Bixa orellana L. pods and seeds: nutritional and chemical characterization, bioactivity studies, and development of a carotenoid-based food colorant

Baha Habboubi

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Supervisors

Doctor Maria Inês Moreira Figueiredo Dias

Doctor Lillian Bouçada de Barros

Doctor Charfi Ichrak

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RESUMO

A *Bixa orellana* L. é mundialmente conhecida como fonte de bixina, um composto carotenoide com alta capacidade corante. A tendência crescente da indústria alimentar em utilizar novas fontes de corantes, aliada à elevada procura de consumidores mais preocupados, torna-se da maior importância encontrar novas matrizes com capacidade corante que sejam seguras para consumo humano e que apresentem também elevada estabilidade nos produtos alimentares a serem aplicados. Além disso, tecnologias de extração sustentáveis são cada vez mais necessárias para maximizar a recuperação de compostos de alto valor agregado, maximizando os rendimentos de extração e usando solventes mais verdes.

O presente trabalho pretendeu aprofundar o estudo do perfil nutricional e químico e do potencial bioativo de sementes de *B. orellana*. Além disso, essas sementes, muito ricas em bixina, foram tratadas para maximizar a extração desse composto por meio de tecnologias assistidas por ultrassons, combinadas com RSM. Para validar a otimização da extração de bixina, foi desenvolvida e implementada uma nova metodologia de cromatografia líquida acoplada a um detetor de matriz de diodos (DAD). Por fim, o extrato à base de carotenoides foi aplicado em massa sem ovo, a fim de atingir a característica cor amarela. Como externalidade deste trabalho, os frutos dessa planta (bio-resíduos resultantes do processamento das sementes) também foram estudados, em relação às suas propriedades bioativas e perfil em compostos fenólicos, avaliando assim a sua possível aplicação em produtos farmacêuticos e nutracêuticos.

Com o estudo da caracterização nutricional das sementes, pôde-se verificar que os macronutrientes mais abundantes encontrados foram os hidratos de carbono, seguidos de gordura, proteínas e cinzas. A sacarose e trealose foram os únicos açúcares encontrados; e o ácido málico foi o principal ácido orgânico encontrado. Os ácidos gordos monoinsaturados (ácido eicosenoico) foram encontrados em maior concentração, seguidos pelos ácidos gordos saturados (ácido heptadecanóico). As amostras também apresentaram maiores quantidades da isoforma α-tocoferol.

O extrato hidroetanólico dos frutos apresentou menores valores de IC_{50} para atividade antioxidante; entretanto, a amostra de sementes revelou menores valores de IC_{50} para $\Delta t = 60$ min e $\Delta t = 120$ min para o ensaio OxHLIA, e menores valores de GI_{50} para os ensaios citotóxicos e hepatotóxicos. Em relação à atividade antimicrobiana, é importante ressalvar observar que ambas as amostras apresentaram concentração mínima inibitória (CMI), concentração mínima bactericida (CMB) e concentração mínima fungicida (CMF) menores quando comparadas com os dois controlos positivos utilizados, contra todas as estirpes

bacterianas e fúngicas. Este resultado é de extrema importância para validar as amostras aqui estudadas, como fontes de compostos antimicrobianos contra patogénicos de origem alimentar.

O perfil de compostos fenólicos entre as amostras de frutos e sementes foi completamente diferente, apresentando nos frutos maiores quantidades de compostos fenólicos, principalmente devido à presença do ácido protocatéquico. O modelo desenvolvido para o processo de extração foi validado, com os resultados das condições ótimas de processamento (sonificação a 348 W por 6 min, utilizando etanol 79% (*v/v*) como solvente de extração), sendo possível obter 27,1 mg de bixina por g de extrato. Por fim, o estudo de aplicação permitiu obter uma massa final sem ovo com uma cor amarela muito semelhante à da amostra comercial.

De maneira geral, este estudo permitiu apresentar resultados inovadores em relação às propriedades nutricionais, químicas e bioativas das sementes, e como externalidade o grande potencial dos frutos quanto à sua atividade biológica. Considerando os bons resultados obtidos no procedimento de otimização, aliados à cor otimista obtida na massa incorporada com extrato à base de carotenoides, vale a pena destacar a forma sustentável como este extrato pode ser obtido, mas também o grande potencial de ser aplicado em produtos alimentares inovadores.

Palavras-chave: *Bixa orellana* L., caracterização nutricional/química; bioatividades; Metodologia de Superfície de Resposta; corante alimentar rico em carotenoides

ABSTRACT

Bixa orellana L. is a worldwide known as a source of bixin, a carotenoid compound with high colorant capacity. The growing tendency in the food industry to use new colorant sources, coupled to the demand of concern consumers, it becomes of the upmost importance to find new colorant matrices that are safe for human consumption and presents high stability in the food products to be applied. Moreover, sustainable extraction technologies are increasingly required to maximize the recovery of high added value compounds, maximizing the extraction yields and using greener solvents.

The present work intended to deepen the study of the nutritional and chemical profile and bioactive potential of *B. orellana* seeds. Furthermore, these seeds, very rich in a carotenoid compound, bixin, were treated to maximize the extraction of this compound by ultrasound-assisted technologies, combined with RSM. To validate the extraction optimization of bixin, a novel liquid chromatographic methodology coupled to a diode array detector (DAD) was developed and implemented. Finally, the carotenoid-based extract was applied in eggless pasta dough, to achieve the characteristic yellow colour. As an externality of this work, the pods of this plant (bio-residues resulting from the processing of seeds) were also studied, in relation to its bioactive properties and profile in phenolic compounds, thus assessing its possible use in pharmaceutical and nutraceutical products.

With the study of nutritional characterization of the seeds, it could be seen that the most abundant macronutrients found were carbohydrates, followed by fat, proteins, and ash. Sucrose and trehalose were the only sugars found; and malic acid was the main organic acid found. Monounsaturated fatty acids (eicosenoic acid) were found in higher amounts, followed by saturated fatty acids (heptadecanoic acid). The samples also presented higher amounts of α-tocopherol isoform.

The pods hydroethanolic extract presented lower IC_{50} values for antioxidant activity; however, the seeds sample revealed lower IC_{50} values for $\Delta t = 60$ min and $\Delta t = 120$ min for OxHLIA assay, and lower GI_{50} values for cytotoxic and hepatotoxic assays. With the regard of the antimicrobial assay, it is important to note that both samples presented lower MIC, MBC, and MFC comparing to the two positive controls used, against all bacterial and fungal strains. This is of the upmost importance to validate the samples studied herein, as sources of antimicrobial compounds against foodborne pathogens.

The phenolic compounds profile between the pods and seed samples was completely different, presenting the pods higher quantities of phenolic compounds, mainly due to the

presence of protocatechuic acid. The model developed for the extraction process was validated, with the results of the optimal processing conditions (sonication at 348 W for 6 min, using 79 % (*v/v*) ethanol as extraction solvent), being possible to obtain 27.1 mg of bixin per g of extract. Finally, the application study allowed to obtain a final eggless pasta dough with a very similar yellow colour when compared to the commercial sample.

Overall, this study allowed to present innovative results in relation to the nutritional, chemical and bioactive properties of the seeds, and as an externality the great potential of the pods regarding its biological activity. Considering the good results obtained in the optimization procedure, combined with the optimistically colour obtained in the pasta dough incorporated with the carotenoid-based extract, it is worth mentioning the sustainable way in which this extract can be obtained, but also the great potential to be applied in innovative food products.

Keywords: *Bixa Orellana* L., nutritional/chemical characterization; bioactivity; Response Surface Methodology; carotenoid-based colorant

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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis (2-amidinopropano)
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATCC	American type culture collection
CFU	Colony-forming unit
DAD	Diode array detector
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DPPH	2,2-difenil-1-picril-hidrazilo/2,2-Diphenyl-1-picrylhydrazyl
dw	Dry weight
IC_{50}	Effective concentration achieving 50% of antioxidant activity
ESI	Electrospray ionization
ex.	Example
FAME	Fatty acids methyl ester
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FID	Flame ionization detector
FL	Fluorescence
GC	Gas-chromatography
GI_{50}	Sample concentration that inhibited 50% of the net cell growth
HBSS	Hank’s balanced salt solution
HPLC	High-performance liquid chromatography
INT	*p*-Iodonitrotetrazolium chloride
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass-to-charge ratio
MA	Malt agar
MBC	Minimum bactericidal concentration
MDA-TBA	Malondialdehyde-thiobarbituric acid
MFC	Minimum fungicidal concentration

MIC	Minimum inhibitory concentration
MTBE	Methyl tert-butyl ether
MS	Mass spectrometry
MS^2	Second stage of mass spectrometry
mu	Mass unit
MUFA	Monounsaturated fatty acids
PDA	Photodiode array detector
PUFA	Polyunsaturated fatty acids
R^2	Coefficient of determination
RI	Refraction index
Rt	Retention time
OxHLIA	Oxidative haemolysis inhibition assay
SD	Standard deviation
SFA	Saturated fatty acids
SPSS	Statistical package for the social sciences
SRB	Sulphorhodamine B
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
tr	Traces
TSB	Tryptic soy broth
UFLC	Ultra-fast liquid chromatography
UV	Ultraviolet radiation
v/v	Volumetric percentage
w/w	Percentage solution
λ_{max}	Wavelength of maximum absorption

INTRODUCTION

1. Introduction

1.1. Introducing *Bixa orellana* L.

Bixa orellana L. (achiote, anatto, annatto, annatto, annatto) is a tree from Amazon region, locally known as "annatto". Annato comes from "*Guaraní (ru-ku)*" the "urucum" which implies red, being the etymological origin in Latin America. Since ancient times, annatto was used in Latin America for the preparation of numerous food dishes, traditional medicinal applications (as antidiabetic and antibacterial) and also for cosmetic preparations. It also played a significant role in numerous traditions of the indigenous cultures. On the other hand, in Europe, annatto has been commercialized relatively recently, despite the knowledge of the potential of this colorant plant (Hagiwara et al., 2003; Lourido Pérez & Sánchez, 2010; Venugopalan, Giridhar, & Ravishankar, 2011). The colorant compounds extracted from the seeds of *B. orellana* is highly stable since it binds with proteins, which has allowed them to be applied in several industrial fields (Priya & Siva, 2014, 2015).

It was during the 1970s that the consumers and the industry took consciousness of the medium long term hazardous effects of the synthetic colours that were being used in for food products, in the consumer's health. In Europe, efforts have been made since then to limited their use by the food and pharmaceutical industry, while in parallel in 1990 the US Food and Drug Administration (FDA) prohibited the use of some of these synthetic colourant compounds. Given this limiting circumstances, researchers and industry have gathered a significant amount of knowledge to substitute the artificial worldwide used colours with natural counterparts (Pérez, Cuen, & Becerra, 2003).

Nowadays, the use of manufactured colorants such as tartrazine (E102), allura ruddy (E129), or nightfall yellow FCF (E110) in food products have been severely questioned in developed nations, since there are reports linking the indiscriminate consumption of these colorants to the upgrading of degenerative illness as also cancer ((Moreno, Hernández, & Velázque, 2005)). As a result, the use of some artificial food colorants such as carmoisine (E122) and Ponceau 4R (E124) have been prohibited in the USA and Europe, whereas there has been a stimulus in the use of natural colorants, as in the case of urucum (E 160b, annatto extricate) (Lima et al., 2001; McCann et al., 2007).

The colouring extract from *B. Orellana* seeds has been reported as innocuous and with low toxicity effects after ingestion or even after dermatological application. The colouring compound bixin represents about 80% of the total amount of the colouring compounds extracted from annatto seeds, which can be further transformed in norbixin, a high added-value compounds with numerous bioactive potential (Satyanarayana, Prabhakara Rao, & Rao, 2003; Vilar et al., 2014). Being one of the most widely used colorant in food, cosmetic, and pharmaceutical products, urucum colorant-based extracts have gained a high economic importance, based also on its high chemical stability and low toxicity for human consumption (Lourido Pérez & Sánchez, 2010; Michelangeli, Medina, Artioli, & Mata, 2002; Smith, 2006)

In this sense, the main objective of this work was to trace a nutritional and chemical profile of the seeds of *B. orellana*, as well as characterizing the bioactive potential of the seeds but also of the pods (by-products of processing stage of the seeds). Furthermore, response surface methodologies were applied to maximize the extraction recovery of bixin from the seeds using ultrasound extraction technologies; and finally, a pasta dough (eggless free) was developed using an enriched carotenoid based extract from *B. orellana*, and compared in terms of colour and shelf-life with commercial fresh pasta dough.

1.2. Botanical and chemical description of *B. Orellana*

1.2.1 Botanical description

Bixa Orellana L. belongs to the Bixaceae family. "*Bixa*" comes from the Latinized form of the Indian word Bija and the name orellana was named after the Spanish discoverer Francisco Orellana, who discovered the plant in 16th century in the amazon river. (Chengaiah, Rao, Kumar, Alagusundaram, & Chetty, 2010; Vilar et al., 2014).

Table 1. Botanical classification of *B. orellana* (Franco et al., 2008).

XVIII Division	Angiosperm
Subdivision:	Angiosperm
Class	Dicotiledoneae
Order:	Parietales
Suborder:	Cistianeae
Family:	Bixaceae
Genre:	Bixa
Species:	Bixa orellana L., Sinónimos: Bixa americana Poir., Bixa purpurea Sweet, Bixa upatensis Ram. Goyena, Bixa tinctaria Salisb.

It is endemic from the tropical regions of America, which is known by the following common names: achiote, annatto, urucu, anatto, orellana, changerica, among others (Franco et al., 2008). It is a shrub with a height between 3 and 5 meters **(Figure 1A),** whose leaves are simple and large. The flowers are arranged in groups and their colours range from whitish to pink, depending on the variety. The petals are dialipetal and spaced apart, with five sepals **(Figure 1B),** appearing at the ends of the branches (Alves & Albuquerque, 2012). The annatto flower is hermaphrodite, but cannot self-fertilize due to the protandria that makes the pollen grains viable or mature before the anthers are receptive. The pollen grain is ripe on the eve of flower opening and the stamens last only one day, thus obstructing self-pollination. Due to this situation fertilization happens only due to cross-pollination, which is usually carried out by winds or insects (entomophilous). This pollination favours the occurrence of a greater diversity of phenotype attributes, characteristics that will influence the content of bixin in grains and pods (C. F. de O. Franco et al., 2008).

A **B**

Figure 1 . *Bixa Orellana* L. shrub (A) and flower (B).

There are flowers in the annatto plant practically throughout the year, however, there is greater intensity in two seasons under normal climate conditions, the first flowering is more intense between February and March, since the main harvest occurs from June to July. The second flowering occurs in July and August, and the harvest takes place in November and December. Both harvests are generally done by hand, as most annatto production comes from family farming. Regarding the duration of flower opening and ripening **(Figure 2),** it may vary from place to place, but may vary from 100 to 140 days (Franco et al., 2008; Franco et al., 2002).

Figure 2 . Young shoot (A), bud with partially exposed petals (B), flower (C), fruit development with 2 days (D) and 7 days (E) of *B. orellana.*

The fruit is a 2 to 6 cm long red capsule with malleable spiny “hair” that evolves from a dark green hue and when matured undergoes a transition to a dark reddish colour. The seeds are 5 mm long and up to 3 mm in diameter, coated with a reddish viscous substance **(Figure 3).** These seeds have a pyramidal to almost conical shape. In the pericarp, 80% of existing pigments consist of a carotenoid by the name of bixin, a dye that can be obtained using vegetable oils as an extraction solvent. However, it is made up of more compounds, even though they are less relevant inert substances. The capsule contains between 30 and 40 seeds (**Figure 3**) on average, exceptionally there may be 70 seeds capsules. Seed colouration can range from dark red, which

means it has a higher colorant concentration to a light pink colour that has a lower colorant concentration (Franco et al., 2008; Franco et al., 2002; Valério, Ramos, Braga Neto, & Macedo, 2015) This seed is of great importance as it is rich in pigments that can be applied in various industries. The annatto dyes may be extracted by an aqueous alkaline solution or by vegetable oil, thereby giving salts of norbixin in water-soluble form and bixin in lypossoluble form (Valério et al., 2015).

Figure 3. Open annatto capsule with exposed seeds.

