Fig tree bioresidues as natural preservatives in fig-based products

Yosra Zbiss

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Supervised by

Doctor Lillian Barros

Doctor Sandrina Heleno

Doctor Feriel Rezouga

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Index

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List of figures

List of tables

List of abbreviations

AAPH: 2,2′-Azobis(2-methylpropionamidine) dihydrochloride

ADI : Applicable daily intake

AGS : Gastric adenocarcinoma cell line

AMPs : Antimicrobial proteins

BBD : Box-Behnken Design

BHA : Butylated hydroxyanisole

BHT : Butylated hydroxy toulene

Bn : Bourjassote noire

CAA : Cellular antioxidant activity

CaCo2: Colorectal adenocarcinoma cell line

CFU : Colony forming unit

d : Day

Da : Dauphine

DCFH : 2',7' Dichlorohydrofluorescein

DCF : Dichlorofluorescin

DM : Dynamic maceration

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl sulfoxide

DW : Dry weight

EC_{50} : Half maximal effective concentration

EMM : Estimated marginal means

FAO : Food and Agriculture Organization

FBS : Fetal Bovine Serum

GC-FID: Gas chromatography with flame ionization detection

GI_{50} : Growth inhibition by 50%

HBSS: Hank's Balanced Salt Solution

HPLC: High performance liquid chromatography

HPLC-DAD/ESI-MS: High performance liquid chromatography coupled with diode array detection with electrospray ionization and mass spectrometry

HPLC-RI: High performance liquid chromatography coupled to a refractive index detector

I : Incorporation

IC_{50} : Half maximum inhibitory concentration

ICE : International Commission on Illumination

ISA : Iron sulphite agar

ISO : International Organization for Standardization

La : Longue d'aout

LOQ : Limit of quantification

LPS : Liposaccharide solution

Ma : Marseille

MBC : Minimum bactericidal concentration

MCF-7: Breast adenocarcinoma cell line

MDA : Malondialdehyde

MHB : Mueller-Hinton Broth

MBC : Minimum bactericidal concentration

MFC : Minimum fungicidial concentration

MIC : Minimum inhibitory concentration

MUFA: Monounsaturated fatty acids

NADH: Nicotinamide Adenine Dinucleotide

NO : Nitric oxide

Pa : Pastilière

PBMC: Peripheral blood mononuclear cells

PCA : Plate Count Agar

PLP2 : Pig liver cells

PUFA : Polyunsaturated fatty acids

ROS : Reactive oxygen species

rpm : Round per minute

RSM : Response surface methodology

S : Solvent

SFA : Saturated fatty acids

SRB : Sulforhodamine-B

ST : Storage Time

TBA : Thiobarbituric acid

TBARS: Thiobarbituric acid reactive substance

TPA : Texture profile analysis

TP : Total phenols

TSB : Tryptic Soy Broth

UAE : Ultrasound assisted extraction

VRBLA: Violet Red Bile Lactose Agar

Abstract

The food processing and storage became imperative to provide the quotidian needs of humans. Therefore, the use of artificial additives started to be mandatory to decrease or postpone the nutritional value losses due to chemical, microbiological and enzymatic changes, thus maintaining the characteristics of the processed food for a longer period.

Nevertheless, the use of synthetic additives has been highly studied due to several scientific alerts about their harmful effects that can include allergic problems, asthma, among others. This situation leads to a deep search for natural alternatives able to serve the same purpose. To do so, food industries started this mission through the extraction of natural compounds from microorganisms, animals, plants and agri-food bio-waste. Indeed, among the food industry, several sectors produce huge amounts of bio residues. Figs, for instance, are known for their richness in minerals and nutrients, which leads to their high consumption, thus generating several fig bio-wastes, which include fig leaves that could be useful for the recovery of bioactive compounds, such phenolic compounds, to act as natural preservatives, hence, valorizing fig-waste and promoting circular economy.

Therefore, in the present work, fig leaves from five different varieties, namely Dauphine (Da), Longue d'Aout (La), Bourjassote Noire (Bn), Marseille (Ma) and Pasteliere (Pa) were screened to determine their phenolic content by HPLC-DAD-ESI-MS as well as their bioactivities, namely antioxidant through the TBARS and CAA assays, antimicrobial by the microdilution method, anti-inflammatory using RAW 264.7 mouse macrophage cell line and cytotoxic through the sulforhodamine B assay. The extraction of total phenols was optimized through response surface methodology and carried out by dynamic maceration and ultrasound assisted extraction, being their determination assessed by the Folin ciocalteu assay.

Finally, the extracts obtained from the optimal conditions of ultrasound assisted extraction, used as a cost effective method, were mixed together searching for possible synergistic effects. When the leaves acted together, the antioxidant activity was higher, since the lowest EC_{50} recorded for Bn was 0.23±0.01 mg/mL, and for the mixture an EC_{50} of 0.12 ±0.01 mg/mL was achieved. Moreover, the mixture also revealed promising results regarding the antimicrobial activity by acting against all the tested bacteria and fungi strains. It was important to showcase that the mixture revealed activity against *Pseudomonas aeruginosa* with a MIC of 10 mg/mL.

In addition, as the extracts showed no toxicity against normal cell line PLP2 with a GI_{50}>400 μg/mL, a concentration of 10mg/mL was incorporated, after the cooking process, at

80°C and 50°C in two formulations of fig jams (extracts with honey and extracts without honey) to determine the efficiency of the developed extracts, acting as natural preservatives, and their stability in the final products. The fig jams were subjected to the evaluation of physical parameters (color, texture, a_w and pH), nutritional (moisture, ash, fat using soxhlet, protein by the kjeldahl method, carbohydrates and energy) and chemical profiles as the free sugars by HPLC-RI and fatty acids through the GC-FID, and microbial load following ISO procedures. The low-sugar fig jam incorporated with natural fig leaf preservatives revealed that the incorporation did not change the overall appearance of the jams. Regarding the nutritional and chemical properties, the formulas presented low sugar, low protein content and high amount of carbohydrates, low fatty acids content with palmitic acid as the major compound. Furthermore, the different temperatures of incorporation showed no discernible changes over time, implying that the molecules of interest present in the extracts are not thermolabile.

Key words: Fig-bio-residues, natural preservatives, fig jam, circular economy.

Resumo

O processamento e armazenamento de alimentos tornou-se imperativo para suprir as necessidades quotidianas do ser humano. Assim, o uso de aditivos artificiais passou a ser obrigatório para diminuir as perdas de valor nutricional por alterações químicas, microbiológicas e enzimáticas, mantendo assim as características do alimento processado por mais tempo.

No entanto, o uso de aditivos artificiais tem sido bastante avaliado devido a vários estudos científicos que alertaram sobre os seus efeitos nocivos, que podem incluir problemas alergénicos, asma, entre outros. Esta situação leva à busca exaustiva por alternativas naturais capazes de servir o mesmo propósito. Para tal, a indústria alimentar iniciou essa missão através da extração de compostos naturais de microrganismos, animais, plantas ou de bio resíduos/desperdício alimentar. De facto, na indústria alimentar, diversos setores produzem grandes quantidades de resíduos, como por exemplo na produção de figos, que podem ser úteis para a recuperação de compostos bioativos para atuar como conservantes naturais. Os figos, por exemplo, são conhecidos pela sua riqueza em minerais e nutrientes e, por isso, o seu consumo aumentou significativamente, levando também à inevitável produção de bioresíduos que incluem folhas de figueira.

Assim, no presente trabalho, foram exploradas folhas de figueira de cinco variedades diferentes, nomeadamente Dauphine, Longue d'Aout, Bourjassote Noire, Marseille e Pasteliere. Estas variedades foram analisadas relativamente ao seu perfil fenólico por HPLC-DAD-ESI-MS, bem como as suas bioatividades, nomeadamente propriedades antioxidantes pelos métodos de TBARs e CAA, antimicrobianas pelo ensaio de microdiluição, anti-inflamatórias utilizando macrógafos de rato e citotóxicas pelo método da sulforrodamina B. A extração dos compostos fenólicos foi otimizada pela metodologia de superfície de resposta e realizada por maceração dinâmica e extração assistida por ultrassons, sendo a sua determinação feita pelo ensaio de Folin ciocalteu.

Finalmente, os extratos obtidos nas condições ótimas de extração pela tecnologia de ultrassons, utilizada por ser mais rápida, com um custo mais baixo, foram misturados de forma a analisar possíveis efeitos sinérgicos. Quando avaliada a atividade antioxidante dos extratos de folhas em conjunto, esta foi mais forte, já que a menor EC_{50} registrada para Bn (0,23 ± 0,01 mg/mL), e para a mistura foi EC_{50} de 0,12 ± 0,01 mg/mL. Além disso, a mistura apresentou resultados promissores quanto à atividade antimicrobiana, pois foram capazes de inibir todas

as estirpes de bactérias e fungos testados. De realçar, que, quando em conjunto, os extratos revelaram atividade contra *Pseudomonas aeruginosa* com valor de CMI de 10 mg/mL.

Como os extratos não apresentaram toxicidade na linha celular PLP2 com GI_{50}>400 μg/mL, estes foram incorporados em duas formulações de compota de figo (com e sem mel), de forma a atuarem como conservantes naturais. Os produtos finais foram avaliados quanto às suas propriedades físicas (cor, textura, aw e pH), perfil nutricionais (humidade, cinzas, gordura por soxhlet, proteína pelo método kjeldahl, hidratos de carbono e energia), e químico, como os açúcares livres por HPLC-RI e ácidos gordos por GC-FID, e aindacarga microbiana. Após o processo de cocção, as diferentes propriedades foram avaliadas em duas temperaturas de incorporação (80°C e 50°C), de forma a determinar a eficiência dos extratos desenvolvidos e a sua estabilidade nos produtos finais. A compota de figo com baixo teor de açúcar incorporada com extratos conservantes naturais obtidos a partir de folhas de figueira, não revelou alterações na aparência geral das compotas.

Em relação às propriedades nutricionais e químicas, as formulações apresentaram baixo teor de açúcar, baixo teor de proteína, alta quantidade de hidratos de carbono, e baixo teor de ácidos gordos (ácido palmítico como composto majoritário). Além disso, as diferentes temperaturas de incorporação não apresentaram alterações discerníveis ao longo do tempo, implicando que as moléculas de interesse presentes nos extratos não são termolábeis.

Palavras-chave: Bioresíduos de figos, conservantes naturais, compota de figo, economia circular.

Introduction

1. Introduction

The consumption of fresh and convenient foods constitutes a problematic topic because of the notable increase in the world's population, as well as the climate change which has affected remarkably the agricultural sector. As a result, food preservatives (synthetic or natural) have been commonly used as a method for maintaining foods at the desirable organoleptic properties to keep the maximum of their benefits and to extend the storage periods to meet the people's daily needs. Actually, the use of synthetic preservatives has adverse effects on human health due to safety and toxicological matters which prompted consumers to opt for natural ingredients, organic foods, thus minimizing the consumption of synthetic additives. Therefore, food industries have started the exploration of natural preservatives that become in high demand because of the consumer's awareness. In addition, these natural components bestow numerous advantageous properties for human health due to their abundance in bioactive compounds such as phenolics, carotenoids, proteins, vitamins, among others, that provide antioxidant, antitumor and antimicrobial properties. To extract these compounds, microorganisms, animals, and plants are the main sources. However, bio-waste can also be explored, especially food waste such as some parts of fruits (leaves, peals etc.) and vegetables which can be outstanding sources of bioactive ingredients (Leichtweis et al., 2021). Moreover, bio-residues generated from the food industry correspond to approximately 194 to 389 kg per person per year around the globe, which leads to environmental pollution (Corrado & Sala., 2018). However, this negative impact can be solved by valorizing food-residues and using them as raw material to extract bio-molecules.

One of the earliest and widely cultivated fruit is fig that is known as a health-beneficial food due to its wealth in nutrients and minerals playing an important role in human nutrition. *Ficus carica* L., is rich in vitamins, amino acids, sugars, and bioactive molecules, responsible for its antioxidant, antitumor, antidiabetic, anti-proliferative, antimicrobial activities, among others, which rises the consumption of this fruit and the exploitation of the tree including its leaves which are considered as fig bio-waste production. Fig leaf is known to be rich in bioactive compounds that can be explored to extract preservative molecules, hence decreasing food waste and using natural preservatives instead of synthetic ones.

The aim of this work is to promote circular economy and sustainability by valorizing, fig leaves of five different varieties cultivated in Portugal, namely Dauphine, Longue d'Aout, Bourjassote Noire, Marseille and Pasteliere, in order to be explored as a raw material to acquire natural preservatives that will be incorporated in a fig jam.

1.1 The use of preservatives in the food industry

Food preservation has been used for centuries in order to elongate storage periods. Back to the 20th century where processed food became an essential part of human nutrition, the use of food additives was crucial (Carocho et al., 2014). To extend the shelf life of food products, preservatives were defined by the Codex Alimentarius, as any substance which is neither consumed as a food nor used as a typical ingredient of it (Food and Agriculture Organization of the United-Nations (FAO), World Health Organization(WHO)., 2019). These additives aim to inhibit the growth of yeasts, molds, and bacteria in foods (Awuchi et al., 2020). Preservatives constitute a group of additives which is divided into 3 subgroups: antibrowning, antioxidant and antimicrobial agents. To reach food preservation, chemical or natural reagents are added, which are ranged from E200 to E399 (Carocho et al., 2014). The international food agencies regulate the authorized amounts of preservatives which should not be superior to the maximum limits (500mg-3000mg/g), so that the daily intake does not exceed the Applicable Daily Intake (ADI) in the processed food to ensure secure products without obvious health risks (Abdelghani & Al-Degs., 2021).

1.2 Artificial vs natural preservatives

Preservatives are divided into two classes: class Ⅰ (natural preservatives) and class Ⅱ (artificial preservatives) (Kumari et al., 2019). These substances are added to food products to stop the growth of the microorganisms, increase the shelf life and maintain the quality and safety by preserving the natural characteristics of processed foods (Seetaramaiah et al., 2011). Both artificial and natural preservatives include antioxidant, antimicrobial and anti-enzymatic agents.

- Artificial preservatives

Synthetic preservatives are a group of chemical substances, as mentioned in **Table1** (Kumari et al., 2019), that are either added to food or sprayed outside of food (Kalpana & Rajeswari., 2019). They are used in one food item aiming to prevent the risk of exposure to more than one chemical substance.

Table 1: Preservatives and their applications (Kumari et al., 2019)

Preservatives	Class	Applications
Nitrites, Nitrates, sulfur dioxide, benzoates and Sorbates	Antimicrobial	Destroy or delay the growth of bacteria, yeast and molds
Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toulene (BHT), ascorbic acid	Antioxidants	Slow or stop the breakdown of fats and oils to prevent rancidity
Erythorbic acid (isoascorbic acid) and citric acid	Anti-enzymatic	Block the process during ripening and harvesting.

Commonly, chemical preservatives are considered safe, however some substances have negative impacts as shown in **Table 2** (Gupta & Yadav., 2021).

Table 2: Negative effects of some artificial preservatives: Negative effects of some artificial preservatives (Gupta & Yadav., 2021)

Chemical Food Preservatives	Where found	Negative Effects
Sodium Benzoate(E211)	Carbonated drinks, Pickles, Sauces	Aggravates asthma and suspected to be a neurotoxin and carcinogen. May cause fetal abnormalities
Sulphur Dioxide (E220)	Carbonated Drinks, Dried Fruits, Juices, Potato Products	May Induce Gastric Irritation Nausea, Diarrhea, Asthma Attacks, Skin Rashes
Potassium Nitrate(E249)	Cured Meats, Canned Meat Products	May lower oxygen caring capacity of blood, may combine with other substances to form nitrosamines that are carcinogens.
Calcium Benzoate(E213)	Drinks, Low Sugar Products, Cereals, Meat Products	May temporarily inhibit digestive enzyme function and may deplete levels of the amino acid glycin.
Aspartame (E951)	Biscuits, confectioneries	May cause neurological damage.
Calcium Sulfite	Burgers, Biscuits, Frozen Mushrooms, Horseraddish.	May cause bronchial problems, flushing, low blood pressure, tingling and anaphylactogen shock.

- Natural preservatives

Sugar, salt, vinegar, honey etc. are natural food preservatives which have been used for centuries. These natural substances or extracts are obtained from plants, animals, or microorganisms. Their consumption has increased because of their enriching components and due to health concerns of the population (Carocho et al., 2014). Natural preservatives present 3 origins: plants, animals, and microorganisms.

