominant in all samples. To facilitate data analysis, only compounds with percentages higher than 2% are presented in **Table 30**. Linoleic acid (C18:2n6) was the major fatty acid found, followed by linolenic acid (C18:3n3) and oleic acid (C18:1n9). Comparing the results obtained for the fatty acids between the individual samples previously studied and the mixtures now analyzed, it was expected that there would be a higher concentration of linoleic acid in the mixtures containing rose and goji, α-linolenic and palmitic acid in mixtures with calendula and apple, respectively. However, the amounts of rose and calendula flowers, goji or apple in the mixtures seemed not sufficient to cause relevant differences in fatty acid distribution in P1, P2, and P3 when compared to P0.

Regarding tocopherols, significant differences among the four samples were determined, probably due to their perishability and the different profiles of the individual components. α-Tocopherol was the most abundant isoform in all samples (ranging from 1.31 ± 0.08 to 4.74 ± 0.05 mg/100 g dw). P1 was the only sample that presented the four isoforms in its composition due to their presence in rose flowers, as previously described in **section 3.1.1** Similarly happens in P2 for the presence of β-tocopherol (absent in P0 sample) and the significant increase in the amount of α-tocopherol, due to the presence of calendula flowers. It was also in P2 samples that it was observed the highest amount of total tocopherol content (6.301 mg/100 g dw), once again attributed to the presence of calendula edible flowers. The incoporation of apple and goji fruits in P3 mixtures had no influence on the tocopherols profile, although there was a decrease in their total content

(2.02 ± 0.08 mg/100 g dw) compared to P0 (3.04 ± 0.12 mg/100 g dw), which could be newly associated to the lower proportion of bilberry in this sample.

3.5.2. Non-anthocyanin and anthocyanin phenolic profiles

Data obtained from the HPLC-DAD-ESI/MS analysis of the non-anthocyanin phenolic compounds in the hydromethanolic extracts of the four different mixtures are presented in **Table 31**. Twenty-four different phenolic compounds were found, from which ten phenolic acids derivatives (peaks **1**^{NA} to **10**^{NA}) and fourteen flavonoids (peaks **11**^{NA} to **24**^{NA}), including thirteen flavonol and one flavanone glycosides.

Flavonols were the major class of phenolic compounds found in bilberry fruits, derived from quercetin isorhamnetin, kaempferol and myricetin. Peaks **11**^{NA} (myricetin-3-*O*-glucoside), **14**^{NA} (quercetin-3-*O*-rutinoside), **15**^{NA} (quercetin-3-*O*-glucoside), **18**^{NA} (kaempferol-3-*O*-rutinoside), **19**^{NA} (isorhamnetin-3-*O*-rutinoside), and **21**^{NA} (isorhamnetin-3-*O*-glucoside) were positively identified from their elution time (Rt) and UV and mass spectra in comparison to commercial standards.

Peak **13**^{NA} presented similar characteristics as peak **14**^{NA} **(**[M-H]$^-$ at *m/z* 609) but a different Rt, so that it was tentatively assigned as a quercetin-*O*-deoxyhexoside-hexoside. Peak **16**^{NA} ([M-H]$^-$ at *m/z* 463) and peak **20**^{NA} ([M-H]$^-$ at *m/z* 447) presented the aglycone fragment of quercetin (*m/z* at 301) from the losses of an hexosyl and deoxyhexosyl moieties**.** Taking into account the previously compounds reported in *V. myrtillus* (Mikulic-Petkovsek et al., 2012b; Vhrosek, Masuero, Palmieri, & Mattivi, 2012; Diaconeasa, Florica, Rugină, Lucian, & Socaciu, 2014), they were tentatively assigned as quercetin-3-*O*-galactoside (compound **16**^{NA}) and quercetin-3-*O*-rhamnoside (**20**^{NA}**)**. Similarly, peak **23**^{NA} ([M-H]$^-$ at *m/z* 431) revealed a unique MS^2 fragment at *m/z* 285, corresponding to the loss of a deoxyhexosyl moiety, being tentatively identified as kaempferol-*O*-deoxyhexoside. The *O*-methylated form of quercetin (isorhamnetin) was also found abundantly in all four samples. Peaks **12**^{NA} ([M-H]$^-$ at *m/z* 623), **22**^{NA} ([M-H]$^-$ at *m/z* 461), and **24**^{NA} ([M-H]$^-$ at *m/z* 491) presented a unique MS^2 fragment at *m/z* 315 (isorhamnetin aglycone), being tentatively identified as isorhamnetin-*O*-deoxyhexosyl-hexoside**,** isorhamnetin-*O*-deoxyhexoside and isorhamnetin-*O*-glucuronide, respectively. The presence of isorhamnetin-3-*O*-rutinoside and isorhamnetin-3-*O*-glucuronide, which may correspond to peaks **12**^{NA} and **24**^{NA}, has been reported by Mikulic-Petkovsek et al. (2012b) in different berries.

Table 31. Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, tentative identification and quantification (mg/g of extract) of non-anthocyanin phenolic compounds present in the hydromethanolic extracts of the four of the four snack mixtures (P0, P1, P2, and P3).

Peak	Rt (min)	λmax (nm)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g of extract)			
						P0	P1	P2	P3
1NA	4.81	263	359	197(100),153(5),135(5)	Syringic acid hexoside$^{(A)}$	0.89 ± 0.01^b	0.84 ± 0.01^c	1.44 ± 0.04^a	0.059 ± 0.001^d
2NA	5.4	330	341	179(100),161(5),135(5)	Caffeic acid hexoside$^{(B)}$	4.347 ± 0.003^b	3.99 ± 0.04^c	5.3 ± 0.1^a	0.229 ± 0.001^d
3NA	5.69	310	325	163(35),119(5)	*p*-Coumaric acid hexoside$^{(C)}$	1.3 ± 0.1^b	1.16 ± 0.01^c	2.49 ± 0.01^a	0.179 ± 0.001^d
4NA	6.67	314	355	193(100),179(5),149(5)	Ferulic acid hexoside$^{(D)}$	3.5 ± 0.1^b	2.9 ± 0.1^c	4.3 ± 0.1^a	0.204 ± 0.002^d
5NA	6.88	314	341	179(100),161(5),135(9)	Caffeic acid hexoside$^{(B)}$	6.8 ± 0.1^b	5.63 ± 0.06^c	8.3 ± 0.2^a	0.24 ± 0.01^d
6NA	7.36	326	353	191(100),179(8),161(5),135(5)	*cis* 5-*O*-Caffeoylquinic acid$^{(E)}$	34 ± 1^b	28.3 ± 0.3^c	39 ± 1^a	1.94 ± 0.04^d
7NA	8.18	295	355	193(100),149(5)	Ferulic acid hexoside$^{(D)}$	0.859 ± 0.001^b	0.80 ± 0.01^c	1.12 ± 0.01^a	0.21 ± 0.01^d
8NA	8.91	320	353	191(100),179(8),161(5),135(5)	*trans* 5-*O*-Caffeoylquinic acid$^{(E)}$	2.51 ± 0.02^b	3.58 ± 0.04^c	4.4 ± 0.2^a	0.414 ± 0.001^d
9NA	9.12	327	385	223(100),205(61),161(32),153(58)	Sinapic acid hexoside$^{(F)}$	2.15 ± 0.01^b	1.7 ± 0.1^c	2.72 ± 0.01^a	tr
10NA	10.35	321	179	135(100)	Caffeic acid$^{(B)}$	0.84 ± 0.02^b	0.52 ± 0.01^c	0.92 ± 0.01^a	tr
11NA	15.22	350	479	317(100)	Myricetin-3-*O*-glucoside$^{(G)}$	2.5 ± 0.1^c	1.82 ± 0.02^d	2.7 ± 0.1^b	4.3 ± 0.1^a
12NA	17.13	351	623	315(100)	Isorhamnetin-*O*-deoxyhexoside-hexoside $^{(H)}$	nd	nd	1.07 ± 0.01^b	1.83 ± 0.03^a
13NA	17.62	336	609	301(100)	Quercetin-*O*-deoxyhexoside-hexoside$^{(H)}$	0.32 ± 0.02^c	0.29 ± 0.01^d	0.55 ± 0.02^b	1.5 ± 0.1^a
14NA	17.85	348	609	301(100)	Quercetin-3-*O*-rutinoside$^{(H)}$	4.4 ± 0.1^c	3.76 ± 0.02^b	6.4 ± 0.1^d	12.925 ± 0.002^a
15NA	18.66	354	463	301(100)	Quercetin-3-*O*-glucoside$^{(I)}$	3.02 ± 0.05^b	2.25 ± 0.03^a	3.93 ± 0.01^c	8.91 ± 0.04^d
16NA	19.06	353	463	301(100)	Quercetin-3-*O*-galactoside$^{(I)}$	1.84 ± 0.01^d	2.06 ± 0.04^c	2.6 ± 0.1^b	4.62 ± 0.03^a
17NA	20.53	283	579	271(100)	Naringenin-*O*-glucuronide$^{(J)}$	5.4 ± 0.1^b	1.43 ± 0.05^c	12.8 ± 0.4^a	tr
18NA	21.3	341	593	285(100)	Kaempferol-3-*O*-rutinoside$^{(I)}$	0.57 ± 0.01^c	0.48 ± 0.01^d	0.72 ± 0.02^b	0.76 ± 0.01^a
19NA	22.3	350	623	315(100)	Isorhamnetin-3-*O*-rutinoside$^{(H)}$	0.639 ± 0.004^c	0.60 ± 0.01^d	2.58 ± 0.02^b	4.5 ± 0.1^a
20NA	22.68	348	447	301(100)	Quercetin-*O*-deoxyhexoside $^{(I)}$	2.4 ± 0.1^c	2.21 ± 0.02^d	3.1 ± 0.1^b	6.8 ± 0.1^a
21NA	23.42	353	477	315(100)	Isorhamnetin-3-*O*-glucoside$^{(I)}$	1.41 ± 0.03^c	1.06 ± 0.01^d	1.75 ± 0.01^b	3.34 ± 0.03^a

Peak	Rt (min)	λmax (nm)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g of extract)			
						P0	**P1**	**P2**	**P3**
22NA	23.9	350	461	315(100)	Isorhamnetin-*O*-deoxyhexoside$^{(I)}$	0.90 ± 0.03^b	0.59 ± 0.01^d	0.83 ± 0.04^c	1.11 ± 0.02^a
23NA	27.38	334	431	285(100)	Kaempferol-*O*-deoxyhexoside $^{(I)}$	nd	0.423 ± 0.001	nd	nd
24NA	28.57	349	491	315(100)	Isorhamnetin-*O*-glucuronide$^{(I)}$	1.04 ± 0.03^c	0.77 ± 0.01^d	1.24 ± 0.02^b	2.4 ± 0.1^a
					Total Phenolic Acids	**58 ± 1^b**	**49.4 ± 0.1^c**	**70.5 ± 0.7^a**	**3.5 ± 0.1^d**
					Total Flavonoids	**24.5 ± 0.2^c**	**17.7 ± 0.1^d**	**40.3 ± 0.7^b**	**52.98 ± 0.02^a**
					Total Phenolic Compounds	**82.0 ± 1^b**	**67.2 ± 0.2^c**	**110.72 ± 0.01^a**	**56.45 ± 0.03^d**

Phenolic acids were the second main group of compounds found in the hydromethanolic extracts of bilberry, being the majority of them linked to sugar moieties, such as peaks **1**^{NA} (syringic acid hexoside, [M-H]$^-$ at *m/z* 359), peak **2**^{NA}/**5**^{NA} (caffeic acid hexoside [M-H]$^-$ at *m/z* 341), **3**^{NA} (*p*-coumaric acid hexoside, [M-H]$^-$ at *m/z* 325), **4**^{NA}/**7**^{NA} **(**ferulic acid hexoside, [M-H]$^-$ at *m/z* 355), and **9**^{NA} **(**sinapic acid hexoside, [M-H]$^-$ at *m/z* 385). Peak **10**^{NA} was positively identified as caffeic acid according to its UV spectra, elution order, and fragmentation pattern in comparison to a commercial standard. Peaks **6**^{NA} and **8**^{NA} were tentatively identified as *cis* and *trans* 5-*O*-caffeoylquinic acid, respectively, accordingly to their characteristic UV spectra, maximum wavelength around 320-326 nm, fragmentation pattern and elution order, by comparison with our database library. Peak **6**^{NA} was the main phenolic acid found in all four samples.

Finally, one flavanone was tentatively identified as naringenin-*O*-glucuronide (peak **17**^{NA}, [M-H]$^-$ at *m/z* 579), with an MS^2 fragment at *m/z* 271 (naringenin aglycone) that corresponded to the loss of a glucuronyl unit. Its UV spectrum was also coherent with that of a flavanone. This was the second main compound in P2 samples (12.78 mg/g of extract), being found in trace amounts in P3 samples. In the authors knowledge, this is the first that naringenin derivatives are reported in bilberry fruits.

The anthocyanin compounds present in the hydromethanolic extracts in all the analysed mixtures (P0, P1, P2, and P3) are presented in **Table 32**. Up to twenty different anthocyanin glycosides were identified derived from six anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin). The major group of anthocyanins were the glycosylated derivatives of pelargonidin, in which six compounds were detected, all of them in the sample P3, whose anthocyanin profile is completely different to the other three mixtures, denoting the influence of goji fruits in the anthocyanin composition of this sample. Actually, no pelargonidin derivatives are present in the blueberry sample (P0) or in its mixtures with rose and calendula flowers (P1 and P2). As discussed in section 3.4.1, the profile of anthocyanin compounds in *V. myrtillus* fruits has already been extensively studied. Eleven (peaks **3**^{A}, **5**^{A}-**7**^{A}, **9**^{A}, **12**^{A}-**15**^{A}, **17**^{A}, **18**^{A}) of the twenty compounds identified were previously identified. The remaining nine anthocyanins were found in mixture P3. Thus, peaks **11**^{A} ([M]$^+$ at *m/z* 433) and **20**^{A} ([M]$^+$ at *m/z* 519) corresponded to pelargonidin derivatives, presenting a characteristic absorption spectra and a unique MS^2 fragment at *m/z* 271, coherent with the loss of hexosyl and malonyl-hexosyl moieties, being tentatively identified as, pelargonidin-*O*-

hexoside and pelargonidin-*O*-malonylhexoside, respectively. Peak **2^{A}** ([M]$^{+}$ at *m/z* 595) presented an additional hexosyl moiety, compared to peak **11^{A}**, being tentatively identified as pelargonidin-*O*-dihexoside. Peaks **8^{A}/10^{A}** ([M]$^{+}$ at *m/z* 681) and **16^{A}** ([M]$^{+}$ at *m/z* 767) were tentatively assigned as pelargonidin-malonyl-dihexoside and pelargonidin-dimalonyl-dihexoside, respectively. Cyanidin derivatives were the second group of anthocyanins found in the mixtures. Peak **19^{A}** ([M]$^{+}$ at *m/z* 535) presented a unique MS^{2} fragment at *m/z* 287, which corresponded to the loss of a malonyl-hexosyl moiety, being tentatively identified as cyanidin-malonyl-hexoside. Peak **1^{A}** ([M]$^{+}$ at *m/z* 611) presented the consecutive release of two hexosyl moieties, being tentatively identified as cyanidin-3,5-*O*-diglucoside, an anthocyanin already identified in rose flowers and goji fruits, components of mixtures P1 and P3, where the compound was detected. Peak **4^{A}** ([M]$^{+}$ at *m/z* 697) presented MS^{2} fragments at *m/z* 535, 449, and 287 which corresponded to the loss of 162 u (hexose), 86 u (malonyl), and 162 u (hexose), respectively, being tentatively identified as cyanidin-malonyl-dihexoside.

As above indicated, the anthocyanin profile in the mixture P3 samples greatly differs from the other three samples, in fact, none of the anthocyanins found in P3 was detected in them (but for peak **1^{A}** in P1), which is explained for goji fruits (and apple in less extent) being the main contributors to their anthocyanin composition; it should also be taken into account that the proportion of bilberries in P3 is lower than in the other mixtures. On the other hand, it is common knowledge that apples have a relatively very low pH value (Li et al., 2013), which could be influencing the extraction of anthocyanins, having already been proven that at lower pH values the extraction of these type of compounds increases. The possible creation of a microenvironment with low pH values and compounds that stabilize anthocyanins may be the explanation for the higher concentration (199.7 ± 0.3 mg/g of extract) of anthocyanins in P3, as it may help to extract compounds attached to matrix structures that are, therefore, less available in a conventional extraction. It is also possible to verify, although it is not as significant, that P1 and P2 extracts also possess greater amounts of anthocyanin compounds (32.2 ± 0.5 and 49 ± 2 mg/g, respectively) than P0 (21.1 ± 0.2 mg/g). Calendula flowers do not have anthocyanins in its composition, so that this component shows no influence in the anthocyanin profile of the mixture, but it might have an influence in the extent of anthocyanin extraction. Mixture P1 has a very similar profile to P0, with the exception of peak **1^{A}** (cyanidin 3,5-di-*O*-glucoside), which was not detected in P0. The presence of this anthocyanin could be expected, since it is the major anthocyanin compound found in rose flowers

Table 32. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (μg/g of extract) of the anthocyanins in the hydromethanolic extracts of the four snack mixtures (P0, P1, P2, and P3).

Peak	Rt (min)	λ_{max} (nm)	$[M]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g of extract)			
						P0	P1	P2	P3
1[A]	11.66	512	611	449(5),287(100)	Cyanidin 3,5-di-*O*-glucoside[A]	nd	0.638±0.003[b]	nd	13.1±0.1[a]
2[A]	13.97	501	595	433(50),271(100)	Pelargonidin-*O*-dihexoside[B]	nd	nd	nd	32.6±0.2
3[A]	14.41	524	465	303(100)	Delphinidin-3-*O*-galactoside[C]	2.653±0.01[c]	4.6±0.1[b]	7.2±0.3[a]	nd
4[A]	15.65	520	697	535(65),449(25),287(100)	Cyanidin-malonyl-dihexoside[A]	nd	nd	nd	3.0±0.1
5[A]	15.85	526	465	303(100)	Delphinidin-3-*O*-glucoside[C]	0.162±0.001[c]	0.358±0.002[b]	0.442±0.004[a]	nd
6[A]	16.93	518	449	287(100)	Cyanidin-3-*O*-galactoside[A]	0.62±0.02[c]	1.01±0.03[b]	1.4±0.1[a]	nd
7[A]	17.68	524	435	303(100)	Delphinidin-3-*O*-arabinoside[C]	1.91±0.02[c]	3.17±0.04[b]	4.9±0.9[a]	nd
8[A]	17.81	514	681	519(50),433(25),271(100)	Pelargonidin-malonyl-dihexoside[B]	nd	nd	nd	67±2
9[A]	19.22	528	479	317(100)	Petunidin-3-*O*-galactoside[D]	2.49±0.02[c]	3.9±0.2[b]	6.2±0.3[a]	nd
10[A]	20.02	514	681	519(90),433(15),271(100)	Pelargonidin-malonyl-dihexoside[B]	nd	nd	nd	54±2
11[A]	20.14	510	433	271(100)	Pelargonidin-*O*-hexoside	nd	nd	nd	16.9±0.1
12[A]	20.24	514	419	287(100)	Cyanidin-3-*O*-arabinoside[A]	0.464±0.004[c]	0.756±0.003[b]	1.05±0.01[a]	nd
13[A]	21.72	518	463	301(100)	Peonidin-3-*O*-galactoside[E]	0.45±0.01[c]	0.67±0.01[b]	1.01±0.01[a]	nd
14[A]	22.64	516	449	317(100)	Petunidin-3-*O*-arabinoside[D]	1.67±0.03[c]	2.5570±0.0003[b]	3.89±0.04[a]	nd
15[A]	22.83	528	493	331(100)	Malvidin-3-*O*-galactoside[D]	5.64±0.05[c]	7.6±0.2[b]	12.5±0.4[a]	nd
16[A]	23.72	501	767	605(100),271(35)	Pelargonidin-dimalonyl-dihexoside[B]	nd	nd	nd	6.56±0.02
17[A]	26.08	522	433	301(100)	Peonidin-3-*O*-arabinoside[E]	0.550±0.004[c]	0.850±0.001[b]	1.22±0.01[a]	nd
18[A]	28.89	528	463	331(100)	Malvidin-3-*O*-arabinoside[D]	4.45±0.02[c]	5.91±0.23[b]	9.0±0.4[a]	nd
19[A]	29.35	520	535	287(100)	Cyanidin-malonylhexoside[A]	nd	nd	nd	2.6±0.1
20[A]	32.43	501	519	271(100)	Pelargonidin-*O*-malonylhexoside[B]	nd	nd	nd	4.4±0.1
					Total Anthocyanins	**21.1±0.2[d]**	**32.2±0.5[c]**	**49±2[b]**	**199.7±0.3[a]**

3.5.3. Bioactivities assessment

Data regarding the antioxidant, antibacterial, and hepatotoxicity activities of the hydromethanolic extracts of P0, P1, P2 and P3 are shown in **Table 33**.

Table 33. Antioxidant, antibacterial and hepatotoxicity activity in bilberry fruits (P0), combined with rose flowers (P1), calendula flowers (P2) and apple and goji berries (P3) (mean ± SD).

	P0	**P1**	**P2**	**P3**
Antioxidant activity[A] EC_{50} values (mg/mL)				
DPPH scavenging activity	2.95 ± 0.03^{b}	2.5 ± 0.1^{c}	2.38 ± 0.04^{d}	3.9 ± 0.1^{a}
Reducing power	1.10 ± 0.02^{d}	1.15 ± 0.03^{c}	1.20 ± 0.02^{b}	1.59 ± 0.01^{a}
β-carotene bleaching inhibition	2.07 ± 0.04^{b}	1.6 ± 0.1^{c}	0.93 ± 0.01^{d}	3.60 ± 0.04^{a}
Antibacterial activity[B] MIC values (mg/mL)				
Gram-negative bacteria				
Acinetobacter baumanii	>20	20	>20	>20
Escherichia coli ESBL1	5	2.5	5	5
Escherichia coli ESBL2	2.5	2.5	2.5	2.5
Escherichia coli	5	5	5	5
Klebsiella pneumoniae	20	20	10	20
Klebsiella pneumoniae ESBL	>20	>20	20	20
Morganella morganii	5	5	5	5
Gram-positive bacteria				
Enterococcus faecalis	2.5	2.5	2.5	2.5
Listeria monocytogenes	2.5	2.5	2.5	2.5
Staphylococcus aureus	2.5	2.5	5	2.5
MRSA	2.5	2.5	5	2.5
MSSA	2.5	2.5	5	2.5
Hepatotoxicity GI_{50} values (μg/mL)				
PLP2	>400	>400	>400	>400

EC_{50} values correspond to the extract concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. A - Trolox EC_{50} values: 43.03 ± 1.71 μg/mL (DDPH), 29.62 ± 3.15 μg/mL (reducing power) and 2.63 ± 0.14 μg/mL (β-carotene bleaching inhibition). B - Ellipticine GI_{50} values: 2.29 mg/mL (PLP2). MIC values correspond to the minimal extract concentration that inhibited the bacterial growth. ESBL - extended spectrum β-lactamases. MRSA - Methicillin-resistant *Staphylococcus aureus*. MSSA - Methicillin-susceptible *Staphylococcus aureus.*

P1 sample was the one that presented the lowest EC_{50} values for all the antioxidant assays performed, i.e., DPPH scavenging activity, reducing power and β-carotene bleaching inhibition (EC_{50} = 2.5, 1.15, and 1.6 mg/mL, respectively). The differences between P0 and P1 samples may be due to the presence of rose samples, that, as previously found, presented a very good antioxidant potential (section 3.1.2). On the contrary, P3 sample was the one that presented the highest EC_{50} values (lowest antioxidant potential), which could be due to the lowest amount of bilberry fruits and the relatively low activity conferred by the apple and goji fruits samples. Comparing the previously described results for the antioxidant activities of rose, calendula (section 3.1.2), apple (section 3.2.3) and goji fruits (section 3.3.3) it was observed that the rose sample presented the lowest EC_{50} values, being therefore in agreement with the results herein obtained.

Regarding the antibacterial activity, the best results were found against Gram-positive bacteria, showing the lowest MIC values (ranging from 2.5 and 5 mg/mL) in all the four studied samples. P2 also showed MIC values of 2.5 mg/mL against the Gram-negative bacteria *Escherichia coli* extended spectrum β-lactamases (ESBL 1 and ESBL 2), and 5 mg/mL against *E. coli* and *Morganella morganii*. These results were in agreement with those reported in previous sections, that showed rose flowers are very active against *E. coli*, although presenting lower MIC values when considered alone (1.25 mg/mL). The previous studies also revealed that the edible flowers, apple and goji fruits were most active against Gram-positive bacterias, with MIC values ranging from of 1.25 to 10 mg/mL for edible flowers, 2.5 to 5 mg/mL for goji fruits, and 2.5 to 5 mg/mL for apple samples.

Regarding the hepatotoxic assays, none of extracts showed hepatotoxicity against the non-tumor PLP2 cell line studied, suggesting that these snacks should not be toxic for human consumption.

CHAPTER 4

Conclusions and Future Perspectives

The present Thesis aimed to deep into the knowledge of different edible flowers, fruits and stems regarding their nutritional characteristics and phytochemical composition and potential beneficial healthy effects, in order to evaluate their suitability to be used either as food or as ingredients in the preparation of functional foods with increased added value.

Among the studied flowers, calendula showed the highest content in total fat, ash, energy, polyunsaturated fatty acids (mainly due to the presence of linolenic acid) and total tocopherols (with the major contribution of α-tocopherol). Rose flowers presented the highest values of total proteins, soluble sugars and organic acids, whereas centaurea revealed the highest carbohydrates concentration and the lowest percentage of saturated fatty acids. Regarding infusions, calendula infusion presented the highest content in sugars, while that of organic acids was found in centaurea infusion. The hydromethanolic extracts and infusions of rose flowers revealed the greatest antioxidant and antibacterial activities, while dahlia's hydromethanolic extract possessed the best for antiproliferative activity against almost all the tumor cell lines tested.

The phenolic composition of the flower samples was characterised in terms of their individual phenolic acids and flavonoids profiles and contents. The different flowers showed distinct phenolic composition, with calendula presenting the highest concentration of total phenolic compounds. Flowers also proved to be interesting natural sources of anthocyanins that might be used to substitute artificial colorants. Nine anthocyanin compounds were detected in dahlia, two in rose and eight in Centaurea extracts. being cyanidin derivatives the most abundant ones.

The incorporation of flowers' extracts in yogurt formulations did not significantly affect their nutritional value and sugars and fatty acids composition. Regarding their potential to be used as yogurt colorants, rose and dahlia extracts led to similar chromatic features as the anthocyanin-based additive E163, suggesting their suitability to be used as alternatives to this commercial colorant, while centaurea extract behave as a less effective coloring agent.

All in all, the obtained results demonstrate that edible flowers can be included in a normal diet as nutrient sources, as well as used to prepare infusions likely to promote healthy effects. Besides, they could be employed as sources of natural pigments alternative to current commercial E163 colorants.

The results obtained in the characterization of the Portuguese apple 'Bravo de Esmolfe' showed that, besides the nutritional and healthy properties already recognized for apples, this variety demonstrated to be a good source of α-tocopherol and phenolic compounds, namely epicatechin-based procyanidins, hydroxycinnamoyl-quinic acids and phloretin derivatives. Furthermore, its hydromethanolic extract revealed significant antioxidant activity and antimicrobial effect against both Gram-positive and Gram-negative bacteria. Overall, 'Bravo de Esmolfe' dried apples could be used in snacks due to their balanced nutritional composition, as well as in the preparation of nutraceutical formulations with potential antioxidant and antimicrobial properties.

The fruits of *L. barbarum* (Goji berries) showed to be good sources of sugars (mainly fructose and glucose), polyunsaturated fatty acids and hydroxycinnamoyl derivatives (*p*-coumaric acid and chlorogenic acid derivatives); they also possessed relevant activity against Gram-positive bacteria. For their part, the stems of this plant presented higher values of energy, fat, proteins and minerals (ash) than the fruit, as well as of monounsaturated fatty acids, tocopherols and flavonols (especially quercetin-3-*O*-rutinoside). Furthermore, the antioxidant and antimicrobial capacities were also greater in the stems. Thus, not only the Goji berry but also the stems constitute interesting sources of compounds with bioactive properties and, therefore, they could be useful for nutraceutical formulations or incorporation into foods. Since stems are by-products, beyond having putative beneficial effects to consumers, they can also contribute to industrial sustainability.

Anthocyanin extracts from bilberry incorporated as colorants to natural yogurts satisfactorily compare with a commercial colorant (E163). Over an assayed period of seven days, the yogurts maintained the nutritional value, fatty acids profile and sugar contents. Although bilberry extracts provided slightly lower coloring intensity than E163, they did provide higher color stability throughout the storage time.

Snacks consisting of dried bilberries combined with different types of fruits and edible flowers were prepared. Bilberry themselves revealed a good nutritional balance with low fat content and high carbohydrates and energetic contribution. However, when supplemented with 2% of dried rose flowers, their protein content and bioactive

potential increased significantly, whereas combination with calendula flowers (0.02%) improved contents in organic acids, tocopherols, phenolic acids, and total non-anthocyanin phenolic compounds. The supplementation of bilberries with dried apple (36%) and goji fruits (4%) increased by 9-fold the anthocyanin content, improving their functional potential. All in all, the combination of dried bilberries with other fruits or edible flowers can be proposed as a feasible way to prepare nutritious and healthy snack choices for the consumer and an added value for the food industry.

Global conclusions

Edible flowers (*Dahlia mignon*, *Rosa damascena* 'Alexandria' and *Rosa gallica* 'Francesa' grafted in *R. canina, Calendula officinalis* L, *Centaurea cyanus* L.) and fruits of *Vaccinium myrtillus* L., have proved to be good natural sources of nutrients, bioactives and pigments, capable of being incorporated into food matrices as functional ingredients and alternative colorants, improving the characteristics of the products and providing added value.

The Portuguese apple variety "Bravo de Esmolfe" has been verified to possess noteworthy nutritional and phytochemical potential, as well as relevant antioxidant and antimicrobial properties, supporting its appreciation by consumers and suitability for its use in the formulation of healthy foods.

Goji berries already have a well-established market, with great interest for consumers, however, their stems were still unexplored by the food industry. The assessment of the Goji stems as sources of bioactive compounds also represents a meaningful breakthrough of this Thesis.

Bilberry fruits when mixed with the previously studied flowers and fruits improved each other nutritious properties. Likewise, beyond their putative health benefits, the anthocyanins of this berry showed good capability to be used as food colorants in yogurts.

Future Perspectives

Taking into account the results obtained in this work, future aims to develop are:

- Optimization of the extraction conditions, using traditional and alternative methods to prepare safe and richer extracts of phenolic compounds.
- Assessment of the stability and toxicity of the prepared extracts.
- Performing sensory and acceptance studies (human panelists) of the novel developed food products.

Especially, the development of novel functional products based on bilberries and edible flowers will continue as a future work in order to offer the industry and final consumer healthier and tastier functionalized products, as well as more appealing foods in terms of color or flavor.

CHAPTER 5

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ANNEXES

ANNEX I

Nutritional and chemical characterization of edible flowers and corresponding infusions: Valorization as a new food ingredients

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Nutritional and chemical characterization of edible petals and corresponding infusions: Valorization as new food ingredients

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ABSTRACT

Edible flowers provide new colours, textures and vibrancy to any dish, and apart from the "glam" factor, they can constitute new sources of bioactive compounds. In the present work, the edible petals and infusions of dahlia, rose, calendula and centaurea, were characterized regarding their nutritional value and composition in terms of hydrophilic and lipophilic compounds. Carbohydrates were the most abundant macronutrients, followed by proteins and ash. Fructose, glucose and sucrose were identified in all the petals and infusions. Rose petals and calendula infusions gave the highest content of organic acids, mainly due to the presence of malic and quinic acids, respectively. Polyunsaturated fatty acids predominated over saturated fatty acids, mainly due to the contribution of linoleic acid. Calendula presented the highest content in tocopherols, with α-tocopherol as the most abundant. These results highlight the interest of edible petals "as" and "in" new food products, representing rich sources of bioactive nutrients.

1. Introduction

Consumption habits are becoming more diversified and directed towards more sustainable food options (Falguera, Aliguer, & Falguera, 2012). The range of plant species used for food is also becoming more varied, seeking to combine new ingredients with some potential health benefits, that could improve the health of the consumers but also with a major importance in ecological sustainability (Leonti, 2012). This search for new food products is also a pursuit for new colours, textures and flavours that can be achieved with the use of edible flowers, such as has been done by several restaurant chefs worldwide (Kelley, Behe, Biernbaum, & Poff, 2001; Łuczaj et al., 2012); leading to the recovery of earlier lifestyles in which flower cookery had an important role in old civilizations (Cunningham, 2015; Rop, Mlcek, Jurikova, Neugebauerova, & Vabkova, 2012).

Apart from the "glam" factor, edible flowers have important nutritional characteristics and can constitute new sources of bioactive compounds (Lara-Cortés et al., 2014; Mlcek & Rop, 2011). They represent an unexplored niche market with great economic and social importance being used since ancient times in culinary preparations, such as sauces, liquors, salads and desserts (Koike et al., 2015; Mlcek & Rop, 2011), and also in the preparation of hot beverages (tisane and infusion), mainly in European countries, due to their medicinal properties (Navarro-González, González-Barrio, García-Valverde, Bautista-Ortín, & Periago, 2015). In ancient Rome, various species of rose flowers (*Rosa* spp.) were used to prepare purée and omelets (Cunningham, 2015). In Medieval France, the flowers of calendula (*Calendula officinalis* L.) were used to prepare omelets but also salads or as an accompaniment cheese (Lara-Cortés et al., 2014). In Mexico, Dahlia flowers are commonly consumed in different type of dishes, for example in dried soups (Lara-Cortés et al., 2014).

The composition on proteins, vitamins, fat and carbohydrates of flowers is not very distinct from other parts of the plant, however protein and fat content are considered to be low (Navarro-González et al., 2015); water represents more than 80% of the flower composition, and carotenoids, phenolic compounds and essential oils have been the most studied bioactive compounds (Navarro-González et al., 2015; Rop et al., 2012). Edible flower consumption is being encouraged, through the sell of packed bunches and boxes, and also through dietary supplements, functional ingredients, and additives (Loizzo et al., 2016; Rop et al., 2012). The innumerous phytochemicals present in edible flowers are related to their health benefits, such as antioxidant, anti-inflammatory, anti-cancer, anti-obesity, hypoglycemic, neuro, hepatic and gastro protective properties (Cunningham, 2015; Loizzo et al., 2016; Lu, Li, & Yin, 2016).