The leaves are in the shape of a heart, arranged in a spiral shape and can measure from 8 to 20 cm in length and 4 to 15 cm in width, having a petiolate structure, that is, they have a ribbed peninerval leaf vein secondary and tertiary along the leaf, having an edge with the wavy limb and small undulations along the leaf **(Figure 4)** (Alves & Albuquerque, 2012)**.**

Figure 4. *B. orellana* leaf.

The trunk has a coffee brown colour, can reach up to 5 meters in height, with a cylindrical shape, having a base diameter between 10 to 15 cm, and some of them may have a diameter greater than 30 cm, as well as their branched roots, where from the coarse main root grow more secondary and tertiary roots that are distributed in the soil, thinner and presenting dark coffee

colour. These roots can reach approximately 30 cm deep and the main root can reach up to 1 m deep (Franco et al., 2008; Franco et al., 2002; Valério et al., 2015).

1.2.2. Main chemical components of usable parts of annatto

The parts used include the flowers, seeds, leaves and roots. In addition to the seed pigment, the annatto plant is also a source of B and C complex vitamins. The plant is rich in riboflavin, cyanidin, niacin, salicylic acid, tomentic acid and ellagic acid, and the leaves also have a volatile fraction where it is possible to find mostly sesquiterpenes. The seeds are rich sources of lipids, minerals (calcium, iron, phosphorus), protein, amino acids (arginine, leucine, lysine, isoleucine, phenylalanine, histidine, threonine, methionine, tryptophan, valine), terpenoids (bixin, norbixin, isobixin). The classification of annatto seeds is made according to several parameters, described in **Table 2.**

Table 2. Parameters for the classification of annatto seeds

(Adapted from Franco et al. 2002).

	Type 1	Type 2	Type 3
Moisture	≤ 10%	>10% ≤ a 14%	>14%
Bixin	> 2,5%	2,0 a 2,5%	<1,8%
Impurities	< 5%	<5%	> 5%
Strange material	Absent	Absent	Absent

For seed standardization purposes the following factors are also considered (Franco et al., 2002):

- Humidity: percentage of water contained in the sample
- Bixin content: percentage of pigment contained in the seed pericarp (raw material in the dye industry);
- Aroma: Typical desired smell, aromatic, penetrating;
- Impurities: debris from the product itself, such as peduncles and leaves;
- Foreign material: grains or seeds of other vegetables, and foreign bodies of any kind not derived from the products and not harmful to human health;
- Mould: mould from the fermentation of the product caused by fungi and / or bacteria.

The seeds of *Bixa orellana* L. are mostly composed by carotenoids compounds, such as bixin and norbixin. First cited in 1825 by Boussingault, crystallization of bixin was successfully achieved in 1878 by Etti. The elemental analysis and exact deliberation of its formula took place in 1917 by Panzer and Heiduschka. In 1928-1933 Kuhn and his collaborators suggested the structural formula, which was later confirmed by Kerrer (Garcia, Bolognesi, Dias, Miguel, & Costa, 2012). Other constituents can be found in annatto, such as amino acids (lysine, phenylalanine, tyrosine, leucine and isoleucine); lipids, including fatty acids linoleic, oleic and α-linolenic acid, as well as minerals (iron, zinc, phosphorus and calcium) (Capella et al., 2016).

- **Bixin ($C_{25}H_{30}O_4$)**

The chemical structure of bixin consists of a 24-carbon isopropenic chain (**Figure 5**) having in its constitution a carboxylic group and a methyl ester at the ends (Lima et al., 2001). What makes this colouring compound one of the most widely used is the possibility that, by extracting the pigment from the seeds, it will obtain a variety of colours from yellow (norbixin) to red (bixin). Depending on the solvent used for the extraction, a water soluble or a fat soluble dye may be obtained (Peter Bilton, 2011). The singularity of bixin is that, among the carotenoids, it is naturally present in the *cis* configuration. It was the first cis-carotenoid to be isolated from natural sources, its molecule contains a carboxylic group and a methyl ester which gives lipossolubility to the molecule. In the cis configuration, both groups are located on the same side of the molecule, which is not the case in the *trans*-bixin form, which is more soluble in oils giving a reddish colour, as opposed to the *cis*-bixin isomer which is orange in colour.

Importantly, this type of annatto compound may undergo some changes, being susceptible to changes in structure due to heat, light and oxidation. It may also change in the presence of some solvents. As soon as the annatto seed becomes isolated, the bixin remains more exposed, hence its deterioration is likely to occur more easily (Constant, Beltrão, Stringheta, & Sandi, 2002; Rivera-Madrid, Aguilar-Espinosa, Cárdenas-Conejo, & Garza-Caligaris, 2016) When enzymes intervene, this will lead to two new carotenoids. When the loss of the methyl ester group of bixin occurs norbixin is obtained.

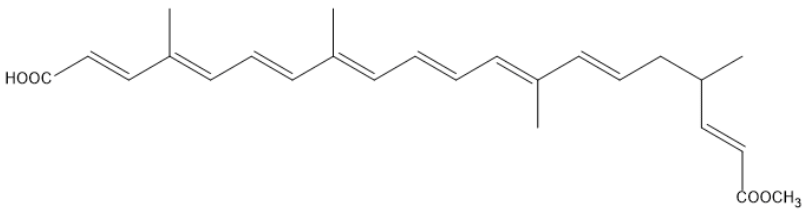

Figure 5. Chemical structure of bixin.

❖ **Norbixin ($C_{24}H_{28}O_4$)**

With the presence of the second carboxyl group in the molecule (**Figure 6**), it will increase the hydrossolubility.

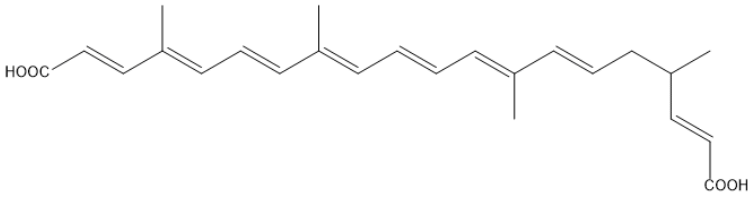

Figure 6. Chemical structure of norbxin.

Conjugated double bonds in either norbixin or bixin may be isomerized. The *cis*-like bixin and norbixin configurations, which are present in the annatto, can be transformed into the *trans* configuration, which is a more stable configuration whenever the molecule is subjected to higher temperatures (Constant et al., 2002) (**Figure 7**). Only natural norbixin pigment reacts with casein, which makes this colorant unique in the cheese and other dairy products segment. The water solubility of bixin and norbixin may be increased by the addition of polysorbates or propylene glycol, however, annatto presents affinity with water and organic solvents, depending on the extraction medium and the type of mixtures, emulsions, dilutions and suspensions used for extraction or obtaining the final product (Garcia et al., 2012; Venugopalan et al., 2011).

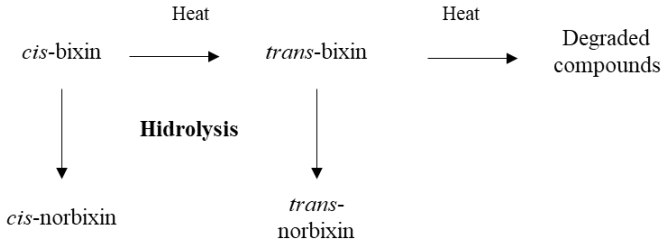

Figure 7. Interrelation of *Bixa orellana* L. colouring compounds.

In short, bixin may occur in the *cis* and *trans* stereochemical configurations. Under normal conditions the most prevalent is *cis*-bixin, or *cis*-norbixin, which is a more unstable configuration than *trans*-bixin. The structural differences between bixin and norbixin make these isomers have distinct solubility, polarity, and shade, and as a result each may have a different application. If these dyes are used according to the US Food Chemical Codex parameters, it is a safe and flavourless colouring compound (Garcia et al., 2012).

1.3. Bioactive properties

In **Table 3** are described some of the biological activities reported for different extracts of *B. Orellana*. Moreover, different parts of the plant were also studied, so the information reviewed is grouped by pharmacological activity.

Among the twenty studies reviewed, antifungal, antibacterial, antimalarial, and mutagenic activities are the most studied ones. Regarding cytotoxic, there are few studies of this plant (Caceres, Cano, Samayoa, & Aguilar, 1990; Freixa et al., 1998; Vilar et al., 2014).

Table 3. Some biological activities studied in different extracts and parts of the plant of *B. Orellana* (adapted from Vilar et al. 2014).

Country/ Biological activity	Part used	Type of extract	Organism tested	Model tested	Dose used	Activity
Argentina						
Antibacterial	LE	EtOH	*Staphylococcusaureu*	*In vitro*	5 mg/mL	Inactive
			Pseudomonasaeruginosa	*In vitro*	5 mg/mL	Inactive
			Micrococcus luteus	*In vitro*	5 mg/mL	Inactive
			Escherichia coli	*In vitro*	5 mg/mL	Inactive
			Bacillus subtilis	*In vitro*	5 mg/mL	Inactive
Antifungal	LE	EtOH	*Aspergillus niger*	*In vitro*	5 mg/mL	Inactive
			Candida albicans	*In vitro*	5 mg/mL	Inactive
Antiviral	SE	EtOH	Virus Herpes simplex 1	Cell culture	0.78 mg/mL	Inactive
Brazil						
Antimalarial	SE	$CHCl_3$	Mouse	*Plasmodium berghei*	100 mg/kg	inactive
Antioxidant	SE	EtOH	*n vitro*	DPPH assay	0.1 g/L	Active
Toxicity	SE	powder	Rat	*In vivo*	500 g/kg	Inactive
Costa Rica						
Anti-inflammatory	SE	EtOH	Rat	Paw edema	100 mg/kg	inactive
Cuba						
Cytotoxic	SE	EtOH	Tumour cell	*In vitro*	250 mg/kg	Active
Ecuador						
Antifungal	LE	MeOH	*Aspergillus niger*	*In vitro*	10 mg/disc	Inactive
			Candida albicans			Inactive
			Cryptococcus neoformans			Inactive
			Fusarium oxysporum			Inactive
			Neurospora crassa			Inactive
			Penicilliumpurpurogenum			Inactive
			Trichophyton mentagrophytes			Active

LE- leaves; AP –aerial parts; SE-seeds.

1.4. Traditional and industrial uses

B. orellana has been widely used since pre-Hispanic times in America in traditional medicine. During the XVII and XVIII centuries it spread to Asia and Africa, where it also became part of the ethnobotanical social legacy. Ethno botanical specialists have documented the utilization of deferent parts of the plant, especially leaves, seeds and roots (Chisté et al., 2011; Venugopalan et al., 2011). **Table 4** shows the large number of applications of *B. orellana* in different countries. In Brazil annatto seeds are used to prepare repellent bug spray and as antimalarial agent. Studies made in Cuba demonstrated the pharmacological activity as antimalarial agent, when tried against *Plasmodium berghei*.

Table 4. Traditional uses of *B. orellana* in different countries of South America.

Country / uses	Plant part
Argentina	
Antipyretic/cardiotonic/antidiarrhea	Seeds
Antidiarrheal/dyes/condiment	Seeds
Brazil	
Body paint	Seeds
Insect repellent	Seeds
Condiment/food colouring	Seeds
Antipyretic Antipyretic/laxatives/burns	Seeds
Colombia	
Snakebite	Seeds
Aphrodisiac	Seeds
Cuba	
Aphrodisiac	Seeds
Honduras	
Aromatic/food colouring	Seeds
Pain/digestive/dissenters	Leaves
Jamaica	
Diabetes	Seeds
Paraguay	
Insecticide/repellent	Seeds
Diabetes	Seeds

1.5. Carotenoids as food colorant

1.5.1. Chemical description and bioactive properties

B. orellana seeds are rich in carotenoids, particularly apocarotenes like bixin, isobixin and norbixina. The carotenoids present in minor concentrations are β-carotene, cryptoxanthin, lutein, zeaxanthin, and methylbixin, among others. *B. orellana* has also been reported has the vegetable source with the most elevated substance of terpenes, for example E-geranylgeraniol, which can represent 57% of all terpenes fraction in the dry seed. Additionally, it's a rich source in fatty acids (linoleic, α-linolenic, and oleic acids), aminoacids (glutamate, aspartate, leucine) and micronutrients (phosphorus, iron, and zinc) (Lourido Pérez & Sánchez, 2010).

Regarding the carotenoid fraction present in annatto seeds, as previously started bixin is the most representative one. Bixin is a lineal apocarotenoid of 25 carbon particles with 9 two-fold bonds and a sub-atomic load of 394.5 g/mole. Is the main carotenoid that exhibits a 9-*cis* arrangement and 2 carboxyl linkages. Its molecular empiric formula is $C_{25}H_{30}O_4$, and its scientific name is methyl hydrogen 9-*cis*-6,60-diapocaroteno-6,60-dioate ester.

Bixin represents around 80% of the complete carotenoids content present in annatto colour extract (mainly in its 9-*cis* formula, with low contents *trans*-bixin and *cis*-norbixin) (Raddatz-Mota et al., 2017).

Bixin has two different stereochemical configurations: *cis*-bixin and *trans*-bixin. *cis*- formula is soluble in polar natural solvents to which it gains an orange shading and is generally insoluble in lipid fractions (Raddatz-Mota et al., 2017). *trans*-Bixin is an more steady isomer, it shows a red shading in solution and is soluble in lipid fractions (Raddatz-Mota et al., 2017)

Phytochemical studies of annatto leaves (other plant part with interesting potential) also showed the presence of bixaghanene, bixein, bixol, crocetin, and isobixin. Its essential oil is mainly constituted by sesquiterpenes being ishwarane the major compound (Venugopalan et al., 2011).

The most reported effect of bixin is its antioxidant activity. *In vitro* tests have showed that seeds extract high ability to inhibit the action of oxygen reactive species (ROS), which has proved to be highly correlated with the presence of bixin (Chisté et al., 2011). It has also been proven the inflammatory effect of this plant (Yong et al., 2013).

1.5.2. Natural versus artificial colorants

Nowadays, there is a great deal of effort on the part of the industry to replace synthetic colorants with natural counterparts. Not only by the current consumer trend, but also by the numerous studies conducted the prove the harmful long-term effects that the consumption of these colouring compounds (e.g., tartrazine (E102), allura red (E129) or dusk yellow FCF (E110)) can have on the health of the consumer (Moreno et al., 2005). In fact, there are already many regulatory agencies that have banned the use of some of these artificial colouring compounds, such as carmoisine (E122) and Ponceau 4R (E124), and allowed the use of colour extracts of *B. orellana* L. (E 160b) (McCann et al., 2007).

With the introduction of annatto colouring extracts in several markets worldwide, this plant has gained an extraordinary monetary significance worldwide, being now one of the most used colouring extracts by the cosmetic, food and pharmaceutical industries (it does not change the flavor of the final product and has no toxicity) (Giridhar, Giridhar, & Parimalan, 2010; Lauro & Francis, 2004; Lourido Pérez & Sánchez, 2010; Michelangeli et al., 2002; Smith, 2006; Van Chuyen, Hoi, & Eun, 2012).

1.5.3. Extraction techniques for carotenoids from natural matrices

The extraction procedures of the annatto seeds carotenoids can be physicochemical, through solvents and chemicals, and/or mechanical, through grinding and scratching (Alves & Albuquerque, 2012). Extraction can also be performed by using natural solvents, for example, ethanol, chloroform, propylene glycol (the one that preserves and guarantees a maximization of the extraction of the colouring compounds), and also antacid extraction (using KOH and NaOH) (Oliveira, 2005). There are a lot of techniques that can be used to extract carotenoid from plant matrices. In **Table 5** its described some of the advantages and disadvantages of each method.

Soxhlet extraction is a fluid extraction, performed at normal pressure and high temperatures (boiling solvents). Soxhlet extraction is a routine method that allows the recuperation of high concentrations of carotenoids. In this sense, many times it serves as control method to assess the efficacy of different techniques for extraction of carotenoids (Macías-Sánchez, Fernandez-Sevilla, Fernández, García, & Grima, 2010). On the other hand, it's a very time consuming method, uses high volumes of solvents (which increases the cost of the extraction method itself,

and in turn, of the final product), and also can lead to an isomerization of the carotenoids extracted (Cardenas-Toro et al., 2015).