- Plants

Bioactive compounds of plants are produced as a defense mechanism, derived from the metabolism of plants, and grouped into three sub-groups: terpenes, phenolic compounds and alkaloids. These compounds present efficient results in the control of pathogens and spoilage microorganisms (Baptista et al., 2020).

- Animals

Lysozymes, lactoferrin, ovotransferrin, lactoperoxidase, antimicrobial proteins (AMPs) from livestock animals, and polysaccharides are preservatives obtained from animal sources. They present antibacterial effects: Lysozyme can suppress several Gram-positive. Lactoperoxidase oxidizes the sulfhydryl groups of proteins present in the bacterial membrane which results in cell lysis (Yu et al., 2021).

- Microorganisms

Lactic acid, organic acids, diacetyl, acetaldehyde, hydrogen peroxide, non-protein compounds of low molecular weight, bacteriocins, among others, contribute to a preservative effect on foods (Baptista et al., 2020). Moreover, varied strains of *Lactococcus lactis* produce nisin which present antimicrobial activities against a vast group of Gram-positive bacteria, among them *Staphylococcus spp*., *Bacillus spp.*, *Listeria spp.*, and *Enterococcus spp*. Pediocin, mainly, produced by *Pediococcus spp*., *Pediococcus acidilactici*, and *Pediococcus pentosaceus* strains revealed antimicrobial activity even at nanomolar concentrations (Yu et al., 2021).

1.3 Natural antioxidants

Antioxidant is any substance that delays, averts, and obstructs the oxidation of a target molecule (Da Silva et al., 2021). The process of preservation of foods demands the use of these compounds to prevent either the oxidation of molecules or elongate the storage life of the products (Da Silva et al., 2021).

The classification of natural antioxidants is based on their origins (plants, animals, or bacteria) and on their chemical structure. Enzymatic and non-enzymatic antioxidants are the two essential groups presented by the system of human antioxidants (**Figure 1**). The enzymatic

antioxidants are divided into two sub-groups primary and secondary products. Moreover, the non-enzymatic group presents an important number of them such as phenolic acids, carotenoids, vitamins, among others (Carocho & Ferreira., 2013).

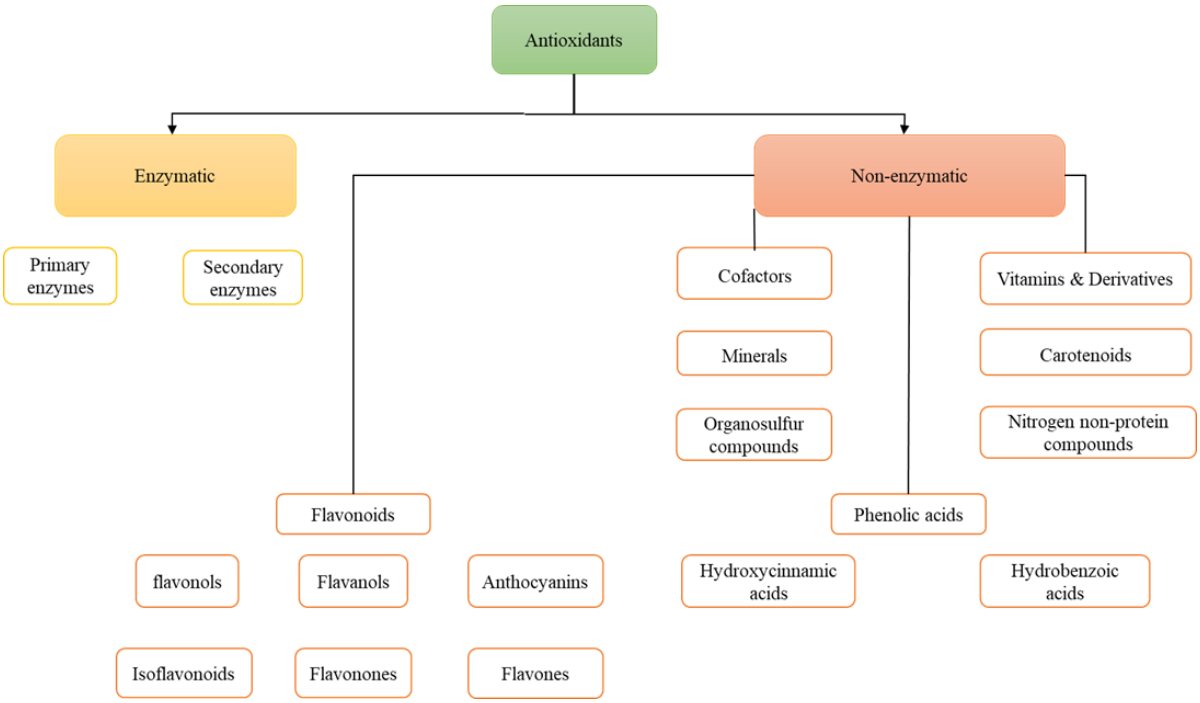


Figure 1: Classes of natural antioxidants (Carocho & Ferreira., 2013)

Among these substances, the major groups are phenols, tannins, flavonoids, and carotenoids.

- Phenols

Phenols (**Figure 2**) are defined as secondary products of plant metabolism that can be obtained from the aromatic amino acid phenylalanine, through the shikimic (phenylpropanoids) and the acetic acids (simple phenols) (Manessis et al., 2020). The classification of these compounds is based on their plant species origin, chemical structure, and solubility in water (Manessis et al., 2020).

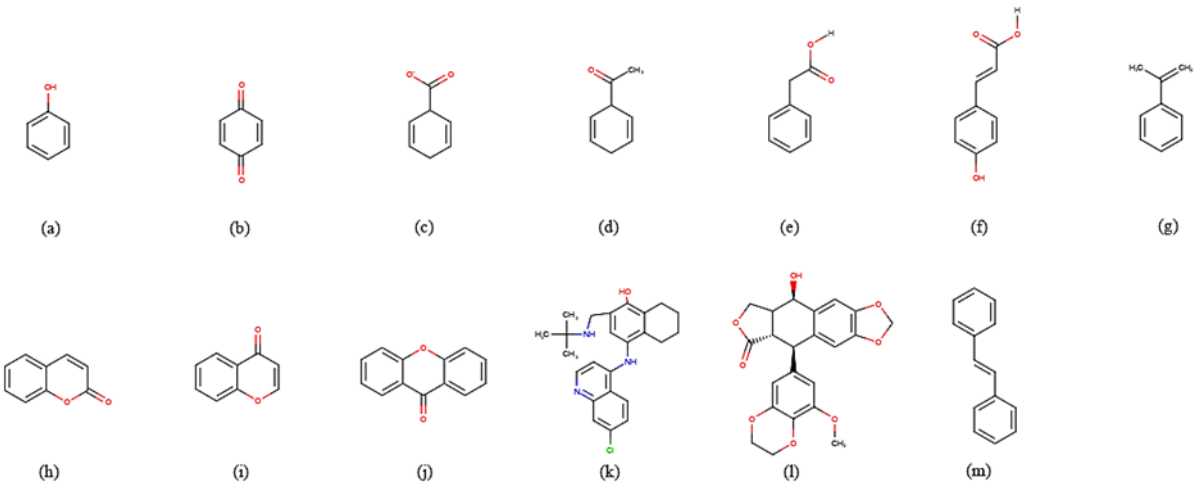


Figure 2: Basic chemical structures of selected phenolics phenols: (a) phenol, (b) benzoquinone, (c) phenolic acid, (d) acetophenone, (e) phenylacetic acid, (f) hydroxycinnamic acid, (g) 2-phenylpropene, (h) coumarin, (i) chromone, (j) xanthone, (k) naphthoquinone, (l) lignans, (m) (Manessis et al., 2020).

- Tannins

Tannins (**Figure 3**) are hydrophilic, astringent, polyphenolic substances, obtained by the polymerization of phenylpropanoid compounds. Tannins are divided into two groups: condensed tannins and hydrolysable tannins (Manessis et al., 2020).

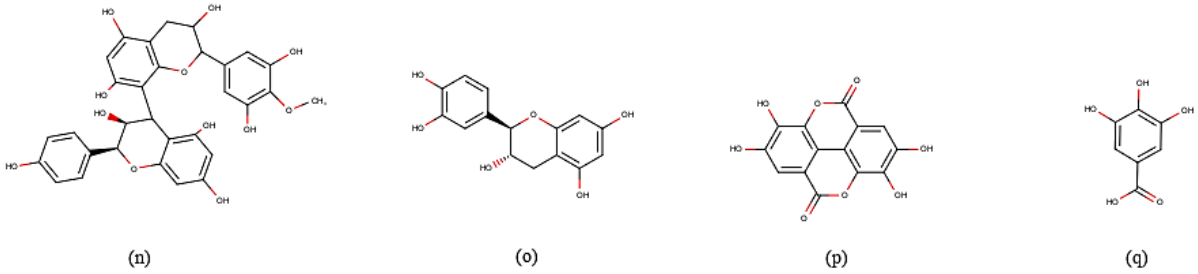


Figure 3: Chemical structures of selected Tannins: (n) proanthocyanidins, (o) catechin, (p) ellagic acid, (q) gallic acid (Manessis et al., 2020).

- Flavonoids

Flavonoids are an antioxidant group of compounds (**Figure 4**) that can be found in various plants. They are derived from the aromatic amino acids phenylalanine and tyrosine, and from malonate. The sub-groups of compounds share the basic structure, which is the flavan nucleus,that consists of the same diphenylpropane ($C_6C_3C_6$) skeleton (Carocho & Ferreira., 2013, Manessis et al., 2020).

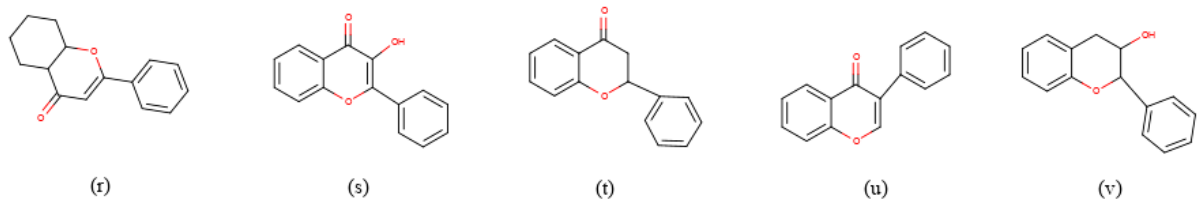


Figure 4: Chemical structures of selected flavonoids: flavone, (s) flavanol, (t) flavanone, (u) isoflavone, (v) flavan-3-ol (Manessis et al., 2020).

- Carotenoids

Carotenoids are pigments in plants, algae and photosynthetic bacteria that cannot be synthetized by animals. The carotenoid hydrocarbons known as the carotenes which contain specific end groups like lycopene and □-carotene; and the oxygenated carotenoids known as xanthophylls, like zeaxanthin and lutein represent the two major groups of carotenoids (Carocho & Ferreira., 2013).

- Mechanism of action of antioxidant agents

The efficiency and the mechanism of action of antioxidant agents depend on the physic-chemical composition of the processed food and beverages, along with the chemical structure of antioxidant molecules (Meyer et al., 2002). Numerous mechanisms of antioxidant activity can work effectively like metal chelators, which transform metal prooxidants into stable products, and radical chain breakers, by accepting radicals or donating hydrogen to prevent propagation of lipid peroxidation, as well as singlet oxygen quenchers, retardants of pro-oxidative enzymes and as inhibitors or reducing agents of the lipid's hydroperoxidation (Márcio Carocho & Ferreira., 2013) (Meyer et al., 2002).

1.4 Natural antimicrobials

Microbiological damage to food products causes a loss of about one-quarter of the world's foods (Searle et al., 2016). The antimicrobials are defined as the inhibitors of microorganism's growth and are known as being beneficial for human health so they can be safely introduced in foods. Natural antimicrobials are derived from plants, animals, and microorganisms.

- Antimicrobials derived from plants

The antimicrobials are synthesized by plants and they can be accumulated in fruit, flowers or buds, leaves, seeds, bulbs, rhizomes or other parts of plants (Bensid et al., 2020), and

belong to the secondary metabolism (Marcio Carocho, 2015). The most common antimicrobial compounds from plant sources are terpenes, steroids, alkaloids, and polyphenols (Carocho et al., 2015). Essential oils, mostly produced by aromatic plants constitute an important group which effectively inhibits the growth of Gram-positive than Gram-negative bacteria (Bensid et al., 2020).

- Antimicrobials derived from animals

Antimicrobials derived from animals include lysozyme, lactoferrin, lactoperoxidase, and chitosan (Bensid et al., 2020).

- Lysozyme

Lysozyme is an enzyme naturally found in mammalian milk and eggs (Searle, 2016). It is applied to inhibit the growth of Gram-negative and Gram-positive bacteria (Bensid et al., 2020).

- Lactoferrin

Lactoferrin is a bioactive glycoprotein isolated from milk and it presents antimicrobial effects on an important group of bacteria, viruses, fungi, and parasites (Yu et al., 2021).

- Lactoperoxidase

Lactoperoxidase present antifungal effects, they act on Gram-negative bacteria to inhibit or eliminate them; however, the action depends on temperature, pH, cell density, as also on the incubation time of the substrate. Moreover, Gram-positive bacteria are usually inhibited, but not necessarily killed (Bensid et al., 2020).

- Chitosan

Chitosan is naturally found in crustacean shells (Searle et al., 2016), it has demonstrated an excellent antibacterial activity on different pathogenic and spoilage microorganisms (Bensid et al., 2020).

- Antimicrobials derived from microorganisms

Some microorganisms and their derivatives present molecules that have impact on others which leads to the prevention or the inhibition of their growth (Batiha et al., 2021). Bacteriocins, organic acids, reuterin, diacetyl, ethanol, CO_2 (as carbonic acid), H_2O_2, and lactic acid derivatives are produced by Lactic acid bacteria (Bensid et al., 2020).. Bacteriocins, for example, obtained by lactic acid bacteria showed an effective control against pathogens (Batiha et al., 2021). Although there are many recognized bacteriocins, only nisin is allowed and authorized as a food preservative. It shows activity against Gram-positive bacteria. However, it

doesn’t show a significant impact on Gram-negative bacteria, yeasts, and molds (Bensid et al., 2020).

- Mechanism of action of antimicrobial agents

Antimicrobial agents added or sprayed on food (Meyer et al, 2002) can act in diverse ways, among the different mechanisms of action they can block the growth of bacteria by tying the cell wall or kill bacteria by cell envelope lysis as well as damaging the outer membrane of Gram- for example (Saeed et al., 2019) (Villalobos-Delgado et al, 2019). Moreover, some antifungal agents destabilize the cell membrane by binding to fungal ergosterol which leads to the loss of solutes (Villalobos-Delgado et al., 2019). Added to that, antimicrobial agents aim at the pathogenic microorganism by the inhibition of NADH oxidation or interference with membrane like the action of organic acids (Saeed et al., 2019), while weak organic acids block aflatoxin production which has an action against yeasts and molds (Villalobos-Delgado et al., 2019).

1.5 Extraction methodologies to obtain natural antioxidants/antimicrobials

1.5.1 Ultrasound assisted extraction (UAE)

UAE is an emerging technique used for numerous food processing targets (Sanwal et al., 2022). Mechanical sound waves can be used in treatments to different states: solid, liquid or gas, with frequencies that exceed 20 kHz (Marić et al., 2018). The propagation of sound waves generate pressure that leads to the creation of vapor bubbles when it transcends the liquid’s tensile strength (Sanwal et al., 2022). These bubbles create cell perturbation that may lead to rupture of cell wall that improves the passage of the solvent into the cells, which intensify the mass transfer (Marić et al., 2018). In addition, UAE is identified depending on the equipment and the microenvironment parameters including temperature, time, frequency, and power. Moreover, the classification of this technique is based on some requirements related to the interaction between ultrasound source and extraction medium, frequency ranges, frequency combinations, and purpose (Dzah et al., 2020). The application of UAE generates an eventual medium to extract bioactive compounds compared to conventional extraction techniques (Chen et al., 2014).

1.5.2 Dynamic maceration (DM)

Among the conventional extraction methods, dynamic maceration is frequently used to extract bioactive compounds (Mohapatra et al., 2021). This technique is applied to weaken and

smash the plant's cell wall to liberate the soluble compounds (Azwanida, 2015). During the process, the sample is maintained in contact with the solvent, which is selected according to the extracted component from the plant, in a clogged holder until dissolution of the soluble matter while keeping agitation (Kharwar et al., 2020). After the necessary period of extraction, the mixture is tightened or filtered to recuperate the bioactive compounds (Azwanida, 2015). This process is recognized as being easy to perform, but it is also known for generating a low extraction yield, a tremendous energy consumption and long extraction times (Kharwar et al., 2020).