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In particular, the nutritional and chemical composition of rose (*Rosa canina* L.) and calendula flowers have already been studied (Barros, Carvalho, & Ferreira, 2011; Miguel et al., 2016), also the fatty acids composition of calendula seeds oils (Dulf, Pamfil, Baciu, & Pintea, 2013) and the crude protein of centaurea (*Centaurea cyanus* L.) flowers (Rop et al., 2012). Despite the existence of some publications regarding edible flowers, it is important to compare their potential to be used in different forms, namely as fresh produces or in infusion preparations. Therefore, in the present work, edible petals of different species (dahlia, rose, calendula and centaurea) were characterized in terms of macronutrients composition, energetic value, fatty acids, soluble sugars, organic acids and tocopherols, and compared to the nutritional composition of their infusions.

2. Materials and methods

2.1. Standards and reagents

HPLC grade acetonitrile, *n*-hexane and ethyl acetate were from Fisher Scientific (Lisbon, Portugal). A reference standard mixture (standard 47885-U) for fatty acids methyl ester (FAME) was purchased from Sigma (St. Louis, MO, USA), as also other standards: α- and δ-tocopherols, sugars and organic acids. The isoforms β- and γ-tocopherols and tocol (50 mg/ml) were purchased from Matreya (Pleasant Gap, PA, USA). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain) and water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and infusion preparation

The samples were kindly supplied by RBR Foods, a farming company producer of fruits and flowers from Castro Daire (Portugal), as dry material to be used directly or for infusion's preparation. Petals of four different species were used in the present study: Dahlia mignon (commercial seeds mixture), *Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*, *Calendula officinalis* L. and *Centaurea cyanus* L. (Fig. 1). These samples are designated throughout the manuscript by their common names: dahlia, rose, calendula and centaurea, respectively. All the samples were reduced to a fine powder (20 mesh) and mixed to obtain homogenate samples.

For infusions preparation, boiling distilled water (100 ml) (pH 6.6) at 100 °C was added to each sample (500 mg) and left to stand at room temperature for 5 min. Afterwards, the infusions were filtered under reduced pressure (0.22 μm) and stored at −5 °C (1 week) until further analysis.

2.3. Nutritional value-proximate composition and energetic value

The samples (dried powdered petals) were analyzed for proteins, fat, carbohydrates and ash according to the AOAC (Association of Official Analytical Chemists) procedures (AOAC, 2005). The crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method (AOAC, 991.02); the crude fat (AOAC, 989.05) was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content (AOAC, 935.42) was determined by incineration

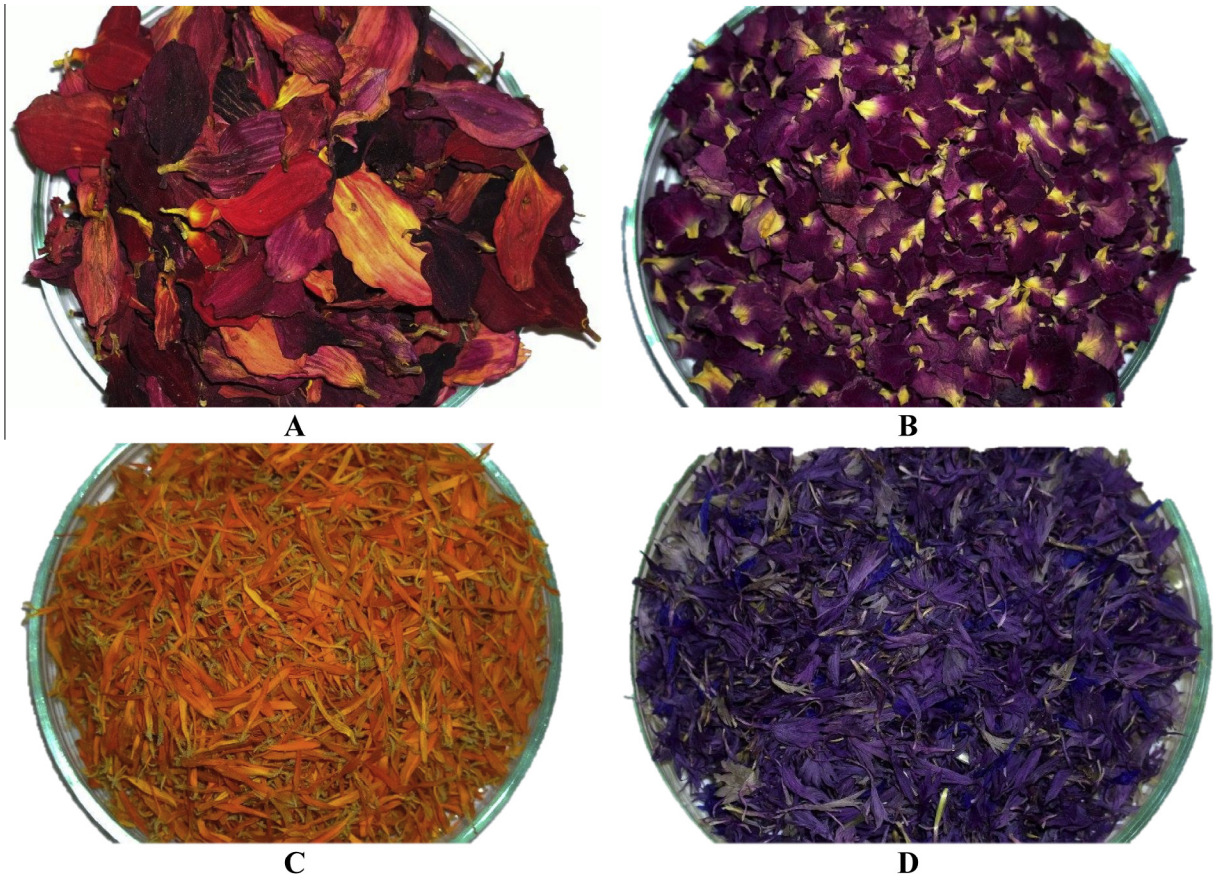

Fig. 1. Petals from (A) Dahlia; (B) Rose; (C) Calendula; (D) Centaurea.

at 550 ± 15 °C. Total carbohydrates (including fibre) were calculated by difference [Total carbohydrates (g/100 g) = 100 − (g fat + g protein + g ash)]. Total energy was calculated according to the following equation: Energy (kcal/100 g) = 4 × (g proteins + g carbohydrates) + 9 × (g fat). For infusions, total carbohydrates were calculated on the basis of total soluble sugars (Section 2.4.1) and the energetic value was calculated taking into account those results.

2.4. Hydrophilic compounds

2.4.1. Soluble sugars

Soluble sugars in dried powdered petals and infusions were determined according to a previously described procedure (Barros et al., 2013), using high performance liquid chromatography system coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany). The quantification was performed using the internal standard (melezitose) method or external standard method for infusions. The results were expressed in g per 100 g of plant dry weight or in g per 100 ml of infusion.

2.4.2. Organic acids

Organic acids were determined in dried powdered petals and infusions by ultra-fast liquid chromatography coupled to photodiode array detector (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan), according to the previously described procedure (Barros, Pereira, & Ferreira, 2013). The quantification was performed by comparison of the peak area recorded at 215 nm as the preferred wavelength. The results were expressed in g per 100 g of plant dry weight or in mg per 100 ml of infusion.

2.5. Lipophilic compounds

2.5.1. Fatty acids

Fatty acids were determined by GC-FID (DANI model GC 1000 instrument, Contone, Switzerland), using dried powdered petals and after a trans-esterification process, according to the previously described procedure (Barros et al., 2013). The results were expressed in relative percentage of each fatty acid.

2.5.2. Tocopherols

The four isoforms of tocopherols were determined in dried powdered petals, according to the previously described procedure (Barros et al., 2013), using HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA), the quantification was based on the fluorescence signal response of each standard, using the internal standard (tocol) method or external standard method for infusions. The results were expressed in mg per 100 g of dry plant weight.

2.6. Statistical analysis

Three samples were used for each species and all the assays were carry out in triplicate. Results were expressed as mean values and standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Proximate composition and energetic value of edible petals and corresponding infusions

Data on the nutritional composition and energetic value of edible petals from four different species-dahlia, rose, calendula and centaurea-, and of the corresponding infusions are shown in Table 1.

Carbohydrates were the most abundant macronutrients in all the dried petals, followed by proteins and ash in dahlia (5.93 and 5.83 g/100 g dw, respectively), rose (7.58 and 4.29 g/100 g dw, respectively) and centaurea (5.79 and 5.68 g/100 g dw, respectively). Rop et al. (2012) presented lower values of crude protein in *C. officinalis* flowers (0.673 g/100 g) originated from Czech Republic. Calendula petals presented a higher amount of fat (5.33 g/100 g dw) and ash (6.93 g/100 g dw) when compared to the other samples, and also a higher energetic contribution (421.58 kcal/100 g). These results are in accordance with the ones described by Miguel et al. (2016) who reported similar values of fat and energy in calendula flowers. Dias et al. (2014) described higher fat (6.56 g/100 g dw) content in dried flowers of *Taraxacum* sect. Ruderalia. Regarding the infusions, rose and dahlia samples presented the highest contribution in carbohydrates (0.19 mg/100 ml), and also the highest energetic value (0.80 and 0.76 kcal/100 ml, respectively). Pereira, Barros, and Ferreira (2015) reported lower energy values and carbohydrates content (0.060 kcal/100 ml and 0.015 g/100 ml, respectively) in the infusions of *Chamaemelum nobile* L., and also lower amounts of sugars, though having a similar profile (fructose, glucose and sucrose). In the same study, no sugars were detected in the infusions of *Gomphrena globosa*, *G. globosa* var. albiflora, *G. haageana* and *Gomphrena* sp., and consequently, carbohydrates content and energetic value could not be calculated.

3.2. Hydrophilic compounds of edible petals and corresponding infusions

Soluble sugars and organic acids composition of the studied dried petals and corresponding infusions is presented in Table 2 and Fig. 2. Dahlia and rose dried petals (10.24 and 10.75 g/100 g dw)

Table 1
Proximate composition and energy of dried petals and corresponding infusions (mean ± SD).

	Dried petals (g/100 g dw)				Infusions (g/100 ml infusion)			
	Dahlia	Rose	Calendula	Centaurea	Dahlia	Rose	Calendula	Centaurea
Nutritional value								
Fat	2.23 ± 0.05b	2.01 ± 0.04b	5.33 ± 0.45a	0.140 ± 0.001	nd	nd	nd	nd
Proteins	5.93 ± 0.2bc	7.58 ± 0.84a	6.43 ± 0.68b	5.79 ± 0.1c	nd	nd	nd	nd
Ash	5.83 ± 0.04b	4.29 ± 0.1d	6.93 ± 0.14a	5.68 ± 0.13c	np	np	np	np
Total available carbohydrates	86.02 ± 0.2b	86.12 ± 0.8b	81.32 ± 0.75c	88.39 ± 0.13a	0.19 ± 0.02a	0.19 ± 0.01a	0.17 ± 0.01b	0.14 ± 0.01c
	Dry petals (kcal/100 g dw)				Infusions (kcal/100 mL infusion)			
Energy	387.83 ± 0.37c	392.87 ± 0.58b	421.58 ± 3.54a	377.99 ± 0.50d	0.76 ± 0.08a	0.80 ± 0.08a	0.68 ± 0.02b	0.56 ± 0.04c

dw – dry weight basis; np – not performed; nd – not detected. In each row and within dry petals or infusions different letters mean significant differences between samples ($p < 0.05$), where "a" and "d" correspond to the highest and lowest values, respectively.

Table 2
Soluble sugars and organic acids composition in dried petals and corresponding infusions (mean ± SD).

	Dried petals (g/100 g dw)				Infusions (mg/100 ml)			
	Dahlia	Rose	Calendula	Centaurea	Dahlia	Rose	Calendula	Centaurea
Soluble sugars								
Fructose	3.87 ± 0.23b	5.14 ± 0.48a	1.47 ± 0.12c	0.65 ± 0.04d	0.10 ± 0.01a	0.10 ± 0.01a	0.066 ± 0.001b	0.07 ± 0.004b
Glucose	3.23 ± 0.25a	3.23 ± 0.41a	0.61 ± 0.07b	0.47 ± 0.02b	0.079 ± 0.02a	0.064 ± 0.004b	0.021 ± 0.001c	0.04 ± 0.001d
Sucrose	3.14 ± 0.15a	2.39 ± 0.17b	1.53 ± 0.18c	0.38 ± 0.01d	0.016 ± 0.001c	0.035 ± 0.001b	0.078 ± 0.001a	0.03 ± 0.01b
Sum	10.24 ± 0.62 a	10.75 ± 1.05a	3.61 ± 0.37b	1.5 ± 0.1c	0.19 ± 0.02a	0.19 ± 0.01a	0.17 ± 0.01b	0.14 ± 0.01c
Organic acids								
Oxalic acid	0.23 ± 0.01c	0.26 ± 0.01b	0.702 ± 0.002a	0.18 ± 0.01d	tr	1.31 ± 0.01	tr	tr
Quinic acid	0.466 ± 0.003b	1.52 ± 0.01a	0.35 ± 0.01b	nd	nd	9.33 ± 0.41b	14.5 ± 0.3a	7.4 ± 0.3c
Malic acid	0.74 ± 0.01c	1.23 ± 0.02a	1.14 ± 0.02b	nd	nd	4.1 ± 0.4a	1.16 ± 0.15b	tr
Shiquimic acid	0.0497 ± 0.0003c	0.062 ± 0.001b	nd	0.108 ± 0.001a	tr	0.368 ± 0.001b	tr	1.05 ± 0.003a
Citric acid	nd	1.2 ± 0.1	nd	nd	nd	nd	nd	15.5 ± 0.5
Succinic acid	nd	nd	1.77 ± 0.03	nd	nd	nd	11.2 ± 0.5	nd
Fumaric acid	tr	0.011 ± 0.001	tr	tr	nd	tr	tr	tr
Sum	1.49 ± 0.01c	4.26 ± 0.13a	3.98 ± 0.02b	0.29 ± 0.01d	tr	15.01 ± 0.1c	26.9 ± 0.3a	23.9 ± 0.8b

dw – dry weight basis; nd – not detected; tr-traces (LOD (μg/mL) and LOQ (μg/mL) for oxalic acid (12.6 and 42, respectively), quinic acid (24 and 81, respectively), malic acid (36 and 1.2 × 10^2, respectively), shiquimic acid (6 and 19, respectively), citric acid (10 and 35, respectively), succinic acid (19 and 64, respectively) and fumaric acid (0.080 and 0.26, respectively). Calibration curves for organic acids: oxalic acid ($y = 9 \times 10^6x + 45973$, $R^2 = 0.9901$); quinic acid ($y = 610607x + 46061$, $R^2 = 0.9995$); malic acid ($y = 912441x + 92665$, $R^2 = 0.999$); shiquimic acid ($y = 7 \times 10^7x + 175156$, $R^2 = 0.9999$); citric acid ($y = 1 \times 10^6x + 45682$, $R^2 = 0.9997$); succinic acid ($y = 592888x + 50689$, $R^2 = 0.9996$) and fumaric acid ($y = 154862x + 1 \times 10^6$, $R^2 = 0.9977$). In each row and within dry petals or infusions different letters mean significant differences between samples ($p < 0.05$), where "a" and "d" correspond to the highest and lowest values, respectively.

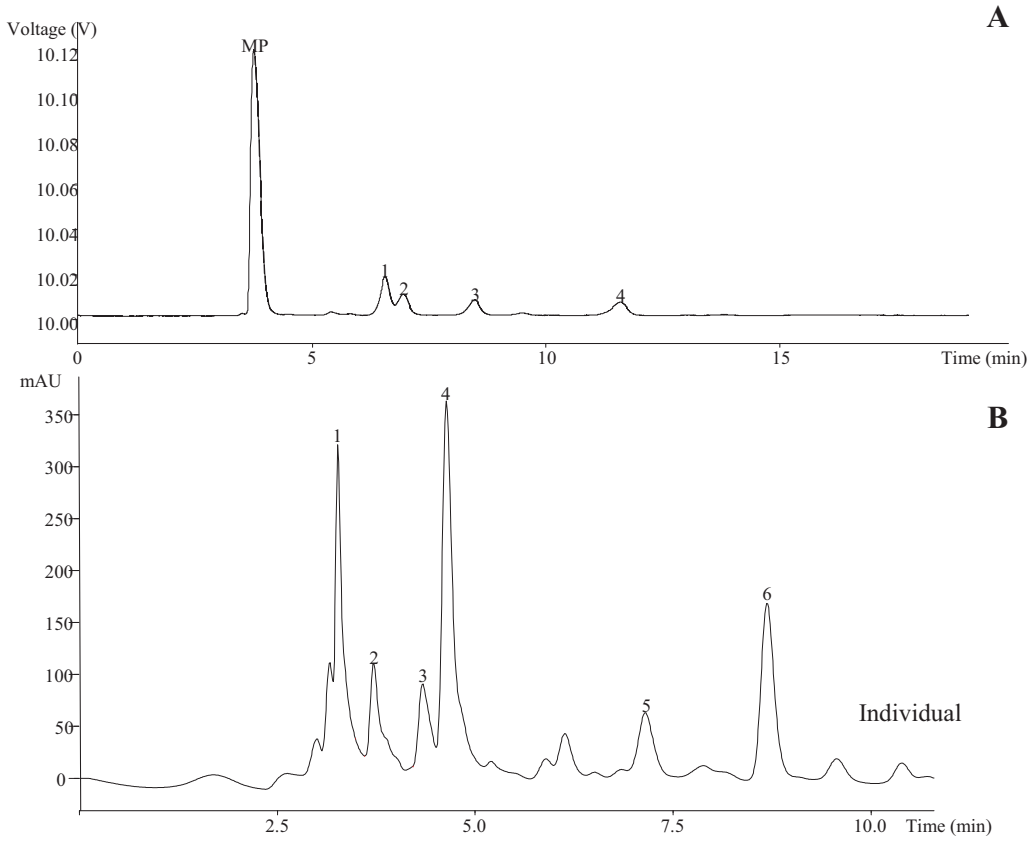

Fig. 2. Individual chromatograms of hydrophilic compounds in rose dried petals. (A) Free sugars profile: 1-fructose; 2-glucose; 3-sucrose; 4-melezitose (IS). (B) Organic acids profile: 1-oxalic acid; 2-quinic acid; 3-malic acid; 4-shiquimic acid; 5-citric acid; 6-fumaric acid. MP-mobile phase.

and infusions (0.19 g/100 ml of infusion) gave the highest total sugars amount, while centaurea dried petals (1.5 g/100 g dw) and infusion (0.14 mg/100 ml) presented the lowest levels of total sugars. Fructose, glucose and sucrose were detected in the dried petals and infusions, being fructose the main sugar present in dahlia and rose samples; with the exception of calendula dry petals and centaurea infusion, where sucrose was predominant. This is in accordance with the results reported by Barros et al. (2011) in *R. canina*. petals, in which fructose was also the main sugar. On the other hand, Dias et al. (2014) reported higher amounts of sugars in flowers of dandelion, despite having a similar profile (fructose, glucose and sucrose). Nonetheless, this tendency was not observed in *C. officinalis* samples analysed by Miguel et al. (2016), where fructose was the main sugar detected, followed by sucrose and xylose. Currently, EFSA does not have a recommended daily dose for sugars intake, since the data on the matter is

insufficient to set an upper limit of consumption for these compounds (EFSA, 2010a). Nonetheless, WHO recommends the reduce of free sugars intake to be less than 10% of total energy intake in a normal daily diet (Brouns, 2015). However, the studied flowers can be used "in" and "as" foods and contribute for sugar's daily intake.

Regarding the organic acids profile, the studied samples presented very distinct profiles (Table 2). The highest amount of organic acids was found in rose dried petals, mainly due to the presence of quinic and malic acids (1.53 and 1.23 g/100 g dw, respectively). Among the infusions, calendula and centaurea presented the highest concentrations, mainly due to the presence of quinic (14.5 mg/100 ml) and citric acids (15.5 mg/100 ml), respectively. The dried petals of calendula also presented high amounts of organic acids, mainly due to the contribution of malic and succinic acids (1.14 and 1.77 g/100 g dw, respectively). The presence of high quantities of malic acid was also detected in *C. officinalis* flowers by Miguel et al. (2016), however the presence of succinic acid was not reported, while citric acid was the main organic acid. The same tendency was also described by Dias et al. (2014) in flowers of dandelion, where malic acid was the most abundant one, showing also the highest level of total organic acids. Fumaric acid was only found in trace amounts in the analysed dahlia and centaurea dried petals. Dahlia revealed the lowest content of organic acids, presenting only traces of oxalic and shiquimic acids.

3.3. Lipophilic compounds of edible petals

The content in lipophilic compounds, namely fatty acids and tocopherols, was determined in the dried petals and the results are shown in Table 3 and Fig. 3. Twenty-four fatty acids were identified, being polyunsaturated fatty acids (PUFA) predominant in all the samples, with the exception of dahlia that showed higher concentration of saturated fatty acids (SFA). Linoleic acid (C18:2n6) was the major fatty acid found in dahlia and rose samples (36.54 and 31.87%, respectively), followed by palmitic acid (C16:0) and linolenic acid (C18:3n3), respectively. Calendula presented linolenic acid (36.90%) as the main fatty acid, followed by palmitic acid (21.70%), while centaurea presented eicosapentaenoic acid (C20:5n3, 26.93%) as the main fatty acid, followed by linolenic acid (18.75%). The results found for *C. officinalis* are in accordance with the ones described by Dulf et al. (2013) in which PUFA content is around 60–64%, and the saturated fraction is mainly consisted by palmitic acid. The same tendency was not reported by Miguel et al. (2016) in calendula samples, that presented a SFA fraction much higher than the PUFA fraction (78% and 21%, respectively). According with the recommendations of EFSA, the recommended daily intake of SFA is the lowest possible (EFSA, 2010b), and therefore, calendula edible flowers are good options presenting the lowest content of SFA. On the other hand, it is recommended a daily intake of 4% of the total dietary energy in linoleic acid and

Table 3
Fatty acids and tocopherols composition in dried petals (mean ± SD).

	Dahlia	Rose	Calendula	Centaurea
Fatty acids (***relative percentage, %***)				
C6:0	0.89 ± 0.07	0.18 ± 0.01	0.27 ± 0.01	0.17 ± 0.01
C8:0	0.90 ± 0.09	0.23 ± 0.02	0.28 ± 0.06	0.07 ± 0.00
C10:0	0.99 ± 0.04	0.33 ± 0.05	0.18 ± 0.08	0.12 ± 0.00
C11:0	nd	nd	0.13 ± 0.03	nd
C12:0	0.74 ± 0.03	1.22 ± 0.05	1.65 ± 0.18	nd
C13:0	nd	0.03 ± 0.00	nd	nd
C14:0	3.11 ± 0.20	2.55 ± 0.14	9.92 ± 0.39	0.89 ± 0.05
C14:1	0.59 ± 0.03	0.31 ± 0.00	nd	0.21 ± 0.02
C15:0	0.66 ± 0.00	0.31 ± 0.01	0.18 ± 0.01	0.37 ± 0.01
C16:0	24.61 ± 0.77	17.10 ± 1.06	21.70 ± 0.10	15.40 ± 0.10
C16:1	0.87 ± 0.00	0.22 ± 0.00	0.23 ± 0.03	0.28 ± 0.02
C17:0	0.91 ± 0.09	0.53 ± 0.04	0.19 ± 0.04	0.82 ± 0.02
C18:0	7.60 ± 0.28	16.80 ± 0.27	3.95 ± 0.08	9.67 ± 0.08
C18:1n9	5.75 ± 0.08	1.95 ± 0.19	1.56 ± 0.06	4.41 ± 0.04
C18:2n6	36.54 ± 0.85	31.87 ± 0.33	20.35 ± 0.14	6.72 ± 0.08
C18:3n3	8.60 ± 0.56	19.54 ± 0.79	36.90 ± 0.55	18.75 ± 0.14
C20:0	1.57 ± 0.08	3.62 ± 0.03	0.63 ± 0.02	5.34 ± 0.05
C20:2	0.40 ± 0.03	nd	nd	nd
C20:3n3	0.63 ± 0.10	0.33 ± 0.00	0.26 ± 0.01	0.51 ± 0.08
C20:5n3	nd	nd	nd	26.93 ± 0.29
C22:0	2.15 ± 0.19	1.81 ± 0.13	0.56 ± 0.04	2.04 ± 0.00
C22:1n9	nd	nd	nd	6.01 ± 0.12
C23:0	0.21 ± 0.02	0.08 ± 0.01	0.13 ± 0.03	0.15 ± 0.00
C24:0	2.31 ± 0.01	1.01 ± 0.07	0.93 ± 0.09	1.14 ± 0.10
SFA	46.64 ± 1.46a	45.79 ± 1.30b	40.70 ± 0.70c	36.18 ± 0.28d
MUFA	7.20 ± 0.11b	2.47 ± 0.19c	1.79 ± 0.02d	10.91 ± 0.13a
PUFA	46.16 ± 1.35d	51.74 ± 1.11c	57.51 ± 0.68a	52.91 ± 0.15b
Tocopherols (***mg/100 g dw***)				
α-Tocopherol	4.36 ± 0.07c	8.16 ± 0.08b	56.78 ± 1.06a	0.55 ± 0.02d
β-Tocopherol	1.77 ± 0.01a	0.18 ± 0.01c	1.16 ± 0.06b	nd
γ-Tocopherol	0.72 ± 0.02b	0.77 ± 0.01b	2.94 ± 0.08a	0.29 ± 0.02c
δ-Tocopherol	0.43 ± 0.01a	0.14 ± 0.01b	nd	nd
Sum	7.28 ± 0.04c	9.25 ± 0.04b	60.88 ± 0.92a	0.84 ± 0.04d

dw – dry weight basis; nd – not detected. C6:0 – Caproic acid; C8:0 – Caprylic acid; C10:0 – Capric acid; C11:0 – Undecylic acid; C12:0 – Lauric acid; C13:0 – Tridecanoic acid; C14:0 – Myristic acid; C14:1 – Myristoleic acid; C15:0 – Pentadecanoic acid; C16:0 – Palmitic acid; C16:1 – Palmitoleic acid; C17:0 – Heptadecanoic acid; C18:0 – Stearic acid; C18:1n9 – Oleic acid; C18:2n6 – Linoleic acid; C18:3n3 – Linolenic acid; C20:0 – Arachidic acid; C20:2 – *cis*-11,14 – Eicosadienoic acid; C20:3n3 – Eicosatrienoic acid; C20:5n3 – Eicosapentaenoic acid; C22:0 – Behenic acid; C22:1n9 – Erucic acid; C23:0 – Tricosanoic acid; C24:0 – Lignoceric acid. SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids. In each row different letters mean significant differences between samples ($p < 0.05$), where "a" and "d" correspond to the highest and lowest values, respectively.

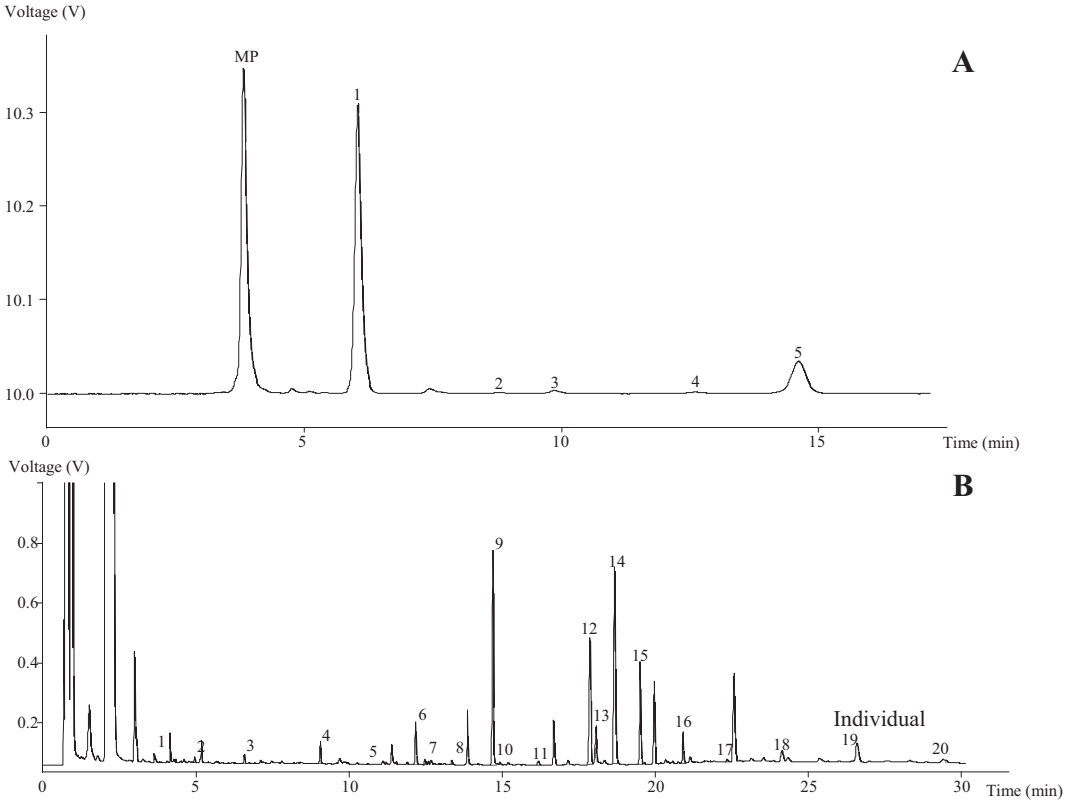

Fig. 3. Individual chromatograms of lipophilic compounds in rose dried petals. (A) Tocopherols profile: 1-α-tocopherol; 2-β-tocopherol; 3-γ-tocopherol; 4-δ-tocopherol; 5-tocol (PI). (B) Fatty acids profile: 1-C6:0; 2-C8:0; 3-C10:0; 4-C12:0; 5-C13:0; 6-C14:0; 7-C14:1; 8-C15:0; 9-C16:0; 10-C16:1; 11-C17:0; 12-C18:0; 13-C18:1n9; 14-C18:2n6; 15-C18:3n3; 16-C20:0; 17-C20:3n3; 18-C22:0; 19-C23:0; 20-C24:0. MP-mobile phase.

also the presence of eicosapentaenoic acid (C20:5n3), especially for pregnant women (EFSA, 2010b); only centaurea samples presented this last compound. For PUFA intake, WHO recommends more than 15% of the total dietary intake for infants (0–24 months) and 11% of the total dietary intake for children (2–18 years) (World Health Organization, 2008).

Regarding tocopherols, *C. officinalis* was the sample that revealed the highest content (60.88 mg/100 g dw), mainly due to the presence of α-tocopherol isoform (56.78 mg/100 g dw). Miguel et al. (2016) also described α-tocopherol as the main isoform in calendula flowers, however, the authors described lower values of total tocopherols. In all the samples, α-tocopherol isoform appears in higher amounts than the remaining isoforms. β- and δ-Tocopherols were not detected in centaurea, being the latter isoform also not present in calendula. The daily recommended dose for tocopherols consumption in adults is 300 mg/day (EFSA, 2008). Despite the lower values of the studied samples, the daily consumption of edible flowers could contribute to supply this vitamin to the organism.

Overall, calendula petals gave the highest content in total fat, ash and energetic contribution, polyunsaturated fatty acids (mainly due to the presence of linolenic acid) and total tocopherols (with the major contribution of α-tocopherol). On the other hand, rose petals presented the highest values of total proteins, soluble sugars and organic acids. Centaurea presented the highest carbohydrates content and the lowest percentage of saturated fatty acids. Regarding the infusions, dahlia and rose showed the highest content in carbohydrates, and the latter the highest energetic contribution. Calendula infusion presented the highest content in sugars, while the highest content in organic acids was found in centaurea infusion. These results demonstrate that edible petals can be consumed in a daily diet as a nutrient source, and could also be used to prepare infusions to be consumed worldwide.

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ANNEX II

Edible flowers as sources of phenolic compounds with bioactive potential

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Edible flowers as sources of phenolic compounds with bioactive potential

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ABSTRACT

The edible flowers are widely used, but there is still a lot to be done in relation to its bioactive potential and its correlation with the presence of phenolic compounds. The aim of this study was determined the individual phenolic profile in the hydromethanolic extracts and infusion preparations of four different flower samples (*Dahlia mignon*, *Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*, *Calendula officinalis* L., and *Centaurea cyanus* L.) and their bioactive potential (antioxidant, antiproliferative, and antibacterial capacity). All the studied flowers presented different profiles regarding their phenolic composition and revealed biological potential. The bioactive potential of the studied flowers was moderate, the hydromethanolic extracts of rose petals showed the best results for antioxidant and antibacterial assays, while the antiproliferative properties were only present in some of the tested cell lines, for the hydromethanolic extracts, in which dahlia and rose showed the best results. These results demonstrate that edible flowers can be used as a source of phenolic compounds with bioactive potential, which can be applied in the food sector, as foods and as sources natural ingredients.

1. Introduction

Chefs around the world strive to go beyond the ordinary as they prepare new recipes, garnishing their dishes with flowers or using them as ingredients in salads, soups, entrees, desserts, and drinks (Kou, Turner, & Luo, 2012). Edible flowers add a fresh and exotic aroma, delicate flavour and a visual appeal that makes them increasingly used in gourmet cuisine. *Dahlia mignon*, *Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*, *Calendula officinalis* L., and *Centaurea cyanus* L. are among the most popular edible flowers (Fernandes, Casal, Pereira, Saraiva, & Ramalhosa, 2017). The consumption of various types of edible flowers provides health benefits to the consumer, since they are a good source of phytochemicals, including phenolic compounds (Fernandes et al., 2017). These compounds have been related to the prevention of chronic degenerative diseases, such as diabetes, cognitive decline, and cardiovascular disease, as well as different types of cancer through the inhibition of their initiation and progression by modulating genes involved in key regulation processes (Anantharaju, Gowda, Vimalambike, & Madhunapantula, 2016; Gutiérrez-Grijalva et al., 2016). The flowers may contain a variety of these natural antioxidants such as phenolic acids and flavonoids, being their presence strongly related to their colour either directly (e.g., anthocyanins and other flavonoid pigments) or indirectly through the copigmentation processes (Brouillard, 1988; Kaisoon, Siriamornpun, Weerapreeyakul, & Meeso, 2011).