Table 5. Advantages/disadvantages of the most common extraction methods of carotenoids from plant matrices.

Extract method	Advantages	Disadvantages
Soxhlet extraction	Simple and conventional method providing the highest recovery of carotenoids.	Can cause thermal degradation and *cis-trans* isomerization of carotenoids.
Microwave-assisted extraction (MAE)	Simple, fast and economical method.	Can cause thermal degradation and *cis-trans* isomerization of carotenoids.
Ultrasound-assisted extraction (UAE)	Rapid, non-thermal and efficient extraction	Aging of the ultrasonic probe surface can change the extraction efficiency
Pulsed electric field (PEF) extraction	High extraction yield, non-thermal process and use of low energy	PEF parameters may differ with change in electrical conductivity of the sample
Enzyme-assisted extraction (EAE)	Rapid and efficient extraction with minimal usage of solvents	High cost of the enzyme

Microwave-assisted extraction (MAE) is a straightforward, quick and economic method for extraction of carotenoids, requiring a short extraction time and low volume of solvents (Cardenas-Toro et al., 2015; Ho, Ferruzzi, Liceaga, & San Martín-González, 2015). MAE can cause thermal degradation and *cis-trans* isomerization of carotenoids, and for that manner, additional steps to prevent the oxidation of carotenoids are needed for an efficient recovery of the compounds (Saini & Keum, 2018).

Ultrasound-assisted extraction (UAE) can be applied in numerous extraction procedures for different types of compounds in different types of matrices. The significant efficacy of UAE is the acoustic cavitation, that prompts cell rupture and upgrades the mass move of in the medium. Power, intensity, and temperature are variables required to be optimized in order to obtain an efficient extraction of the metabolites. UAE has been previously used for the extraction of carotenoids in the green microalgae *Chlorella saccharophila*, where using acetone, 500 W, and extraction time of 13.48 min allowed to obtain high yields of zeaxanthin (11.2 mg/g) and β-carotene (4.98 mg/g) (D. Singh, Barrow, Mathur, Tuli, & Puri, 2015).

Pulsed electric field (PEF) methodology allows to work at low temperatures, low voltages (less than a couple of milliseconds), and can be applied to a numerous of organic material (such as plant, or microbial cells). This procedure enhances the penetrability of the cytoplasmic film, called "electroporation" which empowers the extraction of intracellular carotenoids. The

permeability might be transient (reversible electroporation) or permanent (irreversible electroporation), depending on the applied electric field (Luengo et al., 2015).

Finally, the enzyme-assisted extraction (EAE) techniques use hydrolytic enzymes to to expose intracellular materials for improved extraction yield. EAE for carotenoids extraction have a high potential, and promising industrial applications (despite the enzymes cost) (Sowbhagya & Chitra, 2010). Cellulase and pectinase are commonly used in pre-treatment steps before liquid extraction. Cellulase hydrolyzes the 1,4-β-d-glyosidic linkages of the cellulose. And pectinase separates the pectin substances and pectin found in the plasmatic membrane of plants (Strati, Gogou, & Oreopoulou, 2015).

All of the methodologies mentioned above, uses organic solvents and energy to maximize the extraction of carotenoids from plant matrices. Industry is moving forward towards more environmentally friendly solvents, generated from renewable biomass wastes (e.g., wood, starch, organic products and vegetable oils) or from petrochemical sub-products that are non-lethal and biodegradable (Yara-Varón et al., 2016). Yara-Varón et al., (2016) assessed five green solvents, cyclopentyl methyl ether (CPME), dimethylcarbonate (DMC), ethyl acetic acid derivation (EA), isopropyl alcohol (IPA), and 2-methyltetrahydrofuran (2-MeTHF), for the substitution of *n*-hexane in the extraction of carotenoids from carrots.

1.6. Stabilization procedures and application into food formulations

There are many factors that affect the chemical and structural stability of carotenoids in food matrices (as with many other compounds), being the plan species, geographical origin (characteristic biotic and abiotic factors), maturity and ripening stage of the plant, post-harvest conditions, conditioning steps, and storage time and conditions the most important ones (Yahia, Soto-Zamora, Brecht, & Gardea, 2007).

❖ Genotype and Geographical origin

The genotype the plant matrices highly influences the type and amount of carotenoids within that matrices, and may even be very significant differences intra-species (Ornelas-Paz, Yahia, & Gardea-Bejar, 2007), as it happens with tomato, apricot, cassava, and sweet potato. Mou (2005) study revealed that the carotenoid profile among the 52 different cultivars of lettuce

(crisphead, butterhead, red leaf, Latin, romaine, stem lettuce, crude and wild species) was indeed very different regarding the type and amount of carotenoids compound.

The geographical origin of pods and vegetables is additionally another significant factor that contributes for a high variability in carotenoids fraction in similar plants, mainly dependent on biotic and abiotic factors, but especially climatic conditions. For instance, the carotenoid amount in apricot found to be much higher in plants grown in the Mediterranean zone than the ones from other geographical points (Dragovic-Uzelac, Levaj, Mrkic, Bursac, & Boras, 2007).

❖ Maturity and Ripening Stage

The maturity and ripening stage of plants is found to be the primary factor that influences the type and amount of carotenoids. Carotenoids are usually incremented with the maturity of the plant due to the high metabolic activity of ethylene (Carrillo-Lopez & Yahia, 2010). During the ripening procedure, chlorophylls and chloroplasts ruptures which leads to the development of chromoplasts and consequently, higher amounts of carotenoids.

❖ Effect of Processing on Stability of Carotenoids

Processing of pods and vegetables also influences the stability and concentrations of carotenoids. The structural modification of carotenoid as also its amount can be positively or negatively affected depending on several post-harvest, conditioning and storage conditions of the plant matrices. For instance, the processing of the plants into homogenization or other type of procedures that leads to tissue disruption can lead to a significant diminish of carotenoids (the maceration of green leaves can lead to an 30% loss of β-carotene) (Sahar, Rahman, Aadil, & Ishaq, 2019). Temperature is also a very important factor for carotenoids stability. Despite some carotenoids compounds can resist to slight changes in temperature, the vast majority of them are highly thermolabile. For instance, *cis*-lycopene concentrations increased during heat treatments, while the *trans*-structures are diminished. The same behavior is observed for β-carotene in mango plants after heat treatment (Sahar et al., 2019).

1.6.1. Stabilization Techniques

Due to all of these factors that may affect the chemical structure and concentration of carotenoids after processing the plant matrix, it is necessary to resort to different techniques of stabilization of these molecules. One of the many answers is encapsulation technologies which, by means of a liquid or solid encapsulating material, allow the molecules to be protected from various biotic, abiotic and sample processing factors (Augustin & Sanguansri, 2008; Dias, Ferreira, & Barreiro, 2015).

Different procedures have been used for the encapsulation of carotenoids, for the final intent of maintaining their bioactive functionalities. The most broadly used techniques are freeze-drying, spray drying, liposomes, inclusion complexes, coacervation and extrusion technology (Soukoulis & Bohn, 2018).

1.6.2. Application into food formulations

As a result of the growing health concerns by the consumers, the interest in using carotenoids-rich extracts as colouring agents in novel food products is rising. β-Carotene, astaxanthin, canthaxanthin, lycopene, and lutein are the most required ones, being already used in different food and cosmetic products (Singh, Jayaprakasha, & Patil, 2017). In Asia, the use of red koji rice dates for many years. These red pigments are likewise used food colorants for wine, red soy cheddar, and meat (Dufossé et al., 2005). The French cheddar named "*vieux-dish*" contains the carotenoid produced by *Brevibacterium*, that gives an orange-red-brown shading to the product, increasing its sensory quality for the consumers (Galaup et al., 2015). In Russia, baby formulas products are enriched with natural pigments (lutein in bosom milk) as a functionalizing agent to improve children's health (Kon, Gmoshinskaya, Safronova, Alarcon, & Vandenplas, 2014). Pastry and pasta have likewise been enriched with natural colouring carotenoid compounds. In Japan, *Undaria pinnatifida* (wakame), a palatable ocean rich in fucoxanthin, is popularized as an ingredient for pasta dishes (Prabhasankar et al., 2009).

2. Objectives

The main objective of the present work was to characterize the nutritional profile, chemical composition, and bioactive properties of *Bixa orellana* L.; as also, the maximization of the extraction recovery of bixin from the seeds and the food application of the carotenoid-based extract into pasta dough. To achieve the proposed objective, several specific objectives were defined, described below, with all the methodologies implemented in the present work (**Figure 8**):

1) Nutritional assessment of *B. orellana* seeds by AOAC (Association of Official Analytical Chemists) methods, namely, ash, total fat, proteins, carbohydrates and energetic value;

2) Determination of the chemical composition *B. orellana* seeds in:

a) Sugars by HPLC coupled to an RI detector;

b) Organic acids by UFLC coupled to a DAD detector;

c) Fatty acids by GC coupled to a FID detector;

d) Tocopherols (vitamin E) by HPLC coupled to a fluorescence detector;

3) Bioactive properties and phenolic composition of *B. orellana* seeds and pods:

a) Preparation of hydroethanolic extracts (80:20 *v/v*);

b) Determination of the phenolic profile by HPLC-DAD/ESI-MSn;

c) *In vitro* evaluation of the antioxidant (inhibition of the lipid peroxidation through the assay of reactive species of thiobarbituric acid – TBARS and inhibition of oxidative haemolysis - OxHLIA), hepatotoxic (evaluated using primary cell culture obtained from pig liver cells - PLP2), and antimicrobial (using strains of ATCC food bacteria and fungi through the microdilution method) activities of the hydroethanolic extracts;

4) Development of an experimental design for extraction process optimization of bixin, using ultrasound assisted extraction, chromatographic analysis of carotenoids, and response surface methodology for optimization;

5) Finally, development of an enriched pasta dough with a carotenoid-based extract. Evaluation of physical parameters of colour (CIELab system). Shelf analysis of the obtained product during 30 days, by comparison with fresh pasta dough.

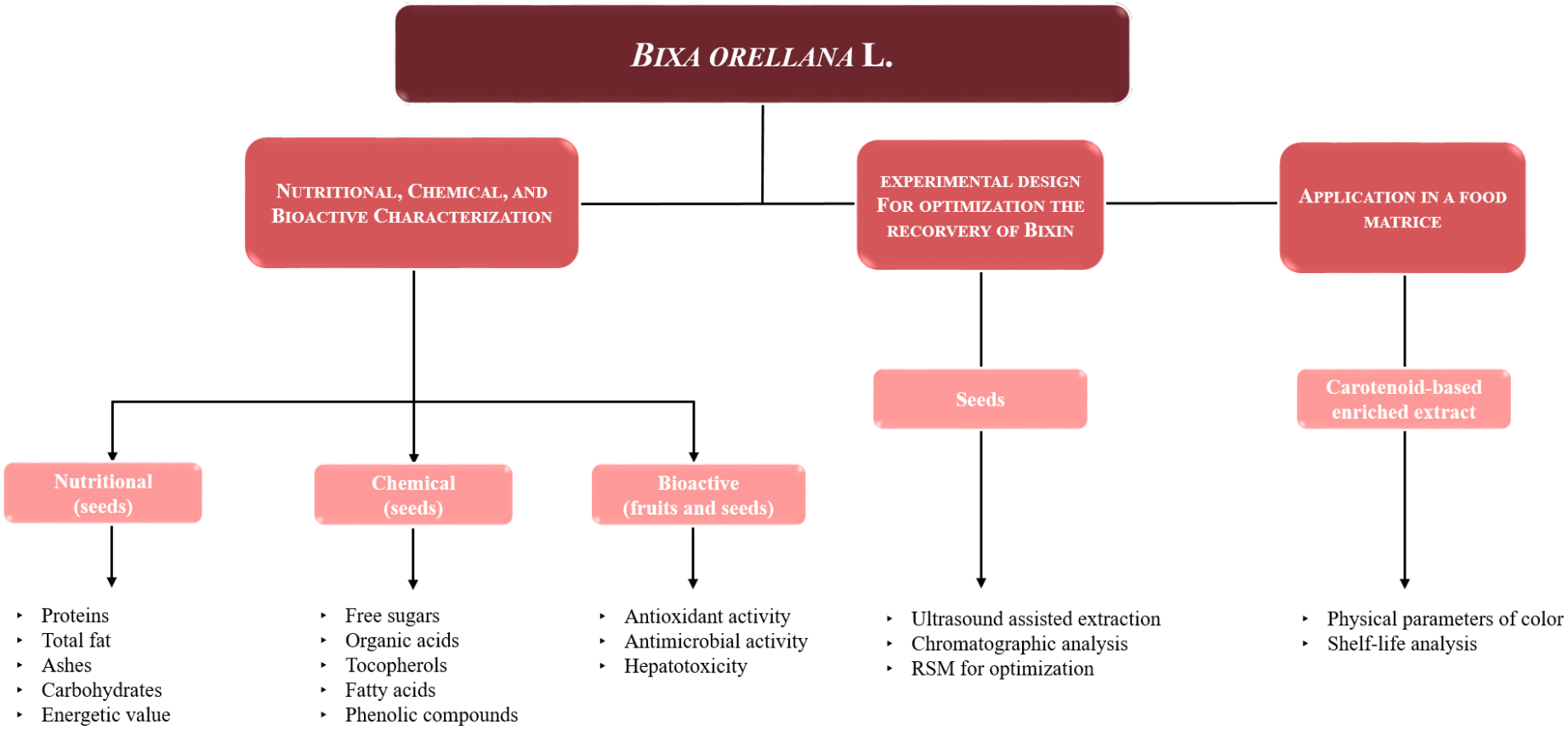

Figure 8. Schematic representation of the work plan developed.

MATERIAL AND METHODS

3. Material and Methods

3.1 Standards and reagents

For the analysis of the phenolic compounds, acetonitrile (99,99%, HPLC) and formic acid were acquired from Fisher Scientific (Lisbon, Portugal) and Sigma-Aldrich (St. Louis, MO, EUA), respectively. The patterns for phenolic compounds (apigenin-6-*C*-glucoside, *p*-coumaric acid, protocatechuic acid, and quercetin-3-*O*-glucosiede) and carotenoids (bixin) in Extrasynthèse (Genay, France). The standard reference mixture of fatty acid methyl ester FAME) 37 (pattern 47885-U) was acquired in the company Sigma-Aldrich (St. Louis, MO, USA), as also individual isomers for tocopherols (α-, β-, λ- e δ-isoforms), sugars (D(+)-sucrose and D(+)- trehalose), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and organic acid patterns (oxalic acid, quinic acid, malic acid, and fumaric acid). Racemic tocol, 50 mg/mL, was bought in Matreya (Pleasant Gap, PA, USA). The solvents *n*-hexane (95%), ethyl acetate (99,8%, HPLC) were acquired in Lab-Scan (Lisbon, Portugal). For the oxidative haemolysis assays the reagent 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was acquired in Sigma-Aldrich (St. Louis, MO, EUA). For the hepatotoxicity assays were used 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), phosphatebuffered saline (PBS), and Tris were acquired from Sigma. For pasta dough preparation, the wheat flour used was purchased from a local supermarket, Bragança, Portugal. Methanol, ethanol and all other solvents and reagents were acquired from scientific retailers. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

3.2. Plant material preparation

The pods and seeds of *Bixa orellana* L, were collected in São Paulo, Brazil, in September of 2017, and packed in bags, protected from light and humidity. After arriving at the laboratory, the seeds were detached from the pods and both samples were subsequently processed. Since the samples already had a high level of dehydration (according to the

manufacter), it was not necessary to resort to freeze-drying processes, such lyophilisation. The samples were then powdered to a size of approximately 35 mesh using a mill and, finally, stored in a cool, dry place until further analysis (**Figure 9**).

A **B**

Figure 9. *B. orellana* seeds (**A**) and pods (**B**) used in the present study.

3.3. Determination of the nutritional profile and chemical composition of *B. orellana* seeds

The following methodologies were only applied to the seed samples of *B. orellana*, since the pods are not edible and its nutritional and chemical input was not the objective of the present work (nonetheless, could be a future perspective to understand the potential of pods for food purposes).

3.3.1. Nutritional profile

The nutritional composition of *B. orellana* seeds was determined using the official methods described by AOAC (2016). For this, the protein, total fat, ash, and carbohydrates content, and energy value were determined.