1.6 Application of natural preservatives in the food industry

1.6.1 Main bottlenecks

The bioactive compounds used as natural preservatives are known for their well-established health promoting benefits. Nowadays, industrial production of natural components is based on plant extraction and chemical synthesis. In contrast, plant extraction acquires noticeable constraints owing to the climatic changes, the elongated growth cycles. In addition to that, extractions are costly because of the excessive energy and the solvent demand (Ofosu et al., 2020). Moreover, it's true that plant metabolites are beneficial but, some of them reveal drawbacks as at high dosages they are pro-oxidant or mutagenic with toxicity (Ofosu et al., 2020). The toxicity of compounds can also be related to its chemical structure and functional groups just as much as its hydrophobic character (Da Silva et al., 2021). For example, a high fat tenor can lower the action of essential oils because the lipid portion of the food can soak up the antimicrobial agent and thus the antimicrobial activity. Furthermore, the high rate of protein in a food product can decrease the activity against microorganisms of essential oils because of the linking that may arise between some of the components of essential oils and proteins. In addition, the incorporation of bioactive compounds requires several studies so that no changes occur, among them the sensory changes that may arise even at low concentrations of essential oils (Da Silva et al., 2021).

Moreover, one of the considerable restraining aspects for the use of natural additives is their sensitiveness to temperature, light, solvents, chemical structure, pH variations and interaction with other food components that may generate destruction, loss or minimization of their functionality (Giaconia et al., 2020, Braga et al., 2018). For example, according to Mederios et al (2019), carotenoids, which can be used by food industries as natural food coloring or a source of antioxidants, are affected by chemical degradation by isomerization and

oxidation as well as presenting low stability of water making their exploration more limited (Medeiros et al., 2019). Furthermore, the instability of bioactive compounds make the choice of food processing, preservation method and storage, a challenge for food industries as its essential and critical at the same time as both bioactive components and the quality of the product must be maintained at good levels (Giaconia et al., 2020, Dalla Nora et al., 2014).

1.7 Fig bioresidues as sources of natural preservatives

1.7.1 Main bioactive compounds present in fig leaves

The fig (*Ficus carica* L.) belongs to the Moracae family, in which milky latex is identified in all parenchymatous tissue, unisexual flowers, anatropous ovules, and aggregated drupes or achenes (Barolo et al., 2014). This fruit is widely cultivated due to its pertinent role in the human health, considering the fact that it is an important source of interesting compounds such as phenolics, organic acids, calcium, antioxidants etc. (Teruel-Andreu et al., 2021, Bankefa et al., 2019). Further, Palmeira et al (2019) indicated that figs present a considerable sources of trace minerals such as iron and potassium, as well as, vitamins (mainly thiamin and riboflavin), fibres and essential amino acids (Palmeira et al., 2019). In addition to bioactive compounds, fig fruits along with leaves are known for multiple biological activities such as antioxidant, anticancer, hepatoprotective, hypoglycemic, hypolipidemic and antimicrobial activities (Bankefa et al., 2019). *F. carica* leaves are rich in phenolic compounds, flavonoids, tannins, alkaloids as well as terpenoids like sterols and triterpenoids (Kiliç et al., 2021). According to a review published in 2021, Teruel-Andrew et al mentioned different phenolic compounds found in fig leaves which belong to diverse chemical families, like phenolic acids (caffeic, chlorogenic, ferulic, coumaric, syringic, quinol and gallic acids) and flavonoids (Kaempferol, catechin, quercetin and myricetin), displayed in Figure4 (Teruel-Andreu et al., 2021). Furthermore, the leaf is recognized as a source of proteins, it comprises also fat, crude fiber, ash, carotenes, bergapten, stigmasterol, sitosterol, and tyrosine (Kiliç et al., 2021). Moreover, studies have shown that fig leaves contain sugars, pectin, and vitamin C (Li et al., 2021). Furthermore, among the major phytochemical components of fig leaves are volatile combinations including aldehydes, alcohols, benzyl alcohol, phenylethyl alcohol, ketone, esters, hexyl acetate, ethyl benzoate, and methyl salicylate, monoterpenes, sesquiterpenes and other combinations (Kiliç et al., 2021).

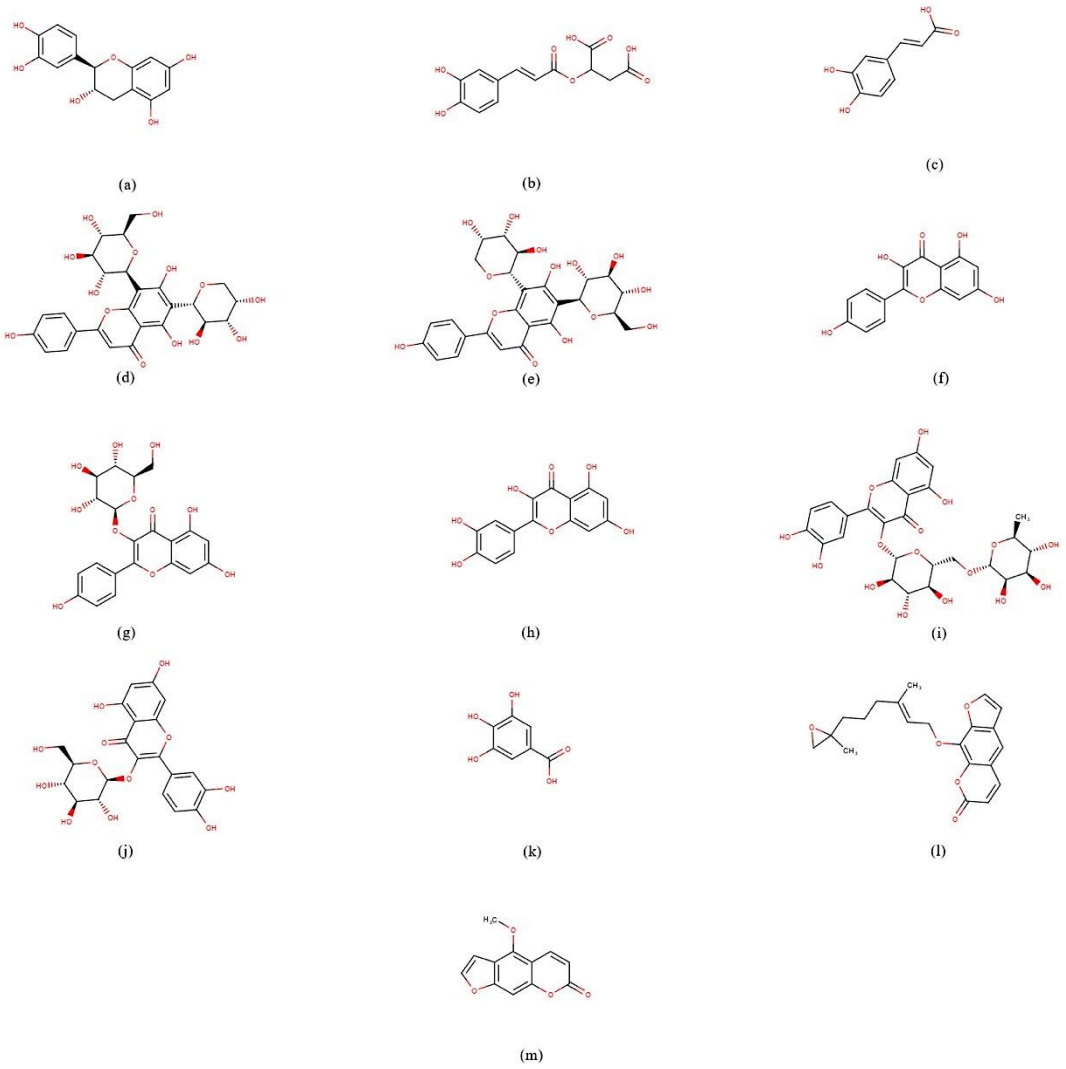


Figure 5: chemical structures fig leaf biocompounds: (a) (+)-catechin, (b) Caffeoylmalic acid, (c) Caffeic acid, (d) Isoschaftoside, (e) Schaftoside , (f) Kampherol, (g) kaempferol 3-O-glucoside (astragalin), (h) Quercetin, (i) Rutin (quercetin-3-O-rutinoside), (j) (j) Quercetin 3-O-glucoside (isoquercetin), (k) Gallic acid, (l) Psoralen and (m) Bergapten (5 methoxypsoralen) (Teruel-Andreu et al., 2021)

1.8 Importance and relevant scientific research on figs

According to a search in Scopus based on the key words "Ficus" AND "Food products", many articles have been published for 38 years that reveal the importance of figs which are carrying more and more interest. However, among the 68 articles, the majority (34) is studying Opuntia ficus and only 18 are focusing on fig studies (**Figure 6**).

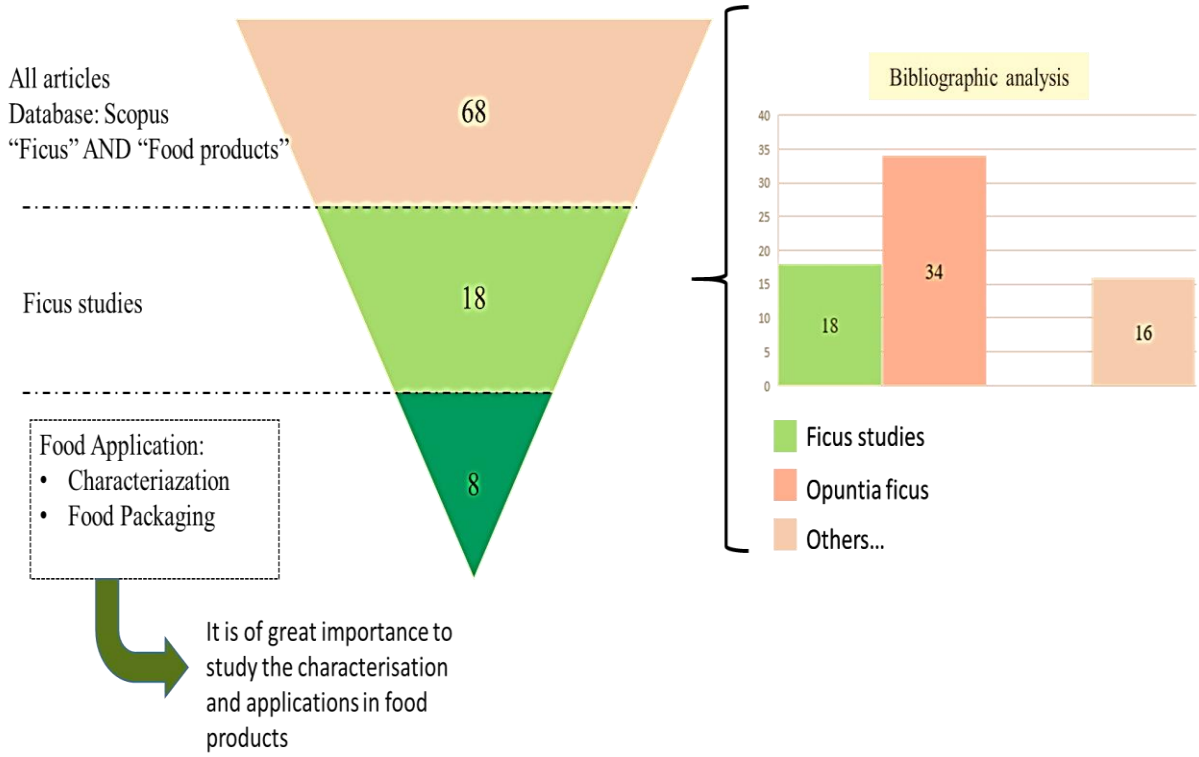


Figure 6: Studies Published in relation to "*Ficus*" AND "Food products"

Conforming to these works, **Table 3** resumes the different researches that have been published in relation to characterization, fig's fermentation, drying methods, food packaging and preferences of consumers that have been studied to explore the benefits of figs along with their by-products to promote the circular economy and sustainability.

Table 3: Different fields of studies on figs

Main objective	Year	Specific objectives	References
Characterization	2021	Microwave drying effect on drying characteristics of *Ficus carica* Linn leaves	Yilmaz et al, (2021)
	2020	Chemical characterization	TURCO et al, 2020
	2018	Chemicals and bioactivity discrimination of syconia of seven varieties of ficus deltoidea	Yunusa et al, 2018.
	2017	Biochemical and genetic characterization of a red fig cultivar (*Ficus carica*)	De Masi et al, 2017
	2015	Physico-chemical and in vitro antioxidant properties of fig (*Ficus carica*) liquid co-products	Viuda-Martos et al, 2015
	2014	Characterization of chemical, nutritional and biological activities of *Ficus carica*.L	Barolo et al, (2014)
	2005	Molecular characterization of figs	Bernardo et al, (2005)
	2003	Determination of Ochratoxin A in dried figs	MacDonald et al, (2003)
Consumer preference	2017	Consumers' preferences for sensory, quality attributes of fresh fig	Ingrassia et al, 2017
Drying methods	2006	Evaluation of thin-layer drying models for describing drying kinetics of figs	Babalis et al, (2006)
	2005	Sun drying of figs	Doymaz, (2005)
	2004	Influence of the drying conditions on the drying constants and moisture diffusivity during the thin-layer drying of figs	Babalis et al, (2004)
Fermentation	2019	Evaluation of different variables influencing to the fermentation of ficus	Minh et al, 2019
Food packaging	2022	Development of *Ficus carica* Linn leaves extract incorporated chitosan films for active food packaging materials	Yilmaz et al, (2022).
	2021	Bioplastic made from Manihot esculenta (Cassava) and *Ficus benjamina*	Quispetera et al, (2021).
	2018	Characterization of nanocomposite films containing methanolic *Ficus carica* extract	Shahbazi, 2018
Review	2019	Review on fresh and dried figs: Chemical analysis and occurrence of phytochemical compounds, antioxidant capacity and health effects	Arvaniti et al, (2019).

2. Objectives

Ficus carica is a fruit extensively cultivated in the Mediterranean region and, as well as the bioresidues resulting from their production, are rich sources of bioactive molecules, namely antioxidants and antimicrobials. To explore these advantages and promote the circular economy; bioresidues obtained from the fig value chain will be valorized to extract bioactive compounds that will allow to obtain a preservative extract for application in a final fig jam.

To accomplish the required goal, specific objectives were determined below with all the methods performed in the current work.

1) Phenolic compounds profile of the fig varieties and bioactive potential evaluation
    a) Phenolic compounds characterization (HPLC-DAD/ESI-MS)
    b) *In vitro* evaluation of antioxidant activity through the Inhibition of lipid peroxidation through reactive substances of thiobarbituric acid (TBARS) and Cellular antioxidant activity (CAA); antibacterial activity (using Gram negative and Gram positive bacteria and antifungal activity; cytotoxicity using tumoral cell lines gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo2), breast adenocarcinoma (MCF-7) and non-tumor cell line pig liver cells (PLP2); anti-inflammatory activity
2) Development of an experimental design for extraction process optimization of fig leaves, using maceration and ultrasound assisted extraction, chromatographic analysis of phenolic compounds, and response surface methodology for optimization.
3) Fig jam preparation and determination of the nutritional value (moisture, ash, protein and fat), chemical composition (free sugars and fatty acids) and physical parameters (color, pH, water activity and texture,), and assessment of the microbial load (plate counting technique) of the final jam.

Material and Methods

3. Material and Methods

3.1 Plant material treatment

The leaves of 5 varieties of figs (**Figure 7**) named “Bourjassote Noire (Bn)”, “Dauphine (Da)”, “Longue d’Aout (La)”, “Marseille (Ma)” and “Pastilière (Pa)”, were collected, in July 2021 in Quinta da Mó de Cima, which is located on the Setúbal peninsula, right on the edge of the Arrábida Natural Park in Portugal, by the industry Mó de Cima, that are specialized in the production of figs. The leaves were packed in bags, protected from light and humidity until their arrival at the laboratory where they were dried at room temperature sheltered from light and moisture until removing the water from the fresh leaves. Finally, the samples were powdered and stored in a dry place until starting the analysis.

Figure 7: Fig leaves from the five analyzed varieties

3.2 Phenolic compounds profile of the fig varieties and bioactive potential evaluation

3.2.1 Hydroethanolic extracts

To prepare the hydroethanolic extracts, 1g of the powdered fig leaves was added to 30mL of ethanol/water (80:20 *v/v*) to be extracted under magnetic stirring for one hour at room temperature. The extract was filtered using a filter paper (Whatman filter paper no. 4) and the residue was extracted once again under the same conditions. Further, the extracts were evaporated by a rotary evaporator (Büchi R-210, Flawil, Switzerland) at 40 °C until the evaporation of the ethanol, and then lyophilized, to remove the remaining water part. Then, the lyophilized extracts were kept in storage, away from light and humidity, for later analysis.