Several plants and their products have been used in foods as a mode of natural preservative, flavoring agent as well as a remedy to treat some of the common illness in humans. This property of curing is attributed mainly to their antimicrobial activities. Use of natural plant derived antimicrobials can be highly effective in reducing the dependence on antibiotics, minimize the chances of antibiotic resistance in food borne pathogenic microorganisms as well as help in controlling cross-contaminations by food-borne pathogens (Mak, Chuah, Ahmad, & Bhat, 2013).

The phenolic profile characterization, as also antioxidant and antitumor activities of calendula samples have been previously studied by (Miguel et al., 2016). As for rose samples (*R. canina*), there are some previous studies on the total phenolic composition (Kuś, Jerković, Tuberoso, Marijanović, & Congiu, 2014), individual phenolic profile (Demir, Yildiz, Alpaslan, & Hayaloglu, 2014; Guimarães et al., 2013), and antioxidant activity of fruits and flowers (Barros, Carvalho, & Ferreira, 2011; Barros, Dueñas, Carvalho, et al., 2012; Hvattum, 2002),

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but there is no report regarding *Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*. Regarding centaurea, only total phenolic composition has been studied (Kuś et al., 2014), while for dahlia, to the author's best knowledge, there are no previous reports on its phenolic composition or bioactive properties.

The main goal of the present study was to establish the phenolic profiles in hydromethanolic extracts and infusion preparations of four different flower samples (*Dahlia mignon*, *Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*, *Calendula officinalis* L., and *Centaurea cyanus* L.), and to evaluate their bioactive potential, including antioxidant, antiproliferative, and antibacterial capacity.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile (99.9%) was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic standards (apigenin-7-*O*-glucoside, caffeic acid, chlorogenic acid, hesperetin, isoliquiritigenin, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-rutioside, naringenin, *p*-coumaric acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside) were from Extrasynthèse (Genay, France). Lipopolysaccharide (LPS), dexamethasone, sulforhodamine B, trypan blue, trichloroacetic acid (TCA), tris(hydroxymethyl)aminomethane (Tris), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). RAW 264.7 cells were from the ECACC ("European Collection of Animal Cell Culture") (Salisbury, UK), and DMEM from Hyclone (Logan, Utah, US). The Griess Reagent System Kit was purchased from Promega (Madison, WI, USA). Dulbecco's modified Eagle's medium (DMEM), hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) were purchased from Hyclone (Logan, Utah, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA), *p*-Iodonitrotetrazolium chloride (INT) from Panreac Applichem (Barcelona, Spain), Tryptic Soy Broth (TSB) and Mueller-Hinton (MH) from Biolab® (Hungary). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and preparation of hydromethanolic extracts and infusions

The flower samples were supplied by the company RBR Foods, from Castro Daire (Portugal), as dry material to be used directly or for preparation of infusions. Petals of four different species were used in the present study: *Dahlia mignon* (commercial seeds mixture), *Rosa damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*, *Calendula officinalis* L., and *Centaurea cyanus* L. These samples are designated throughout the manuscript by their common names: dahlia, rose, calendula, and centaurea, respectively. All the samples were reduced to a fine powder (20 mesh) and mixed to obtain homogenate samples. The authors previously studied their nutritional composition (Pires, Dias, Barros, & Ferreira, 2017).

To prepare the hydromethanolic extracts, 1 g of each sample was submitted to extraction with a methanol:water mixture (80:20, v/v; 30 mL) at 25 °C and 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. Afterwards, the residue was extracted with one additional portion of the hydromethanolic mixture and the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland).

To prepare the infusions, boiling distilled water (100 mL, pH 6.6) at 100 °C was added to each sample (1 g) and left to stand at room temperature for 5 min, Afterwards, the infusions were filtered (0.22 μm).

Both preparations were frozen and then lyophilized in order to perform all the assays described below.

2.3. Analysis of phenolic compounds

The phenolic profile was determined in the lyophilized hydromethanolic extracts and infusions, which were re-dissolved at a concentration of 5 mg/mL in methanol:water (80:20, v/v) and water, respectively. The analysis was performed using a LC-DAD-*ESI/MSn* (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) as previously described by Bessada, Barreira, Barros, Ferreira, and Oliveira (2016). Double online detection was performed using 280, 330 and 370 nm as preferred wavelengths for DAD and in a mass spectrometer (MS). The MS detection was performed in negative mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. The identification of the phenolic compounds was performed based on their chromatographic behaviour, UV–vis and mass spectra by comparison with standard compounds, when available, and by using data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard. The peaks were quantified based on the area of the peak by using a manual quantification methods, which is permitted by the software used. To integrate peaks a perpendicular line was drawn from the valley between the peaks to the baseline extended between the normal baseline before and after the group of peaks. The results were expressed as mg/g of dry weight (dw).

2.4. Evaluation of the bioactivities

2.4.1. Antioxidant activity

The lyophilized hydromethanolic extracts and infusions were re-dissolved in methanol:water (80:20, v/v) and water, respectively, to obtain stock solutions of 2.5 mg/mL and 20 mg/mL, which were further diluted to obtain a range of concentrations (10 mg/mL to 0.07 mg/mL) for antioxidant evaluation by DPPH radical-scavenging (Hatano, Kagawa, Yasuhara, & Okuda, 1988), reducing power (Oyaizu, 1986) and inhibition of β-carotene bleaching assays (Shon, 2003). The final results were expressed as EC_{50} values (μg/mL), sample concentration providing 50% of antioxidant activity (for DPPH assay 50% of radical scavenge, 0.5 of absorbance in the reducing power assay and for β-carotene assay 50% of β-carotene bleaching inhibition). Trolox was used as positive control.

2.4.2. Antibacterial activity

The antibacterial activity was determined in the lyophilized hydromethanolic extracts and infusions, being re-dissolved in water in order to obtain a stock solution of 100 mg/mL, and then submitted to further dilutions. The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal. Seven Gram-negative bacteria (*Escherichia coli*, *E. coli* ESBL (extended spectrum of beta-lactamase), *Klebsiella pneumoniae*, *K. pneumoniae* ESBL, *Morganella morganii*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, isolated from urine and expectoration) and five Gram-positive bacteria (*MRSA*- methicillin-resistant *Staphylococcus aureus*, *MSSA*- methicillin-susceptible *Staphylococcus aureus*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis*) were used to screen the antibacterial activity Minimum Inhibitory Concentrations (MIC) determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay following the methodology proposed by Kuete, Ango, et al. (2011) and Kuete, Kamga, et al. (2011) with some modifications. MIC was defined as the lowest concentration that inhibits the visible bacterial growth. Three negative controls were prepared (one with Mueller-Hinton Broth (MHB)/Tryptic Soy Broth (TSB), another one with the extract, and the third with medium and

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds present in dahlia dry petals.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydromethanolic extracts	Infusions
Phenolic compounds								
1d	6.1	287	653	287(100)	Eriodictyol-acetyldihexoside[C]	DAD/MS	tr	tr
2d	7.4	320	353	191(100), 179(79), 173(20), 161(5), 135(12)	5-*O*-Caffeolquinic acid[B]	DAD/MS; commercial standard	1.82 ± 0.06*	0.53 ± 0.03*
3d	8.8	282	611	449(100), 287(48)	Eriodictyol-dihexoside[C]	Pereira, Peres, Silva, Domingues, and Cardoso (2013)	0.12 ± 0.02*	tr
4d	10.9	283	611	449(100), 287(57)	Eriodictyol-dihexoside[C]	Pereira et al. (2013)	tr	tr
5d	11.4	268	627	465(100), 447(87), 345(9), 285(6)	Pentahydroxyflavanone-dihexoside[G]	Lin, Mukhopadhyay, Robbins, and Harnly (2007)	0.93 ± 0.04*	0.59 ± 0.03*
6d	11.9	274	653	449(100), 287(59)	Eriodictyol-acetyldihexoside[C]	DAD/MS	tr	tr
7d	13.6	269	669	465(38), 447(100), 285(11)	Pentahydroxyflavanone-acetylhexoside-hexoside[G]	Lin et al. (2007)	0.76 ± 0.05*	0.47 ± 0.00*
8d	15.3	285	595	287(100)	Eriodictyol-deoxyhexosyl-hexoside[C]	DAD/MS	1.14 ± 0.06*	0.12 ± 0.07*
9d	16.2	347	771	285(100)	Kaempferol-pentosyl-rhamnosyl-hexoside[E]	Harbaum et al. (2007)	2.23 ± 0.02*	0.11 ± 0.01*
10d	16.3	285	449	287(100)	Eriodictyol-hexoside[C]	Guimarães et al. (2013)	1.56 ± 0.07*	0.18 ± 0.03*
11d	16.7	361	579	417(100), 255(57)	Isoliquiritigenin-dihexoside[D]	DAD/MS	1.57 ± 0.01	tr
12d	17.7	354	609	301(100)	Quercetin-3-*O*-rutinoside[H]	DAD/MS; commercial standard	0.89 ± 0.03*	0.29 ± 0.03*
13d	18.1	350	431	269(100)	Apigenin-hexoside[A]	DAD/MS; commercial standard	2.08 ± 0.05*	0.83 ± 0.04*
14d	20.7	287	637	475(100), 271(52)	Naringenin-hexoside-acetylhexoside[F]	DAD/MS	0.82 ± 0.01*	0.64 ± 0.00*
15d	21.0	284	433	271(100)	Naringenin-3-*O*-glucoside[F]	DAD/MS; commercial standard	2.92 ± 0.03*	0.79 ± 0.01*
16d	21.3	364	621	459(100), 255(48)	Isoliquiritigenin-hexoside-acetylhexoside[D]	DAD/MS	0.10 ± 0.01	tr
17d	21.6	285	579	301(100)	Hesperetin-pentosyl-rhamnoside[C]	DAD/MS	0.24 ± 0.01	tr
18d	23.3	380	433	271(100)	Butein-4′-glucoside (Coreopsin)[C]	Chen et al. (2016); Yang et al. (2016)	0.81 ± 0.05*	0.01 ± 0.00*
19d	26.4	348	563	285(100)	Kaempferol-pentosyl-rhamnoside[E]	Barros et al. (2013)	0.17 ± 0.03*	tr
20d	28.2	381	475	271(100)	Acetylcoreopsin[C]	Chen et al. (2016); Yang et al. (2016)	tr	tr
21d	28.7	377	577	433(100), 271(32)	Coreopsin derivative	DAD/MS	0.70 ± 0.02*	0.17 ± 0.00*
Sum of phenolic acid derivatives							1.817 ± 0.061*	0.53 ± 0.03*
Sum of flavonoids							17.040 ± 0.007*	4.20 ± 0.13*
Sum of phenolic compounds							18.857 ± 0.068*	4.73 ± 0.01 *

Standard calibration curves: A - apigenin-7-*O*-glucoside ($y = 10683x - 45{,}794$, $R^2 = 0.9906$); B - chlorogenic acid ($y = 168823x - 161{,}172$, $R^2 = 0.9999$); C - hesperetin ($y = 34156x + 268{,}027$, $R^2 = 0.9999$); D - isoliquiritigenin ($y = 42820x + 184{,}902$, $R^2 = 0.999$); E - kaempferol-3-*O*-rutioside ($y = 41843x + 220{,}192$, $R^2 = 0.9998$); F - naringenin ($y = 18433x + 78{,}903$, $R^2 = 0.9998$); G - quercetin-3-*O*-glucoside ($y = 34{,}843x - 160{,}173$; $R^2 = 0.9998$); H - quercetin-3-*O*-rutinoside ($y = 13343x + 76{,}751$, $R^2 = 0.9998$). tr - traces; nq - not quantified.

* *t*-Students test *p*-value < 0.001.

Table 2
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds present in rose dry petals.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydromethanolic extracts	Infusions
Phenolic compounds								
1r	18.2	348	477	301(100)	Quercetin-glucuronide[B]	Guimarães et al. (2013)	0.79 ± 0.03*	0.49 ± 0.01*
2r	18.6	355	463	301(100)	Quercetin-hexoside[B]	Guimarães et al. (2013)	1.37 ± 0.04	0.73 ± 0.01
3r	19.0	355	463	301(100)	Quercetin-3-*O*-glucoside[B]	DAD/MS; commercial standard	2.87 ± 0.07*	1.31 ± 0.01*
4r	21.1	348	593	285(100)	Kaempferol-3-*O*-rutinoside[A]	DAD/MS; commercial standard	tr	tr
5r	21.6	353	433	301(100)	Quercetin-pentoside[B]	Guimarães et al. (2013)	0.66 ± 0.01*	0.47 ± 0.01*
6r	22.0	348	461	285(100)	Kaempferol-glucuronide[A]	Guimarães et al. (2013)	tr	tr
7r	22.5	348	447	285(100)	Kaempferol-3-*O*-glucoside[A]	DAD/MS; commercial standard	2.74 ± 0.06*	0.88 ± 0.01*
8r	25.1	348	417	285(100)	Kaempferol-pentoside[A]	Barros, Dueñas, Ferreira, et al. (2011)	tr	tr
9r	25.9	347	417	285(100)	Kaempferol-pentoside[A]	Barros, Dueñas, Ferreira, et al. (2011)	tr	nd
10r	27.2	348	431	285(100)	Kaempferol-rhamnoside[A]	Barros, Dueñas, Ferreira, et al. (2011)	0.29 ± 0.02	tr
11r	30.1	314	609	463(100),301(12)	Quercetin-(*p*-coumaroyl) hexoside[B]	Barros et al. (2013)	0.46 ± 0.01*	0.37 ± 0.00*
12r	33.1	314	593	447(9),285(100)	Kaempferol-(*p*-coumaroyl) hexoside[A]	Guimarães et al. (2013)	tr	tr
Sum of phenolic compounds (flavonoids)							9.18 ± 0.23*	4.24 ± 0.01*

Standard calibration curves: A - kaempferol-3-*O*-rutinoside ($y = 41843x + 220{,}192$, $R^2 = 0.998$); B - quercetin-3-*O*-glucoside ($y = 34843x - 160{,}173$, $R^2 = 0.998$). nq - not quantified; tr-traces.
* *t*-Students test *p*-value < 0.001.

antibiotic). One positive control was prepared with MHB and each inoculum. For the Gram-negative bacteria, antibiotics, such as amikacin, tobramycin, amoxicillin/clavulanic acid, and gentamicin were used. For the Gram-positive bacteria, ampicillin and vancomycin were selected. The antibiotic susceptibility profile of Gram-negative and Gram-positive bacteria has been already described by (Dias et al., 2016) and is provided in supplementary materials (Table A1).

2.4.3. Antiproliferative activity

The lyophilized hydromethanolic extracts and the infusions were redissolved in water to obtain stock solutions of 4 mg/mL, and then submitted to further dilutions. Four human tumor cell lines were tested: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) from DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Sulforhodamine B assay was performed according to a procedure previously described by the authors (Barros et al., 2013).

Each of the cell lines were plated in a 96-well plate, at an appropriate density (1.0×10^4 cells/well) and allowed to attach for 24 h. The cells were then incubated in the presence of different extract concentrations during 48 h. Afterwards, cold trichloroacetic acid (TCA 10%, 100 μL) was added in order to bind the adherent cells and further incubated for 60 min at 4 °C. After the incubation period, the plates were washed with deionized water, dried, sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 μL) was incorporated to each plate well, and incubated for 30 min at room temperature. The plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200 μL) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) (Guimarães et al., 2013).

For evaluation of the hepatotoxicity in non-tumor cells, a cell culture (named as PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by the authors (Abreu et al., 2011). The liver tissues were rinsed in Hank's balanced salt solution containing penicillin (100 U/mL) and streptomycin (100 μg/mL), and divided into 1×1 mm^3 explants. A few of these explants were transferred to tissue flasks (25 cm^2) containing DMEM medium supplemented with fetal bovine serum (FBS, 10%), nonessential amino acids (2 mM), penicillin (100 U/mL), and streptomycin (100 mg/mL) and incubated at 37 °C with a humidified atmosphere (5% CO_2). The medium was changed every two days and the cell cultivation was continuously monitored using a phase contrast microscope. When confluence was reached, the cells were sub-cultured and plated in 96-well plate (density of 1.0×10^4 cells/well) containing DMEM medium supplemented with FBS (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL). Ellipticine was used as positive control and the results were expressed in GI_{50} values (concentration that inhibited 50% of the net cell growth).

2.5. Statistical analysis

Three samples were used for each species and all the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD) and analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $p = 0.05$. When necessary, a Student's *t*-test was used to determine the significant difference between two different samples, with $p = 0.05$. These analyses were carried out using IBM SPSS Statistics for Windows, Version 23.0. (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Individual phenolic profile of the hydromethanolic extracts and infusions of the flower samples

The extraction yields obtained for the hydromethanolic extract were, 47% for dahlia, 39% for rose, 25% for calendula, and 24% for centaurea, while the infusion presented the following extraction yields 37% for dahlia, 34% for rose, 27% for calendula, and 22% for centaurea. Dahlia hydromethanolic extract and infusions showed the most promising yield.

Tables 1–4 presents the compounds characteristic separated using HPLC methodology described above (retention time, λ_{max} in the visible region, mass spectral data), tentative identification and quantification of the phenolic compounds present in the hydromethanolic extracts and infusions prepared from dahlia, rose, centaurea, and calendula petals.

Table 3
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds present in calendula dry petals.

Peak	Rt (min)	λmax (nm)	$[M\text{-}H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydromethanolic extracts	Infusions
Phenolic compounds								
1c	5.8	320	341	179(100)	Caffeic acid hexoside[A]	Miguel et al. (2016)	0.03 ± 0.01	tr
2c	7.4	326	353	191(100),179(79),173(20),161(5),135(8)	5-*O*-Caffeoylquinic acid[B]	Miguel et al. (2016)	0.12 ± 0.01*	0.10 ± 0.00*
3c	10.4	324	179	161(5),135(100)	Caffeic acid[A]	DAD/MS; commercial standard	0.01 ± 0.00	tr
4c	14.7	354	755	301(100)	Quercetin-3-*O*-rhamnosylrutinoside[E]	Miguel et al. (2016)	0.31 ± 0.00*	0.40 ± 0.00*
5c	16.3	354	609	301(100)	Quercetin-deoxyhexosylhexoside[E]	Miguel et al. (2016)	0.33 ± 0.00	tr
6c	16.7	348	739	285(100)	Kaempferol-rhamnosylrutinoside[D]	Miguel et al. (2016)	tr	tr
7c	17.1	355	769	315(100)	Isorhamnetin-3-*O*-rhamnosylrutinoside[C]	Miguel et al. (2016)	3.99 ± 0.04*	2.71 ± 0.01*
8c	18.0	354	609	301(100)	Quercetin-3-*O*-rutinoside[E]	DAD/MS; commercial standard	0.30 ± 0.00*	0.38 ± 0.00*
9c	19.1	354	623	315(100)	Isorhamnetin-3-*O*-neohesperidoside[C]	Miguel et al. (2016)	0.69 ± 0.00*	0.35 ± 0.00*
10c	20.7	352	505	301(100)	Quercetin-3-*O*-(6″-acetyl)-glucoside[E]	Miguel et al. (2016)	0.23 ± 0.00*	0.33 ± 0.00*
11c	20.2	355	623	315(100)	Isorhamnetin-3-*O*-rutinoside[C]	Miguel et al. (2016)	5.40 ± 0.04*	3.20 ± 0.00*
12c	23.7	354	477	315(100)	Isorhamnetin-3-*O*-glucoside[C]	Miguel et al. (2016)	0.04 ± 0.01	tr
13c	26.3	355	519	315(100)	Isorhamnetin-3-*O*-(6″-acetyl)-glucoside[C]	Miguel et al. (2016)	0.16 ± 0.00	tr
Sum of phenolic acid derivatives							0.16 ± 0.03*	0.10 ± 0.00*
Sum of flavonoids							11.15 ± 0.09*	7.37 ± 0.01*
Sum of phenolic compounds							11.31 ± 0.07*	7.47 ± 0.02*

Standard calibration curves: A - caffeic acid ($y = 388345x + 406,369$, $R^2 = 0.994$); B - chlorogenic acid ($y = 168823x - 161,172$, $R2 = 0.9999$); C - isorhamnetin-3-*O*-glucoside ($y = 11117x + 30,861$, $R^2 = 0.9999$); D - kaempferol-3-*O*-rutinoside ($y = 41843x + 220,192$, $R^2 = 0.998$); E - quercetin-3-*O*-glucoside ($y = 34843x - 160,173$, $R^2 = 0.998$). nq - not quantified; tr - traces.
* *t*-Students test *p*-value < 0.001.

Exemplificative phenolic profiles of all the plants are shown in Fig. A1 in supplementary material. One phenolic acid derivative (chlorogenic acid), twenty flavonoids (eryodictiol, kaempferol, quercetin, apigenin, naringenin, hesperetin, butein, and isoliquiritigenin glycoside derivatives) and two unknown compounds were detected in dahlia samples. Rose petals showed twelve flavonoids, all of them derived from kaempferol and quercetin, and did not reveal any phenolic acid derivative. Three caffeic acid derivatives and ten flavonoids (kaempferol, quercetin, and isorhamnetin glycoside derivatives) were identified in calendula samples. Finally, centaurea samples presented five phenolic acid derivatives (derived from caffeic and *p*-coumaric acids), nine flavonoids (quercetin, kaempferol, apigenin, luteolin, and taxifolin glycoside derivatives) and one unknown compound. Due to the complexity of the discussion, letters were attributed to the peak numbers to identify the plant in which they were found: dahlia (d), rose (r), calendula (c), and centaurea (ce). Table A2 in supplementary material shows the main compounds identified in each sample and type of extract.

3.1.1. Flavonoids

3.1.1.1. Flavonols. Quercetin derivatives were detected in the four flower samples. Quercetin-3-*O*-glucoside (peak 3r) and quercetin-3-*O*-rutinoside (peaks 8c and 12d) were identified according to their UV spectra, elution order, and fragmentation pattern in comparison to the commercial standard. Compounds 3r and 8c were previously identified in rose fruits by Guimarães et al. (2013), and in calendula flowers by Miguel et al. (2016), respectively. Peaks 4c and 10c were identified as quercetin-3-*O*-rhamnosylrutinoside and quercetin-3-*O*-(6″-acetyl)-glucoside, respectively, which were also previously reported in *C. officinalis* (Miguel et al., 2016). Peak 12ce corresponded to the same compound as peak 10c. Peak 7ce ($[M\text{-}H]^-$ at *m/z* 667) with three MS^2 fragments at *m/z* 505 (− 162 u, loss of a hexosyl moiety), *m/z* 463 (− 42 u, loss of an acetyl moiety), and *m/z* 301 (− 162 u, loss of a hexosyl moiety) was tentatively identified as quercetin-hexoside-acetylhexoside. Peaks 1r and 5r were tentatively assigned as quercetin-glucuronide and quercetin-pentoside, respectively, being also previously reported by Guimarães et al. (2013) in *R. canina* fruits. Peak 2r presented the same pseudomolecular ion and fragmentation pattern as peak 3r (quercetin-3-*O*-glucoside), but a lower retention time, therefore it was tentatively assigned as a quercetin-hexoside. Similarly, peak 5c showed the same spectral characteristics as peak 8c (quercetin-3-*O*-rutinoside), but different retention time so it could not be identified as the pattern, being for that manner tentatively identified as a quercetin-deoxyhexosylhexoside. Peak 11r ($[M\text{-}H]^-$ at *m/z* 609) also presented the same pseudomolecular ion as 8c, but different UV spectra, fragmentation pattern and retention time. The observation in its MS^2 spectrum of a product ion at *m/z* 463, from the loss of 146 u and the UV maximum at 314 nm, as well as its late elution, were coherent with the presence of a coumaroyl residue. Therefore, this molecule was tentatively assigned as quercetin-(*p*-coumaroyl)hexoside.

Kaempferol derivatives were also observed in the four studied flowers, being especially relevant in the rose sample. Peaks 4r and 7r were identified according to their UV spectra, elution order, fragmentation pattern, and commercial standards, as kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside, respectively. The remaining compounds detected in rose flowers (i.e., peaks 6r, 8r, 9r, 10r, and 12r) were assigned following similar reasoning as for quercetin derivatives. Dahlia samples presented two kaempferol derivatives (peaks 9d and 19d) with pseudomolecular ions $[M\text{-}H]^-$ at *m/z* 771 and *m/z* 563, both releasing a unique MS^2 fragment at *m/z* 285, being associated to kaempferol-pentosyl-rhamnosyl-hexoside and kaempferol-pentosyl-rhamnoside, respectively. Centaurea and calendula samples presented one kaempferol derivative each (peaks 14ce and 6c) that were tentatively identified according to their mass spectral characteristics as kaempferol-acetylhexoside and kaempferol-rhamnosyl-rutinoside, respectively. This latter compound has already been reported in *C. officinalis* flowers

Table 4
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds present in centaurea dry petals.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Hydromethanolic extracts	Infusions
Phenolic compounds								
1ce	5.2	294,320sh	627	465(100), 303(3), 285(3)	Taxifolin derivatives[E]	DAD/MS	0.93 ± 0.02*	0.31 ± 0.00*
2ce	5.7	263	341	179(100), 161(1), 135(1)	Caffeic acid hexoside[B]	Miguel et al. (2016)	0.25 ± 0.01	tr
3ce	7.3	326	353	191(100), 179(80), 173(24), 161(5), 135(10)	*cis*-5-*O*-Caffeoylquinic acid[C]	DAD/MS; Barros, Dueñas, Carvalho, et al. (2012)	1.50 ± 0.18*	0.17 ± 0.01*
4ce	7.4	326	353	191(100), 179(80), 173(42), 161(5), 135(12)	*trans*-5-*O*-Caffeoylquinic acid[C]	DAD/MS; commercial standard	1.40 ± 0.06*	0.24 ± 0.02*
5ce	7.8	346	325	163(100)	*p*-Coumaric hexoside[F]	Barros, Dueñas, Pinela et al. (2012)	0.93 ± 0.01*	0.09 ± 0.01*
6ce	8.2	312	325	163(100)	*p*-Coumaric hexoside[F]	Barros, Dueñas, Pinela, et al. (2012)	0.44 ± 0.06*	0.04 ± 0.00*
7ce	13.0	350	667	505(100), 463(43), 301(14)	Quercetin-hexoside-acetylhexoside[E]	Barros, Dueñas, Carvalho, et al. (2012)	0.78 ± 0.00*	0.26 ± 0.00*
8ce	13.4	274,317sh	627	465(100), 303(3), 285(4)	Taxifolin derivatives[E]	DAD/MS	1.08 ± 0.04*	0.28 ± 0.01*
9ce	13.7	276,316sh	627	465(100), 303(1), 285(3)	Taxifolin derivatives[E]	DAD/MS	1.11 ± 0.12*	0.25 ± 0.01*
10ce	17.8	320	649	473(100), 269(8)	Apigenin-glucuronide-acetylhexoside[A]	DAD/MS	1.25 ± 0.00*	0.46 ± 0.01*
11ce	18.9	346	461	285(100)	Luteolin-glucuronide[E]	Miguel et al. (2016)	0.83 ± 0.01*	0.26 ± 0.00*
12ce	20.4	353	505	463(23), 301(100)	Quercetin-3-*O*-(6″-acetyl)-glucoside[E]	Barros, Dueñas, Carvalho, et al. (2012)	0.83 ± 0.01*	0.25 ± 0.00*
13ce	23.9	337	445	269(100)	Apigenin-glucuronide[A]	Guimarães et al. (2013)	12.22 ± 0.09*	1.52 ± 0.13*
14ce	24.8	330	489	285(100)	Kaempferol-acetylhexoside[D]	Barros, Dueñas, Carvalho, et al. (2012)	tr	tr
Sum of phenolic acid derivatives							4.52 ± 0.17*	0.55 ± 0.02*
Sum of flavonoids							19.03 ± 0.06*	3.59 ± 0.03*
Sum of phenolic compounds							23.55 ± 0.11*	4.14 ± 0.05*

Standard calibration curves: A - apigenin-7-*O*-glucoside ($y = 10683x - 45{,}794$, $R^2 = 0.991$); B - caffeic acid ($y = 388345x + 406{,}369$, $R^2 = 0.994$); chlorogenic acid ($y = 168823x - 161{,}172$, $R^2 = 0.9999$); D - kaempferol-3-*O*-rutinoside ($y = 41843x + 220{,}192$, $R^2 = 0.998$); E - quercetin-3-*O*-glucoside ($y = 34843x - 160{,}173$, $R^2 = 0.998$); F - *p*-coumaric acid ($y = 301950x + 6966.7$, $R^2 = 0.9999$). nq - not quantified; tr - traces.

* *t*-Students test *p*-value < 0.001.

by our group (Miguel et al., 2016).

Isorhamnetin derivatives were only detected in the calendula samples (peaks 7c, 9c, 11c, 12c, and 13c), being identified as isorhametin-3-*O*-rhamnosylrutinoside ($[M-H]^-$ at *m/z* 769), isorhametin-3-*O*-neohesperidoside ($[M-H]^-$ at *m/z* 623), isorhametin-3-*O*-rutinoside ($[M-H]^-$ at *m/z* 623), isorhametin-3-*O*-glucoside ($[M-H]^-$ at *m/z* 477) and isorhametin-3-*O*-(6″-acetyl)-glucoside ($[M-H]^-$ at *m/z* 519), respectively, all of them previously reported in *C. officinalis* flowers by Miguel et al. (2016).

3.1.1.2. Flavones. Peaks 13d ($[M-H]^-$ at *m/z* 431) and 13ce ($[M-H]^-$ at *m/z* 445), presented a unique MS^2 fragment at *m/z* 269 (associated to apigenin), corresponding to the loss of hexosyl (− 162 u) and glucuronyl (− 176 u) moieties, so that they were tentatively identified as apigenin-hexoside and apigenin-glucuronide, respectively. An identity of peak 13d as apigenin-7-*O*-glucoside was discarded by comparison with a standard. Peak 10ce ($[M-H]^-$ at *m/z* 649) released two MS^2 fragments at *m/z* 473 (176 u, loss of a glucuronyl moiety) and *m/z* 269 (162 + 42 u, further loss of an acetylhexosyl residue), being tentatively identified as an apigenin-glucuronide-acetylhexoside. Peak 11ce, with a pseudomolecular ion $[M-H]^-$ at *m/z* 461 releasing a unique MS^2 fragment at *m/z* 285 (loss of 176 u, corresponding to a glucuronyl moiety), was tentatively identified as a luteolin-glucuronide.

3.1.1.3. Flavanones. They were only detected in dahlia samples. Peaks 1d ($[M-H]^-$ at *m/z* 653), 3d and 4d ($[M-H]^-$ at *m/z* 611), 6d ($[M-H]^-$ at *m/z* 653), 8d ($[M-H]^-$ at *m/z* 595), and 10d ($[M-H]^-$ at *m/z* 449]), presenting characteristic UV spectra with λ_{max} around 274 nm and an MS^2 product ion at *m/z* 287, were associated as eriodictyol derivatives. According to their pseudomolecular ions they were tentatively identified as eriodictyol-acetyldihexoside (1d and 6d), eriodictyol-dihexoside (4d), eriodictyol-deoxyhexosyl-hexoside (8d), and eriodictyol-hexoside (10d). Peak 15d ($[M-H]^-$ at *m/z* 433) was identified as naringenin-3-*O*-glucoside based on its LC-MS characteristics in comparison with data available in our compound library. Peak14d ($[M-H]^-$ at *m/z* 637) showing an MS^2 fragmentation pattern at *m/z* 475 ($[M-H-162]^-$) and 271 (further loss of 162 + 42 u) was tentatively assigned as naringenin-hexoside-acetylhexoside. Peak 17d ($[M-H]^-$ at *m/z* 579) presented a unique MS^2 fragment at *m/z* 301 (− 132–146 u, loss of pentosyl rhamnosyl moieties) was tentatively identified as hesperetin-pentosyl-rhamnoside.

Peaks 5d and 7d presented a pseudomolecular ion $[M-H]^-$ at *m/z* 465 that is coherent with a pentahydroxyflavanone structure, also the UV spectra presented by this compounds at λ_{max} 270 nm with a shoulder at 320 nm is also characteristic with dihydroflavonoids, being tentatively assigned as pentahydroxyflavanone-dihexoside (5d) and pentahydroxyflavanone-acetylhexoside-hexoside (7d).

3.1.1.4. Dihydroflavonols. Peaks 1ce, 8ce, and 9ce, all of them showing the same pseudomolecular ion $[M-H]^-$ at *m/z* 627, detected in centaurea samples, were associated to taxifolin (i.e., dihydroquercetin) with hexose and caffeic acid residues (both with 162 mu). This could be explained by the characteristic UV spectra shape with a shoulder over 320 nm and also by the low abundance of the *m/z* 303 fragment. The *m/z* 465 fragment would correspond to the loss of one hexoside moiety ($[M-162]^-$), whereas the loss of the caffeic moiety could be justify the low abundance of the *m/z* 303 fragment.

3.1.1.5. Chalcones. Seven compounds detected in dahlia samples were identified as chalcones.

Peaks 11d and 16d presented an aglycone with *m/z* at 255 that fits both the flavanone liquiritigenin and its corresponding chalcone isoliquiritigenin. However, the flavanone nature was discarded based on their UV spectra showing λ_{max} around 360 nm, characteristic of chalcones, as also checked by comparison with a commercial standard of

isoliquiritigenin. According to their pseudomolecular ions and MS^2 fragmentation patterns, they were tentatively identified as isoliquiritigenin-dihexoside and isoliquiritigenin-hexoside-acetylhexoside.

Peaks 18d, 20d, and 21d showed similar spectra shapes as the previous ones with λ_{max} around 370–380 nm, and a common MS^2 product ion at *m/z* at 271 that matched the chalcone butein. The pseudomolecular ion ($[M-H]^-$ at *m/z* 433) of peak 18d was coherent with butein hexosides, being the first of them (the majority one) assigned as coreopsin (butein-4′-glucoside), described as a main flavonoid in other species of the Asteraceae family, such as *Coreopsis tinctoria* (Chen et al., 2016a; Yang et al., 2016). Peak 20d, possessing a molecular weight 42 u higher that peak 18d, was tentatively assigned as acetylcoreopsin, also reported in *Coreopsis tinctoria* (Yang et al., 2016). Peak 23d should correspond to a coreopsin derivative, although no definite identity could be assigned to it.