The protein content analysis was carried out according to the macro-Kjeldahl method (AOAC 978.04), which is based on the conversion of total nitrogen. In the digestion of organic matter, 15 mL of sulphuric acid was added to the sample (0.5 g), which will retain the nitrogen in the form of $(NH_4)_2SO_4$. In the macro-Kjeldahl apparatus NaOH is added, by backflow volumetry, which will release nitrogen in the form of NH_3, later collected by steam distillation in a solution of 0.1N H_2SO_4. Finally, a titration is made with 0.1N NaOH, using methyl red as

an indicator to calculate the amount of nitrogen, using as correction factor N (amount of nitrogen) x 6.25, and the results expressed in g/100 g dry weight.

The total fat was determined by extracting a known mass from the sample (3g), using a Soxhlet apparatus, with petroleum ether, for approximately 8 hours (between 16 to 20 turns in the Soxhlet apparatus), at a temperature of about 60 ºC (AOAC 920.85). The volatile fraction was evaporated and the lipid fraction was placed in an oven until constant weight. The results were expressed in g/100 g dry weight.

The ash content was determined by incinerating about 0.5 g of muffle sample at 600 ºC (AOAC 923.03) and, finally, the carbohydrate content was calculated by difference of all the parameters obtained previously. In both, the results were expressed in g/100 g dry weight.

The energy value was calculated as described by Regulation (EC) No 1169/2011, (2011) and according to equation (2):

Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat) (2)

3.3.2. Free sugars

From the sample used for Soxhlet extraction (in which the lipid fraction was removed), approximately 1 g of mass was weighed and 1 ml of internal standard solution (melezitose, 25 mg /ml) was added. Subsequently, extraction was carried out, in which 40 mL of 80% ethanol was added to the sample, in a water bath at 80 ºC for 1 hour and 30 min, with alternating agitation every 15 minutes. Subsequently, the samples were centrifuged (K24OR refrigerated centrifuge, Centurion, West Sussex, United Kingdom) for 10 min at 3500 rpm, filtered, and the supernatant transferred to a glass flask in which the ethanol was evaporated at 50 ºC under reduced pressure (Büchi R-210 rotary evaporator, Flawil, Switzerland). The water obtained after evaporation of the supernatant, was then subjected to process to remove the remaining lipids, where it was washed three times with 10 ml of ethyl ether (**Figure 10**). The samples were then placed at 40 ºC to remove residual ethyl ether, and the residue was dissolved in distilled water to be measured at 5 mL and, later, filtered through 0.2 μm nylon filters for vials for later analysis of the sugar profile in the HPLC system.

Figure 10. Lipid fraction removal in the water extract, with ethyl ether.

Chromatographic determination of free sugars the seeds was performed by high-performance liquid chromatography coupled to a refractive index detector (HPLC-RI, Knauer, Smartline 1000 system, Berlin, Germany), as described by the authors Barros et al. (2013). The HPLC system (Knauer, Smartline system) used is equipped with an IR detector (Knauer Smartline 2300) and a 100-5 NH_2 Eurospher column (4.6 × 250 mm, 5 µm, Knauer). The mobile phase used was acetonitrile/deionized water, 70:30 (*v/v*) with a flow rate of 1 mL/min in isocratic mode. The identification of sugars was performed by comparing the relative retention times of the sample peaks with authentic standards and the quantification performed by internal normalization of the peak chromatographic area using the melezitose peak (PI) as a standard. The results were expressed in g per 100 g of dry sample weight.

3.3.3. Organic acids

Approximately 1g of dry seeds sample was weighed into a glass beaker protected from light (previously wrapped in aluminium foil) and 25 ml of metaphosphoric acid (4.5%, *v/v*) was added in a concentration of 10 mg/mL, at a room temperature of about 25 ° C. The mixture was placed under magnetic stirring for 20 minutes and then filtered into a 20 ml test tube. For chromatographic analysis, the samples were filtered into an amber vial (1.5 mL) through a 0.2 µm nylon filter for further analysis on HPLC.

The chromatographic determination of the profile in organic acids was performed by ultra-fast liquid chromatography coupled to a diode detector (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan) as previously described by Barros et al. (2013). The separation of the compounds was carried out through a C_{18} reverse phase column (250 mm x 4.6 mm, 5 µm,

Phenomenex), heated at 35 ºC. The detection occurred through a diode array detector (DAD) programmed to acquire the predefined wavelengths of 215 nm and 245 nm. The elution solvent used in isocratic mode was sulphuric acid (3.6 mM, H_2SO_4). The identification of organic acids and their quantification was determined by comparing retention times and UV spectra with commercial standards. Quantification was performed by comparing the peak area at the programmed wavelength with the corresponding commercial standard, using 7-level calibration lines of: oxalic acid (y = 9x106x + 45973, R^2 = 0.9901, LOD = 6.3 µg / mL; LOQ = 20.8 µg / mL); quinic acid (y = 610607x + 46061, R^2 = 0.99995, LOD = 11.3 µg / mL; LOQ = 37.6 µg / mL); malic acid (y = 950041x + 6255.6, R^2 = 0,9999, LOD = 15.9 µg / mL; LOQ = 59.9 µg / mL); and fumaric acid (y = 154862x + 1x106, R^2 = 0.9977, LOD = 42.5 µg / mL; LOQ = 141.7 µg / mL). The results were expressed in g per 100 g of dry sample weight.

3.3.4. Fatty acids

To determine the fatty acid profile, the lipid extract obtained after Soxhlet extraction (see section 3.3.1.) was subjected to a derivatization process to obtain the volatile fatty acids, called FAME (Fatty acid methyl ester). For this, to the lipid extract was added 5 mL of a 2:1:1 (*v/v/v*) methanol/sulphuric acid/toluene solution, and placed in a water bath at 50 °C for 12 hours with stirring 160 revolutions per minute (rpm). After which, in order to enhance the phase separation, 3 ml of distilled water and 3 ml of ethyl ether were added in which the FAME was dissolved. To remove any water residue, the supernatant was placed in contact with a small portion of anhydrous sodium sulphate, recovering the sample and filtering it (0.2 µm nylon filter) into a vial with a Teflon membrane cover. Fatty acids were determined by gas chromatography coupled to a flame ionization detector (GC-FID, instrument DANI model GC 1000, Milan, Italy), as described by Barros et al. (2013).

The identification of the fatty acid profile was done through a GC system (Model DANI GC 1000) equipped with a split/splitless injector, flame ionization detector (FID, 260 °C) and a Macherey Nagel column (30 m × 0.32 mm × 0.25 µm). The oven was programmed with the following parameters: the initial temperature of the column was 100 ºC, for 2 min; then, the temperature was increased at 10 ºC/min up to 140 ºC, 3 ºC/min up to 190 ºC, 30 ºC/min up to 260 ºC for 2 min. Hydrogen (carrier gas) had a flow rate of 4.0 ml/min (0.61 bar), measured at 50 °C. The split injection (1:50) was performed at 250 ºC. For each analysis, 1 µL of the sample was injected. The identification of fatty acids was made based on the relative retention times of

the peaks of the standard mixture of 37 FAMEs and the samples. For the processing of the results, the software program Clarity 4.0.1.7 (DataApex, Podohradska, Czech Republic) was used, and the results were expressed as a relative percentage (%) of each fatty acid detected.

3.3.5. Tocopherols

To extract the tocopherols, 0.5 g of dry sample was weighed into a falcon tube, to which 100 µL of butylated hydroxytoluene (BHT, 10 mg/mL in hexane), 400 µL of tocol (50 µg/mL in hexane, internal standard) and 4 mL of methanol were added. The mixture was stirred for 1 minute in the vortex, after which 4 ml of hexane and 2 ml of concentrated aqueous sodium chloride solution (NaCl) were added, and vortexed again. The mixture was centrifuged for 5 minutes at 4000 rpm and the supernatant was transferred to a glass vial, to which two spoons of anhydrous sodium sulphate had been added, and the sample was re-extracted two more times with 4 ml of hexane and 2 mL of concentrated aqueous sodium chloride (NaCl) solution. At each extraction, the supernatant was collected into the glass vial.

The obtained extract was then dried and concentrated in a nitrogen stream to remove *n*-hexane, and re-dissolved again in 2 ml of hexane, and filtered into an amber vial (1.5 mL) through a 0.22 µm nylon filter for further HPLC analysis.

Tocopherols were determined using the extraction and chromatographic characterization procedure, following the procedure described by the authors Barros et al. (2013). The chromatographic analysis was performed on an HPLC system coupled to a fluorescence detector, and a column in normal phase of Polyamide II (250 x 4.6 mm, 5 µm, Waters YMC) at 35 ºC (oven 7971 R Grace), being that the system was programmed for excitation at a wavelength of 290 nm and emission at 330 nm. The mobile phase consisted of a mixture of *n*-hexane and ethyl acetate (70:30 *v/v*), and a flow rate of 1 ml/min. The results were analysed using the Clarity 2.4 software (DataApex), the quantification was based on the fluorescence signal by chromatographic comparison with tocol (internal standard). The identification and quantification were based on the response of the fluorescence signal of each standard, using the internal standard method (tocol) and using calibration curves obtained from commercial standards for each compound. The results were expressed in mg per 100 g dry weight.

3.4. Bioactive potential evaluation and phenolic compounds profile of *B. Orellana* seeds and pods

The following methodologies were performed for both pods and seeds of *B. orellana*. Although pods do not have food application potential, it could represent a good source of bioactive molecules, with potential to be applied in other industries, such as the pharmaceutical or nutraceutical. Together with the fact that pods are a resulting by-product of the seeds processing, the sustainability of the use of this by-product, is an asset for the present work.

3.4.1. Hydroethanolic extracts preparation

For hydroethanolic extract preparation, 1 g of the pods and seeds (**Figure 11**) powder was extracted by stirring with 30 mL of ethanol/water (80:20 *v/v*) at room temperature and 150 rpm, for 1 h. The extract was filtered through Whatman filter paper no. 4. The residue was re-extracted once more under the same conditions. Afterwards, the combined extracts were evaporated under vacuum (rotary evaporator Büchi R-210, Flawil, Switzerland) and further lyophilized. The lyophilized extracts were then stored protected from light and humidity for further analysis.

A **B**

Figure 11. Hydroethanolic extraction from the seeds (**A**) and pods (**B**) of *B. orellana*.

3.4.2. Antioxidant activity

3.4.2.1. Inhibition of lipid peroxidation through reactive substances of thiobarbituric acid (TBARS)

For the analysis of antioxidant activity using the TBARS method, was used the protocol described by Sarmento, Barros, Fernandes, Carvalho, & Ferreira (2015) The lyophilized hydroethanolic extracts were re-dissolved in ethanol:water (80:20, *v/v*) to obtain a 0.5 mg / mL stock solution, diluting successively to obtain six concentrations below it.

In parallel, a pig brain suspension (*Sus scrofa*) was prepared, in which a portion of the brain was added together with Tris-HCl buffer (20 mM, pH 7.4) in a ratio of 1:2 (*m/v*), centrifuging the mixture at 3500 rpm for 10 min at a temperature of 10 ºC to avoid rancification of the mixture.

In eppendorf tubes 200 µL of each of the solutions of hydroethanolic extract were placed, to which 100 µL of ascorbic acid (0.1 mM), 100 µL of iron sulfate ($FeSO_4$ - 10 mM) and 100 µL of the supernatant homogenized from pig brain were added and incubated at 37.5 ºC for 1 hour. After incubation, 500 µL of trichloroacetic acid (28% *m/v*) was added to stop the reaction and 380 µL of thiobarbituric acid (2% *w/v*, TBA), and the tubes were placed in a water bath at 80 ºC for 20 minutes, in order to promote the reaction between TBA and malondialdehyde (MDA - reactive oxygen species resulting from lipid peroxidation that occurs in pig brain tissue). Subsequently, the mixture was centrifuged at 3500 rpm for 5 min, to separate the residues from the supernatant. The colour intensity of the MDA - TBA complex was measured at 532 nm. The percentage of inhibition of lipid peroxidation was calculated using the following equation:

% inhibition of lipid peroxidation = (A - B) / A x 100

where A and B refer to the absorbance of the control (water) and the extract solution, respectively. The extract concentration corresponding to 50% inhibition of lipid peroxidation (IC_{50}) was calculated from the graph of the percentage of inhibition of TBARS formation as a function of the extract concentration. As a positive control Trolox was used and the results were expressed in µg/ml.

3.4.2.1. Inhibition of oxidative haemolysis (OxHLIA)

The antihaemolytic activity was assessed using the OxHLIA assay previously described by Lockowandt et al. (2019). Aliquots of sheep's blood were collected from healthy animals and centrifuged at 1000 rpm for 5 min at a temperature of 10 ºC to obtain the erythrocytes (the plasma and buffy coat were discarded). The erythrocytes were first washed with NaCl (150 mM) and three times with phosphate buffered saline (PBS, pH 7.4). The erythrocyte pellet was then re-suspended in PBS 2.8% (*v/v*).

In a 48-well flat bottom plate, 200 µL of the erythrocyte solution was mixed with 400 µL of PBS solution (negative control), of antioxidant samples dissolved in PBS or water (for complete haemolysis). Trolox was used as a positive control. The plates were pre-incubated at 37 ºC for 10 min with shaking, followed by the addition of 2,2'-Azobis dihydrochloride (2-amidinopropane) (AAPH, 160 mM in PBS, 200 µL) and incubated then in the same conditions. The optical density was then measured at 690 nm at 10 min intervals for about 400 min (Takebayashi, Iwahashi, Ishimi, & Tai, 2012). The calculation of the percentage of the erythrocyte population that remains intact (P) was calculated according to the following equation:

$$P\ (\%) = (St - CH0 / S0 - CH0) \times 100$$

where St and S0 correspond to the optical density of the sample at times t and 0 min, respectively, and CH0 is the optical density of complete haemolysis at 0 min. The results were expressed as haemolysis delay time (Δt), which was calculated according to the following equation:

$$\Delta t\ (min) = Ht50\ (sample) - Ht50\ (control)\ (5)$$

where Ht50 corresponds to the haemolytic time of 50% (min) obtained graphically from the haemolysis curve of each concentration of antioxidant sample. The Δt values were subsequently linearly correlated with the different sample concentrations, from which the concentration with the ability to delay haemolysis by 60 min (IC_{50} (60 min), µg/mL) was calculated.

3.4.3. Antimicrobial activity

3.4.3.1. Antibacterial activity

For the determination of antibacterial activity (Carocho et al., 2015), three Gram-positive bacteria (*Staphylococcus aureus* - ATCC 11632; *Bacillus cereus* - clinical isolate; and *Listeria monocytogenes* - ATCC 7973) and three Gram-negative bacteria (*Escherichia coli* - ATCC 25922; *Salmonella typhimurium* - ATCC 13311; and *Enterobacter cloacae* - ATCC 35030) were studied. The six strains of bacteria were acquired by the Mycology Laboratory of the Department of Plant Physiology of the Institute for Biological Research "Siniša Stanković" at the University of Belgrade in Serbia. Using the microdilution method, the minimum inhibitory concentrations (CMI) and minimum bactericidal concentration (CMB) were determined for each bacterium under each extract.

The concentration of the bacterial cultures was adjusted spectrophotometrically at 625 nm to a concentration of 1x105 CFU / mL. The diluted inoculants were grown in a solid medium to verify the absence of contamination. The different dilutions of the hydroethanolic extract were pipetted into a well plate containing 100 μL of triptych soy broth (TSB) and then 10 μL of inoculum was added. The microplates were incubated for 24 h at a temperature of 37 ºC. For the determination of the minimum inhibitory concentration (CMI - lower concentration that produced a significant inhibition (around 50%) of the growth of the bacterium in comparison with the positive control), 40 μL of *p*-iodonitrotetrazolium chloride (INT) (2 mg/ml) and incubated at 37 °C for 30 min. The minimum inhibitory concentrations obtained in the susceptibility test of several bacteria to the extract were also determined by a colorimetric microbial viability assay based on the reduction of the INT colour and compared with a positive control for each bacterial strain. The minimum bactericidal concentration (CMB) was determined by subculture in series, placing 10 μL of each well that showed no colour change in 100 μL of TSB. The lowest concentration that showed no growth after this subculture was considered to be the CMB. The compounds E211 (sodium benzoate) and E224 (potassium metabisulfite), both used as food additives, were the positive controls, and dimethyl sulfoxide (DMSO, 5%) used as a negative control. The results of CMI and CMB were expressed in mg per ml.