3.2.2 Phenolic compounds characterization

In an Eppendorf tube, 10 mg of the lyophilized extract were dissolved in 1 mL of ethanol/water solution (80:20, *v/v*) and filtered in vials to be analyzed with high-performance liquid chromatography coupled with diode array detection with electrospray ionization and mass spectrometry HPLC-DAD/ESI-MS.

According to Bessada et al. (2016), the chromatographic analysis was assessed using a Dionex Ultimate 3000 High Performance Liquid Chromatography (HPLC) 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. A diode detector was used to detect the compounds according to the wavelengths of 280 nm, 330 nm and 370 nm. The separation of the compounds was carried out with a Waters Spherisorb S3 ODS-2 C18 reverse phase column (4.6x150 mm, 3 µm; Milford, USA), thermostatized at 35°C. The mobile phase used was (A) formic acid / water (0.1%) and (B) acetonitrile. The elution gradient 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 was associated to a mass spectrometer (MS). The detection of MS was performed 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 assembled and interpreted using the Xcalibur® program (Thermo Finnigan, San Jose, CA, USA). The determination of the compounds was carried out by comparing the data obtained (retention times, UV-Vis spectra and mass spectra) with data available in the literature and, when available, with the standards. To quantify the compounds, standard solutions with known concentrations were injected to obtain calibration curves.

3.2.3 Antioxidant activity

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

The TBARS method was performed according to the protocol described by Sarmento et al. (2015). The lyophilized leaves extracts were dissolved in water (10 mg/mL) to obtain the stock solution that was further diluted successively to obtain six concentrations below. In addition, a porcine brain suspension (*Sus scrofa*) was prepared: 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*), followed by centrifugation at 3500 rpm for 10 min at a temperature of 10 ºC to avoid rancidification of the mixture. Afterwards, 200 µL of each diluted solution were placed in tubes, to which 100µL of ascorbic acid (0.1 mM), 100 µL of iron sulfate ($FeSO_4$ - 10 mM) and 100µL of the porcine

brain solution were added and incubated in hot bath water at T=37.5 °C during 1h. After incubation, 500 µL of trichloroacetic acid (28% *m/v*) were added to stop the reaction as well as 380 µL of thiobarbituric acid (2% *w/v*, TBA).The tubes were placed once again in hot bath water at T=80 °C during 20 minutes, in order to promote the reaction between TBA and malondialdehyde, MDA - reactive oxygen species resulting from lipid peroxidation that occurs in porcine brain tissue. Afterwards, the mixture was centrifuged at 3500 rpm for 5 min to separate the residues from the supernatant. The measure of the color intensity of the MDA - TBA complex was carried out at 532 nm. To calculate the percentage of inhibition of lipid peroxidation the following equation was used:

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

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 (EC_{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 as EC_{50} in µg/mL.

3.2.3.2 Cellular antioxidant activity (CAA)

The cellular antioxidant activity (CAA) is a cellular method that was performed by following the description of Wolfe & Lui (2007). The CAA was assessed by dissolving the extracts in water to obtain a concentration of 8mg/mL, from which successive dilutions were made with 2',7' dichlorohydrofluorescein (DCFH), easily oxidizable to fluorescent dichlorofluorescin (DCF) by peroxyl radicals ROO•. DCFH was prepared with ethanol and diluted with Hank's Balanced Salt Solution (HBSS) (50 µM), to obtain the tested concentrations (32.5 - 2000 µM).

The Raw 246.7 mouse macrophages were the cells used, because they gave better signal and more reproductive fluorescence. They were incubated at 37 °C in an incubator at humidified atmosphere with 5% CO2, with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), fetal bovine serum (FBS) (10%) and non-essential amino acids (2 mM).

To detach the mouse macrophages, a cell scraper was used and the cells were transferred into falcon tubes. The solution was centrifuged for 5 min at 12000 rpm while the medium was discarded and a new one was added depending on the size of the pellet. An aliquot, of a prepared

solution with a cell density of 70,000 cells/mL, with a volume of 300 µL was transferred to black microplates with clear-bottom (SPL Lifesciences) to be incubated during 48h.

Afterwards, the medium was added and before being incubated for 1h, the cells were washed with HBSS (2x, 100 µL) and the extracts were added at different concentrations (200 µL; 32.5 - 2000 µM). After the incubation, the cells were washed with HBSS (2x, 100 µL) and a 2.2 2'-azobis (2-methylpropionamide) dihydrochloride (AAPH) solution (100 µL; 600 µM), the AAPH is added to induce oxidative stress and generate ROO•. To read the fluorescence, a Biotek FLx800 microplate reader was used, and the reading was performed each 5 min for 1 hour at 485 nm excitation and 538 nm emission. The positive control used was quercetin and the negative control was the dichlorohydroflurescein and DMEM culture medium.

3.2.4 Antimicrobial activity

3.2.4.1 Antibacterial activity

Food bacterial contaminants were used for the determination of antibacterial activity: five Gram-negative bacteria: *Enterobacter Cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterocolitica* (ATCC 13076), *Yersinia enterocolitica* (ATCC8610) and three Gram-positive bacteria: *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), and *Staphylococcus aureus* (ATCC 25923) obtained fromFrilabo, Porto, Portugal.

The incubation of the microorganisms was at 37 ºC for 24h in an appropriate fresh medium before analysis, in order to keep the exponential growth phase.

- Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

According to Pires et al (2018), the samples were initially dissolved in 5% (*v/v*) Dimethyl sulfoxide (DMSO)/Mueller-Hinton Broth (MHB)/Tryptic Soy Broth (TSB) to obtain a final concentration of 20 mg/ ml for the stock solution. Afterwards, 190 µl of this concentration was added in the first well (96-well microplate) in duplicate. In the remaining wells 90 µl of medium MHB or TSB were placed. Then the samples were serially diluted obtaining the concentration ranges of 100 to 0.15 mg/mL. Finally, 10µl of inoculum (standardized at 1.5×108 Colony Forming Unit (CFU) /ml) was added at all the microplate wells. Three negative controls were prepared (one with (MHB)/ (TSB), another one with the extract, and the third with medium, antibiotic, and bacteria). One positive control was prepared with MHB/TSB and each inoculum. Streptomycin and ampicillin were used for all Gram-

negative and Gram-positive bacteria tested except *Bacillus cereus* for which ampicillina was not used as a positive control. Methicillin was used as a positive control for *S. aureus*. The microplates were incubated at 37 °C for 24 h, andthe MIC o was detected following the addition of 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT), a dye used to evidence the bacterial growth, and incubated at 37°C for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determined by change of the coloration from yellow to pink if the microorganisms were viable. For the determination of MBC, 10 µl of liquid from each well that showed no change in color was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth determined the MBC.

3.2.4.2 Antifungal activity

For the determination of antifungal activity, two fungal strains were used: *Aspergillus brasiliensis* (ATCC 16404) and *Aspergillus fumigatus* (ATCC 204305) that were obtained also from Frilabo, Porto, Portugal. According to Heleno et al. (2013), 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 malt agar (MA) to verify the absence of inoculum contamination. Through the successive dilution technique in 96-well microplates, the minimum inhibitory concentration (MIC) was determined. The extract sample was added to the malt medium with the fungal inoculum and the microplates were incubated for 72h at a temperature of 28 °C. The lowest concentrations without visible growth were defined as MIC. The minimum fungicidal concentrations (MCFs) were determined by subculture in series of 2 µL from each well that did not change color, in microplates containing 100 µL of malt broth per well and later incubated for 72h at 28 °C. The lowest concentration without visible growth was defined as MFC, indicating 99.5% of death of the original inoculum. Ketoconazole was used as a positive control, and dimethyl sulfoxide (DMSO, 5%) used as a negative control. The results of MIC and MFC were expressed in mg per ml.

3.2.5 Cytotoxicity

To evaluate the cytotoxicity, three human tumor cell lines were used: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo2) that were purchased from the European Collection of Authenticated Cell Cultures (ECACC), breast adenocarcinoma (MCF-7) was provided by Leibniz-Institute DSMZ and non-tumor cell line pig liver cells (PLP2) that

their primary culture was established in the laboratory (**Figure 8**). All of the cell lines were maintained in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% (FBS), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL). The incubation of the culture flasks was maintained under a humid atmosphere, at 37 ºC and with 5% CO_2. The cells could only be used when their confluence reached 70 to 80%. After incubation the cells were detached using trypsin.

To prepare the stock solutions, 8 mg of each sample were dissolved in 1mL of water to obtain a concentration of 8 mg/mL. Successive dilutions were made from each of the stock solutions, obtaining the concentrations to be tested (0.125 - 8 mg/mL). Afterwards, 10 µL of each of the extract concentrations were incubated with 190µL of the cell suspension of the cell lines tested in 96-well microplates for 72 hours. Once the adherence of the cells was checked the microplates were incubated at 37 ºC and with 5%CO2, in a humid atmosphere. All cell lines were tested at a concentration of 10,000 cells/well. After the incubation period, the cells were corrected: Trichloroacetic acid (TCA) (10% w/v; 100 µL) was previously cooled and plates were incubated for 1 hour at 4 ºC, washed with water and, after drying, a Sulforhodamine B (SRB) solution (0.057%, *m/v*; 100 µL) was added, to couple with the proteins of the cells by electrostatic bonds (Vichai & Kirtikara., 2006), and left to stand at room temperature for 30 minutes. To remove non-adhered SRB, plates were washed three times with a solution of acetic acid (1% *v/v*) and placed to dry. Finally, an adhered SRB was solubilized with Tris (10 mM, 200 µL) and the absorbance at a wavelength of 540 nm was read in the Biotek ELX800 microplate reader. The results were expressed in terms of the concentration of extract with the ability to inhibit cell proliferation by 50% - GI_{50} in µg/mL. As a positive control for this assay it was used ellipticin.

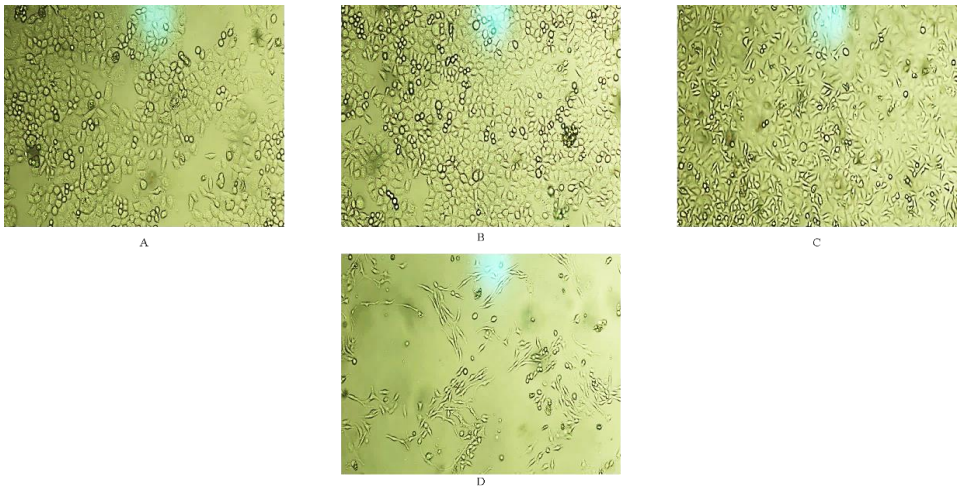


Figure 8: Cytotoxicity: A: MCF7, B: CaCO2, C: AGS, D: PLP2

3.2.6 Anti-inflammatory activity

The anti-inflammatory activity was evaluated by dissolving the extracts in water to obtain a concentration of 8 mg/mL. The RAW 264.7 mouse macrophage cell line, obtained from DMSMZ-Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, was grown in DMEM medium, followed by the addition of heat-inactivated FBS (10%), glutamine and antibiotics, and incubated under a humid atmosphere at 37 °C with 5% CO2. The cells were, then, separated by a cell scraper. According to the Trypan blue exclusion test, a microplate was used and in each well was placed a portion of the macrophages' cell suspension (300µL) with a cell density of 5 * 105 cells/mL and a percentage of dead cells lower than 5%. The microplate was incubated for 24 hours under the conditions mentioned above to enhance the adherence and multiplication of the cells. Afterwards, the cells were treated with different extract concentrations (15 µL, 0.125 – 8 mg/mL) and incubated during one hour, with concentrations varying between 6.25-400 µg/mL. To stimulate the cells, 30µL of the liposaccharide solution – LPS (1 mL/mL) was added and the plates were incubated for supplemental 24 hours. The positive control used was ellipticine and by lack of LPS samples were used as negative control. To quantify the nitric oxide, a Griess reagent system kit (nitrophenamide, ethylenediamine and nitrite solutions), that induces the change of color thus indicating the presence of activity, was used along with the nitrite calibration curve (100 mM sodium at 1.6 mM) prepared in a 96-well plate. The production of the nitric oxide was determined by reading the absorbance at 540 nm (ELX800 Biotek microplate reader, Bio-Tek Instruments, Inc., Winooski, VT, USA) and by comparing the standard calibration line. The determination of the results was performed by the graphical representation of the inhibition percentage of nitric oxide production versus the concentration of the sample and expressed through each extract's concentration that causes the inhibition of 50% of the nitric oxide's production- IC_{50}.

3.3 Optimization procedure for the extraction of phenolic compounds by Response Surface Methodology (RSM)

3.3.1 Optimization conditions

An optimization extraction was applied for both dynamic maceration (DM) and ultrasound assisted extraction (UAE). The Box-Behnken design (BBD) using Design-Expert v.11 software (Stat-Ease, Minneapolis, MN, USA) was chosen for DM and UAE, combining three levels of three independent factors. For DM [A (T, °C), B (t, min) and C (S, %)], where T was the symbol of temperature, t represented the time and S was the solvent percentage

(ethanol percentage). For UAE [A (t, min), B (P, W), and C (S, %), where t represented the time, P was the symbol of power expressed in Watt and S was the percentage of the solvent which was the ethanol. The BBD included 17 independent combinations for which the amount of total phenols (TP) was the response R studied and optimized by the RSM.

3.3.1.1 Dynamic maceration (DM)

The DM extraction process was performed as following, the dried powdered leaf samples (1.5 g) were placed in a flask with 30 mL of required solvent placed in hot water bath at the appropriate temperature, under continuous electromagnetic stirring during the defined extraction time (**Figure 9**). The solutions were then filtered in a tube through a filter paper (Whatman filter paper no. 4) and stored in the refrigerator for further analysis. The TP content was the response R in the optimization procedure.

Figure 9: Hot water bath under continuous electro-magnetic stirring

3.3.1.2 Ultrasound Assisted Extraction (UAE)

The UAE was performed using an ultrasonic system (**Figure 10**) (Ultrasonic homogenizer, model CY-500, Optic Ivymen System, Barcelona, Spain). The dried powdered leaf samples (5 g) were placed in a beaker with 100mL of adequate solvent. After the solutions were filtered using Whatman filter paper no. 4 and stored in the refrigerator until starting the analysis. The TP content served as the optimization process's response R.

Figure 10: Ultrasonic device

Once analyzing all the runs of UAE, the optimal points corresponding to each fig variety were determined, which represented the highest amount of TP content. Further, each variety was extracted separately by applying the conditions of the optimal points using a Qsonica Q500 type ultrasound probe (50 kHz, 500 W maximum power, 203×387×216 mm internal dimensions). The extracts obtained were submitted to a evaporation step, to remove the ethanol part, and further lyophilized to remove the water part (-47 ºC, 0.100 bar; FreeZone 4.5, Labconco, Kansas City, MO, USA) and stored in the dark at cool temperature for further use.

3.3.2 Determination of the total phenols

The determination of the total phenols was performed by a colorimetric assay using the Folin Ciocalteu method. The standards were first prepared by adding gallic acid (750 µL), diluting successively to obtain four concentrations below it. In assay tubes, 500 µL of the extracts, standards and whites were added to 2500 µL Folin Ciocalteu and 2000µL of Sodium Carbonate (an alkali used to oxidize phenols in phenolate ions thus changing the color into a blue pigment spectrophotometrically measured). The mixtures were then placed in hot bath water at 40 °C for 30 min. In the microplates wells, were added 300 µL of each solution to be read at 750 nm.

3.4 Application of the phenolic extracts in a fig jam

After the extraction of the optimal points stemming from the five varieties of the fig leaves separately, only four were used namely Bn, La, Ma and Pa, since I developed a skin allergy against Da, thus avoiding its incorporation.