The most abundant flavonoids in dahlia (except dahlia's infusions), rose, calendula, and centaurea were naringenin-3-*O*-glucoside, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, and apigenin-glucuronide, respectively. Calendula hydromethanolic extracts and infusions presented the highest concentration of flavonoids (11.15 and 7.37 mg/g, respectively) and total phenolic compounds (11.31 and 7.47 mg/g, respectively) from all the studied samples. It also showed higher concentration of these compounds when compared to the ones reported by Miguel et al. (2016) in *C. officinalis* samples. This difference could be related to the type of treatment that were given to the sample (e.g. drying process), as well as the geographic origin of the samples.

3.1.2. Phenolic acids

Phenolic acid derivatives represent the second major class found in the four flower samples, but in rose flowers this kind of compounds were not detected. Caffeic acid (peaks 3c) and 5-*O*-caffeoylquinic acid (chlorogenic acid; peaks 2d, 2c, and 4ce) were positively identified according to their retention, mass, and UV–vis characteristics by comparison with commercial standards. Peak 3ce was assigned as the *cis* form of 5-*O*-caffeoylquinic acid, since the *cis* hydroxycinnamoyl derivatives would be expected to elute before the corresponding *trans* ones (Barros, Dueñas, Carvalho, et al., 2012). Peaks 1c and 2ce ($[M-H]^-$ at *m/z* 341) were tentatively identified as caffeic acid hexoside. The presence of caffeic acid hexoside and 5-*O*-caffeoylquinic acid was already reported in *C. officinalis* by our group (Miguel et al., 2016). Finally, peaks 5ce and 6ce were tentatively identified as *p*-coumaric hexoside based on their pseudomolecular ion ($[M-H]^-$ at *m/z* 325) and the MS^2 fragment at *m/z* 163 ($[\text{coumaric acid-H}]^-$, −162 u, loss of a hexosyl moiety). The observation of two compounds could be explained by a different location of the sugar residue on the coumaric acid or the existence of *cis/trans* isomers.

The hydromethanolic extract from centaurea samples presented the highest concentration in phenolic acids; while for the infusions the highest concentration of phenolic acids was found in centaurea sample. Chlorogenic acid was the most abundant phenolic acid present in all samples, which may be very interesting since this compound has been correlated with various biological effects, including antioxidant, anti-obesity, antiapoptosis, and antitumor activities (Kamiyama, Moon, Jang, & Shibamoto, 2015; Rakshit et al., 2010).

3.2. Bioactivities of the hydromethanolic extracts and infusions of the flower samples

Data regarding antioxidant, antiproliferative, and antibacterial activities of the hydromethanolic extracts and infusions of the four studied flowers are presented in Table 5. The hydromethanolic extracts and the infusions of rose petals were able to inhibit lipid peroxidation and to prevent oxidative damage, as well as, promote free radicals scavenging according to the results obtained for the DPPH radical scavenging assay (0.18 and 0.82 mg/mL, respectively), reducing power (1.29 and 0.64 mg/mL, respectively) and β-carotene bleaching inhibition (0.38 and 1.12 mg/mL, respectively) These results were in accordance with those obtained by Barros et al. (2011) for petals of *Rosa canina* L. Researches revealed that phytochemicals such as anthocyanins, flavonoids, phenolic acids, alkaloids, and glycosides in edible flowers exerted high anti-oxidant activities (Lu, Li, & Yin, 2016).

The hydromethanolic extracts of calendula showed lower EC_{50} values (higher antioxidant activity) in the DPPH and β-carotene bleaching inhibition assays than the ones reported by Miguel et al. (2016). These differences may be related to the presence of total flavonoids present in the samples as described by Lu et al., 2016.

Regarding antiproliferative activity, not all the samples studied inhibit the growth of the studied tumor cell lines. Dahlia hydromethanolic extracts and infusion gave the lowest GI_{50} values against HeLa (223.65 μg/mL) and MCF-7 (361.99 μg/mL, 303.27 μg/mL respectively) cell lines. The presence of flavanones only detected in dahlia samples, may have contributed to antiproliferative activity against more cell line (Manthey and Guthrie, 2002). Rose hydromethanolic extract also presented the capacity to inhibit the growth of tumor cell lines, such cervical and hepatocellular carcinoma. Similar results were also described by Nadpal et al. (2016) for rose sample using a cervical carcinoma cell line (308.5 μg/mL). Centaurea and calendula hydromethanolic extracts and infusions showed hardly or no antiproliferative activity, with GI_{50} values higher than 400 μg/mL in all the tested cells. None of the extracts or infusions presented hepatoxicity toward the non-tumor liver primary culture (PLP2).

The obtained data for antimicrobial activity were presented in Table 5. Our results showed that samples were active against all the microorganisms used, however in most cases, the samples were found to be more active against Gram-positive bacteria with MICs ranging from 0.625 to 10 mg/mL. These results were in accordance with results presented by Nowak et al. (2014). For Gram-positive bacteria, the infusions of rose samples showed the best results for *Staphylococcus aureus* (0.625 mg/mL), MRSA (0.625 mg/mL), and MSSA (1.25 mg/mL). The hydromethanolic extracts of rose sample also showed the best antibacterial activity against Gram-negative bacteria, *E. coli* and *Morganella morganii* (1.25 mg/mL, for both). To obtain this beneficial effect with the consumption of this plant, and considering the mentioned MICs, a portion of 3.9 mg of rose plant/mL (e.g. 0.78 g per cup of infusion) would be necessary. For the remaining plants the necessary portions would be 2.4, 5 and 4.8 mg of dahlia, calendula and centaurea, respectively per mL. Extended-spectrum beta-lactamase-producing (ESBL 2) *Escherichia coli* was not affected by any infusions studied.

Overall, this is an innovative study on the phenolic profile, antioxidant, antiproliferative, and antibacterial activities of dahlia, rose and centaurea petals. Meanwhile, for calendula petals the mentioned bioactivities were previously reported Miguel et al. (2016). Flavonoids were the predominant compounds in all the studied samples, although each of them presented different phenolic profiles, both in terms of phenolic families and contents. The highest concentration of total phenolic compounds was found in calendula, with relevant amounts of isorhamnetin derivatives. The hydromethanolic extracts and infusions of rose petals showed the greatest antioxidant activity, which could be due to the presence of quercetin and kaempferol derivatives.

Flavonoids are also considered safe compounds with low potential to induce organic toxicity (Middleton Jr. et al., 2000). However, they can exhibit prooxidant activity explaining some mutagenic and cytotoxic effect (Galati & O'Brien, 2004). The prooxidant and antioxidant properties of flavonoids such as quercetin and kaempferol derivatives, depend on the environment in which they are inserted as also their chemical structure and concentration (Carocho & Ferreira, 2013).

The hydromethanolic extracts of rose petals showed the best results for the antibacterial activity, and dahlia hydromethanolic extracts for the antiproliferative activity against almost all the tumor cell lines tested, with the exception of NCI-H460. Antiproliferative and antibacterial activities were also highly related with phenolic compounds.

These results support the potential of edible flowers as sources of

Table 5
Antioxidant, antiproliferative, hepatotoxic and antibacterial activities of hydromethanolic extracts and infusions of the studied flowers (mean ± SD).

	Hydromethanolic extracts				Infusions			
	Dahlia	Rose	Calendula	Centaurea	Dahlia	Rose	Calendula	Centaurea
Antioxidant activity EC_{50} values (mg/mL)[A]								
DPPH scavenging activity	0.63 ± 0.01^{c}	0.18 ± 0.02^{d}	1.37 ± 0.08^{a}	0.83 ± 0.03^{b}	1.17 ± 0.05^{c}	0.82 ± 0.01^{d}	16.71 ± 0.29^{a}	10.78 ± 0.26^{b}
Reducing power	1.33 ± 0.07^{b}	1.29 ± 0.09^{b}	7.96 ± 0.08^{a}	8.14 ± 0.18^{a}	0.799 ± 0.001^{d}	0.64 ± 0.01^{c}	10.19 ± 0.12^{a}	5.10 ± 0.03^{b}
β-carotene bleaching inhibition	0.48 ± 0.02^{bc}	0.38 ± 0.03^{c}	0.66 ± 0.08^{b}	1.17 ± 0.01^{a}	2.01 ± 0.07^{c}	1.12 ± 0.04^{d}	8.50 ± 0.08^{a}	8.06 ± 0.30 ^{b}
Antiproliferative activity GI_{50} values (μg/mL)[B]								
MCF-7 (breast carcinoma)	361.99 ± 28.83	> 400	> 400	> 400	303.27 ± 26.13^{b}	377.09 ± 32.09^{a}	> 400	> 400
NCI-H460 (non-small cell lung carcinoma)	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 400
HeLa (cervical carcinoma)	223.65 ± 2.78^{b}	308.45 ± 17.13^{a}	> 400	> 400	> 400	> 400	> 400	> 400
HepG2 (hepatocellular carcinoma)	339.15 ± 15.14^{a}	296.82 ± 23.71^{b}	> 400	> 400	> 400	315.33 ± 19.28	> 400	> 400
Hepatotoxicity GI_{50} values (μg/mL)[B]								
PLP2	> 400	> 400	> 400	> 400	> 400	> 400	> 400	> 400
Antibacterial activity MIC values (mg/mL)								
Gram-negative bacteria								
Acinetobacter baumannii	5	2.5	10	5	10	2.5	> 20	> 20
Escherichia coli ESBL 1	5	5	10	5	10	10	10	20
Escherichia coli ESBL 2	5	1.25	5	5	–	–	–	–
Escherichia coli	2.5	5	20	20	10	5	20	20
Klebsiella pneumoniae	5	2.5	5	10	> 20	10	> 20	> 20
Klebsiella pneumoniae ESBL	5	2.5	10	5	> 20	10	> 20	> 20
Morganella morganii	2.5	1.25	20	10	2.5	1.25	20	20
Pseudomonas aeruginosa	> 20	2.5	5	10	20	2.5	> 20	20
Gram-positive bacteria								
Enterococcus faecalis	2.5	2.5	5	20	5	> 20	20	> 20
Listeria monocytogenes	5	10	5	20	5	> 20	20	> 20
Staphylococcus aureus	2.5	1.25	10	5	1.25	0.625	20	5
MRSA	5	1.25	5	5	2.5	0.625	> 20	20
MSSA	5	1.25	10	10	2.5	1.25	20	10

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. A - Trolox EC_{50} values: 62.98 μg/mL (DDPH), 45.71 μg/mL (reducing power), 10.25 μg/mL (β-carotene bleaching inhibition); B - Ellipticine GI_{50} values: 1.21 mg/mL (MCF-7), 1.03 mg/mL (NCI-H460), 0.91 mg/mL (HeLa), 1.10 mg/mL (HepG2) and 2.29 mg/mL (PLP2). MIC values correspond to the minimal sample concentration that inhibited the bacterial growth. In each row and for the different extraction procedures, different letters mean significant differences ($p < 0.05$).

phenolic compounds with bioactive potential, having a high phytochemical interest for the food industry.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2017.11.014.

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ANNEX III

Antioxidant and antimicrobial properties of dried Portuguese apple variety (Malus domestica Borkh. cv Bravo de Esmolfe)

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Antioxidant and antimicrobial properties of dried Portuguese apple variety (*Malus domestica* Borkh. cv Bravo de Esmolfe)

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ABSTRACT

Malus domestica Borkh apples are one of the most consumed fruits in the world, due to their sweetness and flavour. Herein, 'Bravo de Esmolfe' apple fruits were characterized regarding their nutritional value, chemical composition and bioactive properties. Besides nutrients, flavan-3-ols (i.e., epicatechin and B-type procyanidins) as also hydroxycinnamoyl-quinic acids and phloretin derivatives were identified in the samples. Extracts prepared from 'Bravo de Esmolfe' also proved to have antioxidant activity and antibacterial effects against Gram-positive bacteria, namely methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes* and *Enterococcus faecalis*, and against the Gram-negative bacteria *Escherichia coli*, *Escherichia coli* (ESBL) (producing extended spectrum β-lactamases) and *Morganella morganii*. There is very little information about 'Bravo de Esmolfe' apple, so this study is important to inform consumers about an alternative source of nutritional and bioactive compounds.

1. Introduction

'Bravo de Esmolfe' is a Portuguese apple variety with an intense aroma, highly appreciated by consumers. This apple was recognised as a product with Protected Designation of Origin (PDO), being therefore a high added value product with impact in the local and national economy (N°1107/96, 2001; Reis, Rocha, Barros, Delgadillo, & Coimbra, 2009). In the last few years the 'Bravo de Esmolfe' apple has doubled its price compared to exotic varieties, such as Golden and Starking (Feliciano et al., 2010). Its production is carried out in a restricted and small inland region in northern Portugal, corresponding to a production of 200,000 kg per year, but commercial demand is now increasing, due to its appealing sensory properties, namely sweetness and flavour (Bhatti & Jha, 2010). The regular consumption of fruits and vegetables has been associated with reduced risk of developing chronic diseases. These benefits are often attributed to their high phytochemical content and antioxidant power (Serra et al., 2010). Apple fruits have a wide variety and well-balanced composition, being moderately energetic and well-proportioned in sugar and acid contents, giving it a pleasant taste. The chemical composition of apples varies depending on the cultivar, production region and horticultural practices (Róth et al., 2007). They are mostly constituted by water (84%), minerals, complex B vitamins (Feliciano et al., 2010), monosaccharides, dietary fibre, and various biologically active compounds, such as vitamin C, and certain phenolic compounds (Róth et al., 2007; Wu et al., 2007) Feliciano et al., (Feliciano et al., 2010) studied several nutritional parameters in apple varieties, including the "Bravo de Esmolfe" apple. Wu et al. (Wu et al., 2007) reported the sugars and organic acids composition as also the phenolic profile of different apple cultivars. Various authors (Malec et al., 2014; Mayr, Treutter, Santos-Buelga, Bauer, & Feucht, 1995; Scafuri et al., 2016; Shoji, Masumoto, Moriichi, Kanda, & Ohtake, 2006; Shoji et al., 2003; Verdu et al., 2013; Wojdyło, Oszmiański, & Laskowski, 2008) also presented the phenolic profile of different apple cultivars, but none of the previously mentioned authors have studied the bioactive properties and compounds from the cultivar 'Bravo de Esmolfe'. Therefore, to the best of the author's knowledge, there is still scarce information about this apple variety.

The aim of the present work, was to characterize the nutritional and chemical composition of *Malus domestica* Borkh cv 'Bravo de Esmolfe', as also its bioactive properties in terms of phenolic compounds, antioxidant and antibacterial properties.

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2. Materials and methods

2.1. Standards and reagents

HPLC grade acetonitrile (99.9%), *n*-hexane (95%) and ethyl acetate (99.8%) were purchased from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (standard 47885-U), formic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), L-ascorbic acid, tocopherol, sugars and organic acid standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenolic standards were acquired from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). *p*-Iodonitrotetrazolium chloride (INT) from Panreac Applichem (Barcelona, Spain), Tryptic Soy Broth (TSB) and Mueller-Hinton (MH) from Biolab® (Hungary). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples

Apple samples (*Malus domestica* Borkh. cv 'Bravo de Esmolfe'), were kindly supplied by the RBR foods company from Castro Daire (Portugal), in the dry form (without skin), since the company's objective is to market this as a snack. After reception, the material was reduced to a fine dried powder (20 mesh), mixed to obtain a homogenate sample and stored in a desiccator, protected from light, until further analysis. All the assays were performed in triplicate.

2.3. Nutritional composition

The proximate composition was determined according to AOAC procedures (AOAC, 2016), including protein by the macro-Kjeldahl method (991.02); crude fat using a Soxhlet apparatus and extracting the powdered sample with petroleum ether (989.05) and ash contents (935.42) by incineration at 550 ± 15 °C. The total carbohydrates (including fiber) were calculated by difference ([Total carbohydrates (g/100 g) = 100 − (g fat + g protein + g ash)]) and total energy was calculated according to the following equation: Energy (kcal/100 g) = 4 × (g proteins + g carbohydrates) + 9 × (g fat).

2.4. Fatty acids

Fatty acids were determined after Soxhlet extraction using the powdered sample and after a *trans*-esterification process. The analysis was performed by GC-FID (DANI model GC 1000 instrument, Contone, Switzerland) and separation was achieved using a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm df). The results were expressed in relative percentage of each fatty acid (Barros, Pereira, & Ferreira, 2013; Dias et al., 2015).

2.5. Tocopherols

Tocopherols (four isoforms) were analysed in the powdered sample and analysed by HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) and separation was achieved using a Polyamide II (5 μm, 250 × 4.6 mm) normal-phase column from YMCWaters (YMC America, Inc., Allentown, PA, USA). Tocol was used as an internal standard and the quantification was based on the fluorescence signal response of each standard, The results were expressed in mg per 100 g of dry plant weight. (Barros et al., 2013; Dias et al., 2015).

2.6. Soluble sugars

Soluble sugars were determined in the powdered sample by HPLC coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), as previously described by Barros et al. (2013). Separation was achieved using a Eurospher 100-5 NH2 column (5 μm, 4.6 × 250 mm, Knauer) and quantification was performed using internal standard method (IS, melezitose). The results were expressed in g per 100 g of plant dry weight.

2.7. Organic acids

Organic acids were determined in the powdered samples and analysed by HPLC coupled to photodiode array detector (UFLC-PDA; Shimadzu Corporation, Kyoto, Japan) and separation was performed with a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 × 4.6 mm i.d.). The quantification was performed by comparison of the peak area recorded at 215 nm as preferred wavelength. The results were expressed in g per 100 g of plant dry weight (Barros et al., 2013; Dias et al., 2015).

2.8. Hydromethanolic extracts preparation

The hydromethanolic extracts were prepared by mixing 1 g of the powered dried apple sample with a methanol: water mixture (80:20, *v*/*v*) at 25 °C and 30*g* during 1 h, followed by filtration through a Whatman filter paper No. 4. The remain residue was re-extracted with an additional portion of methanol:water mixture and the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and stored at −20 °C for further analysis.

2.9. Phenolic compounds analysis

The phenolic compounds were determined in the hydromethanolic extract solution (5 mg/ml) by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), following a procedure previously described by the authors (Bessada, Barreira, Barros, Ferreira, & Oliveira, 2016). Chromatographic separation was performed using a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 × 150 mm). For the double online detection, a DAD (280, 330 and 370 nm as preferred wavelengths) and a mass spectrometer performed in negative mode (Linear Ion Trap LTQ XL mass spectrometer equipped with an ESI source, ThermoFinnigan, San Jose, CA, USA) were used and connected to the HPLC system. The identification was performed using standard compounds, when available, by comparing their retention times, UV–vis and mass spectra. If no standard compound was available, phenolic compounds were identified by comparing the obtained information with available data reported in the literature, giving a tentative identification. Quantification was made from the areas of the peaks recorded at 280 nm by comparison with calibration curves obtained from standards. The results were expressed as mg/100 g dry weight (dw).

2.10. Antioxidant and antibacterial activity of the hydromethanolic extracts

The antioxidant activity was evaluated in the extracts re-dissolved in methanol:water mixture (10 to 0.3125 mg/ml) through DPPH radical-scavenging, reducing power, inhibition of β-carotene bleaching and TBARS inhibition assays. Trolox was used as positive control and the results were expressed in EC_{50} values Barros et al. (2013). The antibacterial activity was determined in the extracts re-dissolved in water (stock solution 20 mg/ml). The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal. The antibiotic susceptibility profile was screened previously (Dias et al., 2016) for all the tested bacteria. Microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay were used to determine

Table 1
Nutritional and chemical composition of 'Bravo de Esmolfe' apples (mean ± SD).

Nutritional value (g/100 g dw)		*Soluble sugars (g/100 g dw)*	
Fat	5.9 ± 0.3	Fructose	19.0 ± 0.2
Proteins	2.61 ± 0.02	Glucose	8.4 ± 0.3
Ash	1.84 ± 0.04	Sucrose	1.38 ± 0.02
Total carbohydrates	89.68 ± 0.03	Sum	28.8 ± 0.1
Energy (kcal/100 g dw)	492 ± 1		
Fatty acids (relative percentage, %)		*Organic acids (g/100 g dw)*	
C10:0	0.63 ± 0.01	Oxalic acid	0.101 ± 0.004
C12:0	0.81 ± 0.01	Quinic acid	0.15 ± 0.01
C14:0	1.92 ± 0.07	Malic acid	1.36 ± 0.01
C14:1	2.30 ± 0.05	Shikimic acid	0.0002 ± 0.0001
C15:0	1.04 ± 0.08	Sum	1.6 ± 0.1
C16:0	28.94 ± 0.07		
C17:0	1.8 ± 0.1		
C18:0	16.4 ± 0.1		
C18:1n9	5.89 ± 0.04		
C18:2n6	15.8 ± 0.4		
C18:3n3	7.6 ± 0.3		
C20:0	1.52 ± 0.09		
C20:3n3	0.98 ± 0.04		
C22:0	3.76 ± 0.01		
C23:0	1.02 ± 0.05		
C24:0	9.6 ± 0.1		
SFA	67.4 ± 0.2		
MUFA	8.19 ± 0.08		
PUFA	24.4 ± 0.1		
Tocopherols (mg/100 g dw)			
α-Tocopherol	0.52 ± 0.02		

dw- dry weight basis. C10:0- Capric acid; C12:0- Lauric acid; C14:0- Myristic acid; C14:1 - Myristoleic acid; C15:0- Pentadecanoic acid; C16:0- Palmitic acid; C17:0 - Heptadecanoic acid; C18:0 - Stearic acid; C18:1n9- Oleic acid; C18:2n6- Linoleic acid; C18:3n3- Linolenic acid; C20:0- Arachidic acid; C20:3n3 - Eicosatrienoic acid; C22:0 - Behenic acid; C23:0 - Tricosanoic acid; C24:0 - Lignoceric acid. SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids dw- dry weight basis; Calibration curves for organic acids: oxalic acid ($y = 9 \times 106x + 45973$, $R^2 = 0.9901$); quinic acid ($y = 610607x + 46061$, $R^2 = 0.9995$); malic acid ($y = 912441x + 92665$, $R^2 = 0.999$) and shikimic acid ($y = 7x107x + 175156$, $R^2 = 0.9999$); (< LOD: 12.6, 24, 36 and 0.01 μg/ml for oxalic, quinic, malic and shikimic acids, respectively); (< LOQ: 42, 81, 1.2 × 102 and 0.02, μg/ml for oxalic, quinic, malic and shikimic acids, respectively).

minimum inhibitory concentration (MIC) (Kuete, Ango, et al., 2011; Kuete, Justin, et al., 2011).

3. Results and discussion

Data regarding the proximate composition and energetic value of the 'Bravo de Esmolfe' dried apples are shown in Table 1. Carbohydrates were the most abundant macronutrients, followed by fat, proteins and ash. Feliciano et al. (Feliciano et al., 2010) reported lower values of proteins (0.07 g/100 g dw). However, USDA (United States Department of Agriculture) reported similar values for carbohydrates (95.72 g/100 g dw) and energetic value (360 kcal/100 g dw) in apple raw samples without skin but lower fat content (0.97 g/100 g dw) (USDA, 2016). Sixteen fatty acids were identified with the predominance of saturated fatty acids, mostly palmitic acid, followed by stearic and linoleic acids (28.94%, 16.4% and 15.8%, respectively). Interestingly, previous studies carried out by Wu et al. (Wu et al., 2007) using other apple varieties (Delicious, Golden Delicious, Ralls, Fuji, QinGuan, Granny Smith, Jonagold, Orin and Average), reported linoleic acid as the most abundant fatty acid. α-Tocopherol (0.52 mg/100 g dw) was the only tocopherol isoform found in this sample, which is in agreement with the reported by Feliciano et al., (Feliciano et al., 2010) in 'Bravo de Esmolfe' (0.75 mg/100 g dw). Fructose, glucose and sucrose were the sugars detected in the analysed sample (19.0, 8.4 and 1.38 g/100 g dw, respectively), being fructose the most abundant one; these results are also in agreement with previous studies in 'Bravo de Esmolfe' apple (Feliciano et al., 2010). Regarding organic acids, malic acid was the main molecule present, followed by quinic, oxalic and shikimic acids (1.36, 0.15, 0.101 and 0.0002 mg/100 g dw, respectively). These results are in accordance with those reported by Chinnici, Spinabelli, Riponi, & Amati (Chinnici, Spinabelli, Riponi, & Amati, 2005), where malic acid was also the main organic acid described in apple juices. Feliciano et al. (Feliciano et al., 2010) and Wu et al. (Wu et al., 2007) reported the presence of other organic acids, such as citric, succinic and fumaric acids in different apple varieties, such as Delicious, Golden Delicious, Ralls, Fuji, QinGuan, Granny Smith, Jonagold, Orin and Average. The differences found in the chemical composition of apples could depend on the cultivar, production region and horticultural practices (Róth et al., 2007).

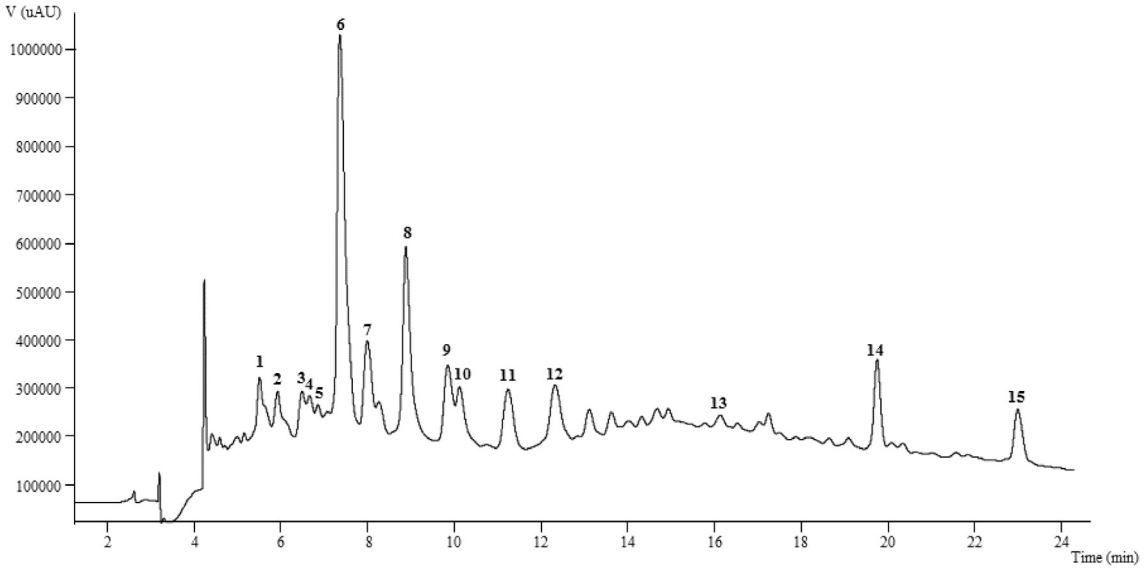

Fig. 1. HPLC chromatogram recorded at 280 nm showing the phenolic profile of the 'Bravo de Esmolfe' hydromethanolic extract (numbers correspond to the compounds mentioned in Table 2).

Table 2
Retention time (Rt), wavelengths of maximum absorption (λ_{max}) mass spectral data, tentative identification and quantification of the phenolic compounds present in the 'Bravo de Esmolfe' hydromethanolic extracts.

Peak	Rt (min)	λ_{max} (nm)	[M−H] (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/100 g dw)	Reference usedfor identification
1	5.56	281	577	451(24),425(100),407(21),289(12)	Procyanidin B1	11.38 ± 0.04	(Mayr et al., 1995; Shoji et al., 2003, 2006; Verdu et al., 2013; Wojdyło et al., 2008)
2	5.98	271	373	327(17),165(100),121(12),93(14)	Unknown	–	
3	6.54	280	865	739(74),713(44),695(100),577(64),575(37),425(10),407(9),289(8),287(7)	B-type epicatechin trimer[1]	11.3 ± 0.1	(Shoji et al., 2003, 2006)
4	6.7	281	865	739(69),713(43),695(1 0 0),577(68),575(36),425(11),407(7),289(6),287(8)	B-type epicatechin trimer[1]	9.6 ± 0.2	(Shoji et al., 2003, 2006)
5	6.96	322	353	191(12),179(1),173(100),161(1),135(2)	4-*O*-Caffeoylquinic acid[2]	5.8 ± 0.1	(Dias et al., 2016)
6	7.4	327	353	191(100),179(6),173(2),161(1),135(1)[3]	5-*O*-Caffeoylquinic acid[2]	51.5 ± 0.5	(Dias et al., 2016)
7	8.09	280	577	451(17),425(100),407(19),289(7)	Procyanidin B2	34.5 ± 0.3	(Mayr et al., 1995; Shoji et al., 2003, 2006; Verdu et al., 2013; Wojdyło et al., 2008)
8	8.95	311	337	191(3),173(95),163(8),145(4),119(3)	4-*p*-Coumaroylquinic acid[3]	14.1 ± 0.6	(Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
9	9.96	281	289	245(100),203(5),187(1),161(2),137(2)	Epicatechin[1]	18.2 ± 0.4	DAD/MS
10	10.19	312	337	191(2),173(100),163(7),119(2)	5-*p*-Coumaroylquinic acid[3]	6.93 ± 0.04	(Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
11	11.34	280	865	739(83),713(53),695(100),577(82),575(43),425(14),407(9),289(8),287(12)	Procyanidin C1	19.56 ± 0.01	(Mayr et al., 1995; Shoji et al., 2003, 2006; Verdu et al., 2013; Wojdyło et al., 2008)
12	12.42	280	1153	865(19),863(18),577(6),575(11),289(3),287(4)	(C4,C8)-epicatechin tetramer[1]	24.77 ± 0.01	(Santos-Buelga, García-Viguera, & Tomás-Barberán, 2003; Shoji et al., 2003, 2006)
13	16.2	280	579	289(56),245(100),203(9)	Unknown biflavonoid[1]	16.7 ± 0.4	
14	19.85	285	567	273(100),167(5),123(5)	Phloretin-2′-*O*-xyloglucoside[4]	7.87 ± 0.02	(Malec et al., 2014; Mayr et al., 1995; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
15	23.09	285	435	273(100),167(4),123(5)	Phlorizin (phloretin-2′-*O*-glucoside)[4]	4.84 ± 0.01	(Malec et al., 2014; Mayr et al., 1995; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008)
					Total phenolic compounds	**237 ± 1**	

Standard calibration curves recorded at 280 nm: (1) catechin ($y = 158.42x + 11.38$, $R^2 = 0.999$); (2) chlorogenic acid ($y = 168823x - 161172$; $R^2 = 0.9999$); (3) *p*-coumaric acid ($y = 706.09x + 1228.1$, $R^2 = 0.9989$); (4) isoliquiritigenin ($y = 42820x + 184902$, $R^2 = 0.999$).

Table 3
Bioactive properties of the 'Bravo de Esmolfe' hydromethanolic extracts (mean ± SD).

Antioxidant activity EC_{50} values (mg/ml) *	
DPPH scavenging activity	0.71 ± 0.05
Reducing power	1.38 ± 0.01
β-carotene bleaching inhibition	7.19 ± 0.04
TBARS inhibition	0.45 ± 0.005
Antibacterial activity MIC values (mg/ml)	
Gram negative bacteria	
Acinetobacter baumannii	> 20
Escherichia coli	5
Escherichia coli ESBL	5
Klebsiella pneumoniae	> 20
Klebsiella pneumoniae ESBL	> 20
Morganella morganii	5
Pseudomonas aeruginosa	> 20
Gram positive bacteria	
Enterococcus faecalis	5
Listeria monocytogenes	5
MRSA	5
MSSA	2.5

EC_{50} values correspond to the extract concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. *Trolox EC_{50} values: 43.03 ± 1.71 μg/ml (DDPH), 29.62 ± 3.15 μg/ml (reducing power), 2.63 ± 0.14 μg/ml (β-carotene bleaching inhibition) and 3.73 ± 1.9 μg/ml (TBARS inhibition). MIC values correspond to the minimal extract concentration that inhibited the bacterial growth. ESBL - extended spectrum β-lactamases. MRSA - Methicillin-resistant *Staphylococcus aureus*. MSSA - Methicillin-susceptible *Staphylococcus aureusii*.

The phenolic profile of the hydromethanolic extract prepared from 'Bravo de Esmolfe' dried apples, recorded at 280 nm is shown in Fig. 1. UV and mass spectra could be obtained for fifteen phenolic compounds (Table 2). The main family of compounds were flavan-3-ols, as also reported for other apple varieties (Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008). Epicatechin (peak 9) was positively identified by comparison with a commercial standard. Two B-type (*epi*)catechin dimers ($[M-H]^-$ at *m/z* 577, peaks 1 and 7, three trimers ($[M-H]^-$ at *m/z* 865, peaks 3, 4 and 11 and one tetramer ($[M-H]^-$ at *m/z* 1153, peak 12 were detected. Peaks 1, 7 and 11 were identified as procyanidins B1 [epicatechin-(4,8)-catechin], B2 [epicatechin-(4,8)-epicatechin], and C1 (5) [epicatechin-(4,8)-epicatechin-(4,8)-epicatechin] by comparison with our database library; these compounds have been consistently reported as majority procyanidins in apple (Mayr et al., 1995; Shoji et al., 2003, 2006; Verdu et al., 2013; Wojdyło et al., 2008). Peak 12 could be assigned as the (4,8)-linked epicatechin tetramer, in coherence with its elution order (Santos-Buelga, García-Viguera, & Tomás-Barberán, 2003) and previous identification in apple (Shoji et al., 2003, 2006). Peaks 3 and 4 might correspond to the trimers epicatechin-(4,8)-epicatechin-(4,8)-catechin and epicatechin-(4,6)-epicatechin-(4,8)-catechin, also founded in apple (Shoji et al., 2003, 2006). Apple procyanidins are known to be mostly based on epicatechin extension units (Mayr et al., 1995; Shoji et al., 2003, 2006). Peaks 5 and 6 were tentatively identified as 4-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid, respectively, based on their fragmentation patterns and elution order (Dias et al., 2016). Similarly, peaks 8 and 10 were tentatively identified as 4-*p*-coumaroylquinic and 5-*p*-coumaroylquinic acids. Similar hydroxycinnamoyl derivatives have already been detected in apple (Malec et al., 2014; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008). Peaks 14 and 15 were identified as phloretin-2′-*O*-xyloglucoside and phlorizin (phloretin-2′-*O*-glucoside), respectively, chalcones that are also commonly present in apple (Malec et al., 2014; Mayr et al., 1995; Scafuri et al., 2016; Verdu et al., 2013; Wojdyło et al., 2008). Peak 13, presenting a pseudomolecular ion $[M-H]^-$ at *m/z* 579, might correspond to a biflavonoid containing an (*epi*)catechin unit, owing to the characteristic MS^2 fragments at *m/z* 289, 245 and 203; no identity could be concluded for peak 2. The most abundant compound present in 'Bravo de Esmolfe' variety was 5-*O*-caffeoylquinic acid (peak 6, 51.5 mg/100 g dw), followed by procyanidin B2 (peak 7, 34.5 mg/100 g dw).