3.4.3.2. Antifungal activity

For the determination of antifungal activity (Carocho et al., 2015), six fungal strains (*Aspergillus fumigatus* - human isolate; *Aspergillus niger* - ATCC 6275; *Aspergillus versicolor* - ATCC 11730; *Penicillium funiculosum* - ATCC 36839; *Trichoderma viride* - IAM 5061; *Penicillium verrucosum* var. *Cyclopium* - food isolate) were used. The fungi were acquired by the Mycology Laboratory of the Department of Plant Physiology of the Institute for Biological Research "Siniša Stanković" at the University of Belgrade in Serbia and kept on agar malt (MA), stored at 4 °C and sub-cultivated once a year. month. The fungal spores were washed from the surface of the agar plates with 0.85% sterile saline solution containing 0.1% Tween 80 (*v/v*). The spore suspension was adjusted with a sterile saline solution to a concentration of approximately 1.0 × 105 in a final volume of 100 μL per well. The inoculums were stored at 4 ° C. The inoculum dilutions were grown in solid MA to verify the absence of inoculum contamination.

Through the successive dilution technique in 96-well microplates, the minimum inhibitory concentration (CMI) was determined. The extract sample was added to the malt medium with the fungal inoculum and the microplates were incubated for 72 h at a temperature of 28 ° C. The lowest concentrations without visible growth (using a binocular microscope) were defined as CMI. The minimum fungicidal concentrations (CMFs) were determined by subculture in series of 2 μL from each well that did not change colour, in microplates containing 100 μL of malt broth per well and later incubated for 72 h at 28 ° C. The lowest concentration without visible growth was defined as CMF, indicating 99.5% of death of the original inoculum. The compounds E211 (sodium benzoate) and E224 (potassium metabisulfite), both used as food additives, were the positive controls, and dimethyl sulfoxide (DMSO, 5%) used as a negative control. The results of CMI and CMF were expressed in mg per ml.

3.4.4. Cytotoxic and Hepatotoxic activity

For cytotoxic evaluation, the lyophilized hydroethanolic extract were dissolved in water in a concentration of 8 to 0.125 mg/mL, and the Sulforrodamine B (SRB) assay was peformed (Abreu et al., 2011). Four human tumour cell lines were used: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo), breast adenocarcinoma (MCF-7), and non-small cell lung cancer (NCI-H460). Each of the cell lines were plated in a 96-well plate, at an appropriate

density 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 deionised water and dried, and 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). Dose-response curves were obtained for each cell line tested and GI_{50} values were calculated, corresponding to the concentration of extract that inhibits 50% of cell proliferation.

For the *in vitro* assessment of hepatotoxic potential, the Sulforrodamine B (SRB) assay was also used (Abreu et al., 2011). The extracts were analysed in concentrations from 8 to 0.125 mg/ mL. Non-tumour culture from African green monkey (Vero) cells were prepared. The tissue as washed with HBSS (Hank's balanced salt solution) solution containing 100 U / mL of penicillin, 100 μg / mL of streptomycin and divided into explants. Some of these explants were placed in 25 cm2 tissue flasks, with DMEM medium supplemented with 10% fetal bovine serum, 2 mM non-essential amino acids and 100 U / mL penicillin, 100 mg / mL streptomycin and incubated at room temperature. 37 ºC with humidified atmosphere with 5% CO2. Cell growth was monitored, renewing the medium every two days. Subsequently, 190 μL of the cell culture with adequate density (1.0 x 10 4 cells / well) were transferred to the 96-well microplates of the cell culture and the analysis performed for each extract dilution following the SRB assay.

Dose-response curves were obtained for each cell line tested and GI_{50} values were calculated, corresponding to the concentration of extract that inhibits 50% of cell proliferation.

3.4.5. Phenolic compounds profile

For the chromatographic analysis of the phenolic compounds, the lyophilized hydroethanolic extracts of the seeds and pods were re-dissolved in an ethanol: water solution (20:80, *v/v*) in order to obtain a final solution of 10mg/mL. The chromatographic analysis was performed accordingly to the previously described by Bessada, Barreira, Barros, Ferreira, & Oliveira (2016), using a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a quaternary pump, an automatic injector (at 5 ºC), a degasser and a

column compartment with an automated thermostat was used. The detection of compounds was carried out with a diode detector (DAD), using the wavelengths of 280 nm, 330 nm and 370 nm. For the separation of the compounds, a Waters Spherisorb S3 ODS-2 C_{18} reverse phase column (4.6 x 150 mm, 3 µm; Milford, USA) was used, thermostatized at 35 °C. The mobile phase used was (A) formic acid / water (0.1%) and (B) acetonitrile. The elution gradient established was isocratic: 10% to 15% B up to 5 min, 15-20% B up to 5 min, 20-25% B 10 min, 25-35% B 10 min, 35-50% B 10 min and rebalancing the column for 10 min, with a flow rate of 0.5 mL / min being defined. The HPLC system described was also connected to a mass spectrometer (MS). The detection of MS was done using an Ion Trap Linear LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an ESI source (electrospray ionization source). The carrier gas used was nitrogen (50 psi). The system worked with a spray voltage of 5 kV, at an initial temperature of 325 ° C and capillary voltage of -20 V. The voltage of the tube lens offset was maintained at -66 V. The spectra were recorded in negative ion mode between 100 and 1500 *m/z*. The collision energy used was 35 (arbitrary units). Data were collected and analyzed using the Xcalibur® program (Thermo Finnigan, San Jose, CA, USA). For the identification of the compounds, the data obtained (retention times, UV-Vis spectra and mass spectra) were compared with data available in the literature and, when available, with the standards. For quantitative analysis, calibration curves were obtained by injection of standard solutions with known concentrations: apigenin-6-*C*-glucoside (y = 107025x + 61531, R^2 = 0,9989; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); *p*-coumaric acid (y = 301950x + 6966,7, R^2 = 1, LOD = 0.68 µg/mL; LOQ = 1.61 µg/mL); protocatechuic acid (y = 214168x + 27102; R^2 = 0.9997; LOD = 0.14 µg/mL; LOQ = 0.52 µg/mL); and quercetin-3-*O*-glucoside (y = 34843x – 160173, R^2 = 0.9998, LOD =17.01 µg/mL; LOQ = 51.54 µg/mL). based on UV-Vis signals and using the length of maximum absorption wave of each standard compound. In the cases where there was no availability of standards for the respective compounds, the quantification was done through calibration curves of compounds of the same phenolic group. The results were expressed in mg of compound per mg / g of extract.

3.5. Experimental design for extraction process optimization in the seeds samples of *B. orellana*

A central composite design (CCD) combining five-level of the independent variables X_1 (time, 1–50 min), X_2 (ultrasonic power, 5–500 W), and X_3 (ethanol proportion, 0–100%, *v/v*)

was implemented to optimize the extraction of bixin from *B. orellana* seeds using RSM (**Table 6**). The Design-Expert software, Version 11 (Stat-Ease, Inc., Minneapolis, USA) was used to generate the 20 experimental points of the CCD design by entering the factor ranges in terms of alphas (α = 1.68). The design included 8 factorial points, 6 axial or star points, and 6 replicated centre points. The 20 experimental runs were randomized to minimize the effects of unexpected variability.

Table 6. Natural and codded values of the independent variables used in the five-level CCD design implemented to optimize the ultrasound-assisted extraction of carotenoids.

Coded values	Natural values		
	Time (min)	Ultrasonic power (W)	Ethanol concentration (%)
-1.68	1	5	0
-1	11	105	20
0	25.5	253	50
+1	40	400	80
+1.68	50	500	100

3.5.1. Ultrasound-assisted extraction

The ultrasound-assisted extraction (UAE) was performed using an ultrasonic system (Ultrasonic homogenizer, model CY-500, Optic Ivymen Systen, Barcelona, Spain) equipped with a titanium probe, following a methodology precisely described (Rocha et al., 2020). The powdered seed samples (~1 g) were placed in beakers with 50 mL of solvent (ethanol/water mixtures) and processed according to the 20 experimental runs of the CCD design. Extractions were performed at 20 g/L at room temperature. The mixtures were then centrifuged at 4000*g* for 10 min and the supernatants were filtered through Whatman filter paper No. 4, and then re-filtered again into an amber vial (1.5 mL) through a 0.22 µm nylon filter for further HPLC analysis. The remaining extract was frozen, for further analysis (if needed).

3.5.2. Chromatographic analysis of carotenoids

For the chromatographic analysis of the carotenoids, Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a quaternary pump, an automatic injector (at 5 ºC), a degasser and a column compartment with an automated

thermostat was used. The detection of compounds was carried out with a diode detector (DAD), using the wavelengths of 400 nm, 430 nm and 470 nm. For the separation of the compounds, a Synergy 4u Hydro-RP (4.6 x 250 mm, 4 μm; Phenomenex, Canada) was used, thermostatized at 30 °C. The mobile phase used was (A) 100 % methanol:BHT (0.1%, 10mg/mL) and (B) 100% methyl tert-butyl ether(MTBE):BHT (0.1%, 10mg/mL). The elution gradient established was isocratic: 5% to 30% B up to 20 min, 30-40% B up to 10 min, 40-50% B 10 min, 50-60% B 10 min, 60-5% B 10 min and rebalancing the column for 10 min, with a flow rate of 0.5 mL / min being defined. Data were collected and analysed using the Xcalibur® program (Thermo Finnigan, San Jose, CA, USA). For the identification of bixin peak, the data obtained (retention time and UV-Vis spectra) was compared with the available standard compound. For quantitative analysis, a 7-level calibration curve of bixin (y = 26270x - 324834 R^2 = 0.9992; LOD = 30 μg/mL and LOQ = 91 μg/mL) was obtained injection of standard solutions with known concentrations based on UV-Vis signal and using the length of maximum absorption wave of the standard compound. The results were expressed in mg of compound per mg/g of extract.

3.5.3. Extraction process optimization by response surface methodology

The carotenoids content was the dependent (response) variable used in the extraction process optimization. The response surface models were fitted by means of least squares calculation using the following second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y is the dependent variable to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of the linear effect, b_{ij} is the coefficient of the interaction effect, b_{ii} is the coefficient of the quadratic effect, and n is the number of variables. Fitting procedures, coefficient estimates, and statistical analysis were performed using Design-Expert software. The analyses of variance (ANOVA) was used to assess the significance of the model generated and of all the terms that make up the model, as well as the lack-of-fit. The method for testing statistical significance was performed by calculating the p-value from the F-value, considering the existence of significance for p <0.05. Only the statistically significant terms were used in construction of the theoretical model. Coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}), and adequate

precision, were used to estimate the adequacy of the polynomial equation to the response (Albuquerque, Pinela, Barros, Oliveira, & Ferreira, 2020a). The lack-of-fit measures the quality of the model's fit to the experimental data. Thus, it must be non-significant (p >0.05).

3.6. Application of the carotenoid-based extract in a food product

The recipe chosen for the preparation of the pasta dough did not contain eggs. This decision was made based on the lack of egg-free food products in themarket, making it impossible for consumers who are allergic to egg proteins to consume these products. Once the colouring extract obtained from annatto, gives a yellow-orange colour to the final product, by incorporating an egg-free pasta dough with this extract, it is possible to obtain a product that is more appealing to the consumer (organoleptic characteristics similar to those of the commercial ones), without the toxic effects of egg proteins, and still a functionalized product with a colouring but also bioactive extract. Three samples were prepared, one commercial sample (fresh pasta obtained from a local supermarket,

Figure 12), one blank sample (without colorant extract), and one annatto sample (containing the colorant extract, 10 mg per 100g of flour recipe). The description of the recipe for pasta dough is described in **Table 7**.

Figure 12. Description of the commercial sample of pasta dough.

After the preparation of the three samples, a colour assessment was performed. To measure the colour of the samples, a colorimeter (model CR-400; Konica Minolta Sensing, Inc., Tokyo, Japan) attached to an adapter for granular materials (model CRA50) was used, following the methodology previously described by Roriz, Barros, Prieto, Morales, & Ferreira (2017). The value of the three-dimensional coordinates CIE L^*, a^*, and b^*, were obtained in a

system computerized with a type C illuminant and an 8 mm diameter diaphragm, for data processing, Spectra Magic Nx software (version CMS100W 2.03.0006, Konica Minolta, Japan). Regarding the coordinates obtained, *L** represents luminosity, *a** represents chromaticity in an axis from green (-) to red (+), and *b** represents chromaticity on an axis from blue (-) to yellow (+). For colour evaluation of the pasta dough, the readings were made at 3 different points for each portion, considering the average values. Previously and after analysis, the instrument used was calibrated with a white standard (Spectra Magic NX Instruction Manual, Konica Minolta Sensing, Inc., 2009, Japan). Finally, to assess the stability of the colorant extract after the application in the fresh pasta dough, all samples were cooked following the indications of the commercial sample. For that, 500 mL of water was placed until boiling, in which the dough was baked for 10 minutes until fully cooked.

Table 7. Formulation for preparation the pasta dough.

Ingredients	Mass (g)
Flour (100% wheat, type 55)	100 g
Olive oil	1 table spoon
Salt	1 tea spoon
Water	~ 50 mL

3.6.1. Shelf-life analysis

For shelf-life analysis, three independent samples of each developed pasta dough were stored individually, protected from light and humidity, at a refrigeration temperature of 4º C (mimic the conditions of refrigeration of the commercial pasta dough). The time chosen for the shelf time analysis was based on the reference for the commercial sample, which would be 30 days. Based on this information, samples were collected from the three dough formulations prepared at time 0 (day of confection), time 15 days and 30 days. For each time, the samples were collected, read the colour (as described above) and frozen for further analysis.

3.7. Statistical analysis

For each analysis with the dry samples and the respective hydroethanolic extracts, all tests were performed in triplicate, the results being expressed as mean values and standard deviation (SD). Significant differences between samples were analysed using Student's t-test with $\alpha = 0.05$, using IBM SPSS Statistics for Windows, Version 23.0. (IBM Corp., Armonk, New York, USA).

RESULTS AND DISCUSSION

4. Results and discussion

4.1. Nutritional profile and chemical composition of *B. orellana* seeds

The results regarding the nutritional value of *B. Orellana* seeds are described in **Table 8**. Carbohydrates were the major macronutrient found in the dry samples (68.1±0.1 g/100g dw), followed by fat (17.7±0.4 g/100g dw), proteins (9.1±0.1 g/100g dw), and finally ash (5.1±0.3 g/100g dw). The higher amounts of carbohydrates present in the samples, originated an energetic value of 469±1 kcal/100g dw.

Valério et al. (2015) in the study performed in annatto seeds residues from Brazil, described lower amounts of lipids and carbohydrates (2.23±0.11 g/100g dw and 42.2±0.69 g/100g dw, respectively), and consequently lower energetic value (234 kcal/100g dw). On the other hand, the protein content was higher (11.50±0.06 g/100g dw). The differences between these results could be explained by the type of studied sample, since the residue sample, studied by Valério, also contained impurities (pieces of branches of the annatto tree).

In another study performed by Prabhakara Rao et al. (2015) in *B. orellana* seeds from India, the nutritional value profile was completely different, presenting a higher percentage of proteins, followed by total lipids and ash (11.2±0.37 %, 6.3±0.1%, and 5.3 ± 0.2%, respectively). This abysmal difference, especially with regard to total lipids, could be due to a number of factors, among which fruit genotype, nutritional status of the plant, postharvest treatments, and storage conditions are among the most important ones.

Comparing the results obtained herein, with other economically important seeds, such as hempseed, pumpkin seed, chia, and sunflower, it is possible to observed that *B. orellana* seeds presented a very distinct nutritional profile. For instance, the proportion between fat and protein content is very similar in pumpkin seeds (*Cucurbita maxima* Linn) from Bangladesh, 36.7% and 34.56%, respectively, (Shahangir, 2015) and 21.31 and 23.45 mg/100 g dw, respectively (Amin et al., 2019), and from Tunisia, 31.57 ± 3.71 and 33.92 ± 3.16 mg/100 g of dry weight flour, respectively (Rezig, Chouaibi, Msaada, & Hamdi, 2012).