3.4.1 Preparation of the mixture

The four varieties were used at a percentage of 25% of each to obtain a mixture. Some bioactive assays were performed (antibacterial + antifungal + toxicity + anti-inflammatory) along with antioxidant activity (TBARs and CAA) in order to determine the concentration in which the extracts mixture could be added, and if the mixture was safe thus, can be used in the fig jam.

3.4.2 Fig jam preparation

Six types of jams were prepared (**Table 4**), with the help of the Tagus Valley team. The entire fig fruits were partially defrosted and then homogenized by blending the entire fruit using 2 kitchen blenders: Bimby Thermomix TM5, Vorwerk and Bimby Thermomix TM 31-1, Vorwerk, with a mix of two varieties of figs: Longue d'Aout (2/3) and Bourjassote Noire (1/3).

The jams developed where divided into three different groups:

- Jams Control:
  - C1-Fig jam with Honey;
  - C2-Fig Jam;
- Fig Jams with extract incorporation at 80 °C
  - A1- Fig jam with Honey and fig leaves enrich extract;
  - A2-Fig jam with fig leaves enrich extract;
- Fig jam with extract incorporated at 50 °C
  - B1- Fig jam with Honey end fig leaves enrich extract;
  - B2-Fig jam with fig leaves enrich extract.

A1, B1 and C1 were prepared with a proportion of 80% figs and 20% honey, while the three other formulas were prepared with 100% figs. The pH of the mix was controlled, using a pH meter (CONSORT C931), by adding lemon juice until an approximate value of pH =3.8, which represented an optimum value for the pectin, that is predominant in the cell wall of fig peels and plays an important role in jellifying the jam, thus improving and keeping its texture (Ayuso et al., 2022). The Brix value, which refers to the amount of solids dissolved in a liquid and usually used to measure the dissolved sugars present in an aqueous solution (in this case jams), was controlled using a refractometer (Hanna Instruments HI 96801), by continuing the cooking process until attending a Brix value of 40, acceptable to the jams. The time of the jam's preparation varies on average between 36 min and 83 min. This difference in time is related to the Brix value and can be explained by the fact that the formulas with honey present a higher amount of sugars dissolved, thus taking lower time of cooking resulting in a final fig jam with high amount of water while the ones with no sugars added take higher time of cooking resulting in a final fig jam presenting a lower water content.

After cooking the jams at 100°C, the preparations were cool down before adding 10 mg of the extracts' mixture at different temperatures; T=80 °C for A1 and B1 and T=50 °C for A2 and B2, in order to study the impact of temperature on the extracts.

Table 4: Preparation of the fig jams

Formulation	Temperature	Honey	Extract	pH	Time
A1	80 °C	+	+	3.65	50 min
A2	80 °C	-	+	3.80	100 min
B1	50 °C	+	+	3.65	23 min
B2	50 °C	-	+	3.80	75 min
C1	80 °C	+	-	3.65	35 min
C2	80 °C	-	-	3.80	75 min

+: with, - without; the value of pH indicated was after adding lemon juice

3.5 Physical parameters

3.5.1 Color

The color of the different jams prepared was measured at t=0d, t=15d and t=30d. This test was carried out by the handheld colorimeter (Konica Minolta, model CR-400, Japan) (**Figure 11**). The D65 illuminant is the standard illuminant for European daylight (daylight illuminant) as defined by the International Commission on Illumination (CIE). The CIE 1976 color space L*a*b was used where L* stands for the lightness, ranging from 0 (black) to 100 (white); a* represents the greenness-redness coordinate, varying from green (-a*) to red (+a*); b*, corresponds to the yellowness with a viewing angle of 10°, and 8 mm aperture.

Figure 11: Colorimeter

3.5.2 Texture

In order to understand the impacts of the extract on the texture profile of the fig jam, a Stable Micro Systems (Vienna Court, Godalming 191 UK) TA. XT Plus Texture Analyzer with a 30 Kg load cell and the P/45 45 mm aluminium cylinder probe was used (**Figure 12**). Using a backward extrusion test in several dimensions could be obtained, namely firmness, consistency, cohesiveness, and work of cohesion. The used speeds were: a pre-test speed of 10 mm/s, test speed of 5 mm/s, and post-test speed of 5 mm/s, while the probe was set to trigger at 20 g and compress for 5 cm.

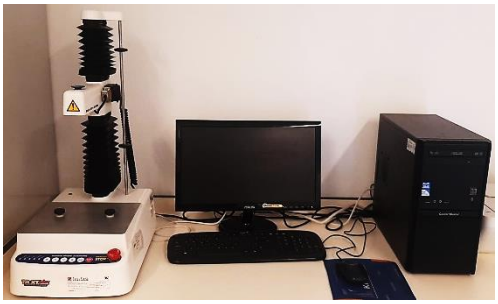


Figure 12: Texture Analyzer

3.5.3 Water activity

The values of the jams' aw were determined using a Dew Point Water Activity Meter 4TE (**Figure 13**). The water activity's value was determined at 20 °C during t=5 min by the dew-point method and with an absolute error of 0.003.

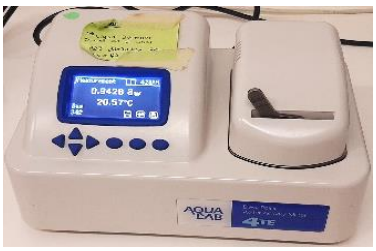


Figure 13*:* Water activity measurement

3.5.4 pH

The pH was determined using the FC 2022/HALO™ pH PROBE (**Figure 14**). The pH probe should be calibrated before using.

Figure 14: pH meter

3.6 Determination of the nutritional value and the chemical composition of the jam

3.6.1 Fat content

The determination of the fat content was performed using a Soxhlet apparatus. A mass of 3 g of fig jam was placed in extraction thimbles (of dimension Ø 26 x 60 mm) and in the extraction vessel (of dimension Ø 51 x 59 mm) was added 50 mL of Petroleum ether. The process took place in three steps as explained in **Figure 15**: the boiling phase (1) consisted of submerging the sample in a heated solvent, where the solvent continuously absorbed the sample's fat content through both immersion and reflux. During the rinsing phase (2) the sample was removed from contact with the condensed solvent. In this step, the only continuous method of sample fat absorption was reflux. The recovery phase (3) included the collection of the

solvent for future reuse, while the fat content was settled in the bottom of the reaction flask. After the extraction (1hour), the fat content was collected in tubes and placed to dry at room temperature.

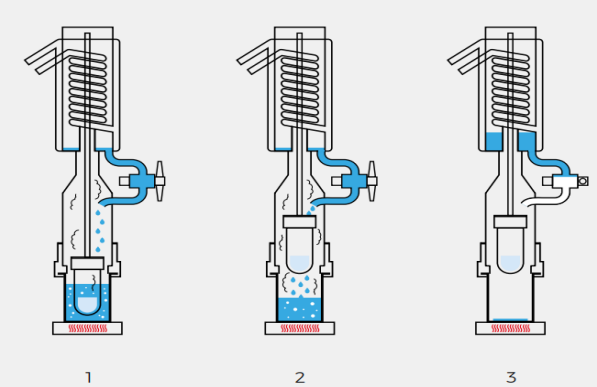


Figure 15*:* Extraction principle of the fat, (1): Boiling phase, (2): Rinsing phase, (3): Recovery phase (Leading Lab Technologies - RAYPA)

3.6.2 Proteins

The protein content of the fig jam was determined conforming to the Kjeldahl method which consists of measuring the nitrogen content of a food. In the mineralization phase,1g of sample was weighed and digested by heating with 15 mL sulphuric acid to retain the nitrogen in the form of $(NH_4)_2SO_4$, and to speed up the reaction a catalyst (K_2SO_4) was added. The tubes were placed in a digester (Foss™ Digestor) at 400 °C for approximately 70 minutes. After the digestion, the cooled tubes were placed in the Kjeldahl apparatus that automatically performs distillation and titration, the solution was made alkaline by adding NaOH to convert the $(NH_4)_2SO_4$ into NH_3 and collected by steam distillation in a known quantity of boric acid H_3BO_3. The reaction that occurred between H_3BO_3 and NH_3 transfer the ammonia gas into solvated ammonium ions (NH_4^+). The titration phase consists of adding HCl until change of color. The amount of protein was determined by multiplying the conversion factor (6.25) by the nitrogen amount read by the equipment.

3.6.3 Moisture

The fig jam samples (2 g) were placed in the balance moisture meter (Adam Equipment, PMB 163), as shown in **Figure 16**. This device gradually raises the temperature to 105 °C to force the moisture to evaporate from the food. If the weight remains constant and no evaporation was recorded, weigh the sample again. The results were obtained using the following equation:

$$\% \, Moisture \; = \; (mi - mf)/mi \; x \; 100$$

Where **mi** is the initial weight and **mf** is the weight after reaching constant weight.

Figure 16: Moisture measurement

3.6.4 Ash content

To determine the ash content a muffle furnace (Nabertherm) was used to incinerate approximately 500 mg of fig jam at 500 °C during 5 hours.

3.6.5 Total carbohydrates and energy

The carbohydrate content was then determined by difference according to the following equation:

$$Total\ carbohydrates = 100 - (g\ ashes + g\ proteins + g\ fats + moisture)$$

And to evaluate the energy content of the fig jam, the Atwater system estimated its value by the equation below:

$$Energy\ (kcal) = 4 \times (g\ protein + g\ carbohydrate) + 9 \times (g\ fats)$$ (Merill & Watt; 1995)

3.6.6 Free sugars

The quantification of the sugars was performed following a study conducted by Barros et al. (2013), with some changes, after the Soxhlet extraction, 1g of sample from which the lipid content was removed, was mixed with 1 mL of melezitose (the standard) and 40 mL of ethanol-water solution (80:20). The tubes were placed in hot bath water for 90min and agitated each 15 min. forwards, the samples were centrifuged for 10 min at 3500 rpm using a K24OR refrigerated centrifuge from Centurion, West Sussex, United Kingdom. After filtering, the supernatant was then transferred to a glass flask, where the ethanol was evaporated at 50 °C under reduced pressure using a Büchi R-210 rotary evaporator from Flawil, Switzerland. After the evaporation, the aqueous phase was washed 3 times using Diethyl ether, then collected in tubes to evaporate the rest of the diethyl ether. After, the solution was adjusted with distilled water to be measured at 5 mL, then filtered and 1.5 mL was transferred in vials to be analyzed by HPLC. The determination of free sugars from the fig jam was carried out using the High-

performance Liquid Chromatography associated to a refractive index detector (HPLC-RI, Knauer, Smartline 1000 system, Berlin, Germany). The HPLC system (Knauer, Smartline system) used is coupled to an IR detector (Knauer Smartline 2300) and the separation was achieved in a 100-5 NH2 Eurospher column (4.6×250 mm, 5 µm, Knauer). The mix acetonitrile/deionized water, 70:30 (*v/v*) with a flow rate of 1 mL/min in isocratic mode was the mobile phase used. The description of the sugars' profile was executed by making a comparison between the relative retention times of the sample peaks and the authentic standards, while an internal normalization of the peak chromatographic area using the melezitose peak (PI) as a standard was used to quantify them. The results were conducted in g per 100 g of fresh weight.

3.6.7 Fatty acids

Following Soxhlet extraction of the lipid fraction, fatty acids were determined according to a study conducted by Pinela et al. (2011) using a transesterification process with 5 mL of methanol: sulfuric acid: toluene 2:1:1 (*v:v:v*), for at least 12 hours in a water bath at 50 °C, 60 rpm. Following esterification, 3 mL of distilled water was added to separate the two phases. The fatty acid methyl esters (FAME) were recovered with 3 mL of diethyl ether, vortexed, and finally collected in flasks containing anhydrous sodium sulfate to remove any remaining water. Following that, the samples were filtered through a 0.2 m nylon filter (Whatman) and analyzed using gas chromatography (DANI 1000, Contone, Switzerland) in conjunction with a flame ionization detection (GC-FID)/capillary column. A split/splitless injector, a FID at 260 °C, and a Zebron-Kame column (30 m x0.25 mm id x0.20 m film thickness, Phenomenex, Torrance, CA, USA) were used for the analysis. At 100 °C, the carrier gas flow rate (hydrogen) was 1.1 mL/min. At 250 °C, a fractional injection (1:50) was performed. The relative retention times of fatty acid methyl ester peaks were compared with standards to identify and quantify fatty acids. The data was recorded and processed using CSW 1.7 software (DataApex 1.7), and the results were expressed as a relative percentage for each fatty acid.

3.7 Microbial assessment

3.7.1 General sample preparation

The fig jam samples were prepared according to the International Organization for Standardization procedure (ISO) 6887-1:2003 according to Carocho et al. (2019). In stomacher bags, the fig jam samples (10 g) were mixed with 90 mL of peptone water (PW) then homogenized in a stomacher equipment (ECN 710-0873, Italy) for 1 min at 300 units. The

suspensions were then diluted to obtain dilutions from 10^{-1} to 10^{-3} that were analyzed in duplicate each.

3.7.2 Microorganism analysis

The microbiological characterization was based on different microorganisms: total aerobic mesophylls, *Escherichia Coli*, yeasts and molds along with *Clostridium perfringens*.

- Total aerobic Mesophils*:* the counting of the Total aerobic Mesophils was carried out by the pour plate method by mixing 1mL of the suspension and 20 mL of Plate Count Agar (PCA) in duplicate (LOQ = 1 log UFC/g, LOQ corresponds to limit of quantification). The plates were incubated at 30°C for 3 days and then counted conforming to ISO 4833-2:2013. The assay was realized at day 0, day 15 and day 30.
- *Escherichia coli:* the determination of the presence or absence of *E.coli* was performed by the pour plate method. In duplicate, 1mL of suspension was pipetted into the plate and 15mL of melted Violet Red Bile Lactose Agar-VRBLA (kept at 50 °C in a water bath) were poured to be homogenized and solidified before adding a top layer of 4mL of VRBLA on the top of the medium and let it solidify, then the plates were incubated in reversed position at 30°C for 48 hours. Conforming to ISO 4832:2006, the plates presenting colonies between 10 and 150 were counted. The *E.coli* colonies were observed in the medium (VRBLA) supplemented with Methylumbelliferyl-b-D-glucuronide MUG (used together as a selective medium to detect *E.coli* in food product), as blue fluorescent colonies under long UV light (365 nm) and counted as follow LOQ = 10 UFC/g.
- Yeasts and molds: In petri dishes containing 20 mL of Agar Dicloran Rosa Bengala Cloranfenicol Base (DRBC) was added 0.2 mL the suspension, in duplicate (LOQ = 1.7 log UFC/g). The plates were incubated at 25°C for 3 days for yeasts and 5 days for molds, afterwards counted in agreement with ISO 21527-2:2008. This assay was realized at day 0, day 15 and day 30.

- *Clostridium perfringens:* To verify the presence of *Clostridium perfringens,* 5mL of the initial suspension were transferred into a 50mL falcon (in duplicate) and dipped in a water bath along with the control, which consisted of transferring 5mL of purified water into a 50mL falcon, at 80°C for 10min (once the

temperature was reached start counting 10min). After the heating process the suspension was immediately cooled in a cold water bath before adding 25mL of Iron sulphite agar (ISA) medium into the falcon and homogenized without creating bubbles, then the tube was transferred in a cold bath to solidify. Once solidified 5mL of ISA medium were added to create anaerobiosis before incubating at 30°C for 24 hours. The spores of *C. perfringens* were observed as black spots that were counted according to ISO 15213:2003, LOQ=2UFC/g.

3.8 Statistical analysis

All data were presented throughout the entire document as mean ± standard deviation. The Design Expert 11 program (Stat-Ease, Minneapolis, MN, USA) was used for the optimization protocol using the Box-Behnken model for the design of experiments followed by an optimization of the TP response. For the extracts analysis a one-way analysis of variance (ANOVA) was used, relying on a Tukey's test for post-hoc classification.

For the jam samples, considering the two factors included, storage time (ST) and incorporation (I), a two-way ANOVA with type III sums of squares using the SPSS Software, version 25 was used. This multivariate general linear model treats the two factors, ST and I as independent, thus allowing the effect of each one to be analyzed independently, providing more insight on their contribution towards the changes. If a significant interaction (<0.05) was recorded among the two factors (ST×I), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM). If there was no significant interaction (>0.05), each factor was evaluated independently using a Tukey's or Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

Results and discussion

4. Results and discussion

According to the FAO (2020), the 17 Sustainable Development Goals cover a variety of subjects, including: (2) Zero Hunger and Sustainable Agriculture; (9) Industry, Innovation, and Infrastructure; along with (12) Responsible Consumption and Production, which are crucial for promoting the circular economy, product development, and conscious production. For this reason, this work was realized under a scope of a project named 100% figo, including industries among them Mó de Cima, specialized in fig production and fig based products in order to valorize fig bio-residues, in our case fig leaves usually discarded by the industry and their incorporation in fig jam, thus emphasizing sustainability. The five varieties of fig leaves were first screened to determine their potency in terms of phenolic compounds and bioactivities as described in the section below.