Data regarding the antioxidant and antibacterial activities of the hydromethanolic extract prepared from 'Bravo de Esmolfe' dehydrated apples are presented in Table 3. The lowest EC_{50} values (highest antioxidant activity) were observed in the TBARS inhibition and DPPH scavenging activity assays (0.45 and 0.71 mg/ml, respectively). However, Hamauzu, Yasui, Inno, Kume, and Omanyuda (2005) have reported lower EC_{50} values when using the DPPH scavenging activity methodology in *Malus domestica* Mill. *var*. Fuji fruits (EC_{50} = 8.4 mg/100 ml), as also Luo, Zhang, Li, & Shah (Luo, Zhang, Li, & Shah, 2016) (EC_{50} = 0.26 mg/ml in a different variety- Fuji). These differences might be related with the studied varieties and the way of preparation of the extracts (e.g., solvents). Regarding the antibacterial results, the hydromethanolic extracts showed the lowest MIC values against Gram-positive bacteria, namely methicillin-susceptible *Staphylococcus aureus* (MSSA) (MIC = 2.5 mg/ml). The methicillin-resistant *staphylococcus aureus* (MRSA) and other Gram-positive bacteria such as *Listeria monocytogenes* and *Enterococcus faecalis* had higher MIC values (MIC = 5 mg/ml). Of the Gram-negative bacteria, *Escherichia coli*, *Escherichia coli* (ESBL) and *Morganella morganii* presented the lowest MIC values (MIC = 5 mg/ml). Luo et al. (Luo et al., 2016), also reported antibacterial activity of polyphenolic apple extracts (but from a different variety: Fuji), with lower MIC values. This would be expected, since the cited work used ATCC reference standard microorganisms, while the bacteria used in the present study were obtained from clinical isolates with multiresistant profiles (Dias et al., 2016). Furthermore, the extract studied herein uses different solvents, which can lead to different results.

4. Conclusion

Dried apple proved to be a good source of fructose, malic acid, palmitic acid and α-tocopherol. Epicatechin and B-type procyanidins, as also hydroxycinnamoyl-quinic acids and phloretin derivatives were the phenolic compounds found in its composition. Furthermore, its hydromethanolic extracts showed antioxidant and antibacterial activity against Gram-positive and Gram-negative bacteria. Overall, this study shows that 'Bravo de Esmolfe' dried apples could be used in snack products with equilibrated nutritional value, and could also be useful in the preparation of nutraceutical formulations with potential antioxidant and antimicrobial properties.

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ANNEX IV

Phenolic compounds profile, nutritional compounds and bioactive properties of Lycium barbarum L.: A comparative study with stems and fruits

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Phenolic compounds profile, nutritional compounds and bioactive properties of *Lycium barbarum* L.: A comparative study with stems and fruits

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ABSTRACT

The increasing awareness of the possible health benefits of berry fruits (*Lycium barbarum* L.) has led to a higher consumption of this type of food products. One of the many examples are the fruits from *Lycium* genus, traditionally used due to their beneficial properties and health benefits associated with liver, kidney, eyesight, immune system, circulation and longevity disorders. In the present study fruits and stems of *Lycium barbarum* L. (goji) were characterized in terms of nutritional profile, sugars, organic acids, fatty acids and tocopherols. Furthermore, a phenolic characterization of their hydromethanolic extracts was performed and correlated with bioactive properties such as antioxidant, hepatotoxic and antibacterial activities. Stems presented higher values of energy, MUFA (monounsaturated fatty acids), tocopherols and flavonols. Stems also showed greater antioxidant and antibacterial (against Gram-negative bacteria) activities. Otherwise, fruits revealed higher contents of sugars, PUFA (polyunsaturated fatty acids) and hydroxycinnamic acid derivatives, and greater activity against Gram-positive bacteria. This is an innovative study that shows the high potential of goji stems and fruits as sources of bioactive compounds, which could be used in nutraceutical formulations, or incorporated into food products with functional properties. Furthermore, the use of stems could bring industrial sustainability as a valuable by-product, which has been scarcely reported.

1. Introduction

The interest in many traditional herbs and plant food supplements, as a source of nutritional antioxidants, is due to the increasing knowledge of the role of antioxidants and free radicals in human health (Dahech et al., 2013). The consumption of plants belonging to the *Lycium* genus has increased exponentially, not only due to their traditionally usage in Chinese medicine, but also because of their wide acceptance as food ingredients (Dahech et al., 2013; Dong et al., 2009). The berries are commonly consumed in soups, as porridge with rice and added to numerous meat and vegetable dishes (Potterat and Food, 2010), eaten raw, as a juice, wine or in tea preparations, as also processed as tinctures, powders, and tablets (Potterat and Food, 2010).

One of the most widely studied species of this genus is *Lycium barbarum* L., which has several vernacular names, being “goji” the most common one (Amagase and Farnsworth, 2011). Since the beginning of the 21th century, goji products have been introduced in Europe and North America and their consumption has increased rapidly due to their claimed beneficial properties for wellbeing and longevity (D’Amato et al., 2013). Goji berries have been associated with a wide range of health benefits, including the treatment of diseases related to liver, kidney, eyesight, immune system, circulation and longevity, as also with sexual activity (Tang et al., 2012). Recent studies also suggest that *L. barbarum* leaves have shown a broad development and application prospects in the food industry due to the rich nutrients, biological active ingredients and trace elements (Liu et al., 2012).

The interest in the composition of berry fruits has been also intensified because of an increased awareness of their possible health benefits, as they are rich sources of micronutrients and phytochemicals such as polyphenols. Some of these phenolic compounds, which can act as antioxidants and antimicrobials, have been identified by different authors (Amagase and Farnsworth, 2011; Dahech et al., 2013; Liu et al., 2017), but to the authors’ best knowledge there is no previous information about the chemical and bioactive characteristics of *L. barbarum* stems. The present study describes and compares the nutritional and chemical composition of *Lycium barbarum* L. stems and fruits; moreover, a phenolic characterization of its hydromethanolic extracts was performed and correlated with bioactive properties (*e.g.*,

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antioxidant, hepatotoxic and antimicrobial). The results of this study might be useful to maximize the potential of stems as by-products with functional properties with interest in food and pharmaceutical industries.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile (99.9%) was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic standards were from Extrasynthèse (Genay, France). Sulforhodamine B, trypan blue, trichloroacetic acid (TCA), tris (hydroxymethyl)aminomethane (Tris), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). *p*-Iodonitrotetrazolium chloride (INT) was from Panreac Applichem (Barcelona, Spain), Tryptic Soy Broth (TSB) and Mueller-Hinton (MH) were purchased from Biolab® (Hungary). All other general laboratorial reagents were from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples

The dried fruits and stems of *Lycium barbarum* L. were supplied by the company RBR foods from Castro Daire (Portugal). After reception, the dried fruits and stems were reduced to a fine dried powder (~20 mesh) and mixed to obtain homogenate samples.

2.3. Nutritional value of L. barbarum fruits and stems

2.3.1. Proximate composition and energetic value

The dehydrated and powdered fruits and stems were analysed for proteins, fat, carbohydrates and ash according to the AOAC (Association of Official Analytical Chemists) procedures (AOAC, 2016). The AOAC 991.02 was followed to determine the crude protein content (N × 6.25, macro-Kjeldahl method); AOAC 989.05 was used to determine crude fat (Soxhlet apparatus with petroleum ether as extraction solvent); AOAC 935.42 was used for ash content determination (incineration at 550 ± 15 °C). The total carbohydrates (including fiber) were calculated by difference, according with the equation: Total carbohydrates (g/100 g) = 100 − (g fat + g protein + g ash). Total energy was calculated according to the following equation: Energy (kcal/100 g) = 4 × (g proteins + g carbohydrates) + 9 × (g fat).

2.3.2. Fatty acids

Fatty acids were determined by using a Soxhlet extraction of the dehydrated and powdered fruits and stems in order to obtain a lipidic fraction and after a trans-esterification process, being further analysed by gas chromatography coupled with a flame ionization detector (GC-FID; DANI model GC 1000 instrument, Contone, Switzerland), according to the procedure previously described by the authors (Dias et al., 2015). The results were expressed in relative percentage of each fatty acid.

2.3.3. Soluble sugars

Soluble sugars were determined in the dehydrated and powdered fruits and stems following a procedure previously described by the authors (Dias et al., 2015). A High performance liquid chromatography system coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany) was used to identify and quantify the soluble sugars. The quantification was performed using the internal standard (melezitose) and the results were expressed in g per 100 g of fruits and stems dry weight.

2.3.4. Organic acids

The dehydrated and powdered fruits and stems were analysed for its organic acids following the procedure previously described by the authors (Dias et al., 2015), using an ultra-fast liquid chromatography coupled to photodiode array detector (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan). The quantification was performed by comparison of the peak area recorded at 215 nm as preferred wavelength. For quantitative analysis, a calibration curve with known concentration (10 − 0.0078 mg/mL) for each available organic acid, was constructed based on the UV signal: oxalic acid ($y = 45{,}973 + 9 \times 10^6 x$, $R^2 = 0.9901$); quinic acid ($y = 46{,}061 + 610607x$, $R^2 = 0.9995$); malic acid ($y = 92{,}665 + 912441x$, $R^2 = 0.999$); citric acid ($y = 45{,}682 + 1 \times 10^6 x$, $R^2 = 0.9997$), and succinic acid ($y = 50{,}689 + 592888x$, $R^2 = 0.9996$). The results were expressed in g per 100 g of fruits and stems dry weight.

2.3.5. Tocopherols

Tocopherols (four vitamers) were determined according with the procedure previously described by the authors (Dias et al., 2015), in the dehydrated and powdered fruits and stems, by HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA). For the quantification, an internal standard (tocol) was used, based on the fluorescence signal response of each standard. The results were expressed in mg per 100 g of fruits and stems dry weight.

2.4. Analysis of phenolic compounds

2.4.1. Preparation of the hydromethanolic extracts

To prepare the hydromethanolic extracts, 1 g of each dehydrated and powdered sample was extracted with a methanol/water mixture (80:20, *v/v*), at 25 °C and 150 rpm, during 1 h, followed by filtration through a Whatman filter paper No. 4. The remaining residue was re-extracted with one additional portion of the methanol/water mixture, and the combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove the methanol; then the extracts were frozen, lyophilized and stored at −5 °C for further analysis.

2.4.2. Phenolic compounds

The hydromethanolic extracts were re-dissolved at a concentration of 5 mg/mL in methanol/water (80:20, v/v). The analysis was performed using a LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) as previously described by (Bessada et al., 2016). The detection was performed using 280, 330 and 370 nm as preferred wavelengths for DAD and in a mass spectrometer equipped with an ESI source and performed in negative mode (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA). The identification of the phenolic compounds was performed based on its chromatographic behaviour and UV–vis and mass spectra by comparison with available standard compounds, and data reported in the literature giving a tentative identification. For quantitative analysis, a calibration curve with known concentration (200–5 µg/mL) for each available phenolic standard: caffeic acid ($y = 406{,}369 + 388345x$, $R^2 = 0.9949$); catequin ($y = -23{,}200 + 84950x$, $R^2 = 0.9999$); chlorogenic acid ($y = -161{,}172 + 168823x$, $R^2 = 0.9999$); ferulic acid ($y = -185{,}462 + 633126x$, $R^2 = 0.9999$); kaempferol-3-*O*-rutinoside ($y = 30{,}861 + 11117x$; $R^2 = 0.9998$); *p*-coumaric acid ($y = 6966.7 + 301950x$, $R^2 = 0.9999$); quercetin-3-*O*-rutinoside ($y = 76{,}751 + 13343x$, $R^2 = 0.9998$); quercetin-3-*O*-glucoside ($y = -160{,}173 + 34843x$, $R^2 = 0.9998$); sinapic acid ($y = 30{,}036 + 197337x$, $R^2 = 0.9997$), was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was carried out through the calibration curve of the most similar available standard. The results were expressed as mg per g of extract.

2.5. Evaluation of bioactive properties

2.5.1. Antioxidant activity

The hydromethanolic extracts were re-dissolved in methanol:water (80:20, v/v) to obtain stock solutions of 2.5 mg/mL, which were further diluted to obtain a range of concentrations (2.5 mg/mL to 0.07 mg/mL) for antioxidant activity evaluation by DPPH radical-scavenging, reducing power, inhibition of β-carotene bleaching, and TBARS inhibition assays (Barros et al., 2013). The final results were expressed as EC_{50} values (mg/mL), which means sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as a positive control.

2.5.2. Antibacterial activity

The hydromethanolic extracts were re-dissolved in water in order to obtain stock solutions of 100 mg/mL, and then submitted to further dilutions. The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal. Seven Gram-negative bacteria (*Escherichia coli*, *E. coli* ESBL (extended spectrum of beta-lactamase), *Klebsiella pneumoniae*, *K. pneumoniae* ESBL, *Morganella morganii*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, isolated from urine and expectoration) and five Gram-positive bacteria (MRSA- methicillin-resistant *Staphylococcus aureus*, MSSA- methicillin-susceptible *Staphylococcus aureus*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis*) were used to screen the antibacterial activity. Minimum inhibitory concentrations (MIC) were determined by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay was used by following the methodology proposed by Kuete et al. (2011a,b) with some modifications (Dias et al., 2016). The antibiotic susceptibility profile was obtained for all the tested bacteria (Table A1, Supplementary material). MIC was defined as the lowest concentration that inhibits the visible bacterial growth.

2.5.3. Hepatotoxicity

The hydromethanolic extracts were re-dissolved in water to obtain stock solutions of 4 mg/mL, and then submitted to further dilutions. For hepatotoxicity evaluation, a porcine liver cells primary culture (PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by the authors (Abreu et al., 2011). Ellipticine was used as positive control and the results were expressed in GI_{50} values, concentration that inhibited 50% of the net cell growth.

2.5.4. Statistical analysis

Three samples were used for each plant part and all the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD), being analysed using a Student's *t*-test, with $\alpha = 0.05$. Furthermore, a Pearson's correlation analysis between the bioactivities and the different groups of phenolic compounds (sum of phenolic acids, sum of flavan-3-ols, sum of flavonols and sum of phenolic compounds) was carried out, with a 95% confidence level. The analyses were carried out using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Nutrient composition

Data on the proximate composition and energetic value of *L. barbarum* are shown in Table 1. Carbohydrates were the most abundant macronutrients in fruits and stems (87 and 78.1 g/100 g dw, respectively). Stems presented the highest contents of ash, proteins and fat (9.9, 7.4, and 4.6 g/100 g dw, respectively), while fruits presented proteins as the second major macronutrient (5.3 g/100 g dw), followed by fat and ash (4.1 and 3.21 g/100 g dw, respectively). Yan et al. (2014) reported different results for goji fruits from China, describing a higher content of proteins and fat (12.1 and 6.89 g/100 g dw, respectively) and a lower ash content (0.95 g/100 g dw). These differences might be explained by the cultivar and/or different edaphic conditions that can lead to variable nutritional contents.

Table 1
Proximate composition, soluble sugars and organic acids in fruits and stems of *Lycium barbarum* L. (mean ± SD).

	Fruits	Stems	*t*-Students test *p*-value
Nutritional value (g/100 g dw)			
Fat	4.1 ± 0.3	4.6 ± 0.3	0.040
Proteins	5.3 ± 0.2	7.4 ± 0.2	< 0.001
Ash	3.21 ± 0.02	9.9 ± 0.1	< 0.001
Total carbohydrates	87 ± 6	78.1 ± 0.4	< 0.001
Energy contribution (kcal/100 g dw)	408 ± 1	383 ± 2	< 0.001
Soluble sugars (g/100 g dw)			
Fructose	12.7 ± 0.4	0.45 ± 0.01	< 0.001
Glucose	14.4 ± 0.4	0.42 ± 0.01	< 0.001
Sucrose	0.8 ± 0.1	0.21 ± 0.02	< 0.001
Sum	27.9 ± 0.9	1.08 ± 0.05	< 0.001
Organic acids (g/100 g dw)			
Oxalic acid	0.010 ± 0.001	0.65 ± 0.001	< 0.001
Quinic acid	nd	0.53 ± 0.03	–
Malic acid	nd	0.899 ± 0.004	–
Citric acid	1.29 ± 0.02	nd	–
Succinic acid	0.77 ± 0.07	nd	–
Sum	2.07 ± 0.01	2.08 ± 0.03	0.677

dw – dry weight basis; nd – not detected.

Soluble sugars and organic acids of the studied fruits and stems are also presented in Table 1. Fructose, glucose and sucrose were the only forms of monosaccharides detected in fruits and stems, being glucose the most abundant one, followed by fructose and sucrose. As expected, fruits presented much higher content in soluble sugars (27.9 g/100 g dw) than stems (1.08 g/100 g dw). These results are in agreement with those obtained by Mikulic-Petkovsek et al. (2012) in goji fruits from Slovenia, where glucose and fructose were also the prevailing sugars detected, although those authors reported a higher total sugars content.

Regarding organic acids (Table 1), fruits and stems presented very different profiles, however, no statistically significant differences were found in the sum of the organic acids between samples. Citric, succinic and oxalic (1.29, 0.77, and 0.010 g/100 g dw, respectively) acids were detected in the fruit; while malic, oxalic and quinic (0.899, 0.65, and 0.53 g/100 g dw, respectively) acids were found in the stems. Oxalic acid was the only organic acid common in both samples. Donno et al. (2015), in goji fruits from Italy, reported the presence of several organic acids, including malic, quinic and tartaric acids that were not detected in samples of this study. These differences might be due to the physical state of the samples and/or the extraction method, as Donno et al. (2015) analysed the organic acids in fresh or semi-fresh samples (stored at 4 °C for a few days) and using ethanol as extraction solvent.

Fatty acids were also determined in fruits and stems of goji fruits and the results are shown in Table 2. Sixteen fatty acids were identified in the fruits, being polyunsaturated fatty acids (PUFA) the predominant group, mainly due to the presence of linoleic acid (C18:2n6, 53.4%), followed by oleic acid (C18:1n9, 16.5%) and palmitic acid (C16:0, 12.77%). Similar results were obtained by Yan et al. (2014) in goji fruits from China, that described linoleic acid (66.81%) and oleic acid (17.13%) as the major fatty acids. In stem samples, eighteen fatty acids were identified, being saturated fatty acids (SFA) predominant, especially palmitic (C16:0, 15.94%) and lignoceric acids (C24:0, 15.3%), followed by linolenic acid (C18:3n3, 14.8%).

Regarding tocopherols (Table 2), both samples presented only two vitamers. The highest content of tocopherols (3.59 mg/100 g dw) was

Table 2
Fatty acids and tocopherols in fruits and stems of *Lycium barbarum* L. (mean ± SD).

	Fruits	Stems	*t*-Students test *p*-value
Fatty acids (relative percentage, %)			
C8:0	0.65 ± 0.04	0.60 ± 0.04	0.020
C10:0	0.10 ± 0.01	0.15 ± 0.01	< 0.001
C12:0	0.19 ± 0.02	0.19 ± 0.02	0.442
C14:0	0.38 ± 0.02	1.7 ± 0.1	< 0.001
C14:1	0.37 ± 0.03	0.35 ± 0.02	0.015
C15:0	0.21 ± 0.02	0.29 ± 0.01	< 0.001
C16:0	12.77 ± 0.07	15.94 ± 0.08	< 0.001
C16:1	0.29 ± 0.02	nd	–
C17:0	0.48 ± 0.05	0.90 ± 0.04	< 0.001
C18:0	7.50 ± 0.06	9.1 ± 0.2	< 0.001
C18:1n9	16.5 ± 0.5	5.12 ± 0.06	< 0.001
C18:2n6	53.4 ± 0.5	9.7 ± 0.2	< 0.001
C18:3n3	1.68 ± 0.02	14.8 ± 0.3	< 0.001
C20:0	1.30 ± 0.07	12.84 ± 0.01	< 0.001
C20:2	nd	1.3 ± 0.2	–
C20:3n3	0.35 ± 0.04	0.73 ± 0.04	0.000
C22:0	2.75 ± 0.08	10.4 ± 0.1	< 0.001
C23:0	nd	0.69 ± 0.01	–
C24:0	nd	15.3 ± 0.3	–
SFA	26.1 ± 0.1	68.0 ± 0.5	< 0.001
MUFA	17.2 ± 0.6	5.46 ± 0.04	< 0.001
PUFA	56.8 ± 0.5	26.6 ± 0.4	< 0.001
Tocopherols (mg/100 g dw)			
α-Tocopherol	0.23 ± 0.02	3.37 ± 0.01	< 0.001
β-Tocopherol	nd	0.22 ± 0.04	–
δ-Tocopherol	0.09 ± 0.01	nd	–
Sum	0.33 ± 0.03	3.59 ± 0.05	< 0.001

dw – dry weight basis; nd – not detected. C8:0 – Caprylic acid; C10:0 – Capric acid; C12:0 – Lauric acid; C14:0 – Myristic acid; C14:1 – Myristoleic acid; C15:0 – Pentadecanoic acid; C16:0 – Palmitic acid; C16:1 – Palmitoleic acid; C17:0 – Heptadecanoic acid; C18:0 – Stearic acid; C18:1n9 – Oleic acid; C18:2n6 – Linoleic acid; C18:3n3 – Linolenic acid; C20:0 – Arachidic acid; C20:2 – *cis*-11,14 - Eicosadienoic acid; C20:3n3 – Eicosatrienoic acid; C22:0 – Behenic acid; C23:0 – Tricosanoic acid; C24:0 – Lignoceric acid. SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids.

determined in the stems, mainly due to the presence of α-tocopherol (3.37 mg/100 g dw), with minor levels of β-tocopherol (0.22 mg/100 g dw). Significant lower concentrations of tocopherols were found in the fruits, also containing α-tocopherol, but with δ-tocopherol as the second vitamer (0.23 and 0.09 mg/100 g dw, respectively). To the authors' best knowledge, there are no previous studies of tocopherols composition in goji fruits and stems.

3.2. Individual phenolic profile

The peak characteristics (retention time, wavelength of maximum absorption and mass spectral data), tentative identification and quantification of phenolic compounds present in the hydromethanolic extracts of *L. barbarum* fruits and stems are presented in Table 3. An exemplificative phenolic profile of the hydromethanolic extracts of both types of samples, recorded at 280 nm, is shown in Fig. 1. Fruits and stems presented different phenolic profile, with the presence of hydroxybenzoic (galloyl derivatives) and hydroxycinnamic (caffeic, *p*-coumaric, ferulic and sinapic acid derivatives) acid derivatives, flavan-3-ols, and flavonols (quercetin and kaempferol derivatives). Sixteen compounds were identified in fruit samples: eight flavonols (peaks **6**, **16**, **17**, **18**, **19**, **20**, **21**, and **22**), seven phenolic acid derivatives (peaks **1**, **2**, **3**, **4**, **7**, **10**, and **14**), and one flavan-3-ol (peak **9**), while eleven compounds were detected in the stems, most of which were phenolic acid derivatives (peaks **2**, **4**, **5**, **7**, **8**, **11**, **12**, and **13**), together with two flavonols (peaks **16** and **20**) and one flavan-3-ol (peak **15**). Only three chlorogenic acids (peaks **2**, **4** and **7**) were common to both samples. Peaks **7**, **9**, **12**, **14**, **16**, **19**, and **20** (5-*O*-caffeoylquinic acid, catechin, caffeic acid, *p*-coumaric acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, and kaempferol-3-*O*-rutinoside, respectively) were identified by its UV and mass spectra, and retention characteristics in comparison with commercial standards. Compounds **19** and **20** had been previously reported by other authors in goji leaves (Mocan et al., 2017) and fruits (Bondia-Pons et al., 2014; Inbaraj et al., 2010).

Flavonols were the most abundant phenolic compounds in goji stems, although mostly due to the presence of quercetin-3-*O*-rutinose (rutin, peak **16**), with minor levels of kaempferol-3-*O*-rutinose (peak **20**). The presence of rutin as a major flavonol in different parts of goji plants has been consistently reported by several authors (Affes et al., 2017; Bondia-Pons et al., 2014; Mocan et al., 2017, 2015a,b, 2014; Protti et al., 2017; Qian et al., 2004; Zhang et al., 2016). Flavonols were less abundant in the fruits, despite they presented greater variety of these compounds. Quercetin-3-*O*-glucoside (isoquercitrin, peak **19**) was positively identified by comparison with a standard. Peak **18** presented the same UV and mass spectral characteristics as compound **19** ($[M-H]^-$ at *m/z* 463), thus corresponding to a quercetin hexoside, which was tentatively assigned as hyperoside (*i.e.*, quercetin-3-*O*-galactoside), owing to the previous identification of both isoquercitrin and hyperoside in goji fruits (*Lycium* spp) by Qian et al. (2004) and Donno et al. (2015). This identity is also coherent with its chromatographic behaviour, as galactosides are expected to elute before its corresponding glucosides (Santos-Buelga et al., 2003). Peak **17** presented a pseudomolecular $[M-H]^-$ at *m/z* 447 releasing an MS^2 fragment at *m/z* 301, allowing its identification as a quercetin-deoxyhexoside, tentatively associated to quercitrin (quercetin-3-*O*-rhamnoside) previously reported in different goji samples (Mocan et al., 2015a, 2014; Protti et al., 2017; Zhou et al., 2017). Peak **6** showed a UV spectra characteristic of a quercetin derivative, and a pseudomolecular ion $[M-H]^-$ at *m/z* 933, yielding fragments at *m/z* 609 ($[M-H\text{-}324]^-$, loss of two hexosyl units) and *m/z* 301 ($[M-H\text{-}308]^-$, loss of a rutinosyl unit), being tentatively identified as quercetin-dihexoside-rutinoside. A compound with the same characteristics (rutin di-hexose) was reported in hydromethanolic extracts of goji fruits from Finland (Bondia-Pons et al., 2014). Other two flavonols derived from kaempferol were also detected in the fruits. As above indicated, peak **21** was identified as kaempferol-3-*O*-glucoside by comparison with a standard, previously reported in goji fruits by Affes et al. (2017) and leaves by Mocan et al. (2017). Peak **22** was tentatively assigned as kaempferol-rhamnoside based on its pseudomolecular ion ($[M-H]^-$ at *m/z* 431) releasing a unique fragment at *m/z* 285, by analogy with the identifications made for quercetin glycosides.

Two flavan-3-ol derivatives were detected in the analysed samples and stems. Catechin (peak **9**) was positively identified in the fruit by comparison with a commercial standard, whereas peak **15**, found in the stems, was associated to a procyanidin dimer according to its UV spectrum, pseudomolecular ion ($[M-H]^-$ at *m/z* 577) and MS^2 fragments at *m/z* 289, 245 and 203.

The remaining compounds detected in goji samples corresponded to phenolic acid derivatives, most of them derivatives of hydroxycinnamic acids, which were the most abundant compounds in the fruits. Three chlorogenic acids, peaks **2**, **4** and **7** showing a pseudomolecular ion $[M-H]^-$ at *m/z* 353 yielding a main product ion at *m/z* 191 (deprotonated quinic acid), were identified as *cis* and *trans* 3-*O*-caffeoylquinic acids and *trans* 5-*O*-caffeoylquinic acid, respectively, based on the hierarchical keys previously described by Clifford et al. (2003, 2005). These type of compounds are among the most common phenolic compounds usually reported in goji samples, although most authors do not indicate the particular derivative, but just refer to them as chlorogenic acid or isomers (Affes et al., 2017; Bondia-Pons et al., 2014; Donno et al., 2015; Mocan et al., 2015a,b, 2014; Qian et al., 2004; Zhang et al., 2016; Zhou et al., 2017). Only Mocan et al. (2017) described the presence of different caffeoylquinic acids in the leaves of cultivated *L. barbarum* from Romania, with particularly high contents of 3-*O*-caffeoylquinic acid. Inbaraj et al. (2010) also reported 3-*O*-caffeoylquinic

Table 3
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (mg/g dw) of the phenolic compounds present in fruits and stems of *Lycium barbarum* L.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Reference used for identification	Fruits	Stems	*t*-Students test *p*-value
1	5.08	262	311	179(100),135(5)	Caftaric acid[A]	Mocan et al. (2015a,Mocan et al., 2015b	0.86 ± 0.04	nd	–
2	5.37	313	353	191(100),179(7),161(3)	*cis* 3-*O*-Caffeoylquinic acid[A]	Clifford et al. (2003, 2005)	2.9 ± 0.1	0.36 ± 0.02	< 0.001
3	5.54	296	487	163(100),119(40)	*p*-Coumaroyl acid dihexoside[B]	Bondia-Pons et al., 2014 and Zhou et al. (2017)	3.6 ± 0.2	nd	–
4	5.74	304	353	191(100),179(7),161(3)	*trans* 3-*O*-Caffeoylquinic acid[C]	Clifford et al. (2003, 2005)	8.87 ± 0.01	0.59 ± 0.02	< 0.001
5	5.82	264	343	191(3),169(100),125(3)	Galloylquinic acid[A]	Guimarães et al. (2013)	nd	1.59 ± 0.01	–
6	7.16	324	933	609(100),301(5)	Quercetin-dihexoside-rutinoside[D]	Bondia-Pons et al. (2014)	3.73 ± 0.03	nd	–
7	7.47	315	353	191(100),179(3),161(3)	*trans* 5-*O*-Caffeoylquinic acid[A]	DAD/MS, standard	3.3 ± 0.1	8.03 ± 0.01	< 0.001
8	7.92	284	385	223(100),207(50),179(40),163(14),149(3)	Sinapic acid hexoside[E]	Chahdoura et al. (2014)	nd	2.8 ± 0.1	–
9	8.07	315	289	245(2),20(13),137(20)	Catechin[F]	DAD/MS, standard	10.4 ± 0.4	nd	–
10	8.75	318	517	193(100), 179(5),149(20)	Ferulic acid dihexoside[G]	Dias et al. (2016)	0.9 ± 0.1	nd	–
11	9.36	284	385	223(100),207(40),179(2),161(19),153(36),149(2)	Sinapic acid hexoside[E]	Chahdoura et al. (2014)	nd	0.9 ± 0.1	–
12	10.36	322	179	161(5),159(4),135(100)	Caffeic acid [A]	DAD/MS, standard	nd	0.52 ± 0.01	–
13	14.31	272	787	635(12),617(14),483(3),465(4),447(5),423(20),313(2),271(10)	Tetragalloyl-glucose[C]	Rached et al. (2016)	nd	2.4 ± 0.1	–
14	15.79	310	163	119(100)	*p*-Coumaric acid[B]	DAD/MS, standard	12.3 ± 0.4	nd	–
15	15.84	290	577	289(76),245(14),203(18)	Procyanidin dimer[F]	Pires et al. (2017)	nd	6.2 ± 0.1	–
16	17.71	352	609	301(100)	Quercetin-3-*O*-rutinoside (rutin)[D]	DAD/MS, standard	16.6 ± 0.1	48 ± 1	–
17	18.42	nd	447	301(100)	Quercetin-3-*O*-rhamnoside (quercitrin)[H]	Mocan et al. (2014), Protti et al. (2017) and Zhou et al. (2017)	tr	nd	–
18	18.84	355	463	301(100)	Quercetin-3-*O*-galactoside (hyperoside)[H]	Qian et al. (2004) and Donno et al. (2015)	0.70 ± 0.01	nd	–
19	19.11	353	463	301(100)	Quercetin-3-*O*-glucoside (isoquercitrin)[H]	DAD/MS, standard	2.42 ± 0.04	nd	–
20	21.12	348	593	285(100)	Kaempferol-3-*O*-rutinoside[G]	DAD/MS, standard	tr	0.83 ± 0.01	–
21	22.27	343	447	285(100)	Kaempferol-3-*O*-glucoside[G]	DAD/MS, standard	4.21 ± 0.04	nd	–
22	27.41	nd	431	285(100)	Kaempferol-rhamnoside[G]	MS	tr	nd	–
					Sum of phenolic acid		32.7 ± 0.8	17.2 ± 0.2	< 0.001
					Sum of flavan-3-ols		10.4 ± 0.4	6.2 ± 0.1	< 0.001
					Sum of flavonols		27.6 ± 0.1	48.5 ± 0.6	< 0.001
					Sum of phenolic compounds		71 ± 1	71.9 ± 0.9	0.113

tr-trace amounts; nd- not detected. Standard calibration curves: A - caffeic acid ($y = 406{,}369 + 388345x$, $R^2 = 0.9949$); B - *p*-coumaric acid ($y = 6966.7 + 301950x$, $R^2 = 0.9999$); C - chlorogenic acid ($y = -161{,}172 + 168823x$, $R^2 = 0.9999$); D - quercetin-3-*O*-rutinoside ($y = 76{,}751 + 13343x$, $R^2 = 0.9998$); E - sinapic acid ($y = 30{,}036 + 197337x$, $R^2 = 0.9997$); F - catequin ($y = -23{,}200 + 84950x$, $R^2 = 0.9999$); G - ferulic acid ($y = -185{,}462 + 633126x$, $R^2 = 0.9999$); H - quercetin-3-*O*-glucoside ($y = -160{,}173 + 34843x$, $R^2 = 0.9998$); G - kaempferol-3-*O*-rutinoside ($y = 30{,}861 + 11117x$; $R^2 = 0.9998$).

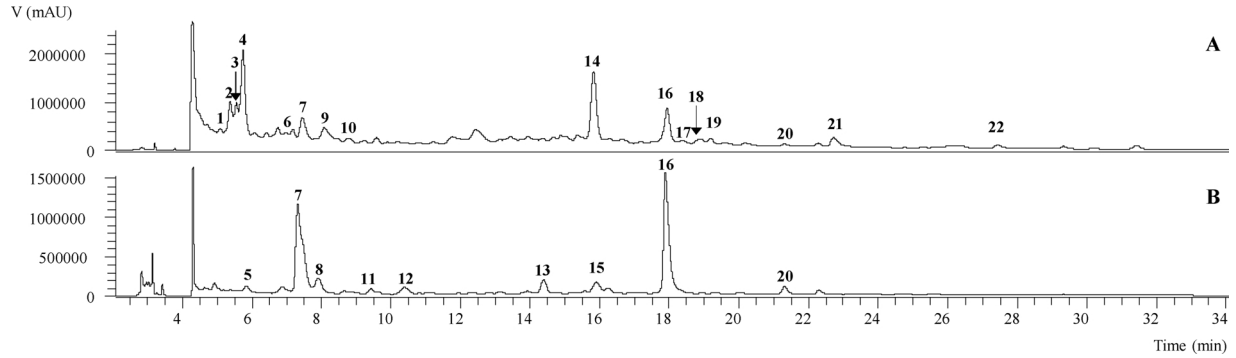

Fig. 1. HPLC phenolic profile recorded at 280 nm of the hydromethanolic extracts of fruits (**A**) and stems (**B**) of *L. barbarum*. Peak numbering is according to Table 3.