On the other hand, hempseed presents higher amounts of lipid fraction, followed by carbohydrates and protein (35.5, 27.6, and 24.8%, respectively) (Callaway, 2004), being this lipid fraction largely comprised by n3 and n6 fatty acids, giving it high biological importance (Crescente et al., 2018).

The same profile was verified in sunflower seed, containing 37.47% of crude fat, 18.72% of protein percent, 6.11% of carbohydrates, and 3.49% of ash (Anjum, Nadeem, Khan, & Hussain, 2012), and therefore there is a greater expression of total fat than in the seeds of *B. orellana*.

In chia seeds, the results are not so coherent, having described a higher percentage of carbohydrates in relation to total fat (42.1 and 30.7 g/100g, respectively, Kulczyński, Kobus-Cisowska, Taczanowski, Kmiecik, & Gramza-Michałowska, 2019) and, on the other hand, similar values of protein, fibre and fat (25.07, 28.96, and 26.24%, respectively, Segura-Campos, Ciau-Solís, Rosado-Rubio, Chel-Guerrero, & Betancur-Ancona, 2014) were described.

This comparison showed that, despite the lower content in fat, *B. orellana* seeds contains higher amounts of carbohydrates, that could be translated to a higher content in dietary fibre, and thus could be indicated for a balanced diet intake as a source of fibre compounds. Further studies need to be conducted to assess the content in fibre in *B. orellana* seeds.

Table 8. Nutritional value of *B. orellana* seeds (mean ± SD).

Nutritional value	Quantification
Fat (g/100 g dw)	17.7± 0.4
Proteins (g/100 g dw)	9.1±0.1
Ash (g/100 g dw)	5.1± 0.3
Total available carbohydrates (g/100 g dw)	68.1± 0.1
Energy contribution (kcal/100 g dw)	469±1

dw - dry weight.

Data regarding the sugar and organic acid composition in *B. orellana* seeds are presented in **Table 9**. A total amount of 3.78±0.01 g/100g dw of sugars were found, divided between to different sugars, sucrose and trehalose (1.13±0.01 g/100g dw and 1.65±0.01 g/100g dw, respectively). In the study conducted by Valério et al. (2015) in annatto seeds residues from Brazil, it was only identified one type of sugar compound, sucrose (11.78 ± 0.02 g/100g dw). The profile in sugar content could change accordingly with the genotype and maturation stage of the seed, which could explain the results obtained by Valério and herein.

In what concerns organic acids profile (**Table 9**), a total amount of 1.09±0.01 g/100g dw was found in the seeds, due to the presence of malic acid (0.90±0.04 g/100g dw), followed by oxalic acid (0.17±0.01 g/100g dw), and quinic acid (0.020±0.001 g/100g dw). Fumaric acid was found in trace amounts, which means that the compound was in a concentration below the limits of quantification (LOQ).

Table 9. Soluble sugars and organic acids composition of *B. orellana* seeds (mean ± SD).

Soluble sugars (g/ 100g dw)	Quantification
Sucrose	1.13 ± 0.01
Trehalose	1.65 ± 0.01
Sum	3.78 ± 0.01
Organic acids (g/100g dw)	
Oxalic acid	0.17±0.01
Quinic acid	0.020±0.001
Malic acid	0.90±0.04
Fumaric acid	tr
Sum	1.09±0.01

dw - dry weight; tr – trace amounts. Calibration curves for organic acids: oxalic acid (y = 9x106x + 45973, R^2 = 0.9901, LOD = 6.3 µg / mL; LOQ = 20.8 µg / mL); quinic acid (y = 610607x + 46061, R^2 = 0.99995, LOD = 11.3 µg / mL; LOQ = 37.6 µg / mL); malic acid acid (y = 950041x + 6255.6, R^2 = 0,9999, LOD = 11.3 µg / mL; LOQ = 37.6 µg / mL); and fumaric acid (y = 154862x + 1x106, R^2 = 0.9977, LOD = 42.5 µg / mL; LOQ = 141.7 µg / mL).

A total amount of 18 fatty acid compounds were found in *B. orellana* seeds lipid fraction (**Table 10**). The samples revealed higher amounts of monounsatured fatty acids (MUFA, 38.4±0.5 %), followed by saturated fatty acids (SFA, 36.1±0.5%), and polyunsaturated fatty acid (PUFA, 25.4±0.4%). The higher MUFA content is mainly due to the presence of the eicosenoic acid (C20:1, 34.03±0.27), while for SFA is due to the presence of the heptadecanoic acid (C17:0, 26.6±0.7%), and for PUFA the presence of α-linolenic acid (C18:3n3, 13.56±0.18%). Despite not being the major compound found, the bioactive properties of α-linolenic acid are very well-known and reported, such as the anti-inflammatory effects (Pauls et al., 2018) or even in the prevention of non-alcoholic fatty liver disease (Jeyapal et al., 2018).

As previously stated, hempseed presents higher amounts of lipids, comprised by n3 and n6 fatty acids, mainly linoleic acid and α-linolenic acid (Callaway, 2004; Crescente et al., 2018). On the other hand, pumpkin seeds fatty acid profile is mainly constituted by saturated and monounsaturated fatty acids (Dotto & Chacha, 2020), specially oleic acid, stearic acid, palmitic and linoleic acid (41, 27, 17, and 15 %, respectively) (Shahangir, 2015).

A different profile is obtained for chia seeds, where PUFA constituted between 72-83% of the fatty acid profile, mainly due to the presence of linolenic acid, linoleic acid, and in smaller amounts eicosadienoic acid (Kulczyński et al., 2019).

Table 10. Fatty acids and tocopherols composition of *B. orellana* seeds (mean ± SD).

Fatty acids (relative percentage, %)	
Capric acid (C10:0)	0.034±0.002
Undecylic acid (C11:0)	0.03±0.01
Tridecylic acid (C13:0)	0.07±0.01
Myristic acid (C14:0)	1.35±0.05
Myristoleic Acid (C14:1)	1.3±0.2
Pentadecenoic acid (C15:1)	1.9±0.1
Palmitoleic acid (C16:1)	0.56±0.07
Heptadecanoic acid (C17:0)	26.6±0.7
cis10-heptadanoic acid (C17:1)	0.64±0.02
Stearic acid acid (C18:0)	0.57±0.04
Linolenic acid (C18:2n6c)	7.9±0.1
γ-Linolenic acid (C18:3n6)	1.536±0.002
α-linolenic acid (C18:3n3)	13.56±0.18
Arachidic acid (C20 :0)	1.67±0.15
Eicosenoic acid (C20:1)	34.03±0.27
Heneicosanoic acid (C21:0)	5.69±0.16
Dihomo-gamma-linolenic acid (C20:3n6)	1.35±0.12
Eicosapentaenoic acid (C20:5n3)	0.97±0.05
SFA	**36.1±0.5**
MUFA	**38.4±0.5**
PUFA	**25.4±0.4**
Tocopherols (mg/100 g dw)	
α-Tocopherol	7.6±0.6
β-Tocopherol	6.1±0.7
δ-Tocopherol	0.39±0.03
Sum	14.01±1.24

dw- dry weigh. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

In **Table 10** is also described the composition of tocopherols in *B. orellana* seeds, having been found only three isomers of the vitamin E, in a total amount of 14.01±1.24 mg/100g dw. The α-tocopherol isomer was found in higher amounts (7.6±0.6 mg/100g dw), followed by β-tocopherol isomer (6.1±0.7 mg/100g dw) and δ-tocopherol isomer (0.39±0.03 mg/100g dw). There has been described that *B. orellana* contained high amounts of vitamin E (Rodrigues et al., 2007), in which the α-tocopherol isomer stands out, being an asset for this sample since this isoform of vitamin E has the highest biological activity, focusing on the direct

repair of oxidizing radicals, preventing the spread of lipid peroxidation and thereby preventing the spread of many diseases (Traber & Atkinson, 2007).

4.2. Bioactive properties evaluation and phenolic compounds profile of *B. orellana* seeds and pods

In the following sub-chapter, all tests were performed on the seeds but also on the pods of B. orellana. As previously stated in this thesis, the pods of B. orellana have no food purpose, however, being a by-product of the processing of seeds, it is interesting to study its composition in compounds with high added value (such as phenolic compounds), but also its bioactive properties, thus being able to consider its potential use in industrial level.

4.2.1. Antioxidant, antimicrobial, cytotoxic, and hepatocellular activities

Antioxidants are compounds that inhibit or prevent oxidative stress, through mechanisms such as inhibition of lipid peroxidation and/or elimination of free radicals, depending on the oxidative agent (Carocho, Morales, & Ferreira, 2018). There are several *in vitro* methods in order to identify the antioxidant capacity of a sample. In this work it was proposed to evaluate the antioxidant *in vitro* properties by inhibiting lipid peroxidation in pig brain tissues through the formation of reactive substances of thiobarbituric acid (TBARS) and inhibition of oxidative haemolysis in erythrocytes isolated from sheep blood (OxHLIA). The results of the antioxidant activity tests are shown in **Table 11** and are expressed as IC_{50} (µg/mL). From the obtained results for TBARS assay, pods presented lower IC_{50} values (86±6 µg/mL), corresponding to a higher antioxidant capacity. However, the same was not observed for OxHLIA assay, where the seeds hydroethanolic extracts presented lower IC_{50} values for Δt = 60 min and Δt = 120 min (5.9±0.3 and 15.4 ± 0.8 µg/mL, respectively). The difference in results obtained between the two tests of antioxidant activity, is further proof that the evaluation of this activity has to be done using various methodologies, because the type of compounds that make up the sample can greatly influence its activity and behaviour within the cells, thereby influencing their biological activity.

It should also be noted that both samples for OXHLIA 60 min and the seeds samples for OXHLIA 120 min, showed lower IC_{50} values than the ones presented by the positive control used, Trolox, further validating the use of these extracts as sources of compounds with high

capacity to inhibit the lipid peroxidation and with high haemolytic activity, and, therefore, with high potential to be used in pharmaceutical and nutraceutical applications.

Few studies have been carried out on the dry mass of these seeds in relation to its antioxidant activity. There are reports in the literature, of studies with other parts, using reducing power and DPPH assays. The most significant results regarding the DPPH assay, was that combining annatto extracts with ascorbic acid, decreased its antioxidant action (Abayomi, Adebayo, Bennett, Porter, & Shelly-Campbell, 2014). The authors also concluded that the antioxidant action may be due to the presence of tannins and flavonoid found. Annatto extract holds a few guarantee for use as a plant-derived antioxidant for therapeutic application. Its suitability as pharmaceutical colorant in any case requires careful evaluation as this may be influenced by its biological action as antioxidant (Abayomi et al., 2014).

Table 11. Antioxidant activity of the hydroethanolic of *B. orellana* seeds and pods (mean ± SD).

	TBARS (IC_{50} values, µg/mL)	**OxHLIA** (IC_{50} values, µg/mL)	
		Δt = 60 min	**Δt = 120 min**
Seeds	95 ± 4	5.9 ± 0.3	15.4 ± 0.8
Pods	86 ± 6	16 ± 1	41 ± 1
p-value	> 0.001	> 0.001	> 0.001
Trolox	5.4 ± 0.3	19.6 ± 0.7	41 ± 1

The IC_{50} values correspond to the concentration of the extract that reaches 50% of antioxidant activity.

Many studies have been carried out in recent years with the intention of identifying and exploring antimicrobial agents to combat microbial resistance. Thus, the seeds and pods of *B. orellana* were subjected to analysis of antimicrobial activity in eleven strains in total, in order to find the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC), **Table 12**. Before comparison results among the two samples studied, it is important to note that both presented lowest MIC, MBC, and MFC comparing to the two positive controls used, against all bacterial and fungal strains. This is of the upmost importance to validate the samples studied herein, as sources of antimicrobial compounds against foodborne pathogens.

Table 12. Antibacterial and antifungal activities of *B. orellana* seeds and pods (mean ± SD).

	Seeds		Pods		E211		E224	
Antibacterial activity (mg/mL)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus	0.12	0.25	0.12	0.25	0,5	0,5	2	4
Staphylococcus aureus	0.25	0.5	0.25	0.5	4	4	1	1
Listeria monocytogenes	0.25	0.5	0.25	0.5	1	2	0,5	1
Escherichia coli	0.15	0.25	0.15	0.25	1	2	0,5	1
Enterobacter cloacae	0.25	0.5	0.25	0.5	2	4	0,5	0,5
Salmonella typhimurium	0.25	0.5	0.25	0.5	1	2	1	1
Antifungal activity (mg/mL)	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus fumigatus	0.06	0.12	0.06	0.12	1	2	1	1
Aspergillus versicolor	0.06	0.12	0.06	0.12	2	2	1	1
Aspergillus níger	0.1	0.12	0.12	0.25	1	2	1	1
Penicillium funiculosum	0.1	0.12	0.06	0.12	1	2	0,5	0,5
Penicillium verrucosum var. cyclopium	0.06	0.12	0.04	0.06	2	4	1	1

MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration; MFC - minimum fungicidal concentration; Positive controls: E211 (sodium benzoate) and E224 (potassium metabisulfite).

Regarding the samples itself, the antibacterial and antifungal activity of both samples revealed the same MIC, MBC, and MFC values, except pods presented lowest MIC values against *Penicillium funiculosum* and *Penicillium verrucosum* var. *cyclopium* (0.06 and 0.04 mg/mL, respectively).

Finally, the last biological assays performed were the cytotoxicity and hepatotoxicity activities, being both assessed as the ability of the extract to inhibit 50% of cell growth (GI_{50}) and the results are described in **Table 13**. From the results obtained, the abysmal difference between the two samples is unquestionable, as it can be seen that the hydroethanolic extracts of fruit presented only the capacity to inhibit 50% of colorectal adenocarcinoma (CaCo) cell growth, with a GI_{50} of 177 ± 4 mg/mL. For the remaining four cell lines the pods hydroethanolic extracts did not show activity at the maximum tested concentration. Still in the pods samples, the concentration required to inhibit the growth of the non-tumour culture from African green monkey **(**VERO), was higher than for CaCo cell line, which indicates that this extract could damage normal cell lines before reaches the concentration needed to inhibit the growth of tumour cell lines, indicating its toxicity. Regarding the seeds hydroethanolic extracts, presented cytotoxic activity against all studied cell lines, gastric adenocarcinoma (AGS, 157±3 mg/mL), CaCo (167±10 mg/mL), breast adenocarcinoma (MCF-7, 287±27 mg/mL), and non-small cell lung cancer (NCI-H460, 237.9±9 mg/mL). It also showed Hepatotoxic activity in VERO cell

lines, presenting only a lower concentration than that obtained for MCF-7 cell line, revealing for that manner also some toxicity effects.

Table 13. Cytotoxic and hepatotoxic activities *B. orellana* seeds and pods (mean ± SD).

	Pods	Seeds
Citotoxic activity (mg/mL)		
Gastric adenocarcinoma (AGS)	>400	157 ± 3
Colorectal adenocarcinoma (CaCo)	177 ± 4	167 ± 10
Breast adenocarcinoma (MCF-7)	>400	287 ± 27
Non-small cell lung cancer (NCI-H460)	>400	237.9 ± 9
Hepatotoxic activity (mg/mL)		
Non-tumour culture from African green monkey **(**VERO)	180 ± 2	279 ± 22

>400 – did not show activity at the maximum tested concentration. Ellipticine GI_{50} values: 0.9±0.1 µg/mL (AGS), 0.8±0.1 µg/mL (CaCo), 1.020±0.004 µg/mL (MCF-7), and 0.6±0.1 µg/mL (Vero).

4.2.2. Phenolic compounds profile

The chromatographic results obtained by HPLC-DAD/ESI-MSn regarding the retention time, wavelengths of maximum absorption in the visible region, mass spectrum data, tentative of identification and quantification of the phenolic compounds present in the hydroethanolic extracts of *B. orellana* seeds and pods are present in **Table 14**.