4.1 Phenolic compounds profile of the fig varieties and bioactive potential evaluation

4.1.1 Phenolic compounds' profile

The chromatographic data of the phenolic compounds present in the hydroethanolic extracts of the five varieties of fig leaves, were ascertained by HPLC-DAD/ESI-MS through the retention time, wavelengths of maximum absorption in the visible region, mass spectrum data, tentative of identification and quantification were displayed in **Table 5**. According to the chromatographic results 12 phenolic compounds were identified for Bn, La, Pa and Ma, while 13 compounds were determined for Da that can be classified as flavonoids and phenolic acids. As mentioned in **Table 5** polyphenols' content varied greatly among fig varieties and was specific to each one. La variety presented the highest amount of total phenolic compounds, (42.442±0.27 mg/g of extract) followed by Da, Pa, Ma and Bn. The major compounds in the hydroethanolic extracts were Apigenin-*C*-hexoside-*C*-pentoside, ranged between 6.19±0.08 mg/g of extract (Bn) and 13.623±0.107 mg/g of extract (La) followed by the Quercetin-*O*-deoxyhexosyl-hexoside, varied from 5.293±0.006 mg/g of extract (Bn**)** to 9.334±0.039 mg/g of extract (La). The predominant phenolic acids were caffeic acid and *cis* 5-O-caffeoylquinic acid that were respectively highly presented in La and Pa.

The quantitative variations in fig varieties have been reported by several writers. Petruccelli et al. (2018) mentioned that from 10 varieties of Italian fig leaves, 18 phenolic compounds were identified using data from HPLC-DAD-TOF-MS, the total phenol content varied from 26.9 mg/g of DW (Dry Weight) to 7.50 mg/g of DW. Abdel-Aziz et al. (2020) documented that from different fig leaves collected in Egypt, 21 phenolic compounds were analyzed using HPLC with

a total content of 63.972 mg/g DW. While according to Teruel-Andreu et al. (2021), *Ficus carica*.L leaves presented 40 phenolic compounds according to 41 studies that were conducted in which total phenols were analyzed. Petruccelli et al. (2018) documented that 5-O-caffeoylquinic acid ranged from 0.405±0.04 mg/g DW to 2.061±0.02 mg/g DW. While Teruel-Andreu et al. (2021) reported that the kaempferol 3-O-glucoside was the main compound in fig leaves.

The variations between the different results may be explained by the different experimental factors used while the extraction of the phenolic compounds such as the type of the solvent and the method of extraction. Bruni and Sacchetti. (2009), mentioned that genetic control and physiological factors as well as differing growth periods, geographic location and horticultural practices are factors that could impact the biosynthesis of polyphenols.

Table 5: Tentative chromatographic identification of phenolic compounds present in fig leaves (retention time (Rt), wavelengths of maximum absorption in the visible region (max), and spectral mass, quantification data (mg/g extract).

Peak	Rt (min)	lmax (nm)	$[M-H]^-$ (*m/z*)	MS2 (*m/z*)	Tentative identification	Quantification				
						Pa	La	Da	Bn	Ma
1'	4.92	273	343	181(100),137(12),121(8)	Homovanillic acid hexoside	nd	nd	nd	nd	nd
1	5.99	320	341	179(100),161(18),135(5)	Caffeic acid hexoside	0.747±0.01 [b]	0.559±0.027 [c]	1.08±0.028 [a]	0.427±0.008 [d]	0.535±0.014 [c]
2	7.08	324	353	191(100),179(12),161(7),135(5)	*cis* 5-O-caffeoylquinic acid	1.524±0.056 [a]	1.221±0.036 [b]	1.275±0.067 [b]	0.548±0.01 [e]	0.667±0.027 [d]
3	7.26	324	353	191(100),179(9),161(8),135(5)	*trans* 5-*O*-Caffeoylquinic acid	nd	nd	2.361 ± 0.004	nd	nd
3'	8.6	253/292	459	167(100),151(12),123(7)	Vanillic acid di-deoxyhexoside	nd	nd	nd	nd	nd
4	10.12	328	579	459(22),429(83),357(63),327(100),309(54)	Luteolin *O*-pentosyl-C-hexoside	3.539±0.149 [c]	7.696±0.202 [a]	3.986±0.093 [b]	0.092±0.001 [e]	3.086±0.199 [d]
5	10.54	324	179	163(100)	Caffeic acid	2.547±0.039 [b]	3.682±0.049 [a]	2.163±0.071 [c]	0.706±0.02 [f]	1.022±0.056 [e]
7	11.7	283	337	191(100),163(12),119(10)	5-*O*-*p*-Coumaroylquinic acid	3.624±0.003 [a]	0.947±0.045 [c]	0.923±0.025 [c]	0.599±0.001 [e]	0.68±0.023 [d]
8	12.88	337	563	473(58),443(100),383(15),353(20),311(5),297(5)	Apigenin-*C*-hexoside-*C*-pentoside	6.631±0.054 [d]	13.623±0.107 [a]	8.831±0.128 [b]	6.19±0.08 [f]	6.402±0.023 [e]
9	14.37	338	563	473(58),443(100),383(15),353(20),311(5),297(5)	Apigenin-*C*-hexoside-*C*-pentoside	0.562±0.002 [d]	0.867±0.028 [b]	1.009±0.018 [a]	0.592±0.013 [c]	0.601±0.015 [c]
10	15.72	287	545	501(100),459(13),313(5),167(98)	Vanillic acid -malonyl-rhamnoside-rhamnoside	0.104±0.001 [e]	0.237±0.005 [a]	0.181±0.001 [b]	0.175±0.001 [c]	0.178±0.004 [b,c]
11	16.82	355	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside	6.716±0.038 [d]	9.334±0.039 [b]	9.903±0.007 [a]	5.293±0.006 [f]	5.966±0.023 [e]
11'	17.4	341	609	301(100)	Quercetin-*O*-deoxyhexosyl-hexoside	nd	nd	nd	nd	nd
12	18.21	351	463	301(100)	Quercetin-*O*-hexoside	1.426±0.007 [b]	0.998±0.022 [c]	1.160±0.03 [a]	0.586±0.004 [f]	0.668±0.002 [e]
13	19.58	355	549	505(),463(),301()	Quercetin-*O*-malonyl-hexoside	3.640±0.007 [a]	1.895±0.042 [b]	1.386±0.032 [d]	0.826±0.001 [f]	1.279±0.014 [e]
14	20	359	593	285(100)	Kaempherol-O-deoxyhexosyl-hexoside	0.895±0.013 [d]	1.383±0.026 [a]	1.047±0.02 [b]	0.718±0.009 [e]	0.735±0.012 [e]
					TPC	31.954±0.168 [c]	42.442±0.27 [a]	35.304±0.293 [b]	16.754±0.067 [f]	21.82±0.288 [e]
					TPA	8.545±0.024 [a]	6.646±0.153 [c]	7.983±0.08 [b]	2.455±0.002 [f]	3.083±0.05 [e]
					TF	23.41±0.144 [c]	35.796±0.117 [a]	27.321±0.213 [b]	14.299±0.069 [e]	18.738±0.238 [d]

nd: not detected, TPC: Total Phenolic Compounds; TPA: Total Phenolic Acids; TF: Total Flavonoids ; for table rows, significant differences are represented by different letters

4.1.2 Antioxidant activity

To the best of our knowledge, the current work was the first to describe how to assess the antioxidant capacity of *Ficus carica* L. leaves using a combination of TBARS and CAA. Antioxidants are substances that, depending on the oxidative agent, either inhibit or prevent oxidative stress through mechanisms like reduction of lipid peroxidation and/or elimination of free radicals (Carocho, Morales & Ferreira., 2018). It was suggested in the current work to assess the antioxidant in vitro characteristics by preventing lipid peroxidation in pig brain tissues by producing reactive thiobarbituric acid compounds (TBARS) and the evaluation of antioxidant properties by reducing the reactive oxygen species (ROS) by the CAA method. The results of antioxidant activity were expressed as EC_{50} (µg/mL) for TBARS assay and as % inhibition max tested for CAA as what was mentioned in **Table 6**. Indeed, Bn was the variety that highly prevented lipid peroxidation with the lowest EC_{50} (230±10 µg/mL) followed by Da (350±10 µg/mL) and Ma (450±30 µg/mL), while Pa showed the highest EC_{50} (780±20 µg/mL) thus the lowest antioxidant activity. As for the CAA, Pa expressed the maximal capacity to reduce the ROS (% inhibition max tested 65±11), however Bn revealed a lower % inhibition max tested (40±3 %). For TBARS and CAA assays, Trolox and quercetin were used as positive controls, respectively, displaying better inhibition of thiobarbituric acid reactive substances and reactive oxygen substances in comparison with the tested varieties and the mixture.

According to Palmeira et al. (2019), fig peel and pulp were used to study their antioxidant activity by the TBARS method, found that the peel presented an IC_{50} of 1.14± 0.04 mg/mL and the pulp 1.24±0.04 mg/mL. These results revealed better capacity when compared with the ones verified by Viuda-Martos et al. (2015) that explored the peel and pulp powders. Although their antioxidant potentials, our fig leaves measured by the TBARS method were more promising. The antioxidant activity of the fig leaves were shown to be highly correlated with their phenolic content including Apigenin-*C*-hexoside-*C*-pentoside (Farooq et al., 2020) and quercetin isomers that revealed strong antioxidant capacity through its effect on reactive oxygen species (Xu et al., 2019). Since the type of compounds in the sample can greatly affect its activity and behavior within the cells, thereby influencing their biological activity, the difference in results between the two tests of antioxidant activity is further evidence that this activity must be evaluated using different methodologies. In addition, the difference in results could be explained by the different mechanisms of actions of the two methods

Table 6: Antioxidant activity by thiobarbituric acid reactive substances inhibition assay method (TBARS) and cellular antioxidant activity (CAA) of leaf samples of different fig varieties

Leaves	TBARS	CAA	
	EC_{50} (µg/mL)	max tested [] (µg/mL)	% inhibition max tested []
Pa	780±20^{e}	2000	65±11
La	740±20^{d}	2000	60±4
Da	350±10^{b}	2000	57±5
Bn	230±10^{a}	2000	40±3
Ma	450±30^{c}	2000	33±3
Positive Control	9.1±0.3 (Trolox µg/mL)	0.3 (Quercetin µg/mL)	95±5

(For table rows, significant differences are represented by different letters, []: concentration)

4.1.3 Antimicrobial activity

The antimicrobial potential of the five varieties of fig leaves was studied through the microdilution method against selected food borne bacterial strains and fungi, to determine the minimum inhibition concentration (MIC), the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) as shown in **Table 7**.

Considering **Table 7**, all the varieties revealed activity against some of the Gram-positive and Gram-negative bacteria as well as a moderate antifungal activity. Pa was proved to be the variety with the highest capacity to inhibit all the bacteria except *Pseudomonas aeruginosa* followed by La, while Da, Bn and Ma acted against five bacteria strains. *Yersinia enterocolitica* showed higher sensitivity to Bn and Ma presenting MIC values of 0.6 mg/mL, however *Listeria monocytogenes* was only inhibited by the action of Pa with a MIC value of 5 mg/mL. Among the different varieties, Da presented the lowest MIC values against *Salmonella enterocolitica*, *Escherichia coli* and *Staphylococcus aureus* (5 mg/mL and 2.5 mg/mL, respectively). Actually *Pseudomonas aeruginosa* was the most resistant bacteria as no activity was shown. In addition, all the varieties had activity against *Aspergillus brasiliensis* and *Aspergillus fumigatus* with MIC values of 10 mg/mL except for Pa that showed highest antifungal potential against *A. fumigatus* with MIC value of 5mg/mL. Besides, the MBC and MFC values of all the varieties were the same (>10 mg/mL).

The antimicrobial potential of *Ficus carica* leaves was carried out by several studies through the agar disc diffusion method. Weli et al (2015), reported that the highest antibacterial activity was obtained from ethyl acetate extract against *E.Coli* and *P. aeruginosa* and moderate

potential against *S. aureus*. Another study conducted by Mahmoudi et al. (2016) reported the MIC values of ten Algerian varieties of fig leaves against *B. cereus and S. aureus* ranged from 2.19-8.75 mg/mL and 8.75-17.5 mg/mL, respectively. In comparison with our results *S. aureus* was more sensitive to our varieties with MIC values ranging from 2.5-10 mg/mL, whereas comparing the MIC values of the studied extracts of the Algerian fig leaves with our extracts (MICε10 mg/mL), we noticed that *B. cereus* was less susceptible to our leaves. Furthermore, our extracts were noticed to be more efficient on Gram negative bacteria as they acted against 80% of the total strains while Gram positive bacteria were less susceptible (approximately 53% of the total bacteria tested). The same results were described by Weli et al. (2015), mentioning that this behavior could be explained by an unusual mechanism of the analyzed extracts active compounds.

Table 7: Antimicrobial activity of fig leaves

	Fig leaves										Positive Control					
	Pa		La		Da		Bn		Ma		Streptomicina 1 mg/mL		Methicillin 1 mg/mL		Ampicillin 20 mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria																
Enterobacter Cloacae	10	>10	10	>10	10	>10	10	>10	10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	10	>10	10	>10	2,5	>10	5	>10	5	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterocolitica	10	>10	10	>10	5	>10	10	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	10	>10	5	>10	1.25	>10	0.6	>10	0.6	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria																
Bacillus cereus	10	>10	10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	5	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	10	>10	10	>10	2,5	>10	10	>10	10	>10	0.007	0.007	0.007	0.007	0.15	0.15
Fungi											Ketoconazole					
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC				MFC	
Aspergillus brasiliensis	10	>10	10	>10	10	>10	10	>10	10	>10	0.06				0.125	
Aspergillus fumigatus	5	>10	10	>10	>10	>10	10	>10	10	>10	0.5				1	

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MFC: Minimum fungicidal concentration; (for table rows, significant differences are represented by different letters

4.1.4 Cytotoxicity

The evaluation of cytotoxicity and hepatotoxicity were based on the ability of the extract to inhibit 50% of cell proliferation (GI_{50}) as described in **Table 8**. From results obtained, Da showed the highest capacity to inhibit 50% of gastric adenocarcinoma (AGS) cell proliferation with a GI_{50} of 158±13 μg/mL, followed by La (GI_{50} of 173±12 μg/mL) and Bn (GI_{50} of 235±23 μg/mL). While Pa and Ma did not show activity. For the breast adenocarcinoma (MCF-7) cell line all varieties, except for Bn (GI_{50} >400 μg/mL), had the capacity to inhibit 50% of cell proliferation and the maximal activity was revealed by La (GI_{50} of 207.5±6.9 μg/mL). All the extracts from the five varieties of figs had a similar behavior (GI_{50} >400 μg/mL), which indicated that they did not inhibit the cell proliferation of colorectal adenocarcinoma (CaCO2). Regarding the hepatotoxicity, the varieties Pa, Bn and Ma did not show capacity to inhibit the proliferation of non-tumor cell line (PLP2) with a GI_{50} >400 μg/mL, thus confirming the safety of these varieties extracts to be incorporated in food products.

However, La and Da revealed the capacity to inhibit non-tumor cell proliferation. The concentration required to inhibit the proliferation of PLP2 was 225 ± 11 μg/mL for La and 248 ± 10 μg/mL for Da. Nonetheless those obtained GI_{50} values were higher than the ones obtained for the tumor cells. This fact constitutes a security window, on which we can safely apply the recovered extracts without the risk of toxic effects.

The results obtained in this work were compared to a research conducted by Abdel-Rahman et al. (2021) who studied the cytotoxicity of fig leaves, collected from local farms in Egypt, using non-tumor cells by treating peripheral blood mononuclear cells (PBMC) and tumor-cell lines including CaCo-2 and MCF-7. The results of PBMC showed that at the lowest concentrations of *Ficus carica* extract (5000, 2500, 1250, 625, 312.5, and 156 μg/ml), there were no cytotoxic effects. With regards to CaCo-2 cell lines, the highest inhibition of cell proliferation was recorded under the highest concentration of fig leaves extract while the 50% of inhibition was shown under a concentration of approximately 1250 μg/mL. In the case of the cancer line MCF-7, 50% of growth inhibition was obtained when concentration of the extracts was at 312.5 μg/mL.