Table 4
Antioxidant, hepatotoxic and antimicrobial activity of fruits and stems of *Lycium barbarum* L. (mean ± SD).

	Fruits	Stems	*t*-Students test *p*-value	Correlation factor r^2			
				Phenolic acids	Flavan-3-ols	Flavonols	Phenolic compounds
Antioxidant activity EC_{50} values (mg/mL)[A]							
DPPH scavenging activity	6.25 ± 0.2	0.28 ± 0.02	< 0.001	0.998	0.996	0.999	0.880
Reducing power	1.09 ± 0.02	0.23 ± 0.01	< 0.001	0.999	0.997	0.999	0.880
β-carotene bleaching inhibition	1.9 ± 0.3	0.26 ± 0.02	< 0.001	0.973	0.971	0.974	0.857
TBARS inhibition	3.9 ± 0.2	0.07 ± 0.02	< 0.001	0.995	0.993	0.996	0.877
Hepatotoxicity GI_{50} values (μg/mL)[B]							
PLP2	> 400	> 400	–	–	–	–	–
Antimicrobial activity MIC values (mg/mL)							
Gram-negative bacteria							
Acinetobacter baumannii	> 20	20	–	–	–	–	–
Escherichia coli ESBL 1	5	5	–	–	–	–	–
Escherichia coli ESBL 2	5	10	–	0.999	0.997	0.999	0.880
Escherichia coli	2.5	2.5	–	–	–	–	–
Klebsiella pneumoniae	20	10	–	0.999	0.997	0.999	0.880
Klebsiella pneumoniae ESBL	20	20	–	–	–	–	–
Morganella morganii	5	5	–	–	–	–	–
Pseudomonas aeruginosa	20	10	–	0.999	0.997	0.999	0.880
Gram-positive bacteria							
Enterococcus faecalis	2.5	10	–	0.999	0.997	0.999	0.880
Listeria monocytogenes	5	5	–	–	–	–	–
Staphylococcus aureus	2.5	2.5	–	–	–	–	–
MRSA	2.5	5	–	0.999	0.997	0.999	0.880
MSSA	2.5	10	–	0.999	0.997	0.999	0.880

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. A- Trolox EC_{50} values: 43.03 ± 1.71 μg/mL (DDPH), 29.62 ± 3.15 μg/mL (reducing power), 2.63 ± 0.14 μg/mL (β-carotene bleaching inhibition) and 3.73 ± 1.9 μg/mL (TBARS inhibition); B - Ellipticine GI_{50} values: 2.29 mg/mL (PLP2). MIC values correspond to the minimal extract concentration that inhibited the bacterial growth. ESBL - extended spectrum β-lactamases. MRSA - Methicillin-resistant *Staphylococcus aureus*. MSSA - Methicillin-susceptible *Staphylococcus aureus*.

acid in the fruits of *L. barbarum*, although in lower amounts than the ones reported in this paper.

Peak **14**, identified as *p*-coumaric acid by comparison with a standard, was the majority phenolic acid derivative in the fruits, whereas lower levels of caffeic acid (peak **12**) were present in the stems. Other hydroxycinnamoyl derivatives detected in the samples were caftaric acid (peak **1**), previously described in the leaves of *L. barbarum* (Mocan et al., 2015a,b), and different glycosides (peaks **3**, **8, 10** and **11**). Peak **3** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 487 releasing fragments at *m/z* 163 (−324 mu, loss of two hexosyl moieties) and 119, which is coherent with a *p*-coumaroyl acid dihexoside, as reported in goji fruits from Finland and Spain (Bondia-Pons et al., 2014); 6-*O*-trans-p-coumaroyl-2-*O*-glucopyranosyl-a-D-glucopyranoside was also recently identified by Zhou et al. (2017) in wolfberries from China. Similarly, peak **10**, with a pseudomolecular ion $[M-H]^-$ at *m/z* 517 and a main MS^2 product ion at *m/z* 193 from the loss of 324 mu, could be tentatively identified as a ferulic acid dihexoside. Peaks **8** and **11** presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 385 and an MS^2 fragment at *m/z* 223 (sinapic acid aglycone), corresponding to the loss of an hexosyl unit, so that they were tentatively identified as sinapic acid hexosides.

Finally, peaks **5** and **13** were associated to galloyl derivatives. The first one was identified as galloylquinic acid based on its pseudomolecular ion ($[M-H]^-$ at *m/z* 343) and the major MS^2 fragment at *m/z* 169 [gallic acid-H]$^-$, from the loss of quinic acid moiety (−152 mu). Peak **13** was assigned as tetragalloyl-glucose from its pseudomolecular ion $[M-H]^-$ at *m/z* 787 and fragment ions at *m/z* 635, 483, and 313 from the consecutive loss of three gallic acid units. The identification of both compounds was supported by its comparison with previously obtained data available in a compound library (Guimarães et al., 2013; Rached et al., 2016). To the authors' best knowledge, these compounds have not been previously cited in goji samples.

The total content of phenolic compounds did not show any statistically significant difference between fruits and stems of goji samples. However, significant differences were found between samples when considering each family of phenolic compounds, being phenolic acid derivatives the majority compounds in the fruits (32.7 mg/g *vs* 17.2 mg/g in the stems) and flavonols in the stems (48.5 mg/g *vs*

27.6 mg/g in the fruits).

Quercetin-3-*O*-rutinoside was by far the major phenolic compound in stems (48 mg/g extract) and fruits (16.6 mg/g extract), followed in these latter by *p*-coumaric acid (12.3 mg/g extract). The differences between fruits and stems are explained by the clear difference in plant tissues. Although a greater amount of flavonols might be expected in the fruits, the obtained results could be explained by the edafoclimatic characteristics, degree of ripening and storage conditions (Haminiuk et al., 2012).

3.3. Bioactivities of fruit and stem hydromethanolic extracts

Data regarding the antioxidant, hepatotoxic and antibacterial activities are presented in Table 4. The hydromethanolic extracts of *L. barbarum* stems showed the highest antioxidant activity in all assays: DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS inhibition (EC_{50} = 0.28, 0.23, 0.26, and 0.07 mg/mL, respectively). Similar results were reported by Liu et al. (2017) in ethanolic extracts of *L. barbarum* stems from Taiwan, namely DPPH scavenging activity and reducing power (0.102 and 0.167 mg/mL, respectively). On the other hand, Jabbar et al. (2014) reported lower EC_{50} values in methanolic extracts of goji fruits from China, regarding DPPH scavenging activity and reducing power (0.042 and 0.121 mg/mL, respectively), in comparison with the herein analysed hydromethanolic extract.

The antioxidant activity revealed by the herein study samples could be due to their high content in phenolic acids derivatives and flavonoids. The antioxidant activity of phenolic acid derivatives depends on the number of hydroxyl groups in the molecule, that would be strengthened by steric hindrance. Moreover, the electron-withdrawing properties of the carboxylate group in benzoic acids has a negative influence on the H-donating abilities of the hydroxy benzoates, being hydroxylated cinnamates more effective than benzoate counterpart (Rice-Evans et al., 1996). The presence of multiple hydroxyl groups in flavonoids and other phenolics structures gives them a reducing character. In fact, it has been shown in *in vitro* assays that many of these compounds possess a strong antioxidant activity. This activity is particularly high, three to four fold higher in ortho-dihydroxy flavonoids (those containing a catechol group in their aromatic rings) such as flavonols or flavanol (Vicente and Boscaiu, 2018). Thus, the differences in the phenolic compounds family present in each of the study plant part (phenolic acids for fruits and flavonols in stems), could explain the greater antioxidant capacity of the stems.

Neither fruits nor stems revealed toxicity towards a porcine liver primary culture (PLP2).

Regarding antibacterial activity (Table 4), both samples showed better results against Gram-positive than against Gram-negative bacteria, with MIC values ranging between 2.5 and 10 mg/mL. The lowest MIC values were determined for *E. faecalis* (2.5 mg/mL), *L. monocytogenes* (5 mg/mL), *S. aureus* (2.5 mg/mL), MRSA (2.5 mg/mL), and MSSA (2.5 mg/mL). As for Gram-negative bacteria, the stems presented higher activity against *A. baumannii* (20 mg/mL), *K. pneumonia* (10 mg/mL), and *P. aeruginosa* (10 mg/mL). Mocan et al. (2017) and Mocan et al. (2015b) reported lower MIC values in goji leaves and flowers, respectively, against both Gram-negative and positive bacteria. A possible explanation could be that the bacteria used by those authors were ATCC (with no resistance profile), so that lower concentrations of extracts could be needed to inhibit the growth of the bacterial strains.

As it can also be seen in Table 4 good correlations were obtained between the different families of analysed phenolic compounds and the antioxidant activity (r^2 < 0.8). Regarding antibacterial assays, good correlation coefficients were observed for *Escherichia coli* ESBL 2, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*.

The presence of phenolic compounds, namely flavonoids and phenolic acids (*e.g.* chlorogenic acids derivatives) could be related to the antibacterial potential of the study samples. Flavonoids are known to be synthesized by plants in response to microbial infection, thus explaining the *in vitro* antimicrobial activity of these substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins, which may disrupt the microbial membrane (Cushnie and Lamb, 2005; Kabir et al., 2014). The antimicrobial activity of polyphenols has also been attributed to their structural features, as well as pH and sodium chloride concentration, resulting in physiological changes in the microorganisms and eventual cell death (Kabir et al., 2014). Chlorogenic acid is a phenolic ester of caffeic acid and (−)-quinic acid (Chiang et al., 2004), which is metabolized into active compounds, such as quinic, caffeic, benzoic, hippuric, ferulic, isoferulic, and hydroxybenzoic acids. Studies carried out by Kabir et al. (2014) confirm that chlorogenic acids and related compounds exhibited a potent antimicrobial activity, and a synergistic effect between compounds. Thus, these compounds could be related to the antimicrobial potential revealed in these plant parts.

Overall, the stems of *L. barbarum* showed higher values of energy, fat, proteins and ash, as also monounsaturated fatty acids, tocopherols, and flavonols (*i.e.*, quercetin-3-*O*-rutinoside). They also presented greater antioxidant capacity and higher activity against Gram-negative bacteria. The fruits of *L. barbarum* possessed higher contents of sugars (mainly fructose and glucose), as expected, polyunsaturated fatty acids, hydroxycinnamoyl derivatives (*p*-coumaric acid and chlorogenic acid derivatives), and flavan-3-ols (catechin); they also showed higher activity against Gram-positive bacteria.

All in all, this study allowed verifying that not only the fruit but also goji stems can be sources of compounds with interesting nutritional and bioactive properties and, therefore, they could be useful for nutraceutical formulations or its incorporation into foods with functional properties. Since stems are by-products, besides its possible beneficial effects to consumers, they also provide industrial sustainability and could be used as an add value by-product scarcely noticed up to now.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2018.06.046.

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Annex V

Incorporation of natural colorants obtained from edible flowers in yogurts

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Incorporation of natural colorants obtained from edible flowers in yogurts

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ABSTRACT

The substitution of artificial dyes by natural colouring agents is among the top concerns of food industry to fulfil current consuming trends, justifying the prospection of novel natural sources of these compounds. Herein, the hydrophilic extracts from rose, cornflower and dahlia were tested as potential substitutes to E163 (anthocyanin extract). Besides comparing the colouring capacity, the potential occurrence of changes in the chemical composition of yogurts (nutritional parameters, free sugars and fatty acids) was also assessed throughout storage (up to 7 days) and compared with a "blank" (free of any additive) yogurt formulation. In general, yogurts prepared with flower extracts, presented similar nutritional value and free sugars profile to those prepared with E163 and to the "blank" yogurt. Nevertheless, rose extract turned out to be the most suitable alternative to E163 as these two groups of yogurts had similar nutritional composition, free sugars and fatty acids composition, besides presenting close scores in colour parameters.

1. Introduction

Fermented milk is a dairy product processed by lactic fermentation, which ends up by coagulating milk casein due to the acidification process (pH values around 4.6). Among different fermented dairy products, yogurt is certainly one of the most popular, being widely consumed all over the world due to its organoleptic and nutritional properties (Arioui, Ait Saada, & Cheriguene, 2017; Caleja et al., 2016).

Some yogurt formulations are prepared using specific additives, such as exemplified by colorants. However, the recent concerns about the safety of artificial colorants in food products, has encouraged the development and application of natural colorants, which are generally considered safer than artificial ones (Pop, Lupea, Popa, & Gruescu, 2010). Anthocyanins are authorised food colorants (E163 in EU) and have previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1982 and by the EU Scientific Committee for Food (SCF) in 1975 and 1997 (Pop et al., 2010; Rodriguez-Amaya, 2016). Anthocyanins are water-soluble pigments isolated from plants, being responsible for the blue, purple, and red colour of many plant tissues. These phenolic compounds are widely found in fruits (especially berries), as well as flowers and leaves, mainly linked to sugar units. Their sugar-free counterparts (anthocyanidins) are based on the flavylium cation, which might present different substitution patterns originating the diversity of anthocyanidins found in nature (Hidalgo & Almajano, 2017). Among the 17 natural anthocyanidins, cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin, are the major forms in most species (Hidalgo & Almajano, 2017).

In what concerns the application of anthocyanins in food products, there are some previous reports describing the incorporation of rose (*Rosa damascena*) petals extracts in yogurt (*e.g.*, Chanukya & Rastogi, 2016). Owing to the previously evidenced suitability of *R. damascena* as a colour ring agent in yogurt, we selected that species as one of the plant sources of anthocyanins to be incorporated in the yogurt formulations prepared in the lab. Likewise, we selected the flowers of *Centaurea cyanus* L. (cornflower), mainly due to its richness in cyanidin 3-*O*-(6-*O*-succinylglucoside)-5-*O*-glucoside (Takeda et al., 2005), but also in other bioactive phenolic compounds such as apigenin-glucuronide (Pires, Dias, Barros, & Ferreira, 2017) and *Dahlia mignon* (dahlia), which also presents a rich composition in different phenolic compounds like naringenin-3-*O*-glucoside, kaempferol-pentosyl-rhamnosyl-hexoside or apigenin-hexoside (Deguchi, Ohno, Hosokawa, Tatsuzawa, & Doi, 2013; Pires et al., 2018).

The selection and purchase of food products are greatly influenced by sensory expectations (Spence, Levitan, Shankar, & Zampini, 2010). Visual perception deliver so called quality cues, perceived prior to actual consumption and give hints of the quality attributes that are apparent during the consumption (Jantathai, Sungsri-in, Mukprasirt, &

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Duerrschmid, 2014; Spence et al., 2010). Colour plays an important role in the development of food preferences and sensory perception (Jantathai et al., 2014).

Nevertheless, colour is not important only in what concerns the product appearance. In fact, some colouring agents may have important functions beyond their primary effect. Anthocyanins, for instance, might have beneficial health effects due to their antioxidant, anti-inflammatory, anticancer, and anti-diabetic properties, thereby being of great interest to the food industry (Rodriguez-Amaya, 2016). However, it is also necessary to take into account that anthocyanins might degrade or react in food systems to form complex reaction products, leading to a mixture of products in addition to the parent anthocyanins (Rodriguez-Amaya, 2016). The intensity and stability of anthocyanins when used as food additives are influenced by pH, structure, concentration, co-pigmentation and metal complexing, as well as temperature, light, oxygen, acetaldehyde, ascorbic acid, sugars and their degradation products, sulphur dioxide, amino acids and catechins. Still, when low pH conditions are maintained, anthocyanins are relatively stable (EFSA, 2013; Rodriguez-Amaya, 2016).

Accordingly, the aim of the present study was to develop a new colouring strategy in yogurt products using natural anthocyanin rich extracts obtained from edible flower petals of *Dalia mignon*, *Centaurea cyanus* L. and *Rosa damascena* “Alexandria” mixed with *Rosa gallica* “French” draft in *Rosa canina*. These flowers were firstly characterized and quantified regarding the anthocyanin content, thought an HPLC-DAD-ESI/MS system. Additionally, the chromatic stability was evaluated by performing the evaluation studies (nutritional parameters, free sugars, fatty acids, anthocyanin content, and colour parameter) in yogurt formulation at two different periods (preparation day and after 7 days of storage).

2. Materials and methods

2.1. Samples

Dried commercial samples of petals of *Dahlia mignon*, rose resulting from *R. damascena* 'Alexandria' and *R. gallica* 'Francesa' draft in *R. canina*, and *Centaurea cyanus* L. were provided by RBR foods (Castro D'aire, Portugal).

In order to prepare the extracts, samples were reduced to powder (20 mesh) and were extracted by maceration (25 °C, 150 rpm, 1 h) using a stirring plate (VELP scientific, Keyland Court, NY, USA) by adding 1 g of dry material to 50 mL of distilled water. Afterwards, the mixture was filtered through Whatman filter paper No. 4, frozen and lyophilized. The lyophilized extracts obtained were used as natural additives.

2.2. Anthocyanin compounds identification by HPLC-DAD-ESI/MS

The chromatographic data of anthocyanin compounds were acquired from a Dionex Ultimate 3000 system (Thermo Scientific, San Jose, CA, USA), coupled to diode array, using 520 nm as preference wavelength, and to a mass spectrometer (MS, Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) operating in the positive mode (Gonçalves et al., 2017). Retention times, UV–Vis and mass spectra were compared with available standards and with literature data to identify the anthocyanin's. Calibration curves of the available anthocyanin standards were constructed based on the UV signal to perform quantitative analysis, in case of an unavailable commercial standards, the compounds were quantified *via* the calibration curves of the most similar available standards. The results were expressed as μg/g of dry extract.

2.3. Fortification of yogurts with natural and commercial colorant additives

2.3.1. Incorporation process

The base formulation yogurts (fat 3.8%; protein 5.0% and carbohydrates 4.7%) were purchased at the local market. Five groups (three samples/group) of yogurts (70 g each) were prepared, with three replicates of each: i) control samples (BY); ii) yogurts with commercial colorant, E 163 (AY); iii) yogurts with rose petals extract (RY); iv) yogurts with *Centaurea cyanus* L. petals extract (CY); v) yogurts with *Dahlia mignon* petals extract (DY). All colorants were added to a portion of 70 g of yogurt and were prepared in duplicate. The E163 colorant was added at a 0.02% concentration; in the case of yogurts added with petals extracts, slightly higher concentrations of each extract (0.05% for dahlia extract; 0.15% for rose extract; 0.10% for centaurea extract) were added (the quantity was added until an evident change in colour was obtained).

2.3.2. Nutritional and chemical composition

The proximate composition was determined according to AOAC procedures (AOAC, 2016), including protein (991.02), crude fat (989.05) and ash (935.42) contents. Crude protein (N × 6.25) was determined by the Kjeldahl method; ash content was estimated by subjecting the sample to incineration at 600 ± 15 °C for 5 h, while crude fat was determined using a Soxhlet apparatus with petroleum ether as recycling solvent and total carbohydrate was estimated by difference. The total energy was calculated using the following equation: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI; Knauer, Smartline system 1000, Berlin, Germany), using melezitose as an internal standard. All the mentioned procedures were previously described by the authors (Barros, Pereira, & Ferreira, 2013; Dias et al., 2015).

The fatty acids were determined by gas chromatography coupled with a flame ionization detector (GC-FID/capillary column, DANI model GC 1000, Contone, Switzerland), a split/splitless injector and a Macherey–Nagel column. The identification of fatty acids was performed by comparing the relative retention times of FAME peaks from samples with commercial standards (Barros et al., 2013; Dias et al., 2015).

Anthocyanins were determined in the yogurt sample by extracting 3 g of dry yogurt with water at 25 °C, 150 rpm during 1 h, followed by filtration through a Whatman filter paper No. 4. The remaining residue was re-extracted with an additional portion of water mixture, stored at −20 °C and lyophilized for further analysis. The lyophilized extracts were analysed using the HPLC-DAD-ESI/MS system mentioned above.

2.4. Physico-chemical parameters

The colour was measured in triplicate for each sample using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The CIE L^*, a^* and b^* colour space values were registered using a data software “Spectra Magic Nx” (version CM-S100W 2.03.0006), using the illuminant C and diaphragm aperture of 8 mm (Fernandes et al., 2012). The pH values of the samples was measured directly with a HI 99161 pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA).

2.5. Statistical analysis

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

Data were expressed as mean ± standard deviation, maintaining the significant numbers allowed by the magnitude of the corresponding standard deviation.

An analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model (GLM) procedure to compare the parameters evaluated in the prepared yogurts. The dependent variables were analysed using 2-way ANOVA with the factors “yogurt formulation” (YF) and “storage” (SE). When a statistically significant

interaction was detected among the two factors, their effect was evaluated by checking estimated marginal means plots for all levels of each factor. On the contrary, if no statistical significant interaction was found, means were compared using Tukey's multiple comparison test, after verifying the homogeneity of variances through Levene's test.

In addition, a linear discriminant analysis (LDA) was used to have a better understanding about the YF overall effect. A stepwise technique was applied, considering the Wilks' Λ test with the usual probabilities of F (3.84 to enter and 2.71 to be removed) for variable selection. Only variables with a statistically significant classification performance ($p < 0.050$) were maintained by the statistical model. The significant independent variables were selected following the stepwise method of LDA. This procedure is based in sequential forward selection and backward elimination steps, where the inclusion of a new variable requires verifying the significance of all previously selected variables (Zielinski et al., 2014). The main purpose was estimating the relationship between the single categorical dependent variables (yogurt formulations) and the quantitative independent variables (results obtained in the laboratorial assays). The LDA outputs allowed determining which independent variables contributed more to the differences in the average score profiles of different YF. A leaving-one-out cross validation procedure was carried out to assess the model performance.

3. Results and discussion

3.1. Anthocyanin profile characterization

Owing to the powerful colouring capacity of anthocyanins, these compounds were thoroughly characterized in the extracts obtained from the petals of each selected species. The extraction yields (mg of anthocyanin per 100 g of petals) obtained for each sample extract were: ~53% for dahlia; ~46% for rose; and ~23% for centaurea samples.

Nine anthocyanin compounds were detected in dahlia, two in rose and eight in centaurea extracts. Peak characteristics, tentative identification and compound quantification are presented in Table 1. Cyanidin (Cy; peaks **1**, **2**, **3**, **4**, **6**, **10**, **11**, **13**, **15**, and **17**), pelargonidin (Pg; peaks **8**, **9**, **12**, **16**, and **18**), and delphinidin (Dp; peaks **5** and **7**) were identified as main aglycones, based on the observation of their characteristic fragments in MS^2 spectra. As reviewed by Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, and Galán-Vidal (2009), these non-methylated anthocyanidins are the most commonly found in flowers, being cyanidin derivatives the most abundant in the analysed samples.

The conjugated bonds of anthocyanins, the glycosylated form of anthocyanidins, result in red, blue, and purple-coloured plants (Khoo, Azlan, Tang, & Lim, 2017). Several foods, like yoghurt, are considered healthy, but they lack important components such as phenolic compounds. Therefore, the incorporation of plant extracts rich in anthocyanins in these fermented products might impart a desirable red colour, while enhancing their potential health effect (Mourtzinos et al., 2018).

Before incorporating the flower extracts in yogurts, their profiles in anthocyanins were thoroughly characterized. Peak **1**, detected in rose and centaurea samples, was positively identified as cyanidin 3,5-di-*O*-glucoside based on the HPLC-DAD-MS results and comparison with our database library. This compound was already described as the main anthocyanin in petals of *R. damascena* (Velioglu & Mazza, 1991) and *R. hybrida* (Lee, Lee, & Choung, 2011) used with edible purposes, as well as in flowers from different *Centaurea* species (Mishio, Takeda, & Iwashina, 2015), highlighting its suitability to be incorporated in yogurt formulations. Peak **2**, found in rose samples, was also positively identified as cyanidin-3-*O*-glucoside according with its retention time and mass spectral data by comparison with a standard. The presence of this reddish-purple anthocyanin was also reported in rose hips (*R. canina*) previously Hvattum (2002).

Peak **4** ($[M]^+$ at m/z 711) was the majority anthocyanin in centaurea samples. Its MS^2 spectra yielded fragments at m/z 549 (−162 mu, loss of a hexose), 449 (−262 mu, loss of succinylhexose) and 287 (cyanidin), coherent with an identity as Cy-3-*O*-(6″-succinylglucoside)-5-*O*-glucoside, a compound consistently identified in centaurea flowers also referred to as centaurocyanin (Mishio et al., 2015; Kôsaku; Takeda & Tominaga, 1983), and whose combination with a flavone glycoside

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification, and quantification of anthocyanins in dahlia, rose, and centaurea extracts. Results are presented as mean ± standard deviation.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion (m/z)	MS^2 (m/z)	Tentative identification	Quantification (μg/g extract)
Rose						
1	11.5	514	611	449(10),287(100)	Cyanidin 3,5-di-*O*-glucoside[A]	13.19 ± 0.01
2	18.5	516	449	287 (100)	Cyanidin-3-*O*-glucoside[A]	0.131 ± 0.004
					Total Anthocyanins	**13.326 ± 0.002**
Centaurea						
1	11.7	512	611	449(5),287(100)	Cyanidin 3,5-di-*O*-glucoside[A]	5.5 ± 0.2
3	18.03	516	697	535(62),449(8),287(46)	Cyanidin 3-*O*-(6″-malonylglucoside)-5-*O*-glucoside[A]	6.2 ± 0.3
4	20.38	516	711	549(3),449(48),287(100)	Cyanidin 3-*O*-(6″-succinylglucoside)-5-*O*-glucoside[A]	11.2 ± 0.5
5	29.6	518	465	303 (100)	Delphinidin-hexoside[C]	1.5 ± 0.2
6	31.5	518	463	287 (100)	Cyanidin-glucuronide[A]	0.85 ± 0.06
7	32.6	518	561	303 (100)	Delphinidin-malonylhexoside[C]	tr
8	38.1	501	695	609(9),433(2),271(82)	Pelargonidin 3-*O*-(6″-succinylglucoside)-5-*O*-glucoside[B]	0.18 ± 0.01
9	39.2	502	519	271 (100)	Pelargonidin-malonylhexoside[B]	0.17 ± 0.01
					Total Anthocyanins	**26 ± 1**
Dahlia						
10	11.6	516	449	287 (100)	Cyanidin-hexoside[A]	2.98 ± 0.01
11	13.4	504	449	287 (100)	Cyanidin-hexoside[A]	2.654 ± 0.001
12	15.1	514	579	271 (100)	Pelargonidin-rutinoside[B]	1.4 ± 0.1
13	17.2	514	491	287 (100)	Cyanidin-acetylhexoside[A]	5.36 ± 0.01
14	19.4	501	431	269 (100)	Methylapigeninidin-hexoside[A]	4.1 ± 0.1
15	20.8	518	595	287 (100)	Cyanidin-rutinoside[A]	0.8 ± 0.1
16	28.5	504	595	271 (100)	Pelargonidin 3,5-di-*O*-glucoside[B]	0.8 ± 0.1
17	31.5	518	491	287 (100)	Cyanidin-acetylhexoside[A]	0.33 ± 0.02
18	32.7	516	433	271 (100)	Pelargonidin-hexoside[B]	0.450 ± 0.001
					Total Anthocyanins	**18.8 ± 0.2**

tr-trace amounts; Standard calibration curves: A – cyanidin-3-*O*-glucoside ($y = 243287x$ - 1E+06; $R^2 = 0.995$); B – pelargonidin-3-*O*-glucoside ($y = 276117x$ – 480418; $R^2 = 0.9979$); C– delphinidin-3-*O*-glucoside ($y = 557274x + 126.24$; $R^2 = 0.997$).

Table 2
Nutritional composition (g/100 g fresh weight) and energy values (kcal/100 g fresh weight) for different yogurt formulations (YF) and storage effect (SE). Results are presented as mean ± standard deviation.[a]

		Water	Fat	Protein	Ash	Carbohydrates	Galactose	Lactose	Energy
YF	**BY**	85.0 ± 0.4	3.3 ± 0.1	5.3 ± 0.3	0.79 ± 0.03	5.6 ± 0.1	0.69 ± 0.01[c]	4.7 ± 0.1	73 ± 2
	RY	84.8 ± 0.4	3.3 ± 0.2	5.3 ± 0.2	0.85 ± 0.01	5.8 ± 0.2	0.71 ± 0.04[bc]	4.7 ± 0.2	74 ± 3
	DY	85.0 ± 0.1	3.4 ± 0.1	5.4 ± 0.1	0.86 ± 0.02	5.4 ± 0.1	0.71 ± 0.01[b]	4.8 ± 0.1	73 ± 1
	CY	84.8 ± 0.1	3.2 ± 0.1	5.4 ± 0.1	0.86 ± 0.02	5.7 ± 0.1	0.76 ± 0.02[a]	4.8 ± 0.1	74 ± 1
	AY	84.9 ± 0.2	3.4 ± 0.1	5.3 ± 0.1	0.82 ± 0.02	5.5 ± 0.1	0.72 ± 0.02[b]	4.9 ± 0.1	74 ± 1
	ANOVA *p*-value (n = 18)[b]	0.083	0.001	0.039	< 0.001	< 0.001	< 0.001	< 0.001	0.632
SE	**0 days**	85.0 ± 0.2	3.3 ± 0.1	5.3 ± 0.2	0.84 ± 0.04	5.6 ± 0.2	0.73 ± 0.03	4.8 ± 0.1	73 ± 1
	7 days	84.8 ± 0.3	3.4 ± 0.1	5.4 ± 0.1	0.84 ± 0.03	5.6 ± 0.2	0.71 ± 0.03	4.8 ± 0.1	74 ± 2
	ANOVA *p*-value (n = 45)[c]	0.056	0.061	0.119	0.763	0.258	0.100	0.408	0.081
YF × SE	*p*-value (n = 90)[d]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.272	< 0.001	< 0.001

[a] Results are reported as mean values of each YF, aggregating results from 0 to 7 days, and mean values of SE, combining all YF.
[b] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one YF.
[c] If $p < 0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts.
[d] In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

Table 3
Physicochemical parameters (CIE L^*, a^* and b^* and pH values) for different yogurt formulations (YF) and storage effect (SE). Results are presented as mean ± standard deviation.[a]

		L^*	a^*	b^*	pH
YF	**BY**	93 ± 1	−3.5 ± 0.1	9.8 ± 0.4	4.3 ± 0.1
	RY	88 ± 1	2.2 ± 0.1	9.0 ± 0.3	4.3 ± 0.1
	DY	84 ± 1	2.1 ± 0.3	17.7 ± 0.4	4.4 ± 0.1
	CY	90 ± 1	−1.1 ± 0.2	9.5 ± 0.5	4.2 ± 0.1
	AY	89 ± 1	3.1 ± 0.5	6.5 ± 0.5	4.8 ± 0.1
	ANOVA *p*-value (n = 18)[b]	< 0.001	< 0.001	< 0.001	< 0.001
SE	**0 days**	88 ± 3	1 ± 3	10 ± 3	4.4 ± 0.2
	7 days	89 ± 3	0 ± 2	11 ± 3	4.4 ± 0.2
	ANOVA *p*-value (n = 45)[c]	0.056	0.250	0.312	0.946
IF × ST	*p*-value (n = 90)[d]	< 0.001	< 0.001	< 0.001	0.867

[a] Results are reported as mean values of each YF, aggregating results from 0 to 7 days, and mean values of SE, combining all YF.
[b] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one YF.
[c] If $p < 0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts.
[d] In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

and metal ions give rise to protocyanin, a stable complex pigment considered to be the main responsible for the blue colour of *Centaurea cyanus* flowers (Kosaku Takeda et al., 2005). This compound could have interesting colouring properties to be used as a natural additive in food products. Similarly, mass spectral characteristics of peak **3**, with a molecular ion $[M]^+$ at *m/z* 697 and MS^2 fragments at *m/z* 535 (−162 mu, loss of a hexose), 449 (−248 mu, loss of malonylhexose) and 287 (cyanidin), allowed tentatively assigning it as Cy-3-*O*-(6″-malonylglucoside)-5-*O*-glucoside owing to its previous identification in flowers from different *Centaurea* species (Mishio et al., 2015). Peak **6** ($[M]^+$ at *m/z* 463) was another cyanidin derivative, tentatively identified as Cy-*O*-glucuronide based on the loss of 176 mu (a glucuronyl moiety) to yield the unique MS^2 product ion at *m/z* 287.

Peaks **8** and **9** in centaurea samples were associated to pelargonidin derivatives based on their characteristic absorption spectra showing λ_{max} at 501 nm and the fragment ion observed at *m/z* 271 (Pg). Peak **8** ($[M]^+$ at *m/z* 695), with similar fragmentation behaviour as peak 4, was identified as Pg-3-*O*-(6″-succinylglucoside)-5-*O*-glucoside, previously described in *Centaurea cyanus* flowers by Kosaku Takeda, Kumegawa, Harborne, and Self (1988). Peak **9** ($[M]^+$ at *m/z* 519) was tentatively assigned as a Pg-*O*-malonylhexoside based on the loss of 248 mu (malonylhexoside) to yield the aglycone ion at *m/z* 271. Pelargonidin differs from most anthocyanidins as it might provide an orange hue to flowers and red to some of the fruits and berries (Jaakola, 2013; Khoo et al., 2017), having also demonstrated a notable anti-inflammatory effect (Duarte et al., 2018). In a similar way, peak **7** ($[M]^+$ at *m/z* 561), yielding a unique MS^2 fragment at *m/z* 303 (−248 mu) was associated to delphinidin-*O*-malonylhexoside, whereas **p**eak **5** ($[M]^+$ at *m/z* 465) was assigned as a Dp-*O*-hexoside; a possible identity as Dp-3-*O*-glucoside was excluded by comparison with peak characteristics with our database library. Delphinidin appears as a purple pigment in the nature, and the blue hue of flowers is often due this pigment, which was previously reported for its anti-inflammatory, antioxidant, and anti-tumorigenic activities (Ko et al., 2015), making it specially interesting as an ingredient of innovative food formulations (Khoo et al., 2017).