Regarding seeds sample, 5 compounds were tentatively identified, one carboxylic acid, three *C*-glycosylated derivatives, and one coumarin compound. As far as the authors knowledge, there are no previous reports on the phenolic composition on the *B. orellana* seeds, so the identification of these compounds relied on literature descriptions. Peak **1f** ($[M-H]^-$ at *m/z* 291) was tentatively identified as brevifolin-carboxylic acid accordingly to the previously reported by Santos et al. (2013); peaks **2f**, **3f**, and **4f** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 723, being tentatively identified as *C*-glycosil derivative accordingly to the previously reported by Prakash, Baskaran, & Kudachikar (2019); and finally peak **5f** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 329, being tentatively identified as trihydroxy-octadecenoic acid accordingly to the previously reported by Llorent-Martinez, Spinola, Gouveia, & Castilho (2015). The three *C*-glycosil derivatives represented the major compounds found in the samples, while peak **5f** could not be quantified since no available similar standard compound was available. The low number of phenolic compounds found in this sample, may be due to the high fat content present in it. Since the deslipidification of this extract was not carried out, there

may have been an under quantification of the compounds. Further studies are needed to evaluate this hypothesis.

For the fruit samples, six compounds were tentatively identified, one phenolic acid, one carboxylic acid, and four flavonoids. Peak **1s**, presented a pseudomolecular ion $[M-H]^-$ at *m/z* 153 and a characteristic MS^2 fragment at *m/z* 119, being identified as protocatechuic acid, in comparison its retention time and UV spectra with the available standard compound. Peak **2s** (equal to peak **1f**) was tentatively identified as brevifolin-carboxylic acid following Santos et al. (2013). Peak **3s** was tentatively identified as apigenin-*C*-dihexoside, with a pseudomolecular ion at *m/z* 593, and a base peak at *m/z* 473 (typical fragmentation of di-*C*-glycosyl flavones) (Tahir et al., 2012). Peaks **4s** ($[M-H]^-$ at m/z 477), **5s** ($[M-H]^-$ at m/z 461), and **6s** ($[M-H]^-$ at m/z 461), presented a unique MS^2 fragment at *m/z* 301, 315, and 285, being tentatively identified as quercetin-*O*-glucuronide, isorhamnetin-*O*-deoxyhexoside, and kaempherol-O-glucuronide, respectively.

Overall, the profile between the samples was completely different (only one compound in common, peak 1f and 2s), However, the pods samples revealed higher quantities of phenolic compounds (2.83±0.01 mg/g extract), mainly due to the presence of protocatechuic acid (1.75±0.01 mg/g extract).

Table 14. Retention time (Tr), wavelengths of maximum absorption in the visible region (λmax), mass spectrum data, tentative of identification and quantification (mg / g extract) of the phenolic compounds in the hydroethanolic extracts of *B. orellana* seeds and pods (mean±SD).

Seeds						
Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification
1f	6.9	277	291	247(100),203(31),177(81)	Brevifolin-carboxylic acid	0.029±0.002
2f	31.99	324	723	677(199),451(34)	*C*-glycosyl derivative	0.02±0.01
3f	33.23	324	723	677(199),451(34)	*C*-glycosyl derivative	0.59±0.02
4f	34.64	324	713	677(100),451(22)	*C*-glycosyl derivative	0.31±0.03
5f	37.86	373	329	311(61),293(34),229(100),211(59),171(40)	Trihydroxy-octadecenoic acid	nq
					Total Phenolic compounds	**0.959±0.002**
Pods						
Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification
1s	5.06	261	153	109(100)	Protocatechuic acid	1.75±0.01
2s	6.9	277	291	247(100),203(31),177(81)	Brevifolin-carboxylic acid	0.81±0.01
3s	9.14	335	593	503(35),473(100),383(12),353(21)	Apigenin-*C*-dihexoside	0.26±0.02
4s	21.76	326	477	301(100)	Quercetin-*O*-glucuronide	tr
5s	24.35	346	461	315(100)	Isorhamnetin-*O*-deoxyhexoside	tr
6s	25.84	329	461	285(100)	Kaempherol-*O*-glucuronide	tr
					Total Phenolic compounds	**2.83±0.01**

tr – trace amounts; nq – not quantifiable. Standard calibration curves used for quantification: apigenin-6-*C*-glucoside ($y = 107025x + 61531$, $R^2 = 0{,}9989$; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL, peaks 2f, 3f, 4f, and 3s); *p*-coumaric acid ($y = 301950x + 6966{,}7$, $R^2 = 1$, LOD = 0.68 µg/mL; LOQ = 1.61 µg/mL, peaks 1f and 2s); protocatechuic acid ($y = 214168x + 27102$; $R^2 = 0.9997$; LOD = 0.14 µg/mL; LOQ = 0.52 µg/mL, peak 1s); and quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$, LOD =17.01 µg/mL; LOQ = 51.54 µg/mL, peaks 4s, 5s, and 6s).

4.3. Evaluation of the Extraction process optimization by response surface methodology

4.3.1. Chromatographic identification of bixin by HPLC-DAD

The chromatographic results obtained by HPLC-DAD regarding the retention time, wavelengths of maximum absorption in the visible region, and identification of bixin in the hydroethanolic extracts of *B. orellana* seeds are present in **Table 15**. An exemplificative chromatogram profile is present in **Figure 13**. The hydroethanolic extracts of *B. orellana* seeds presented only one peak ate the retention time of 7 min, with UV spectra with a maximum absorption at 456 nm, coherent with results obtained for the commercial standard compound of bixin, allowing the identification of the compound as bixin. This results are in accordance with the reported by several authors, that stated that more than 80% of the carotenoid composition of *B. orellana* is bixin (C. F. O. Franco et al., 2002; Satyanarayana et al., 2003; Vilar et al., 2014)

Table 15. Retention time (Tr), wavelengths of maximum absorption in the visible region (λmax) and identification of bixin in hydroethanolic extracts of *B. orellana* seeds.

Peak	Rt (min)	λmax (nm)	Identification
1	7	456	Bixin

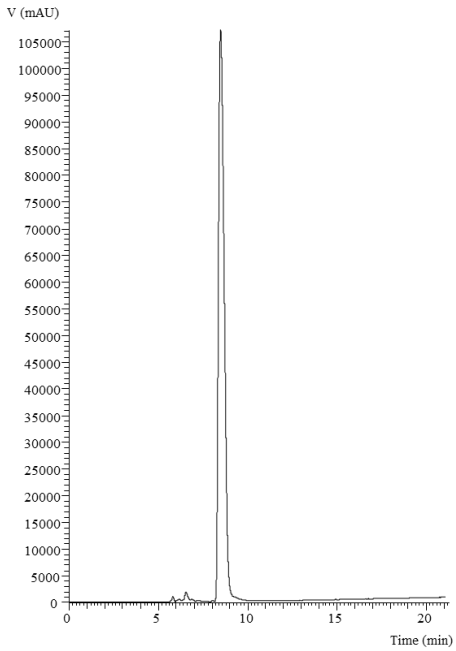

Figure 13. Exemplificative chromatogram profile recorded at 430 nm.

4.3.2. Evaluation of the extraction process optimization by response surface methodology

RMS is a statistical tool suitable for optimization of extraction processes involving one or more response variables and allows to determine the optimal processing conditions with a reduced number of experimental trials, when compared with conventional one-factor-at-a-time approaches, which do not allow the evaluation of interactions between variables (Pinela et al., 2016). As presented in **Table 16**, the carotenoids content ranged from 0.24 to 36.46 mg/g extract with the runs 11 and 14, which combined low levels of ultrasonic power and high levels of ethanol concentration with medium conditions of the other two variables, respectively.

Table 16. Experimental responses obtained under the extraction conditions defined by the CCD design matrix for the citric acid content.

Runs	Space type	Experimental design matrix			Experimental responses
		Time (min)	Ultrasonic power (W)	Ethanol concentration (%)	Carotenoids content (mg/g extract)
1	Factorial	11	105	20	0.64
2	Factorial	40	105	20	0.44
3	Factorial	11	400	20	0.76
4	Factorial	40	400	20	0.75
5	Factorial	11	105	80	7.13
6	Factorial	40	105	80	7.61
7	Factorial	11	400	80	32.87
8	Factorial	40	400	80	35.69
9	Axial	1	253	50	0.91
10	Axial	50	253	50	0.72
11	Axial	25.5	5	50	0.24
12	Axial	25.5	500	50	21.23
13	Axial	25.5	253	0	0.75
14	Axial	25.5	253	100	36.46
15	Center	25.5	253	50	3.70
16	Center	25.5	253	50	0.74
17	Center	25.5	253	50	1.11
18	Center	25.5	253	50	1.34
19	Center	25.5	253	50	0.98
20	Center	25.5	253	50	0.78

To develop polynomial models capable of predicting the effects of the process independent variables on a given response, it is necessary to assess the accuracy of their fitting to the experimental data. In this study, the response data in **Table 16** were fitted to the Eq. (1) polynomial model, but not all parameters were used in the models construction since some

coefficients were non-significant (*ns*) (**Table 17**). The parametric values presented in **Table 17** represent the expected change in response per unit change in factor value when all remaining factors are held constant. The higher the parametric value, the more significant is the weight of the variable. A synergistic effect is indicated by a positive sign, while a negative sign indicates an antagonism (Pinela et al., 2019). The parametric values were used to construct the Eq. (2) model, which illustrates the complexity of the extraction trends. The theoretical model presented a *ns* lack-of-fit and an adequate precision greater than 68, which indicates that the model equation adequately describe the effects of the independent variables on the response (Iberahim, Sethupathi, Goh, Bashir, & Ahmad, 2019). The coefficients R^2 and R^2_{adj} were greater than 0.99 in both cases (**Table 17**), thus indicating that the response variability can be explained by the extraction variables. The Eq. (2) model was statistically validated and used to predict optimal processing conditions.

Table 17. Parametric values of the polynomial equation and statistical information of the model fitting procedure. Parametric superscripted 1, 2 and 3 stand for the independent variables time, ultrasonic power and ethanol concentration, respectively.

Effect		Carotenoids content
Intercept	b_0	1.3±0.3
Linear effects	b_1	ns
	b_2	6.6±0.3
	b_3	10.3±0.3
Quadratic effects	b_{11}	ns
	b_{22}	3.3±0.3
	b_{33}	6.1±0.3
Interactive effects	b_{12}	ns
	b_{13}	6.7±0.3
	b_{23}	ns
Statistics		
Model F-value		659.92
Lack of Fit		ns
R^2		0.9958
R^2_{adj}		0.9943
Adequate Precision		68.77

R^2: coefficient of determination; *R^2_{ajd}*: adjusted coefficient of determination.

$$Y_{carotenoids} = 1.3 - 6.6P + 10.3S + 6.7P^2 + 6.1S^2 + 6.7PS \quad (2)$$

Certain features regarding the overall effects of the independent variables on the extraction of carotenoids from achiote can be inferred from the complexity of the Eq. (2) polynomial model. The intercept corresponds to the overall average response of all the runs of the RSM design. The extraction of carotenoids was affected mostly through positive linear and quadratic effects of the ethanol proportion, which means that by increasing the ethanol proportion, increases the amount of carotenoids extracted (**Figure 14** and **Figure** 15). This variable also interacted positively with the time. The ultrasonic power ranked second, also causing linear and quadratic effects in the extraction. Overall, the effect of the independent variables on extraction can be ordered as follows: *S* > *P* > *t*. The observed interactive effect also supports the used of RSM as an optimization tool.

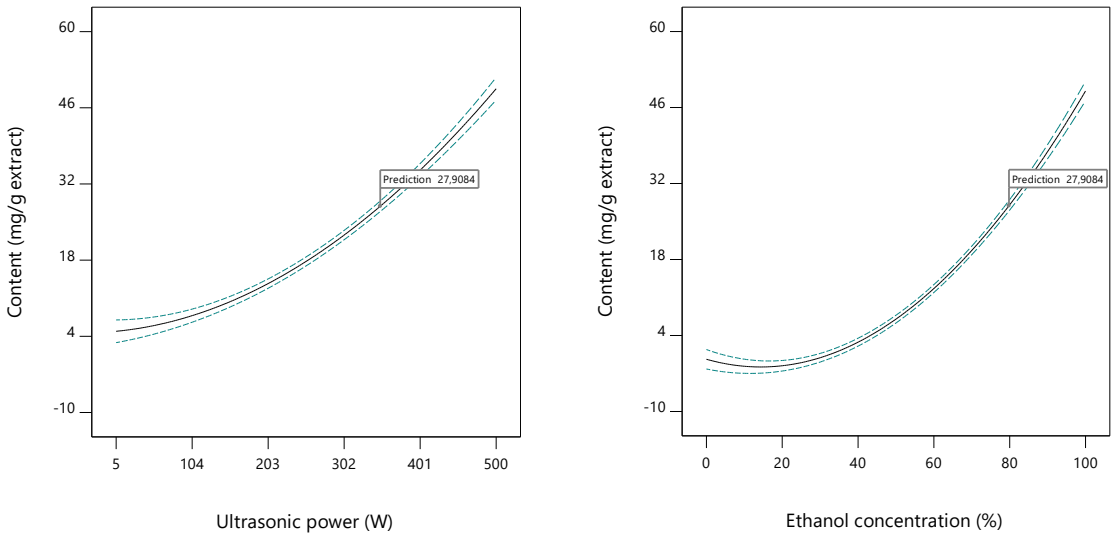

Figure 14. 2D response graphs for the effects of the independent variables ultrasonic power and ethanol concentration on the carotenoids content (mg/g extract) extracted from achiote. In each graph, the excluded variables were fixed at their optimal value.

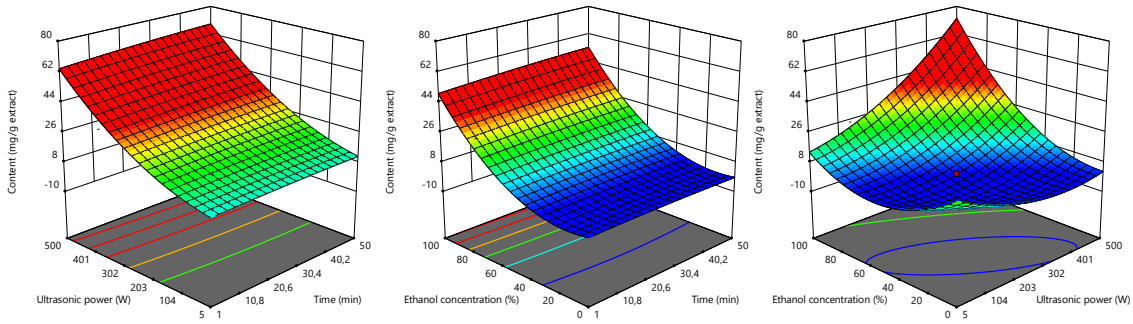

Figure 15. Response surface graphs for the combined effects of the independent variables on the carotenoids content (mg/g extract) obtained from *B. Orellana*. In each graph, the excluded variable was positioned at its optimal value.

Table 4 shows the UAE conditions that maximize the recovery of carotenoids from *B. orellana*. When applying experimentally the optimal processing conditions (sonication at 348 W for 6 min, using 79 % (*v/v*) ethanol as extraction solvent), it was possible to obtain 27.1 mg of bixin per g of extract, a value similar to that predicted by the model. This result experimentally validates the model developed for the extraction process.

A previous study, performed by Yolmeh, Habibi Najafi, & Farhoosh, (2014) in *B. orellana* seeds from India, the authors also optimize the extraction of bixin using ultrasound methodologies. The optimal processing obtained was an extraction time of 7.25 min, with a solvent (chloroform) of 14%, temperature of 72.7°C, and duty cycle of 0.8. The concentration of bixin in the optimal point, was given in absorbance value (0.865%), so it is not comparable with our results (Yolmeh et al., 2014).

Comparing with the results obtained by Yolmeh et al. (2014), the herein obtained are more optimistic, since it uses lower time of extractions, greener solvents (despite in higher concentrations, ethanol can be used for food application), and high extraction yields.

Other type of optimization methodology has also been applied for the optimal extraction of bixin, such as microwave assisted extraction. Quiroz et al. (2019) reported similar extraction time (5 min), but higher amounts of ethanol (96%) in the optimal processing conditions for bixin extraction in *B. orellana* seeds from Colombia.

Table 18. Optimal processing conditions that maximize the extraction of carotenoids from *B. orellana* and model-predicted response optimum.