Table 8: Cytotoxicity of leaves of different fig varieties

	Pa	La	Da	Bn	Ma	Ellipticine (positive control)
Cytotoxicity (GI_{50}, µg/mL)						
AGS	>400	173±12	158±13	235±23	>400	1.23±0.03
MCF-7	253±12	207.5±6.9	223±21	>400	279±24	1.02±0.02
CaCo2	>400	>400	>400	>400	>400	1.21±0.02
Hepatotoxicity (GI_{50}, µg/mL)						
PLP2	>400	225±11	248±10	>400	>400	1.4±0.1

>400 – did not show activity at the maximum tested concentration; gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo2), breast adenocarcinoma (MCF-7) pig liver cells (PLP2)

4.1.5 Anti-inflammatory activity

The anti-inflammatory activity was performed to determine the capacity of a substance to inhibit the production of nitric oxide, considered as a pro-inflammatory mediator that in abnormal situations its production became higher (Sharma et al., 2007). In this assay, NO was stimulated by adding LPS and the results were expressed as IC_{50}. Da was the variety that showed the highest anti-inflammatory activity with an IC_{50} of 19.6±5.2 µg/mL, followed by Pa and Ma. While La and Bn did not reveal activity with a value of IC_{50}> 400 µg/mL (**Table 9**).

Andrade et al. (2019) studied the leaf extract from *Ficus curtipes* that presented a decrease in NO levels with concentrations ranged from 31.25 to 500 µg/mL and found also that the highest concentration caused a significant NO reduction (39.32±8.69%) compared to the control. Although, *F.curtipes* leaf extract presented promising results the leaves of some varieties of *F.carica* showed higher anti-inflammatory potentials.

The different varieties of fig leaves were shown to be a potential source of phenolic compounds such as Apigenin-*C*-hexoside-*C*-pentoside and Quercetin-*O*-deoxyhexosyl-hexoside, the two major molecules that could have an impact on the bioactivities along with the other phenolic acids and flavonoids as presented above.

Table 9: Anti-inflammatory activity of leaves of different fig species

	Pa	La	Da	Bn	Ma	Ellipticine (positive control)
RAW (IC_{50},µg/mL)	82±8	>400	19.6±5.2	>400	89±3	6.3±0.4

>400 – did not show activity at the maximum tested concentration

4.2 Extraction optimization

After screening the fig leaves, two methods of extraction were optimized in order to maximize the amount of total phenols while optimizing the extraction's conditions before studying the impact of mixing the leaves together on the bioactivities.

4.2.1 Response surface methodology for the extraction of total phenols

The extraction was carried out using two methods: UAE and DM, each revealed different results of the total phenol content as shown below.

4.2.1.1 Dynamic maceration (DM) extraction optimization

The experimental design for DM of the five varieties of fig leaves was carried out using the Box Behnken model, which only took into account points with the defined range of the different factors, which were: extraction temperature, ranging from 20 to 80 °C (A); extraction time, varying between 5 to 90 minutes (B) and the percentage of the solvent, which extended from 0% (only water) to 100% ethanol. The studied response (R) was the amount of total phenols (TP) obtained by the Folin Ciocalteu method (**Table 10**).

Table 10 presented the 17 conditions applied, the response (R) of the varieties studied along with the appropriate p-value of the model and the lack-of-fit.

Table 10: Experimental design of the extraction's conditions of the amount of total phenols of Pa, La, Da, Bn and Ma using DM

				Response – Total Phenols				
Run	Temperature (°C) (A)	Time (min) (B)	Solvent (%) (C)	Pa	La	Da	Bn	Ma
1	50	47.5	50	10.6417	20.3519	1.5432	14.4103	6.60828
2	20	90	50	6.57869	14.8077	1.42059	~~12.4104~~	5.28071
3	50	5	0	4.89174	20.087	1.20074	14.5949	16.365
4	50	47.5	50	10.6713	21.7007	1.72359	14.6287	7.22838
5	80	47.5	0	5.79934	21.9346	1.72923	15.4701	14.7795
6	80	90	50	7.7907	15.828	3.68536	13.7888	5.5964
7	50	90	100	1.91103	3.11318	0.524266	0.646876	1.07954
8	50	47.5	50	11.0124	18.9553	1.56293	14.4835	5.99382
9	50	47.5	50	10.3162	~~9.81307~~	2.06606	15.5574	6.67311
10	80	5	50	8.10216	11.9228	1.64608	11.6015	4.91851
11	20	5	50	4.8565	5.50902	0.768077	9.13801	4.0687
12	50	47.5	50	~~3.8277~~	16.434	1.96036	14.4399	7.77238
13	50	5	100	1.18383	1.43327	0.262133	0.236765	0.938605
14	20	47.5	100	0.796264	~~0.140932~~	0.218444	0.0690565	0.675063
15	20	47.5	0	6.98598	16.5482	1.40509	11.4197	17.443
16	80	47.5	100	2.5987	1.17537	0.606006	0.858274	1.49669
17	50	90	0	6.45467	19.9052	2.56355	13.2109	13.6873
***p*-value**				< 0.0001	0.0058	< 0.0001	< 0.0001	< 0.0001
Lack-of-fit				0.0722	0.3153	0.6130	0.9664	0.3399

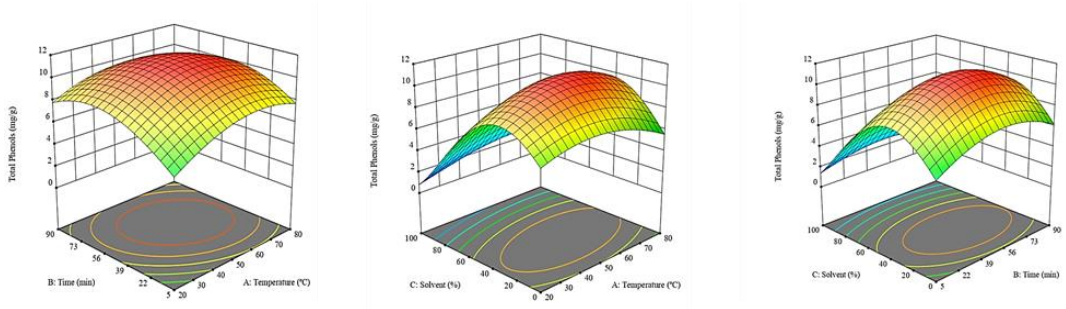
To check the quality of the quadratic model suggested by the software the *p*-value of the model and the lack-of-fit test, which is used to evaluate the model's "fitness", by demonstrating the model's appropriateness to correctly estimate the variation, were evaluated (Prasard et al., 2011). A good fit was assessed as the model's *p*-value <0.05 and a p-value for the lack of fit higher than 0.05. For this reason, the studied responses were optimized by applying transformations of natural Log, and base 10 Log for Ma and Da respectively, and a square root transformation with ignoring the second run for Bn, and with ignoring the twelfth run for Pa. As for La no transformation was needed, however the runs 9 and 14 were ignored to make the model fit better. Furthermore, the combination of the 17 runs were performed to fit the second order polynomial equation model for each variety:

- Pa: $3.26 + 0.1692A + 0.1161B - 0.0636C - 0.1041AB + 0.2387AC - 0.0086BC - 0.29A^2 – 0.3732B^2 -1.09C^2$
- La: $19.36 + 1.69A + 1.84B – 9.03C – 1.35AB – 1.18AC + 0.4654BC – 4.22\ A^2 – 3.12B^2 - 5.10C^2$
- Ma: $1.92 + 0.1098A + 0.0439B – 1.37C – 0.0329AB + 0.2405AC + 0.0796BC – 0.1455A^2 – 0.18B^2 -0.3849C^2$
- Bn: $3.83 + 0.3063A + 0.0350B – 1.54C + 0.1167AB + 0.0274AC + 0.1258BC – 0.3219A^2 – 0.3732B^2 – 1.39C^2$
- Ma: $1.92 + 0.1089A + 0.0439B – 1.37C – 0.0329AB + 0.2405AC + 0.0796BC – 0.1455A^2 – 0.18B^2 -0.3849C^2$

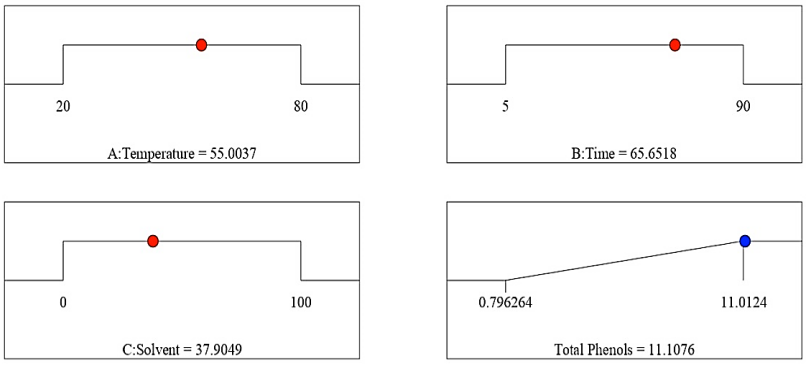
To better understand the optimization process, the 3D response surface plots were established to visually interpret the effect of the temperature-time- % of solvent combination on the analyzed response. In each 3D graph, the excluded value was fixed at its optimal value.

As revealed in **Figure 17a**, for **Pa**, by fixing the solvent (C) at its optimal value, the impact of temperature (A) and time (B) on the amount of TP was emphasized (Response – R), on the left hand side plot. The red area was large and the range of temperature (A) that yielded in the highest amount of TP was between 50 °C and 70 °C (precisely at 55 °C) followed by time (B) in which the highest yield extended from 56 to 73 min (precisely at 66 min).To showcase the effect of temperature (A) and solvent (C) on TP, the time (B) was fixed at its optimal value (middle plot). Both factors interacted very well, with values varying from 55-70 °C for temperature (precisely at 55 °C) and a % of solvent of 40 to 50% (precisely at 38%) that generated the maximum amount of the response. The interaction between the time (B) and solvent (C) had the same profile as (AC), for higher amount of TP the temperature was found effective between 56-73 °C (precisely at 55 °C) and the solvent varied from 30% to 50% (precisely at 38%). Combining the optimal values of the 3 factors together, the optimal point

was set at A = 55 °C, B = 66min and S= 38% ethanol with a predicted amount of TP of 11.1 mg/g similar to the experimental response obtained being 11 mg/g (**Figure 17b**).



(a)

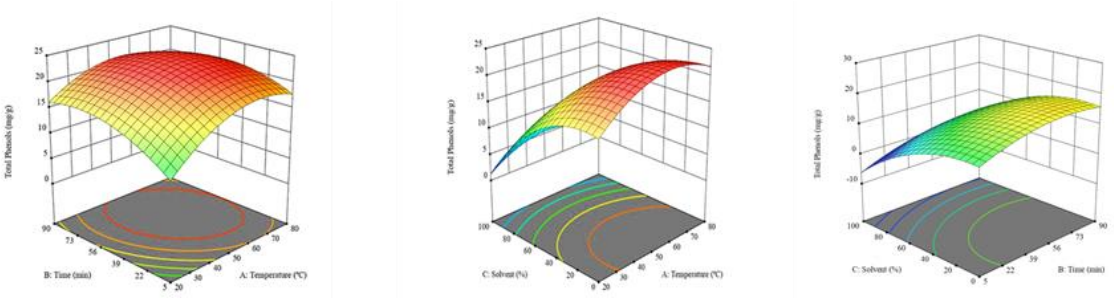


(b)

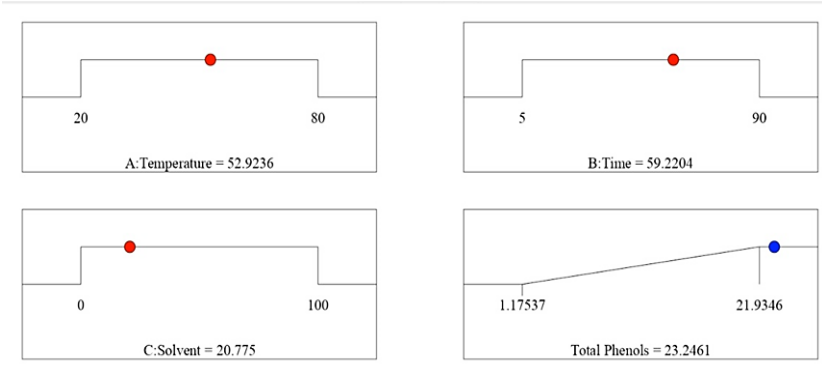
Figure 17: Graphical representation of the optimal points of **Pa** for the DM extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

The optimal conditions applied to **La** variety were shown in **Figure 18a**, revealing that a long range of time and temperature yielded good results.

It was noteworthy, studying the impact of temperature (A) and solvent (C) on TP, showing that, the increase of solvents helped promote the yield, while temperature had a low effect (center plot). The association of both factors B and C showed that once again the lower amounts of solvent promoted the yield, while a small variation was sought over time. The combination of the 3 factors together set at their optimal values, gave a predicted amount of TP of 23 mg/g which was slightly higher than the experimental value 22 mg/g at A = 53 °C, B = 59 min and S = 21% ethanol (**Figure 18b**).



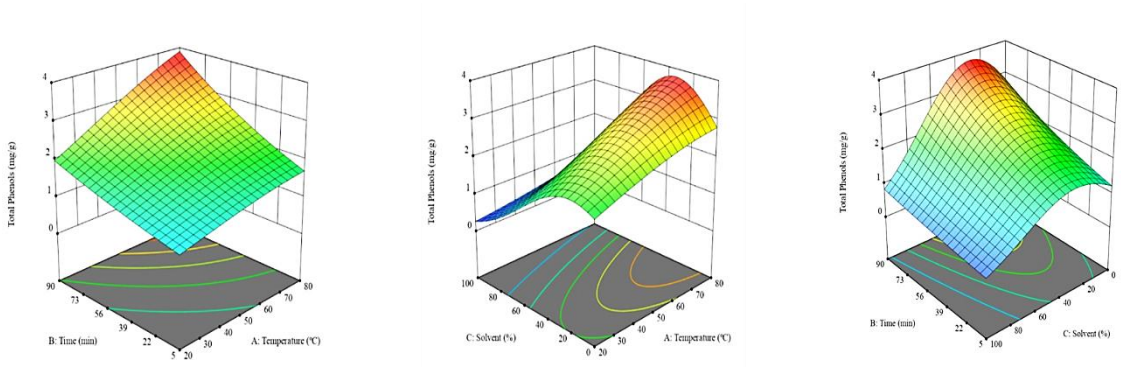
(a)



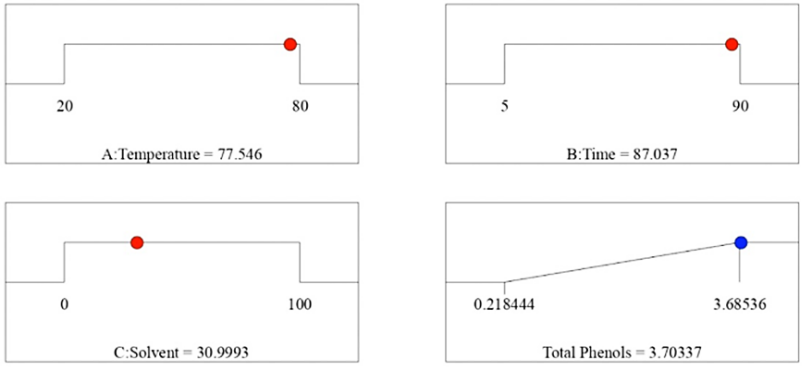
(b)

Figure 18: Graphical representation of the optimal points of **La** for the DM extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

The favorable conditions for the extraction of the maximum amount of TP from **Da** were represented in **Figure 19a**, the influence of temperature (A) and time (B) was highlighted. The higher temperature (A) (70 – 80 °C) and longer extraction time (B) (73 - 90 min) generated maximum extraction of TP (precisely at 77 °C and 87 min). The AC plot showed that high extractability of TP, set at temperatures between 70 – 80 °C and 20 – 40% of solvent (precisely 77 °C and 31%, respectively). The solvent (C) vs time (B) (right plot) revealed that 20 – 40% of ethanol with maximum extraction time resulted in high amount of TP. In **Figure 19b**, the optimal conditions were shown, in which the predicted content of TP was equal to the experimented one (3.7 mg/g) once testing the optimal conditions A = 77 °C, B= 87 min and S= 31% ethanol.