Similar reasoning was applied to identify anthocyanins in dahlia samples as cyanidin (peaks **10**, **11**, **13**, **15** and **17**) and pelargonidin derivatives (peaks **12**, **16** and **18),** which were previously reported in dahlia flowers (Deguchi et al., 2013; Kosaku; Takeda, Harborne, & Self, 1986; Yamaguchi et al., 1999). The presence of Pg-3,5-diglucoside in flowers of *Dahlia variabilis* was identified by Yamaguchi et al. (1999) and Deguchi et al. (2013), which could correspond to peak **16** ($[M]^+$ at *m/z* 595) in our samples. For the remaining compounds (peaks **10**, **11**, **12**, **13**, **15**, **17, and 18**), no conclusions about the precise identity of the anthocyanins could be obtained, and the glycoside moieties were assigned based on the mass losses observed in the MS^2 spectra, as hexosides (−162 mu), acetylhexosides (−204 mu) or deoxyhexosylhexosides (−308 mu). Curiously, none of the observed peak losses indicates the presence of malonylglucosides, a type of derivatives usually reported in dahlia flowers (Deguchi et al., 2013; Kosaku; Takeda et al., 1986; Yamaguchi et al., 1999). Cy-acetylhexoside (peak **13**) was the most abundant compound in dahlia, representing the main responsible for the coloration of this edible flowers. In addition to improve the sensory characteristics of yogurt, that cyanidin might be a promising antiglycation agent for preventing or ameliorating AGEs-mediated diabetic complications (Suantawee, Cheng, & Adisakwattana, 2016). Finally, peak **14** presented a molecular ion $[M]^+$ at *m/z* 431 and a unique MS^2 fragment at *m/z* 269, which could match the mass of methylapigeninidin, so that it might be associated to a methylapigeninidin-hexoside, a pigment reported in red sorghum (Wu & Prior, 2005). Nevertheless, the absorption spectrum of peak **14** would not be coherent with such an identity, as the maximum absorption in the visible region of that compound should be expected around 470 nm (Awika, 2008; Yang, Dykes, & Awika, 2014). Thus, the identity of this peak as a 3-deoxyanthocyanin is uncertain, although in case it is confirmed it would be the first description of this type of pigments in dahlia flowers.

In general, these flowers have a great potential to be used as natural

Table 4
Fatty acids profile (relative percentage) of yogurt formulations (YF) and storage effect (SE). Results are presented as mean ± standard deviation.[a]

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n3	SFA	MUFA	PUFA
YF	**BY**	1.3 ± 0.2	1.7 ± 0.1	1.3 ± 0.1	2.9 ± 0.2	3.6 ± 0.1	11.9 ± 0.4	1.4 ± 0.1	35 ± 1	1.4 ± 0.1	11.0 ± 0.2	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	71 ± 1	23 ± 2	5.0 ± 0.1
	RY	0.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.6 ± 0.1	12.2 ± 0.2	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.1	20 ± 1	2.2 ± 0.1	1.3 ± 0.1	72 ± 1	23 ± 1	4.6 ± 0.2
	DY	1.0 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.5 ± 0.1	12.1 ± 0.1	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.2	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	72 ± 1	23 ± 1	5.1 ± 0.1
	CY	1.1 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.6 ± 0.1	12.0 ± 0.1	1.5 ± 0.1	36 ± 1	1.5 ± 0.1	11.5 ± 0.1	20 ± 1	2.5 ± 0.2	1.5 ± 0.1	72 ± 1	23 ± 1	5.3 ± 0.2
	AY	1.2 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.7 ± 0.1	12.0 ± 0.3	1.5 ± 0.1	35 ± 1	1.4 ± 0.1	11.3 ± 0.1	21 ± 1	2.3 ± 0.1	1.4 ± 0.1	72 ± 1	23 ± 1	4.9 ± 0.2
	ANOVA *p*-value (n = 18)[b]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001	0.133	< 0.001	< 0.001	0.180	0.125	< 0.001
SE	**0 days**	1.1 ± 0.2	1.4 ± 0.1	1.1 ± 0.1	2.7 ± 0.1	3.5 ± 0.1	11.9 ± 0.3	1.5 ± 0.1	35 ± 1	1.5 ± 0.1	11.3 ± 0.3	21 ± 1	2.3 ± 0.1	1.5 ± 0.1	71 ± 1	23 ± 1	5.0 ± 0.2
	7 days	1.0 ± 0.3	1.6 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	3.6 ± 0.1	12.2 ± 0.2	1.5 ± 0.1	36 ± 2	1.5 ± 0.1	11.4 ± 0.1	20 ± 1	2.3 ± 0.1	1.4 ± 0.1	72 ± 1	22 ± 1	5.0 ± 0.1
	t-student *p*-value (n = 45)[c]	0.018	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.737	0.001	0.984	0.213	< 0.001	0.063	< 0.001	< 0.001	< 0.001	0.532
IF × ST	*p*-value (n = 90)[d]	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a] Results are reported as mean values of each YF, aggregating results from 0 to 7 days, and mean values of SE, combining all YF.
[b] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one YF.
[c] If $p < 0.05$, the corresponding parameter presented a significant difference among stored and non-stored yogurts.
[d] In this table, the interaction among factors was significant in all cases; thereby no multiple comparisons could be performed.

colorants, being an excellent source of anthocyanins to develop innovative products with new sensorial and bioactive characteristics.

3.2. Characterization of different fortified yogurts

Natural additives are generally considered as producing no harmful effects on consumers' health, contrarily to some artificial compounds (Carocho, Barreiro, Morales, & Ferreira, 2014). Nevertheless, the acceptability of these products is highly dependent on their appearance and rheological properties (Caleja et al., 2016; Santillán-Urquiza, Méndez-Rojas, & Vélez-Ruiz, 2017). In what concerns yogurt, similarly to several other food products, colour is a determining factor. Bearing this in mind, different plant species were selected as potential sources of colourants to be incorporated in yogurt. Yogurts prepared with different flower extracts were compared with each other and also with yogurts added with a commercial anthocyanin extract (E163, authorised by EFSA). In addition, a set of yogurts were used exactly as bought (free of any colouring agent), functioning as the "blank" yogurt control.

Besides comparing different yogurt formulations (YF), their stability throughout storage was also evaluated, specifically by performing the same evaluation assays on the preparation day and after 7 days of storage (SE).

Since the effect of each factor (YF or SE) might be affected by the second factor level (*i.e.*, different storage effects according on each YF, or *vice versa*), the interaction (YF × SE) was also evaluated. In all cases where a significant interaction was found ($p < 0.050$), the multiple comparisons could not be performed. In those cases, the overall conclusions were obtained from the corresponding estimated marginal means (EMM) plots.

Starting by analysing the results for nutritional parameters (Table 2), a significant interaction among YF and SE (YF × SE) was found in all cases, thereby indicating that each YF reacted differently to storage. Considering each factor individually, YF-related differences were significant in most cases, except water and energy, while SE had no significant effect in any case. In either case, the nutritional profile is very similar among all tested samples, with water as the main component (≈85 g/100 g), followed by carbohydrates (slightly higher in RY and CY and lower in DY) and protein (a bit higher in CY and lower in RY), both corresponding to ≈5.5 g/100 g), fat (≈3.2 g/100 g in CY to 3.4 g/100 g DY and AY) and ash (< 0.9 g/100 g in all yogurts). This profile resulted in energy values around 74 kcal/100 g in all cases. Actually, owing to the low quantity of colorant added, it was not expectable to have differences of high magnitude among different YF, particularly in what concerns fat amounts (the flower extracts were prepared with water). Nevertheless, the plant species used in the extraction procedures had different nutritional composition (Pires et al., 2017), causing some minor changes in the corresponding yogurts. Even so, these results validate the maintenance of the nutritional quality of natural yogurt (herein identified as BY).

In what concerns individual sugars, lactose was the main compound (≈4.8 g/100 g, with slightly higher values in AY). Minor levels of galactose were also quantified, varying form the maximum values detected in CY (0.76 g/100 g) to the lowest in BY (0.69 g/100 g).

More significant differences were, as observable in Table 3, obtained in the case of colour parameters, which is in line with the main purpose of this work. Yogurts free of any additive (BY) showed the highest L^* values, followed by CY, AY, RY and DY. On the contrary, BY presented the lowest a^* values, while AY, RY and DY reached the highest (without significantly different values among them). On the other hand, the absence of significant differences for a^* values among AY, RY and DY, indicate that rose and dahlia extracts might be potential alternatives to E163.

Fatty acids profiles, especially for their potential usefulness as indicators of suitable conservation conditions, were also characterized (Barreira, Pereira, Oliveira, & Ferreira, 2010; Pereira et al., 2016). All fatty acids quantified in relative percentages above 1% are presented in

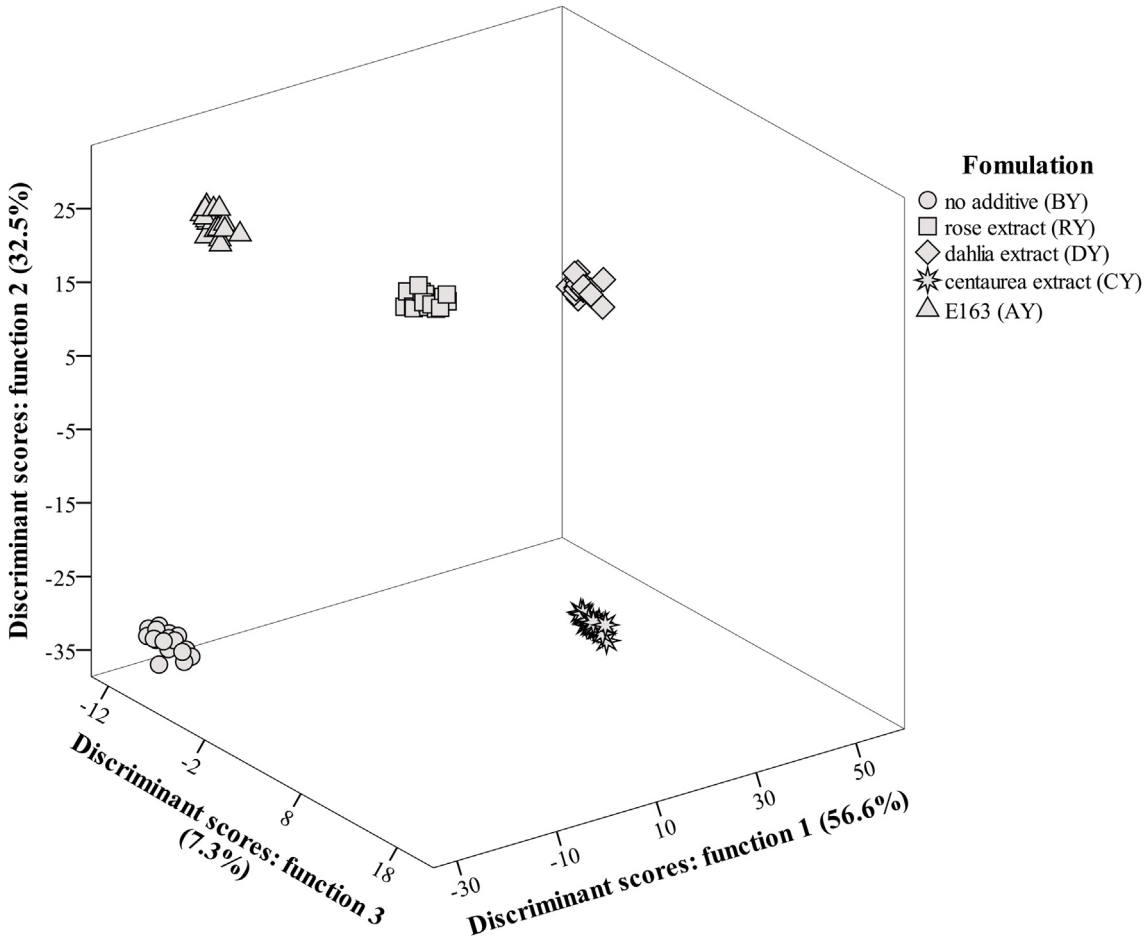

Fig. 1. Three-dimensional distribution of YF markers according to the canonical discriminant functions coefficients defined from different yogurt parameters.

Table 4, but the complete profiles included also C11:0, C13:0, C14:1, C17:0, C17:1, C18:3n6, C20:0, C20:1, C20:4n6, C20:5n3, C22:0, C23:0, C24:0 (however, all fatty acids were included in the Linear Discussion Analysis discussed in the next section).

Since milk was the main source of fatty acids in yogurt, and bearing in mind, once again, that the added extracts were aqueous, the high similarity among YF is coherent. Nevertheless, C18:1n9 ($p = 0.133$), SFA ($p = 0.180$) and MUFA ($p = 0.125$) were the only cases with no significant differences among tested YF, most likely because the added extracts might have different effectiveness in preventing the oxidation of specific fatty acids throughout time.

Since the interaction among factors (YF × SE) the next conclusions were obtained from the EMM plots (data not shown): BY presented higher percentages of C4:0 (1.3%), C6:0 (1.7%), C8:0 (1.3%), and C10:0 (2.9%), while C15:0 (1.5%), C16:1 (1.5%), C18:0 (11.5%), C18:2n6 (2.5%) and PUFA (5.3%) showed the highest values in CY. Yogurts prepared with rose extract (RY), on the other hand, had the highest percentages of C14:0 (12.2%) and C16:0 (36%), whilst C12:0 was slightly higher in AY (3.7%).

In what concerns SE effect, almost all tabled fatty acids showed significant differences, except in the cases of C15:0 ($p = 0.737$), C16:1 ($p = 0.984$), C18:0 ($p = 0.213$), C18:2n6 ($p = 0.063$) and PUFA ($p = 0.532$). In stored samples, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0 and SFA were quantified in higher percentages, while C4:0, C18:1n9, C19:3n3 and MUFA tended to present higher values in non-stored samples, thereby generally corroborating the higher resistance of saturated forms to storage.

3.3. Linear discriminant analysis

Despite the statistical significance of differences among different YF, we decided to verify if the magnitude of the detected differences was high enough to discriminate each YF. Accordingly, a linear discriminant analysis (LDA) was applied to find the variables with highest contribution to discriminate each YF.

The first three discriminant functions included 97.7% (first function: 61.4%; second function: 30.0%; third function: 6.3%) of the observed variance (Fig. 1). From the 41 variables under analysis, the discriminant model selected b^*, a^*, L^*, pH, C4:0, C8:0, C13:0, C16:1, C17:1, C18:3n3, C18:3n6, C20:1, C20:4n6, C20:5n3, C23:0, C24:0 and PUFA as those having discriminant ability, which clearly indicates that fatty acids and colour parameters were the variables with highest dissimilarity among the prepared YF.

In what concerns the correlations among functions and variables, function 1 was highly correlated with b^* and L^*, placing markers corresponding to DY and BY in the farthest positions due to their differences in both parameters (the highest b^* value was measured in DY, while the maximum L^* was measured in BY). Function 2, in turn, was mostly correlated with a^*, mostly separating markers corresponding to AY and RY (positive end of the axis) from BY (negative end of the axis). Function 3 also contributed to separate the markers of each YF, being especially effective in separating BY and CY. Owing to the higher proximity of their markers according to the three plotted discriminant functions RY and AY showed the highest similarity among the assayed parameters.

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

validated grouped cases.

4. Conclusion

Overall, the natural extracts with highest potential as alternatives to E163 resulted to be RY, considering the main purpose of colouring yogurts in the yellow-orange series. In addition to the provided colour, these groups of yogurts (AY and RY) showed very similar nutritional value, free sugars and fatty acids composition.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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ANNEX VI

Edible flowers: Emerging components in the diet- review

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Review

Edible flowers: Emerging components in the diet

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ABSTRACT

Background: The search for a healthier lifestyles and changing eating habits, have placed viable and safer alternatives products in the market. In particular, edible flowers are used to make dishes more attractive, by adding color, flavor and other sensory characteristics, thus also presenting in their composition bioactive compounds, such as polyphenols, that may provide beneficial health effects.

Scope and approach: This review deals with the production, harvesting and storage of wild edible flowers, as well as aspects concerning their processing, packaging and consumption. Moreover, the most abundant bioactive molecules, namely phenolic compounds and more particularly anthocyanins, are also reviewed. Some extraction techniques, such as Solid-Liquid extraction (SLE), Supercritical Fluid Extraction (SFE), Ultrasound Assisted Extraction (UAE), Pressurized Liquid Extraction (PLE) and Microwave Assisted Extraction (MAE) are discussed, as also the most convenient solvents used. Particular focus is employed on the anthocyanins present in edible flowers.

Key findings and conclusions: The presence of phenolic compounds has attracted the interest not only of the consumers, but also of the food industry, due to their potential to be used as natural additives, namely as preservatives and colorants, that can be applied as an alternative to substitute their artificial counterparts. The major phenolic acids found in edible flowers are caffeoylquinic acids, while cyanidin-3-*O*-glucoside is the main anthocyanin. Methanol and acetone were the most common solvents to extract polyphenols and solid-liquid extractions are the most common methodology applied. Only a small part of edible flowers has been explored, being required more studies, so that they can be used with total efficiency.

1. Introduction

Since ancient times, edible flowers have been used in the human diet (He et al., 2015; Lu, Li, & Yin, 2015; Rop, Mlcek, Jurikova, Neugebauerova, & Vabkova, 2012), being their practice very well documented worldwide, from ancient Greece and Rome, medieval France, Europe, Victorian England, or the Middle Eastern region (He et al., 2015; Lu et al., 2015) to Asian countries like China and Japan, where the consumption of edible flowers has been reported for thousands of years (Rop et al., 2012). Nowadays, globalization and awareness of consumers have contributed in the improvement and comeback of earlier lifestyles, where edible flowers have an important role (Liu & Long, 2002). This plant part is an abundant natural resource and most of them contain phytochemicals with putative health effects, which have increasingly attracted attention (Lu et al., 2015). Edible flowers have been long used in folk medicine to treat diseases, but recent studies have supported these traditional health benefits, revealing their rich composition in bioactive compounds, which have been correlated to functional properties (He et al., 2015; Lu et al., 2015; Rop et al., 2012; Wetzel et al., 2010). Also, research has also been focused on the safety of common edible flowers, so as to ensure their safe use and dosage by different industries, with different purposes (Koike et al., 2015a; Wetzel et al., 2010). There are numerous edible flowers all over the world and only a small part of them have been studied. Therefore, more detailed information regarding this natural matrix is needed, in order to increase their acceptability as food ingredients and to avoid potential risks (Lu et al., 2015). Since not all flowers meet the essential requirements, in order to be considered edible, such as being non-toxic and innocuous and should have nutritional properties, they cannot be included in the human diet (Lara-Cortés, Osorio-Díaz, Jiménez-Aparicio, & Bautista-Baños, 2013). Some flower species have toxic or antinutritional substances, such as trypsin inhibitors, hemagglutinins, oxalic acid, cyanogenic glycosides or alkaloids. Some of these compounds have been found, for instance, in flowers of *Agave salmiana* Otto ex Salm-Dyck, *Erythrina Americana* Mill., *Erythrina caribaea* Krukoff & Barneby or *Yucca filifera* Chabaut (Lara-Cortés et al., 2013; Navarro-

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González, González-Barrio, García-Valverde, Bautista-Ortín, & Periago, 2015; Sotelo, López-García, & Basurto-Peña, 2007). Consequently, those flowers must be considered inedible and hence cannot be included in the human diet.

2. From farm to table

2.1. Production and safety

The appearance of edible flowers in no way differs from the ornamental species and it is crucial to differentiate them by using chemical and biological parameters, so that they could be distinguished for their edibility (Mlcek & Rop, 2011). Purely decorative flowers may have toxic components that can lead to intoxication and be even fatal in certain cases. In many cases, the cultivation of these plants involves the use of harmful chemicals, whereas edible flowers are usually the result of an organic production, intended for food purposes (Fernandes, Casal, Pereira, Saraiva, & Ramalhosa, 2017).

Bringing innovative products with nutraceutical properties and health benefits to the market is one of the current challenges of producing edible flowers. In the 21st century, the agro-food industry faces several challenges, namely Food Security (having enough to eat) and Food Safety (safe to eat) (Scotter, 2015). These challenges should be understood not only in an environment of tremendous technological progress and evolution of consumer's life-styles, but also in economic terms, in which the food industry is called to operate under seemingly contradictory market demands (Behe et al., 2010; Chen & Wei, 2017). Consumers preference for natural products, minimally processed foods, packaging without preservatives and free of negative effects (e.g., low in fat, salt and sugar) is gaining a great attention by the food industry. This is also affected by recently emerged issues, such as climatic changes, financial crisis and breakthrough regarding technology information (Chen & Wei, 2017; Scotter, 2015).

2.2. Processing and packaging

The marketing of fresh edible plants and their acceptance by the consumers is an important factor regarding their commercialization and post-harvest performance. Although external quality-related attributes, such as appearance, color and odour, are the main criteria for attracting consumers preference and decision-making, there is a great interest in foods with bioactive and nutraceutical components (Behe et al., 2010; Chen & Wei, 2017). The packaging of these flowers is usually performed using small and rigid plastic containers, because they are highly perishable (could suffer oxidation) and are easily contaminated by insects, which compromises their nutritional and bioactive characteristics, as also decreasing their attractiveness (Fernandes et al., 2018; Villavicencio et al., 2018). Hence, it is essential to develop improved techniques to aid quality retention and extend shelf life of edible flowers. The most common methods used to improve postharvest storage of flowers quality include refrigeration, drying, canning in sugar and preservation in distillates (Fernandes et al., 2017). However, these methods may cause undesirable biochemical and nutritional changes in the processed product that may affect its overall quality. Food irradiation is an economically viable technology to extend shelf life of perishable commodities, which allows the disinfestation of insects, improves hygiene and helps maintain quality, in addition to preserving the bioactive characteristics and phytochemicals of the irradiated products (Farkas & Mohácsi-Farkas, 2011; Koike et al., 2015a). Koike et al. (2015a) studied the effects of electron-beam and gamma irradiation on the phenolic profile and antioxidant activity of edible flowers of *Viola tricolor* L. They concluded that irradiation allows increasing the shelf life of these flowers without negatively affecting the levels of phenolic compounds and antioxidant activity, making this technology a suitable commercial alternative. Modified Atmosphere Packaging (MAP) is another technology that has been extensively used to maintain the quality, extend shelf life and decrease microbial growth in perishable products, such as edible flowers, as demonstrated by Kou, Turner, and Luo (2012). These authors investigated the effect of the treatment with the synthetic plant growth regulator 1-methylcyclopropene together with modified atmosphere packaging (MAP) on the shelf life of *Dianthus caryophyllus* L. and *Antirrhinum majus* L. and concluded that this technology significantly extended the storage time of both edible flowers, reducing the weight loss and helping to maintain the visual quality (fresh appearance) (Kou et al., 2012).

Edible coatings are another methodology used to extend shelf life. The coatings could be derived from proteins, lipids and polysaccharides and can be used to protect perishable food products from deterioration by providing a selective barrier to moisture, oxygen and carbon dioxide. This allows delaying dehydration, suppressing respiration and improving textural quality, while helps to retain volatile flavour compounds and reduces microbial growth, thus making this methodology appropriate to be applied in edible flowers, as demonstrated by Fernandes et al. (2018). Those authors evaluated the effects of alginate coating on the physico-chemical and microbiological quality of *Viola* x *wittrockiana* edible flower under cold storage. The flowers coated with alginate revealed a good appearance until 14 days of storage, 7 days more than the uncoated sample, and also presented a significant reduction of yeasts and moulds counts (Fernandes et al., 2018).

2.3. Consumption

Cultural differences and patterns of consumption may determine the acceptance by consumers of a “new food” or “unfamiliar food”. The consumption of edible flowers in the Asian cuisine is already a common practice, and over time they have gained more common usage in other cultures, including Europe (Rodrigues et al., 2017). Their aroma, taste and appearance turns meals more attractive, together with their nutritional properties and low fat and energetic content have promoted their consumption worldwide (Rodrigues et al., 2017).

2.3.1. Nutritional and chemical composition

Edible flowers are usually composed by 70–95% of water. The composition and levels of other nutrients and phytochemicals depend on the part of the flower; for example, pollen is a source of proteins, carbohydrates, saturated and unsaturated lipids, carotenoids and flavonoids, while the nectar is made up of amino acids, free sugars, proteins, inorganic ions, lipids, organic acids, phenolic compounds, alkaloids and terpenoids, among others. Petals and other parts of the flowers are richer in vitamins, minerals and antioxidants (Fernandes et al., 2017; Mlcek & Rop, 2011). Carbohydrates are the most abundant macronutrients in edible flowers; values of 88.39 and 86.12 g/100 g dry weight (DW) were determined in *Centaurea cyanus* L. and Rose, respectively (Pires, Dias, Barros, & Ferreira, 2017). Fiber content is quite variable, ranging from 6.1 to 55.4 g/100 g dry weight, as determined for flowers of *Allium schoenoprasum* L., and *Spilanthes oleracea* L. and *Tagetes erecta* L. (Fernandes et al., 2017). Potassium, phosphorus, calcium and magnesium are the main minerals present, with potassium levels being higher than calcium, bringing greater benefits relative to the prevention of cardiovascular diseases (Fernandes et al., 2017; Rop et al., 2012). The use of edible flowers in the development of children's snacks has a dated application. In Poland the favourite children snacks are developed using flowers of *Trifolium spp*., *Lamium album* and *Robinia pseudacacia* (Łuczaj et al., 2012). The demand for healthier eating habits and low-calorie foods, has promoted the development of new functional products obtained from natural sources, which is the case of the study developed by Pires, Dias, Barros, Barreira, et al. (2018), which incorporated different edible flower extracts into yogurts, adding an additional colour capacity to the developed product.

2.3.2. General and medicinal uses of edible flowers by consumers

Edible flowers are usually used to add color (*C. cyanus* and *V.*

Table 1
Edible and medicinal uses of some flowers.

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
Anchusa azurea P. Mill.	Garden anchusa and Italian bugloss	Boraginaceae	Violet light-blue  **Source: by Miguel Martínez in www.flickr.com**	Soup, boil, fries and salad	Depurative, antitussive, diaphoretic, and diuretic	(Loizzo et al., 2016)
Antirrhinum majus L.	Snapdragon	Plantaginaceae	Red, Rose, White  **Source: by naturguker.de in www.flickr.com**	Salad	Antiphlogistic, resolvent and stimulant; liver disorders, treatment of scurvy, tumours and as detergent, astringent and diuretic	(Loizzo et al., 2016)
Bauhinia purpurea L.	Orchid Tree, Purple Butterfly Tree, Mountain Ebony, Geranium Tree, Purple Bauhinia	Leguminosae	Purple  **Source: by Carlos Beutelspacher in www.flickr.com**	Salad	Nephroprotective and thyroid hormone regulating; antibacterial, antidiabetic, analgesic, anti-inflammatory, anti-diarrheal and antitumor activities	(Lai et al., 2010)
Bombax malabaricum L.	Cotton tree	Bombacaceae	Orange, red  **Source: by Ilya Borovok in www.flickr.com**	Cooked and accompanied with meat and rice	Treatment of chronic inflammation, fever, diarrhoea, hepatitis, and contused wounds	(Zhang et al., 2015)
Calendula officinalis L.	Marigold or Scotch marigold	Asteraceae	Orange  **Source: by Marco Ottaviani in www.flickr.com**	Salads, omelettes or as an accompaniment cheese	Antioxidant, anti-inflammatory, antitumor, anti-edematous, anti-HIV, antibacterial and antifungal activities ; immunomodulatory and immuno-stimulating, spasmolytic, spasmogenic and gastroprotective, insecticidal, heart rate decrease, cardioprotective, genotoxic and antigenotoxic dose-dependent	(Benvenuti, Bortolotti, & Maggini, 2016; Lara-Cortés et al., 2013)
Capparis spinosa L.	Flinders rose	Capparaceae	White-violet 	Preserved in vinegar and salt and salad	Antiseptic, diuretic, and protective of capillary vessels	(Loizzo et al., 2016)

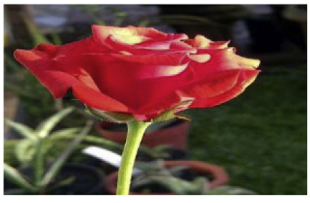
(*continued on next page*)

Table 1 (*continued*)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
			Source: by C. E. Timothy Paine in www.flickr.com			
Carthamus tinctorius L.	Safflower	Asteraceae	Red 	Infusions and cakes	Restoring menstrual flow and promoting blood circulation	(Wang et al., 2016)
			Source: by Färberdistel oder Saflor in www.flickr.com			
Centaurea cyanus L.	Cornflower and bachelor's button	Asteraceae	Blue 	Infusions, garnish and natural food colorant	Antioxidant activity, soothing, and used in ocular inflammation	(L. Fernandes et al., 2017)
			Source: by Atanue D. in www.flickr.com			
Chrysanthemum morifolium Ramat	Florist's daisy and hardy garden mum	Asteraceae	Yellow-white 	Infusions and cakes	Detoxifying and heat-clearing effects	(Wang et al., 2016)
			Source: by Inthemind Ofnature in www.flickr.com			
Cichorium intybus L.	Chicory	Astereacea	Light blue 	Soup, boil, potage and salad	Depurative, diuretic, laxative, hypoglycaemic, , disinfectant of urinary tract and hepatoprotective	(Loizzo et al., 2016)
			Source: by Hiro. Morison in www.flickr.com			
Gardenia jasminoides J.Ellis	Gardenia, cape jasmine, cape jessamine, danh-danh and jasmin	Rubiaceae	White 	Infusions and soup	Promoting diuresis and heat-clearing	(Wang et al., 2016)
			Source: by Stefano in www.flickr.com			
Hedysarum coronarium L.	Sweetvetch	Fabaceae		Soups, fries with eggs, and salad	Hypocholesterolemic and laxative effects	(Loizzo et al., 2016)

(*continued on next page*)

Table 1 (*continued*)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
			Purple  **Source: by María García in www.flickr.com**			
Hibiscus rosa sinensis L.	Chinese hibiscus, China rose, Hawaiian hibiscus, rose mallow and shoeblackplant	Malvaceae	Rose  **Source: by P.L. Tandon in www.flickr.com**	Infusions and food supplement	Genito-urinary troubles, bronchial catarrh, fever and cough	(Lu et al., 2015)
Hibiscus sabdariffa L.	Roselle	Malvaceae	Red  **Source: by Oleksandr Reva in www.flickr.com**	Flavouring agents, beverage (hot and cold), jams preparation of herbal drinks, fermented drinks, wine, ice cream, chocolates, puddings and cakes	Hypertension, abscesses, dysuria, fever and scurvy	(Lu et al., 2015; Xiong et al., 2014)
Jasminum sambac L.	Arabian jasmine and Sambac jasmine	Oleaceae	White  **Source: by Robert Sarkisian in www.flickr.com**	Infusions and porridge	Skin diseases, cancer, uterine bleeding, ulceration, leprosy and wound healing	(Wang et al., 2016; Xiong et al., 2014)
Lonicera japonica Thunb.	Japanese honeysuckle and golden and silver honeysuckle	Caprifoliaceae	Yellow-green  **Source: by Ebroh in www.flickr.com**	Infusions and soup	Heat-clearing and detoxifying	(Lu et al., 2015; Wang et al., 2016)
Rosa chinensis Jacq	China rose and Chinese rose	Rosaceae	Red 	Flavour extract, jams and infusions	Homeostasis, menstruation disorders, trauma and diarrheal	(Lu et al., 2015)

(*continued on next page*)

Table 1 (*continued*)

Scientific Name	Common name	Botanical family	Coloration	Edible use	Medicinal use	References
			Source: by Bunga Mawar in www.flickr.com			
Tropaeolum majus L.	Garden nasturtium, Indian cress, and monks cress	Tropaeolaceae	Yellow. Orange, Red 	Ingredients in meals, salads, foodstuffs and drinks	Antibacterial, antitumor and antithrombotic activities, diuretic and hypotensive effects	(Benvenuti et al., 2016; Navarro-González et al., 2015)
			Source: by Taylor World in www.flickr.com			
Viola tricolor L.	Johnny Jump up and heartsease	Violaceae	Yellow, orange, purple, violet  **Source: by Nora Caracci and Albert_zsolt in www.flickr.com**	Food colorants, sweets, salads, soups, vinegars and drinks	Prevention in Alzheimer, Parkinson, atherosclerosis and various cancers; antiallergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective and vasodilator effects.	(Koike et al., 2015a; Navarro-González et al., 2015)

tricolor), fragrance and flavour (*Hibiscus sabdariffa* L.) to food products such as salads, soups, entrees, desserts and drinks. In addition to these characteristics, different flowers have also been described to possess anti-inflammatory, anti-edematous, anti-HIV, antimicrobial (anti-bacterial and antifungal), immuno-stimulating and immunomodulatory, spasmolytic, spasmogenic, gastroprotective, insecticidal, genotoxic, antioxidant, or anti-tumour activities (Benvenuti, Bortolotti, & Maggini, 2016; Lara-Cortés et al., 2013). Table 1 describes some edible flowers and their food and medicinal uses.

The most frequent form to consume edible flowers is in fresh salads, for example *A. majus* and *Bauhinia purpurea* L. are widely employed in this type of meals, but they can also be consumed dried or canned in sugar, as well as incorporated in cocktails in ice cubes (Lai, Lim, & Kim, 2010; Loizzo et al., 2016). Moreover, edible flowers may also be preserved in distillate products or as pickles in vinegar and salt, such as the buds of *Capparis spinosa* L., commonly consumed in Mediterranean countries as seasoning or garnish and that have been related to possess diuretic, antiseptic, and capillary vessels protective properties (Loizzo et al., 2016). *Tropaeolum majus* L. is often consumed as an ingredient in different meals like salads and also in beverages, being its consumption associated to different health benefits, namely antibacterial, antitumor, antithrombotic, diuretic, and hypotensive effects (Benvenuti et al., 2016; Navarro-González et al., 2015). The flowers of *Carthamus tinctorius* L., *Chrysanthemum morifolium* ramat., *Gardenia jasminoides* J. Ellis, *Lonicera japonica* Thunb., and *Rosa chinensis* Jacq are commonly consumed as infusions and also incorporated into cakes; they have been reported to present significant bioactive properties, such as promoting blood circulation, restoring menstrual flow, and heat-clearing and detoxifying activities (Wang et al., 2016).