Optimal extraction conditions			Optimum	
Time (min)	Ultrasonic power (W)	Ethanol concentration (%)	Model-predicted value	Experimental value
5.99	347.96	79.46	27.5±0.4 mg/g extract	27.1±0.2 mg/g extract

4.4. Application study in pasta dough

Food colour, from the consumer's perception, is many times associated with the flavour, safety, and nutritional value of the product. With the growing awareness of the public about synthetic colorants and their hazard long term effects on human health, natural pigments arise as safer response to the public's demand (Sigurdson, Tang, & Giusti, 2017).

Anthocyanins, carotenoids, betalains, and chlorophylls, are the main groups of natural colorants used and intensively studied for their application in food formulations. In fact, anthocyanins group, capable of providing colours that goes from the red, purple, blue, and green, depending on the medium pH, are generally the most studied group. There are many studies compromising the application of anthocyanin rich extracts in bakery products, such as the cyanidin-3-*O*-glucoside rich extract from *Arbutus unedo* L. in wafers (López et al., 2019), pomegranate epicarp extracts (da Silva Veloso et al., 2020), *Rubus ulmifolius Schott* (Da Silva et al., 2019), and *jabuticaba* epicarp (Albuquerque, Pinela, Barros, Oliveira, & Ferreira, 2020b). Natural anthocyanin rich extract from edible flowers has also been incorporated in dairy products (Pires et al., 2018) and soft drinks formulations have also been developed from purple and red potatoes anthocyanin rich-extract (Sampaio et al., 2020) and from *Lonicera caerulea* L. var. *Kamtschatica* (Molina et al., 2019).

However, studies of carotenoid-based extract application in food matrices are scarce, and normally involved commercial carotenoids, to verify the chemical behaviour in the food product, as in the study of different formulations of curcumin (E100) in a hydrophilic matrix (yogurt) (Almeida et al., 2018). Since carotenoids are lipophilic compounds, its application is limited by the chemical structure of the food product, and normally involves stabilization procedures, as spray-drying processes, to make the compounds more compatible with the food product.

The yellow colour provided by carotenoids, also allows the replacement of the egg in certain food products (where the egg is not a crucial part of the product), in an attempt to minimize the allergic effects that some consumers have to the egg proteins. With this, it would

be possible to have an organically very similar product to the initial one, but without allergic effects for the final consumer.

With this aim in mind, an eggless pasta dough was developed, incorporated with the carotenoid-rich extract of *B. orellana* (**Figure 16**). This final product was then compared to a control, with no added carotenoid-rich extract, and a commercial pasta dough.

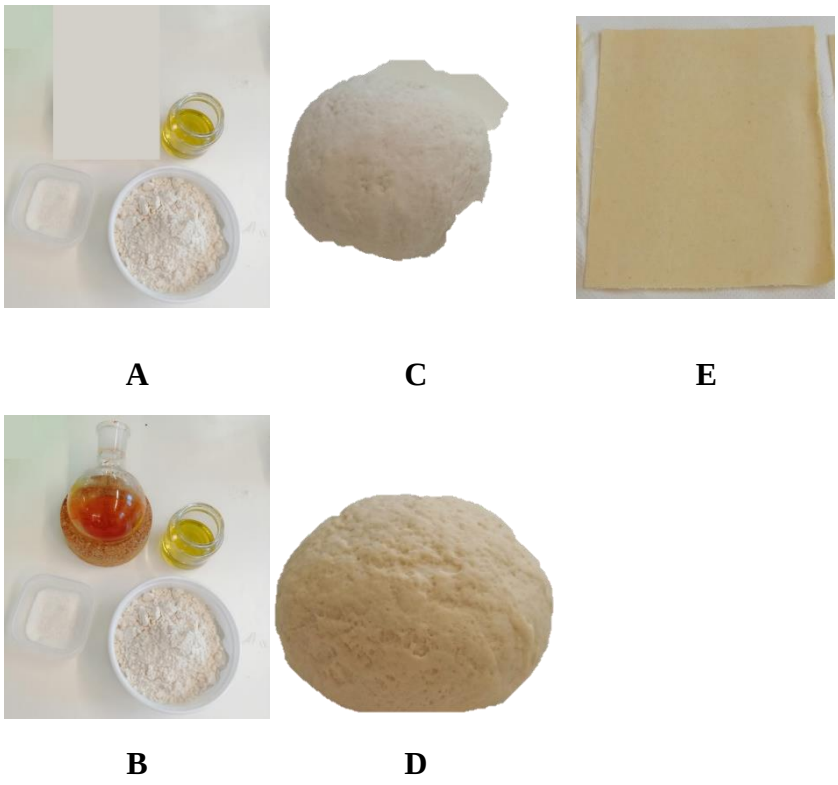

Figure 16. Ingredients for the preparation of the control and carotenoid-based enriched pasta dough (**A** and **B**, respectively) and the corresponding final fresh product (**C** and **D**, respectively). Commercial fresh pasta dough (**E**).

As it is possible to verify in **Figure 16**, the obtained final fresh product incorporated with the *B. orellana* extract (**Figure 16 - D**), presented a very appealing yellow colour, very similar to the commercial sample (**Figure 16 – E**).

The results of the colorimetric evaluation are described in **Table 19**, with the *L* a* b ** parameters obtained for the commercial pasta dough, but also for the two developed products (control and carotenoid-based extract), and also the exemplification of the obtained colour converted into RGB colour system. As expected, the control sample present the most different CIELab parameters, with lowest luminosity (62.5±0.6) and a *b** chromaticity pending more to

the blue, as expected. The final pasta dough presented, to the naked eye, a very subtle shade of grey, that when converted to the RGB colour system, and represented in a computer screen (opacity effect), turns the grey colour, darker. Comparing the commercial and the carotenoid-base extract pasta dough, both presented high *L** (86.1±2.3 and 79.8±0.3, respectively), and almost *b** chromaticity to the yellow colour (39.9±0.7 and 39.8±0.6, respectively). The carotenoid-base extract pasta dough presented an *a** chromaticity a little bit higher, pending to the red spectrum, obtaining a darker yellow.

Table 19. Physical parameter of (CIE *L* a* b **) at Time 0 (confection day) of the commercial, control, and carotenoid-based enriched pasta dough.

	Commercial	Control	Carotenoid-based
L*	86.1 ± 2.3^{a}	62.5 ± 0.6^{c}	79.8 ± 0.3^{b}
a*	-1.6 ± 0.7^{c}	0.36 ± 0.01^{b}	3.2 ± 0.2^{a}
b*	39.9 ± 0.7^{a}	0.38 ± 0.01^{b}	39.8 ± 0.6^{a}
RGB colour system			

4.4.1. Shelf life analysis

The addition of additive compounds to foodstuff, provided a considerable improvement of the organoleptic characteristics, as also increased the shelf life of the products, with microbiological quality and security (Martins, Roriz, Morales, Barros, & Ferreira, 2016). The sensorial attractiveness (visual perception, colour and smell) is of the upmost importance for any food product succeed in the global market. On the other hand, modern food processing and packaging techniques have been also developed to improve the shelf life of natural products, contributing to reduce the use of preserving compounds in the food products (López et al., 2019; Martins et al., 2016).

This duality of ideas, which, in one hand, the use of natural preserving additives is promoted, on the other, the processing of the foods samples or additives, can already be a treatment to prolong the shelf life of the product (without the need of adding additives). That is why it is urgent to find a balance between the additives that are added to the food and the type of processing that can be done to prolong the shelf life and thus obtain an attractive and safe product for the final consumer.

In the present work, and as mentioned above, it was aimed to study the shelf life of the developed pasta dough samples at 0, 15, and 30 days. Based on **Figure 17**, it is possible to

observe that at shelf time 15 days, all samples showed contamination, except for the commercial pasta dough.

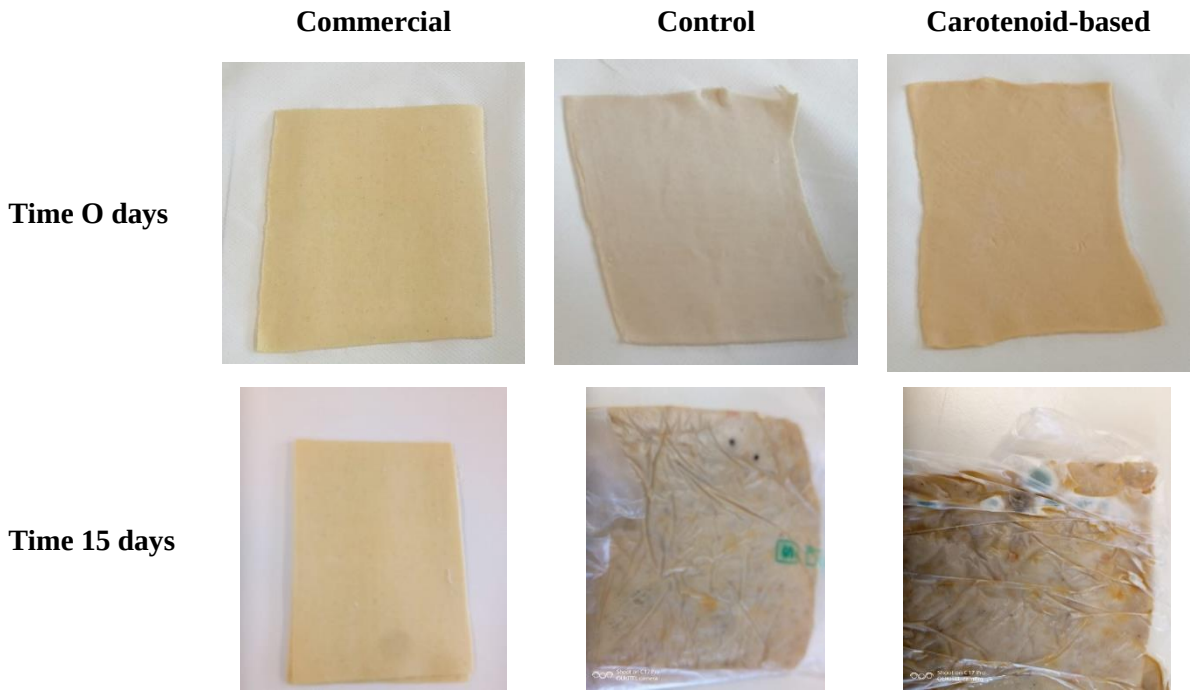

Figure 17. Schematic representation of the three samples of pasta dough in Time 0 (confection day) and Time 15 days.

With these negative results, the shelf life assessment could not be continued, and also for reasons of time constriction, it was also not possible to repeat the assays. However, there are a number of conclusions that can be drawn from these results and that can serve as a basis for future work.

The first conclusion to be taken would be the use of microbial load studies to certify the shelf life of the products developed. The microbial load studies would have to be carried out not only on the products developed, but more importantly, on the carotenoid-based extract used for incorporation. With this, we would have been possibility to understand if a potential contamination would be the result of the natural extract used or of the processing steps when preparing the pasta dough (manipulation of the pasta dough and use of inadequate equipment).

Then, a possible thermal treatment of the extract would also be necessary, such as pasteurization or lyophilisation (under very controlled conditions of temperature and pressure), to reduce the microbial load, without loss of the colouring capacity.

Finally, the packaging of the samples could also be carried out under other conditions that would better mimic the conditions of the commercial pasta dough.

CONCLUSION AND FUTURE PERSPECTIVES

5. Conclusions and future perspectives

The present work intended to deepen the study of the nutritional and chemical profile and bioactive potential of *B. orellana* seeds. Furthermore, these seeds, very rich in a carotenoid compound, bixin, were treated to maximize the extraction of this compound by ultrasound-assisted technologies, combined with RSM. Finally, the carotenoid-based extract was applied in eggless pasta dough, to achieve the characteristic yellow colour. As an externality of this work, the pods of this plant (bio-residues resulting from the processing of seeds) were also studied, in relation to its bioactive properties and profile in phenolic compounds, thus assessing its possible use in pharmaceutical and nutraceutical products.

With the study of nutritional characterization of the seeds, which included the determination of protein and lipid content, ash content, carbohydrates and energy value, it could be seen that the most abundant macronutrients found were carbohydrates, followed by fat, proteins, and ash. Sucrose and trehalose were the only sugars found; while for organic acids, malic, oxalic, quinic, and fumaric acids were found. Regarding the lipid fraction, monounsaturated fatty acids were found in higher amounts, mainly due to the presence of heneicosanoic acid, followed by saturated fatty acids, mainly the heptadecanoic acid. The tocopherols profile registered the presence of three isoforms, α-, β-, and δ-, where α- presented as the most abundant.

With regard to the antioxidant assays conducted in the present study in the hydroethanolic extracts of the seeds and pods of *B. orellana*, it was possible to observed that the pods sample presented lower IC_{50} values, corresponding to a higher antioxidant capacity. However, the same was not observed for OxHLIA assay, where the seeds hydroethanolic extracts presented lower IC_{50} values for $\Delta t = 60$ min and $\Delta t = 120$ min. The difference in results obtained between the two tests of antioxidant activity, is further proof that the evaluation of this activity has to be done using various methodologies, because the type of compounds that make up the sample can greatly influence its activity and behaviour within the cells, thereby influencing their biological activity. It should also be noted that both samples showed lower IC_{50} values than the ones presented by the positive control used, Trolox, further validating the use of these extracts as sources of compounds with high capacity to inhibit the lipid peroxidation and with high haemolytic activity, and, therefore, with high potential to be used in pharmaceutical and nutraceutical applications. For the antimicrobial assay, it is important to note that both samples presented lower MIC, MBC, and MFC comparing to the two positive

controls used, against all bacterial and fungal strains. This is of the upmost importance to validate the samples studied herein, as sources of antimicrobial compounds against foodborne pathogens. Finally, for the cytotoxic activity assays, the fruit hydroethanolic extracts did not show very promising results, revealing only the capacity to inhibit the growth of the colorectal adenocarcinoma (CaCo) cell line, and in a lower concentration than the one required to inhibit the non-tumour culture from African green monkey **(**VERO) cell line, indicating some toxicity. The seeds hydroethanolic extract revealed the capacity to inhibit all the tumour cell lines studied, but also with some toxicity associated. The phenolic compounds profile between the pods and seed samples was completely different (only one compound in common); However, the pods samples revealed higher quantities of phenolic compounds, mainly due to the presence of protocatechuic acid.

To maximize the recovery of bixin from *B. orellana*, the optimal processing conditions were sonication at 348 W for 6 min, using 79 % (*v/v*) ethanol as extraction solvent, being possible to obtain 27.1 mg of bixin per g of extract, a value similar to that predicted by the model. This result experimentally validates the model developed for the extraction process.

Finally, the application study allowed to obtain a final eggless pasta dough with a very similar yellow colour when compared to the commercial sample. Nevertheless, the shelf life assessment of the final developed products and commercial samples, was not possible to realize, due to contamination of the food products at time 15 days.

Overall, this study allowed to present innovative results in relation to the nutritional, chemical and bioactive properties of the seeds, and as an externality the great potential of the pods regarding its biological activity. Considering the good results obtained in the optimization procedure, combined with the optimistically colour obtained in the pasta dough incorporated with the carotenoid-based extract, it is worth mentioning the sustainable way in which this extract can be obtained, but also the great potential to be applied in innovative food products.

AS FUTURE PERSPECTIVES OF THIS WORK WOULD BE IMPORTANT TO STUDY THE:

1. Bioactive activity of the extracts obtained in the optimal processing conditions by ultrasound assisted extraction, to validate its use as colorant extract to be incorporated in a food matrix, but also to infer some biological activity and add value for future applications;

2. Performed microbial load studies to certify the shelf life of the products developed. The microbial load studies would have to be performed in the final products but also in the flour used to prepare the pasta dough and in the carotenoid-based extract;
3. Study the influence of a thermal treatment in the carotenoid-based such as pasteurization or lyophilisation (under very controlled conditions of temperature and pressure), to reduce the microbial load, without loss of the colouring capacity;
4. Study the influence of the packaging of the samples for the prevention of the degradation of the final products; as also perform the study of some physical parameters, such as, texture, and the nutritional profile of the developed products through the shelf-life analysis;
5. Study the population acceptance through a sensory analysis of the elaborated products;
6. Test other food matrices to applied the carotenoid-base extract.

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