In general, edible flowers are eaten whole, but there are some flower species where only some parts should be consumed (petals of *Tulipa*, *Chrysanthemum*, *Rosa* spp. or the flower buds of daisies (*Bellis perenis*) or garden nasturtium (*Tropaeolum majus*)) due to their bitterness or other unpleasant characteristics. The acceptability of edible flowers depends on a number of factors, such as the social group, species of flowers, characteristics (taste, texture and appearance), consumers profile (education, gender, annual income), or presentation (composition of flowers, size and price) (Fernandes et al., 2017).

2.4. Toxicity of edible flowers

Currently there are not many studies regarding the toxicity of edible flowers, thus those that are found associate that the toxic effects depends on the plants parts (Egebjerg et al., 2018). For instance, the leaves of *Allium schoenoprasum,* have presented in their composition sulphurous substances, which are non-existent in the flowers, which is a non-common plant part consumed (Sobolewska, Podolak, & Makowska-Wąs, 2015). Moreover, the presence of some compounds in plant parts, such as hydrogen cyanide, erucic acid, coumarin and thujone, have to follow a guidance value table set by EFSA or JECFA, which contemplates the Tolerable Daily Intake or Acceptable Daily Intake of these compounds, which will determine the toxicity effects of plant parts (Egebjerg et al., 2018). Some of flowers presented in this review, namely *Tropaeolum majus,* when ingested in amounts higher than 39.5 g of fresh flowers, will exceed the Tolerable Daily Intake in erucic acid (Egebjerg et al., 2018). Moreover, the ingestion of more than 18 g of *Achillea millefolium* flower will exceed the Acceptable Daily Intake for thujone and the ingestion of 7 g of *Galium odoratum* flowers will exceed the Tolerable Daily Intake for coumarin, for adults (Egebjerg et al., 2018; Kalemba-Drożdż, 2019).

Taking into account all these facts, these compounds may be present in other food sources, therefore further studies focusing on the recommended doses are need and should be developed, although most often these doses are not exceeded if the plant is used as an edible decoration part in food products, such as cakes or desserts (Egebjerg et al., 2018; Kalemba-Drożdż, 2019).

3. Bioactive compounds present in edible flowers

Phenolic compounds are a group of secondary metabolites found in different plant parts that are considered to have numerous bioactive properties (Kucekova, Mlcek, Humpolicek, & Rop, 2013). These compounds are classified according to the number of phenol subunits present, in polyphenols and simple phenols (Vuolo, Lima, & Maróstica

Junior, 2019), and can be further divided into flavonoids and non-flavonoid compounds. Phenolic acids and derivatives are the main non-flavonoid compounds found in plants, but there are other compounds that are considered in this class, such as stilbenes, lignins, lignans, coumarins, naphtoquinones, xanthones, and anthraquinones, which could also be found in natural sources (Huang et al., 2017; Li & Sun, 2017).

Phenolic acids occur naturally in plants and can be divided into hydroxybenzoic benzoic acids (C6–C1) and hydroxycinnamic acids (C6–C3); they may occur as free acids and as derivatives usually combined with sugars or organic acids (e.g., quinic acid). Caffeic, *p*-coumaric, vanillic, ferulic and protocatechuic acids are present in almost all plants, and their presence in the diet has been linked to the prevention of aging-related diseases, such as cancer, cardiovascular and neurodegenerative diseases (Butts-Wilmsmeyer et al., 2018; Kucekova et al., 2013).

Flavonoids are characterized by their C6–C3–C6 skeleton and are constituted by main 6 subgroups: flavan-3-ols, flavonols (e.g. quercetin, kaempferol, myricetin), flavones (e.g. apigenin, luteolin, chrysin), flavanones (e.g. hesperidin, naringenin, eriodictyol), isoflavones (e.g. genistein, daidzein, glycitein) and anthocyanins (e.g. cyanidin, delphinidin, malvidin) (Dias, Carocho, Barros, & Ferreira, 2019; Li & Sun, 2017). Flavan-3-ols may occur in their monomeric forms (catechins) or as polymers (condensed tannins or proanthocyanidins), for which very different bioactive properties have been reported, being related with cardiovascular protection (Dias et al., 2019). Flavones differ from other flavonoids by their double bond between C2 and C3 in the flavonoid skeleton, with no substitution at the C3 position, a keto group at C4 position (Hostetler, Ralston, & Schwartz, 2017). Biological activities, such as anticancer, cytotoxic, hepatoprotective, antidiabetic, anti-inflammatory, antiviral and anti-ageing properties have been descried for these compounds (Dias et al., 2019). Flavonols are similar to flavones but they possess a hydroxy group at C3 position; quercetin and kaempferol and their glycosylated and methylated forms are the major representatives of this group. A range of beneficial health effects have been described for flavonols, including anti-inflammatory, genotoxic and antioxidant capacities, as also effects against cardiovascular, Parkinson's and Alzheimer's diseases (Dias et al., 2019). Flavanones are mainly made up of three main abundant aglycones (hesperetin, naringenin, and eriodictyol), being mostly present in citrus fruits as glycosylated forms; their presence has been associated with anti-inflammatory, anti-cancer, anti-mutagenic, cardiovascular, anti-proliferative, vasorelaxant, and vasoprotective effects (Dias et al., 2019). Isoflavonoids are mainly found in legumes and have been related to estrogenic, antibacterial, antiviral, anti-inflammatory and anti-ischemic activities (Zheng, Deng, Guo, Chen, & Fu, 2019). Anthocyanins occur in nature mainly in the form of heterosides whose aglycone (or anthocyanidin) consists of a 2-phenylbenzopyrylium (flavylium) skeleton diversely hydroxylated and methoxylated. Currently more than 700 anthocyanins have been described in nature and above 200 have been tentatively identified (Santos-Buelga & González-Paramás, 2019). These compounds are water-soluble pigments highly recognised as colorant molecules, being responsible for the coloration of many fruits and vegetables and the petals in most flowers, and they are also acknowledged to have a high bioactive potential (Pires, Dias, Barros, Barreira, et al., 2018).

Flowers may contain a variety of all these phenolic compounds, which are recognised as natural antioxidants, being their presence strongly related to their colour, either directly (e.g., anthocyanins and other flavonoid pigments) or indirectly through co-pigmentation processes (Kaisoon, Siriamornpun, Weerapreeyakul, & Meeso, 2011; Skrajda, 2017).

Table 2 presents the main non-anthocyanin phenolic compounds found in some edible flowers. Among them, phenolic acid derivatives, especially hydroxycinnamic acids, are found in relevant amounts, with caffeoylquinic acids being majority phenolic compounds in many species (e.g. *Achillea millefolium* L., *H. sabdariffa* and *Tropaelum majus* L.). Regarding flavonoids, flavonols are within the main phenolics present in edible flowers, in particular quercetin and kaempferol derivatives (Table 2). Quercetin was the main phenolic compound quantified in *V. tricolor* and *Hemerocallis fulva* L. (46 mg/g DW and 273 mg/g DW, respectively (Koike et al., 2015a; Wu, Mong, Yang, Wang, & Yin, 2018), while caffeoylquinic acids were the best represented compounds in four of the twelve flowers analysed by Guimarães et al. (2013), being *Matricaria recutita* L., the flower with the highest amount of these derivatives, namely 3,4-*O*-dicaffeolyquinic acid (730 mg/100 g DW).

Moreover, solid-liquid extraction systems (maceration, ultrasound assisted extraction, among others) using different organic solvents and mixture of organic/water solvents (water, mixture of water and ethanol or methanol and acetone) are the most common methodology applied in the extraction of non-anthocyanin compounds, as it can be visualized in Table 2.

3.1. Anthocyanins composition in edible flowers

The presence of anthocyanins confers the flowers a great diversity of colours, touching practically all the visible spectra, from orange and red to purple and blue hues, making these matrices a potential source of these natural pigments, which can provide new colours and flavours, attracting the attention of consumers. Edible flowers are used to garnish and/or decorate meals, sweets, ice-creams or drinks improving not only the aesthetic effect, but also adding a specific taste and smell to the food dishes. Further, nutritional and bioactive features of edible flowers represent an additional value for their consumption, in particular, phenolic acids and flavonoids have been recognised as the most representative biologically active compounds found in petals of fresh edible flowers (Pires, Dias, Barros, Calhelha, et al., 2018; Pires et al., 2017). Many edible flowers have begun to arouse interest in the food industry due to the important amounts of anthocyanins present in their composition. Anthocyanins have been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1982 and by the EU Scientific Committee for Food (SCF) in 1975 and 1997 (Pop, Lupea, Popa, & Gruescu, 2010; Rodriguez-Amaya, 2016), and are authorised as food colorants in the European Union with the common code E − 163 regardless of their origin, indicating that, at least from a regulatory point of view, they are looked upon as a group of harmless compounds (Santos-Buelga & González-Paramás, 2019).

The growing concern about the substitution of artificial colorants for natural counterparts has promoted the interest in the search of new alternatives, and in this case edible flowers could be interesting sources of these natural molecules. Table 3 presents some edible flowers and their composition in anthocyanins. It can also be found that the most common anthocyanins present in the majority of the flowers are cyanidin derivatives, namely cyanidin-3-*O*-glucoside. However, other major compounds can also found, such as malvidin-3-*O*-glucoside (202.1 mg/kg fresh weight (FW) and delphinidin-3-*O*-glucoside (109 mg/kg FW) in *Nelumbo nucifera* (Gaertn.) (Deng et al., 2013), delphinidin-3,7-*O*-diglucoside (3936 μg/g DW) in *Crocus sativus* L. (Goupy, Vian, Chemat, & Caris-Veyrat, 2013), delphinidin-3-*O*-(4"*p*-coumaroyl)-rutinoside-5-*O*-glucoside (10.2 mg/g DW) in *V. tricolor* (Koike et al., 2015a), or pelargonidin-3-*O*-sophoroside (591.6 mg/g DW) in *Tropaeolum majus* L. red variety (Garzón, Manns, Riedl, Schwartz, & Padilla-Zakour, 2015).

Anthocyanins have been described to provide a range of health benefits, including antioxidant, anti-inflammatory and anti-proliferative effects. Many fruits and vegetables have been demonstrated to inhibit the initiation, promotion, and progression of several cancers, such as breast, prostate, liver, colorectal, intestinal, blood, or cervical cancers, which has been related to their anthocyanin composition (Hidalgo & Almajano, 2017; Khoo, Azlan, Tang, & Lim, 2017; Li, Wang, Luo, Zhao, & Chen, 2017). Anthocyanin-rich extracts from *Hibiscus* have shown to be able to significantly suppress rotenone-induced

Table 2
Content and extraction methodology of the main non-anthocyanin phenolic compounds in edible flowers.

Edible Flowers	Origin	Main phenolic compounds	Identification	Content	Extraction methodology	Reference
Achillea millefolium L.	Bulgaria	Isorhamnetin-3-*O*-(6-*O*-rhamosyl-galactoside)	HPLC-PAD-ESI-QTOF-MS	12.6 mg/g DW	Ultrasound-assisted extraction, with a power of 200W and frequencies of 60 kHz, using ethanol (1:10 plant/solvent ratio), 30 min time, 40 °C	(Villalva et al., 2019)
		Luteolin-7-*O*-glucoside		7.69 mg/g DW		
		Luteolin		4.47 mg/g DW		
		3,5-*O*-Dicaffeoylquinic acid		3.62 mg/g DW		
Hemerocallis fulva L.	China and Taiwan	Quercetin	HPLC- DAD	273 mg/g DW	Solid-liquid extraction with 300 g of sample in 2000 ml boiling distilled water for 1 h.	(Wu et al., 2018)
		Ellagic acid		205 mg/g DW		
		Chlorogenic acid		175 mg/g DW		
Hibiscus sabdariffa L.	Alfândega da Fé, Bragança, Portugal	5-(Hydroxymethyl)furfural	HPLC-DAD-ESI/MSn	5.75 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of ethanol/water (80:20 v/v), 25 °C at 150 rpm for 1 h	(Jabeur et al., 2017)
		3-*O*-Caffeoylquinic acid		2.88 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of water, 25 °C at 150 rpm for 1 h	
		5-*O*-Caffeoylquinic acid		1.53 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of ethanol/water (80:20 v/v), 25 °C at 150 rpm for 1 h	
Lavandula pedunculata Mill.	Ponte de Sôr , Portalegre	Salvianolic acid B	HPLC-DAD-ESI/MSn	582 mg/g DW	Solid-liquid extraction with 1 g in 30 mL of ethanol/water (80:20 v/v), 25 °C at 150 rpm for 1 h	(Lopes et al., 2018)
		Rosmarinic acid		550 mg/g DW		
		Luteolin-7-*O*-glucuronide		84.1 mg/g DW		
Lonicera japonica Thunb	China	Chlorogenic acid	HPLC-DAD	16.0 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
		Rutin		0.62 mg/g DW		
		Protocatechuic acid		0.25 mg/g DW		
Matricaria recutita L.	Trás-os-Montes, North-eastern Portugal.	Luteolin-*O*-acylhexoside	HPLC-DAD–ESI/MS	1290 mg/ 100g DW	Solid-liquid extraction with 1 g in 30 mL of methanol:water (80:20 v/v) for 1 h.	(Guimarães et al., 2013)
		3,4-*O*-Dicaffeolyquinic acid		730 mg/100g DW		
		Feruloyl hexoside acid dimer		590 mg/100g DW		
Paeonia suffruticosa Andrews	China	Rutin	HPLC-DAD	18.1 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
		Gallic acid		7.99 mg/g DW		
		Quercetin		7.24 mg/g DW		
Prunus persica L.	China	Chlorogenic acid	HPLC-DAD	6.54 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
		Kaempferol		2.78 mg/g DW		
		Rutin		0.70 mg/g DW		
Rosa chinensis Jacq.	China	Gallic acid	HPLC-DAD	6.87 mg/g DW	Solid-liquid extraction with 2 g in 50 mL of 80% acetone for 5 min.	(Xiong et al., 2014)
		Chlorogenic acid		2.66 mg/g DW		
		3-Hydroxy-4-methoxybenzaldehyde thiosemicarbazone		1.09 mg/g DW		

(*continued on next page*)

Table 2 (*continued*)

Edible Flowers		Origin	Main phenolic compounds	Identification	Content	Extraction methodology	Reference
Rosa rugosa Thunb.		Poland	Ellagitannin	UPLC-PDA-Q/TOF-MS	1072 mg/100 g FW	Solid-liquid extraction with 50 g in 500 mL of ethyl alcohol for 18 days.	(Cendrowski, Scibisz, Kieliszek, Kolniak-Ostek, & Mitek, 2017)
			(+)-Catechin		178 mg/100 g FW		
			Sanguine H-2		166 mg/100 g FW		
Trapaeolum majus L.	Yellow	Geneva, NY, USA	*cis* 5-*p*-Coumaroylquinic acid	HPLC-PDA	639 mg/100g DW	Solid-liquid extraction with 0.5 g in 1:1 (w/v) 70% aqueous acetone under a nitrogen atmosphere* during 10 min.	(Garzón, Riedl, & Schwartz, 2009; Navarro-González et al., 2015)
			3-*O*-Caffeoylquinic acid		283 mg/100g DW		
			5-*O*-Caffeoylquinic acid		247 mg/100g DW		
	Orange		Kaempferol dihexoside		1199 mg/ 100g DW		
			5-*O*-Caffeoylquinic acid		233 mg/100g DW		
			3-*O*-Caffeoylquinic acid		182 mg/100g DW		
	Red		Myricetin dihexoside		2265 mg/ 100g DW		
			Kaempferol dihexoside		268 mg/100g DW		
			5-*O*-Caffeoylquinic acid		138 mg/100g DW		
Viola tricolor L.		São Paulo, Brazil	Quercetin-3-*O*-(6-*O*-rhamnosylglucoside)-7-*O*-rhamnoside	HPLC–DAD–ESI/MS	46 mg/g extract DW	Solid-liquid extraction with 0.5 g in 20 mL of methanol/ water 80:20 (v/v), at room temperature, 150 rpm, for 1h	(Koike et al., 2015a)
			Quercetin-3-*O*-rutinoside		28 mg/g extract DW		
			Isorhamnetin-3-*O*-(6-*O*-rhamosyl-galactoside)		12.6 mg/g extract DW		

* ultrasound-assisted extraction; DW- dry weight; FW- fresh weight

Table 3
Anthocyanin content of edible flowers.

Edible flower	Main anthocyanins	Identification	Content	Extraction methodology	Reference
Bauhinia purpurea L.	Cyanidin-3-*O*-glucoside	HPLC- PAD	59.8 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Bombax malabaricum D.C.	Cyanidin-3-*O*-glucoside	HPLC- PAD	63.4 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Brunfelsia acuminate L.	Cyanidin-3-*O*-glucoside	HPLC- PAD	61.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Calliandra haematocephala Hassk.	Cyanidin-3-*O*-glucoside	HPLC- PAD	517 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Centaurea cyanus L.	Cyanidin-3,5-di-*O*-glucoside	HPLC-DAD-ESI/MS	5.5 μg/g DW	Solid-liquid extraction with 1 g of plant material in 50 mL of water at 25 °C using a stirring plate, during 2h.	(Pires, Dias, Barros, Barreira, et al., 2018)
	Cyanidin-3-*O*-(6″-malonylglucoside)-5-*O*-glucoside		6.2 μg/g DW		
	Cyanidin-3-*O*-(6″-succinylglucoside)-5-*O*-glucoside		11.2 μg/g DW		
	Delphinidin-*O*-hexoside		1.50 μg/g DW		
	Cyanidin-*O*-glucuronide		0.85 μg/g DW		
	Pelargonidin-3-*O*-(6″-succinylglucoside)-5-*O*-glucoside		0.18 μg/g DW		
	Pelargonidin-*O*-malonylhexoside		0.17 μg/g DW		
Crocus sativus L.	Delphinidin-3,7-*O*-diglucoside	UPLC-DAD-ESI/MS	3936 μg/g DW	Ultrasound-assisted extraction with 2 g for 5 min in 20 mL methanol/water (50:50, v/v) containing hydrochloric acid 1%, followed by a magnetic stirring for 30 min.	(Goupy et al., 2013)
	Petunidin-3,7-*O*-diglucoside		380 μg/g DW		
	Petunidin-3-*O*-glucoside		475 μg/g DW		
	Malvidin-*O*-glucoside		13 μg/g DW		
Dahlia mignon (commercial mixture)	Cyanidin-*O*-hexoside	HPLC-DAD-ESI/MS	2.98 μg/g DW	Solid-liquid extraction with 1 g of plant material in 50 mL of water at 25 °C using a stirring plate, during 2h.	(Pires, Dias, Barros, Barreira, et al., 2018)
	Pelargonidin-*O*-rutinoside		1.40 μg/g DW		
	Cyanidin-*O*-acetylhexoside		5.36 μg/g DW		
	Methylapigeninidin-*O*-hexoside		4.10 μg/g DW		
	Cyanidin-*O*-rutinoside		0.80 μg/g DW		
	Pelargonidin-3,5-di-*O*-glucoside		0.80 μg/g DW		
	Pelargonidin-*O*-hexoside		0.45 μg/g DW		
Dendranthema grandiflorum Ramat.	Cyanidin-3-*O*-(3″malonoyl)-glucoside	HPLC-ESI/MS	11.3 mg/g DW	Ultrasound-assisted extraction with 0.1 g of plant material in 2 mL of water-formic acid, 95:5 (v/v) for 5 min	(Park et al., 2015)
	Cyanidin-3-*O*-glucoside		1.06 mg/g DW		
Dianthus caryophyllus L.	Cyanidin-3-*O*-glucoside	HPLC-PAD	52.4 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Rhododendron spp L.	Cyanidin-3-*O*-glucoside	HPLC-PAD	65.9 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Gerbera jamesonii Bollus ex Hooker F.	Cyanidin-3-*O*-glucoside	HPLC-PAD	60.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Hibiscus rosa-sinensis L.	Cyanidin-3-*O*-glucoside	HPLC-PAD	72.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)

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Table 3 (*continued*)

Edible flower		Main anthocyanins	Identification	Content	Extraction methodology	Reference
Hibiscus sabdariffa L.		Delphinidin-3-*O*-sambubioside	HPLC- LC–MS	4.11 mg/g DW	Solid-liquid extraction with 0.1 g of plant material in 10 mL of water at 100 °C for 10 min	(Sindi, Marshall, & Morgan, 2014)
		Delphinidin-3-*O*-glucoside		0.15 mg/g DW		
		Cyanidin-3-*O*-sambubioside		3.81 mg/g DW		
		Cyanidin-3-*O*-glucoside		0.46 mg/g DW		
Ipomoea cairica (L.) Sweet		Cyanidin-3-*O*-glucoside	HPLC-PAD	11.0 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Jatropha integerrima Jacq.		Cyanidin-3-*O*-glucoside	HPLC-PAD	641.5 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Lantana camara L.		Cyanidin-3-*O*-glucoside	HPLC-PAD	48.6 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Lilium brownie A.Poit.		Cyanidin-3-*O*-glucoside	HPLC-PAD	10.7 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Nelumbo nucifera (Gaertn.)	Red	Malvidin-3-*O*-glucoside	HPLC–MS	202 mg/kg FW	Solid-liquid extraction with 0.7 g of plant material in 5 mL of 70% methanol aqueous solution containing 2% of formic acid, at 4 °C in dark during 24h	(Deng et al., 2013)
		Delphinidin-3-*O*-glucoside		109 mg/kg FW		
		Petunidin-3-*O*-glucoside		55.6 mg/kg FW		
		Cyanidin-3-*O*-glucoside		30.6 mg/kg FW		
		Peonidin-3-*O*-glucoside		28.2 mg/kg FW		
	Pink	Malvidin-3-*O*-glucoside		81.9 mg/kg FW		
		Delphinidin-3-*O*-glucoside		23.8 mg/kg FW		
		Petunidin-3-*O*-glucoside		13.3 mg/kg FW		
		Cyanidin-3-*O*-glucoside		5.88 mg/kg FW		
		Peonidin-3-*O*-glucoside		8.56 mg/kg FW		
	Yellow	Malvidin-3-*O*-glucoside		2.53 mg/Kg FW		
		Delphinidin-3-*O*-glucoside		0.14 mg/Kg FW		
Oncidium varicosum		Cyanidin-3-*O*-glucoside	HPLC-PAD	52.1 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Orostachys fimbriata (Turcz.)		Cyanidin-3-*O*-glucoside	HPLC-PAD	160 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014
Pelargonium × *hortorum*		Cyanidin-3-*O*-glucoside	HPLC-PAD	497 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014
Rosa hybrida cv. Noblered		Cyanidin-3,5-di-*O*-glucoside		375 mg /g DW	Solid-liquid extraction with 5 g of plant material in 500 mL of methanol with 1% (v/v) of TFA during 48h at 4 °C, in the dark	(Lee, Lee, & Choung, 2011)

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Table 3 (*continued*)

Edible flower		Main anthocyanins	Identification	Content	Extraction methodology	Reference
Rosa damascena 'Alexandria' and *Rosa gallica* 'Francesa' draft in *Rosa canina*		Cyanidin-3,5-di-O-glucoside Cyanidin-3-*O*-glucoside	HPLC-DAD-ESI/MS	13.2 μg/g DW 0.13 μg/g DW	Solid-liquid extraction with 1 g of plant material in 50 mL of water at 25 °C using a stirring plate, during 2h	(Pires, Dias, Barros, Barreira, et al., 2018)
Salvia splendens Sellow ex J.A. Schultes		Cyanidin-3-*O*-glucoside	HPLC-PAD	30.6 mg/g FW	Solid-liquid extraction with 1 g of plant material in 5 mL of methanol/acetic acid/water (50:3.7:46.3, v/v/v) at 37 °C for 30 min in a shaking water bath	(Li et al., 2014)
Tropaeolum majus L.	Yellow	Pelargonidin-3-*O*-sophoroside Delphinidin-3-*O*-dihexoside Cyanidin-3-*O*-sophoroside	HPLC-PDA	126 mg/g DW 95.1 mg/g DW 24.8 mg/g DW	Ultrasound-assisted extraction with 0.5 g of plant material with 1:1 (w/v) 70% aqueous acetone under a nitrogen atmosphere for 10 min in a chilled water bath	(Garzón et al., 2015)
	Orange	Pelargonidin-3-sophoroside Delphinidin-3-*O*-dihexoside Cyanidin-3-*O*-sophoroside		439.6 mg/g DW 35.9 mg/g DW 10 mg/g DW		
	Red	Delphinidin-3-*O*-dihexoside Pelargonidin-3-*O*-sophoroside Cyanidin-3-*O*-sophoroside		592 mg/g DW 213 mg/g DW 76.0 mg/g DW		
Tropaeolum majus L.		Pelargonidin-3-*O*-sophoroside Delphinidin-*O*-dihexoside Cyanidin-*O*-dihexoside	HPLC-DAD-ESI-MS	5.8 mg/g DW 3.2 mg/g DW 0.21 mg/g DW	Solid-liquid extraction with 0.5 g of plant material with 20 mL of methanol, containing 0.5% TFA during 2h.	(Koike et al., 2015b)
Viola x *wittrockiana*	Yellow	Delphinidin-3-*O*-(4″- *p*-coumaroyl)-rutinoside-5-glucoside Petunidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside Cyanidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside Malvidin-3-*O*-(4′′-*p*-coumaroyl)-rutinoside-5-glucoside	HPLC-DAD-ESI-MS	4.69 mg/g DW 2.08 mg/g DW 4.39 mg/g DW 2.72 mg/g DW	Solid-liquid extraction with 1.5 g of plant material in 50 mL of ethanol 50% (pH = 2)	(Skowyra, Calvo, Gallego, Azman, & Almajano, 2014)
	Red	Delphinidin-3-*O*-(4″- *p*-coumaroyl)-rutinoside-5-glucoside Petunidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside Cyanidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside Malvidin-3-*O*-(4′′-*p*-coumaroyl)-rutinoside-5-glucoside		11.40 mg/g DW 7.72 mg/g DW 7.25 mg/g DW 4.74 mg/g DW		
	Violet	Delphinidin-3-*O*-(4″- *p*-coumaroyl)-rutinoside-5-glucoside Petunidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside Cyanidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside Malvidin-3-*O*-(4′′-*p*-coumaroyl)-rutinoside-5-glucoside		11.6 mg/g DW 9.89 mg/g DW 7.74 mg/g DW 9.07 mg/g DW		
Viola tricolor L.		Delphinidin-3-*O*-(4″-*p*-coumaroyl)-rutinoside-5-glucoside Delphinidin-3-*O*-rutinoside Cyanidin-3-*O*-rutinoside	HPLC-DAD-ESI-MS	10.2 mg/g DW 3.30 mg/g DW 0.70 mg/g DW	Solid-liquid extraction with 0.5 g of plant material in 20 mL of methanol containing 0.5% trifluoroacetic acid, during 2h	(Koike et al., 2015a)

DW- dry weight; FW- Fresh weight

dopaminergic cell death via interference with microglial activation and amelioration of mitochondrial dysfunction, suggesting their neuroprotective activity and ability to improve cognitive, memory and motor performances, which may have potential application in the prevention of neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases (Li et al., 2017). Cyanidin-3-*O*-glucoside has demonstrated to be able to inhibit carrageenan-induced acute inflammation and peritonitis through downregulation of cyclooxygenase-2 expression and inhibition of prostaglandin E2 production, indicating its anti-inflammatory potential (Li et al., 2017).

Although several *in vitro* and *in vivo* studies have been carried out to try to demonstrate the biological activity of anthocyanins, a major drawback for their use is their low bioavailability, as they are considered to be poorly absorbed and highly largely metabolised, being found in blood under the form of metabolites (Fernandes et al., 2019). In this respect, the use of nanotechnology can provide promising tools for solving the problems of bioavailability (Sharif, Shah, Butt, & Sharif, 2016). On the other hand, anthocyanin structure may also influence their activity and the molecular mechanisms involved, so that the isolation and purification of specific molecules is required in order to determine their effects (Li et al., 2017).

3.2. Flower anthocyanins and extraction methodologies

Anthocyanin contents and composition in edible flowers can present high variability depending on the species (Table 3), but also due to edafo-climatic and abiotic factors. Furthermore, the different variables in the extraction process, such as the extraction methodology, employed solvents, solid/liquid ratio, or temperature can also have a great influence in the composition of the obtained extracts.

The polar characteristics of the anthocyanins allow them to be extracted by different polar solvents, such as methanol, acetone, water and ethanol. Methanol and acetone and their aqueous mixtures are among the solvents most commonly used to extract polyphenols, including anthocyanins (Santos-Buelga, Gonzalez-Manzano, Dueñas, & Gonzalez-Paramas, 2012). However, the replacement with greener solvents (water, ethanol or a mixture of both), considering the requirements for food and pharmaceutical industries, in order to obtain "environmentally friendly" products, have been gaining a great consideration (Machado, Pereira, Barbero, & Martínez, 2017).

Several parameters need to be considered for an efficient anthocyanin extraction. In Table 3 a summary of the solvents and extraction methods used in edible flowers is shown.

Acidified methanol (Deng et al., 2013; Lee, Lee, & Choung, 2011; Skowyra, Calvo, Gallego, Azman, & Almajano, 2014; Koike et al., 2015a), acidified mixtures of methanol/water (Li et al., 2014; Goupy et al., 2013), water (Pires et al., 2018a; Sindi, Marshall, & Morgan, 2014), acidified water (Park et al., 2015), and acetone (Garzón et al., 2015) are within the most common extraction solvents. The ability to extract some flavonoids, increases with acidification of the solvent, especially when methanol or ethanol (protic polar solvents) are used, in which the change of the phenol-phenolate equilibrium for polar phenyl form, improves the extraction of these molecules (Atta-ur-Rahman, Iqbal Choudhary, & Perry, 2015; Hostettmann, 2014). In the case of the anthocyanins, acidification of the solvents is necessary, since they are structurally dependent of the pH of the medium, modifying their solubility and stability (Santos-Buelga, C. & González-Paramás, 2014). Nevertheless, as it can be seen in Table 3, the solvents applied in anthocyanins extraction from edible flowers include the use of organic solvents with or without acidic agents, such as acetic, hydrochloric, formic and trifluoroacetic acids. The type of the acid used in the extraction solvents also influences the extraction yields of these compounds. For instance, Oancea, Stoia, and Coman (2012) demonstrated that using hydrochloric acid instead of acetic acid improves the extraction yield of anthocyanins. Soft acidic conditions must be used to prevent cleavage of the sugar moieties and labile conjugated residues (Santos-Buelga, C. & González-Paramás, 2014). Anthocyanins are highly susceptible to degradation. In this way it is fundamental to use methodologies that allow their extraction with the least number of interferers, making a strict control of pH and temperature (Silva, Morais, Costa, Pintado, & Calhau, 2015). The most common methodology applied in the extraction of anthocyanins is conventional solid-liquid extractions (SLE), due to their simplicity and non-required specific equipment as it can be verified in Table 3 (Silva et al., 2015).

Supercritical Fluid Extraction (SFE), Ultrasound Assisted Extraction (UAE), Pressurized Liquid Extraction (PLE) and Microwave Assisted Extraction (MAE) are alternative methodologies that can be applied in the extraction of anthocyanins.

Supercritical fluid extraction (SFE) is used to obtain bioactive compounds from natural matrices, applying low viscosity solvents (near the gas), significantly reducing the extraction time and with a higher penetration of the fluids in the solid pores, resulting in a faster and more efficient process. (Otero-Pareja, Casas, Fernández-Ponce, Mantell, & De La Ossa, 2015). Supercritical CO_2 is the usual solvent of choice, although it is only suitable for non-polar or low polarity compounds, so that it is no suitable as such for anthocyanin extraction, for which some percentages of solvent modifiers are required, such as methanol or ethanol (Santos-Buelga, C. & González-Paramás, 2014). This technique has been used by different authors obtaining good yields (Santos-Buelga, C. & González-Paramás, 2014). Ultrasound assisted extraction (UAE) is a key technology for sustainable "green" extraction. This extraction system presents high reproducibility, reducing the consumption of solvent, simplifying the manipulation and the processing, and conferring a greater degree of purity to the final product (Silva et al., 2015). While, Pressurized Liquid Extraction (PLE) is characterized by the use of liquid solvents, using high temperatures and pressures (Mustafa & Turner, 2011). The solubility, amount of extracted compounds, solid-liquid bonds and the mass transfer rises with increasing temperature, while viscosity and interfacial tension decreases (Machado et al., 2017). Although this methodology presents some advantages, such as low solvent consumption, low extraction time and possibility of process automation, it requires high temperatures that can lead to anthocyanin degradation, therefore, it is not often applied as an alternative extraction method for this type of molecules (Silva et al., 2015). Another system that can be applied to extract anthocyanins is Microwave-assisted extraction (MAE). This technique promotes rapid heating of the solvent and sample, because the microwave energy causes molecular movement and rotation of liquids with a permanent dipole (Yang & Zhai, 2010). It has advantages over conventional extraction methodologies, such as improved efficiency, reduction of extraction time, low solvent consumption, and high level of automation (Silva et al., 2015).

In general, there is not an ideal extraction system and conditions that can be used for anthocyanin extraction. A simple or a more technological extraction methodology may be applied, although the lack of data on the direct comparison among technologies, using the same type of samples under the same conditions, limits the selection of an infallible extraction method. Therefore, the choice of the extraction process depends on different factors, among which the final application of the extract obtained and the type of sample used (Ongkowijoyo, Luna-Vital, & Gonzalez de Mejia, 2018; Silva et al., 2015). Some general guidelines can be taken into account for the extraction of anthocyanins in order to select the most adequate extraction process, not forgetting that methodologies involving the use of high temperatures may induce the degradation of these compounds (Sarkis, Jaeschke, Tessaro, & Marczak, 2013), Methodologies with better extraction yields, such as MAE and UAE, which mainly use water as solvent, present economic and ecological advantages, although the costs of production deserve a comparison with the cost of the equipment.

4. Concluding remarks

Edible flowers are increasingly used to color and enhance the visual appearance of various dishes, having special interest in culinary and for the food industry. They are receiving more and more attention by consumers in the search for more attractive and healthier alternatives, with less environmental impact in terms of production and processing. Besides, edible flowers are also appreciated by their nutritional characteristics, associated to a low content in fat and energetic value, and can also be sought as a natural source of bioactive compounds, such as phenolic compounds, which may play an important role in health promotion and disease prevention. Among them, flavonoids and anthocyanins, which are in the focus of the pharmaceutical and food industries for their putative healthy effects and as promising new colorant molecules, that can replace the artificial counterparts. There are numerous species of plants all over the world whose flowers can be used with edible purposes and only a small part of them have been explored. More studies are thus required, so that they can be used in a safe way and with total efficiency.

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