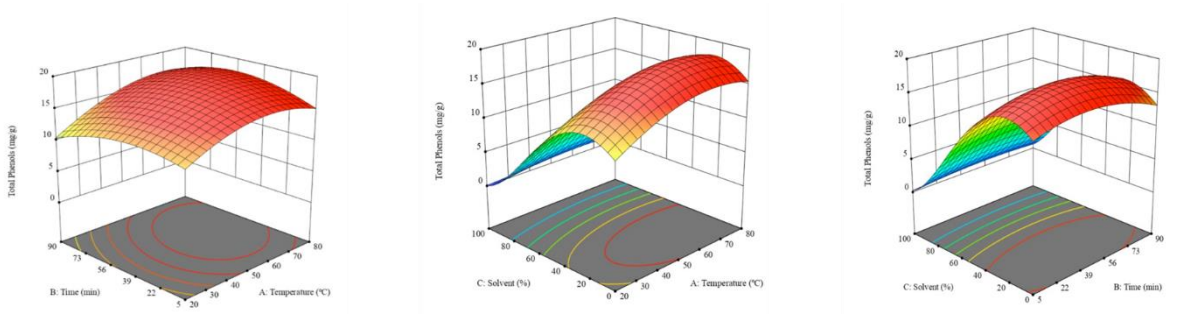
(a)



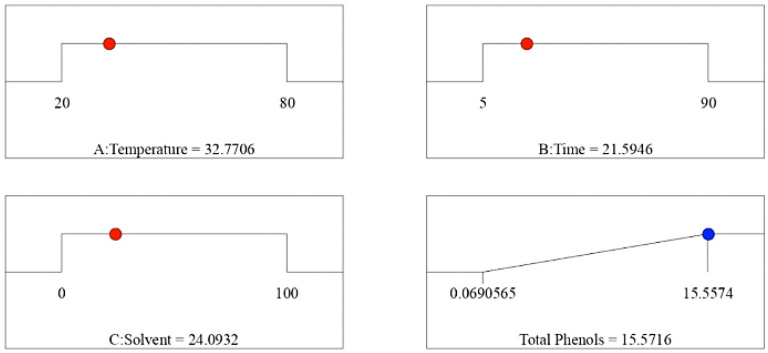
(b)

Figure 19: Graphical representation of the optimal points of **Da** for the DM extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

The 3D plots represented the influence of the three factors on the response (**Figure 20a**). As for **Bn**, through varying temperature (A) and time (B) a notable interaction between the two factors was observed which was highlighted by a large red zone, meaning that these two factors had low contributions to the yield. To study the interaction between temperature (A) and solvent (C), the time was adjusted at its optimal value. High TP content was observed at temperatures between 30 °C and 80 °C (precisely 33 °C) with a percentage of ethanol ranging from 0% to 40% (ideally 24 %). The BC graph displayed that the maximum extractability of TP was at time of 22min and solvent extending from 20 to 40% (precisely 24%). Overall low ethanol percentages were the most important factor to improve the yields of total phenols. Combining the three factors together set at their optimal values (A = 33 °C, B = 22 min and C = 24%), both the predicted and the experiment amounts of total phenols were equal as shown in **Figure 20b**.



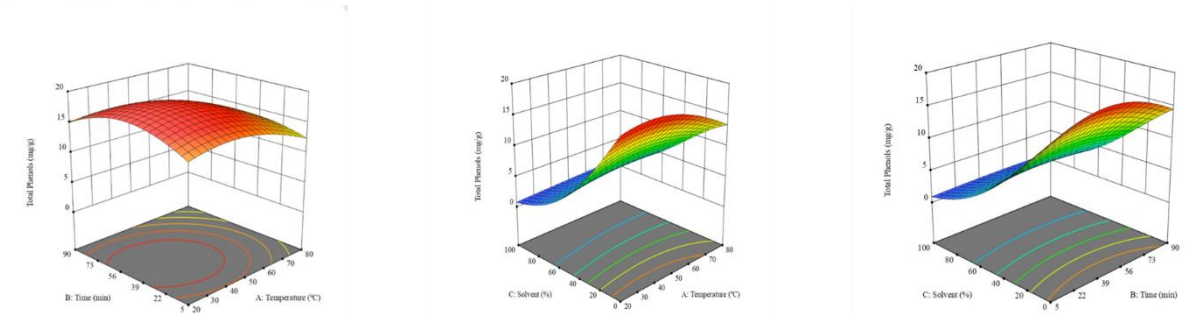
(a)



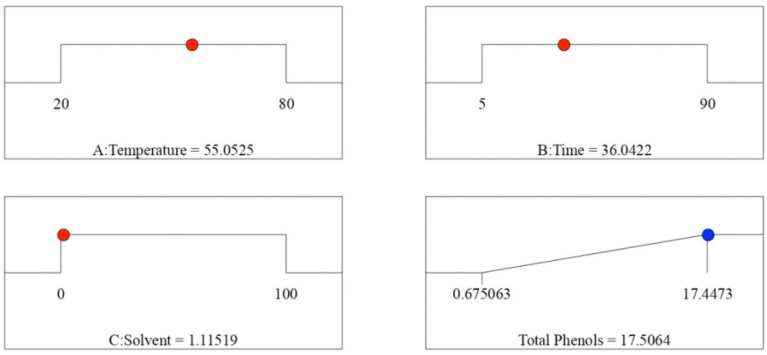
(b)

Figure 20: Graphical representation of the optimal points of **Bn** for the DM extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

The **Ma**'s 3D plots revealed the relation between the different factors studied (**Figure 21a**). High amount of TP was registered with a large red zone where temperatures (A) varied between 20 °C and 50 °C (precisely at 55°C) while time (B) was between 22 – 73 min. Once again, these two factoes did not show much influence to the improvement of higher yields. The adjustment of time (B) at its optimal value while varying the temperature (A) and solvent (C) showed that the highest TP content was at 30 °C< A < 50 °C and 0%< S <10%. As for BC the maximum of TP was showcased at 39 min< B <73 min and 0%< C <10%. Actually, when applying the optimal conditions (A= 55 °C, B = 36 min, C = 1%) the predicted amount of TP was almost equal to the experimental (**Figure 21b**). The profile of Ma was similar to Bn, in which low solvent amount promoted the extraction of total phenols.



(a)



(b)

Figure 21: Graphical representation of the optimal points of **Ma** for the DM extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

According to the results cited above the optimal conditions established for DM were temperature varying from 33 - 77 °C, extraction time ranging between 22 - 87 min and % of solvent between 21 - 38% of ethanol to obtain higher amount of TP being between 3.7 mg/g (Da) and 23mg/g (La).

4.2.1.2 Ultrasound assisted extraction (UAE) optimization

The Box Behnken model was used to carry out the experimental design for UAE of the five varieties of fig leaves. The factors were extraction time, ranging from 3 to 30 min (A), extraction power varying from 5 to 75 % (between 25-375W being their equivalent in Watt) (B), and the percentage of the solvent, which extended from 0% (only water) to 100% ethanol. The amount of total phenols (R) as determined by the Folin Ciocalteu technique was the response under study. The goal of the UAE extraction's optimization process was to maximize the total phenol content in order to achieve the promising level of food preservation.

Table 11 represented the 17 conditions applied, the response obtained (R) of the varieties studied along with the appropriate p-value of the model and the lack-of-fit.

Table 11: Experimental design of the extraction's conditions of the amount of total phenols of Pa, La, Da, Bn and Ma using UAE

				R				
Run	Time (min)	Power (%)	Solvent (%)	Pa	La	Da	Bn	Ma
1	3	40	0	1.20462	3.35455	1.55127	5.78285	1.24942
2	30	75	50	2.41771	4.94735	0.916286	6.60059	2.06858
3	16.5	40	50	0.877888	2.91048	0.929796	3.88536	0.927663
4	3	40	100	0.0374023	0.0779334	0.524841	0.239346	0.0249586
5	16.5	5	0	1.33724	3.41286	1.96476	6.36807	1.47234
6	30	5	50	0.992726	2.23923	1.03895	4.16552	0.718964
7	30	40	0	1.60496	3.33784	2.37505	5.67868	1.42897
8	16.5	40	50	0.678077	2.15319	0.937973	4.86806	0.956461
9	16.5	75	100	0.134819	0.34494	0.756295	1.04463	0.104599
10	30	40	100	0.0601566	0.0989099	0.365206	0.252857	0.0178479
11	16.5	40	50	0.910597	1.40017	0.964283	4.67429	0.665278
12	16.5	40	50	0.760206	2.9695	0.814247	4.03895	0.934418
13	3	5	50	0.583149	2.41558	~~0.917352~~	3.72786	0.701898
14	16.5	75	0	1.68211	3.98847	2.70143	6.38904	1.71446
15	3	75	50	1.24409	3.42494	1.05814	4.69527	1.04215
16	16.5	5	100	0.0470018	0.0893105	0.294099	0.326453	0.00327093
17	16.5	40	50	0.69692	2.24385	1.03183	3.69942	0.576394
***p*-value**				< 0.0001	0.0052	< 0.0001	< 0.0001	0.0001
Lack-of-fit				0.1899	0.4640	0.0913	0.3848	0.3888

The *p*-value of the model and the lack-of-fit test were assessed to ensure the accuracy of the quadratic model recommended by the software. To better fit the model (*p* value <0.05 and a *p*-value for the lack of fit higher than 0.05) some modifications were considered: base 10 Log for Pa and square root for both Ma and Bn, whereas no transformation was suggested for La and Da while to fit the model the 13 run was ignored for Da.

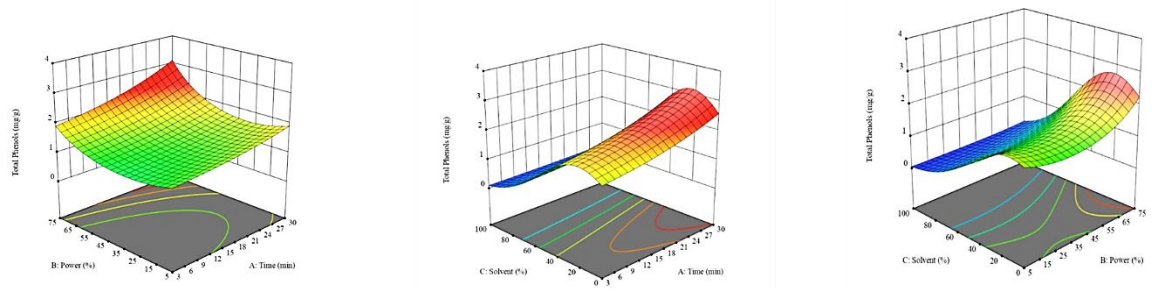
As well, for each variety, the 17 runs were combined to fit the second order polynomial equation model:

- Pa: $-0.1084A + 0.1063A + 0.1591B – 0.6855C + 0.0144AB + 0.0204AC + 0.0895BC + 0.0199A^2 + 0.1487B^2 – 0.5019C^2$
- La: $2.34 + 0.1688A + 0.5686B – 1.69C + 0.4247AB + 0.0094AC – 0.08BC + 0.3399A^2\ 0.5815B^2 – 0.9580C^2$
- Da: $0.9356 + 0.1971A + 0.2687B – 0.8315C – 0.2990AB – 0.2459AC -0.0686BC – 0.2056A^2 + 0.0195B^2 + 0.4740C^2$
- Bn: $2.05 + 0.0630A + 0.1524B + -0.9067C + 0.0730AB+ 0.0088AC + 0.1116BC – 0.0469A^2 + 0.1693B^2 – 0.5627C^2$

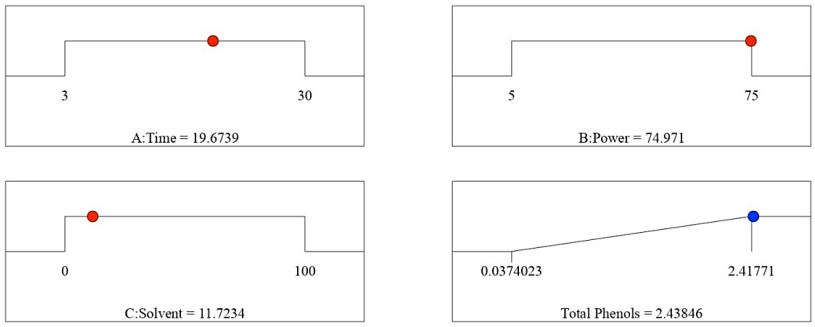
- Ma: $0.8965 + 0.0601A + 0.1419B – 0.5205C + 0.1018AB -0.0255AC + 0.0426BC$ $0.0325A^2 + 0.1072B^2 – 0.2779C^2$

The 3D response surface graphs were developed to visually assess the impact of the time-power-% of solvent combination on the analyzed response in order to better perceive the optimization process. The excluded value was set to its optimal value in each 3D plot.

In an attempt to highlight the impact of the time (A) and power (B) (plot on the left) on the TP content, the solvent was fixed at its optimal point. Time between 18-21min and maximum of power revealed a high amount of TP (**Figure 22a**). As for the middle plot, by fixing the power (B) at its optimal value, it seemed that between 21-30min (precisely 20min) and solvent of 0-20% (precisely 12%), the recovery of TP was the highest, meaning that lower solvent amounts promote high TP. By Fixing the time (A) at its optimal value, the influence of power (B) and solvent (C) was showcased on the right plot. The highest amount of TP was obtained at almost maximum power and solvent between 0 - 20 %. As shown in **Figure 22b** the predicted amount of TP (2.4 mg/g) was equal to the experimental value (2.42 mg/g) obtained under optimal conditions A = 20 min, B = 75% and C = 12%.



(a)

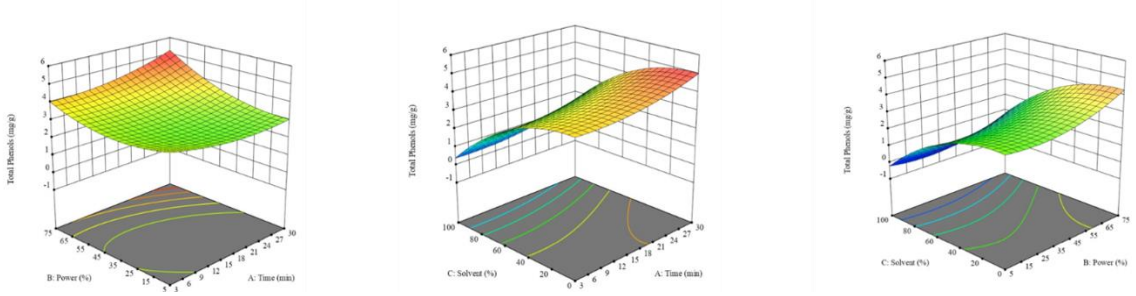


(b)

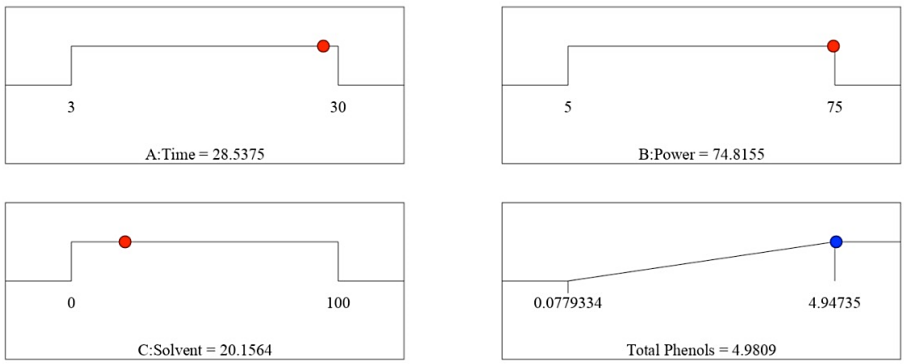
Figure 22: Graphical representation of the optimal points of **Pa** for the UAE extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

For **La**, the ideal point that produced the greatest amount of total phenols was shown graphically in **Figure 23a**. The influence of time (A) and power (B) on the content of TP was

highlighted by setting the solvent (C) at its optimal value. The time range (A) that produced the greatest amount of TP was between 27 min and 30min (exactly at 28 min), followed by power (B), ranging from 65 to 75%. The power (B) was set at its optimal value to demonstrate how time (A) and solvent (C) affect TP. Time varied between 27 and 30 min while the solvent content was between 0-20%. For higher amount of TP power (B) and solvent (C) were set at values extending from 65-75% (precisely 75%) and 0-20% (precisely 20%), respectively. Overall, solvent once again was the most important factor, with its quantity highly influencing the yield of the TP. Combining the three factors at their optimal conditions A = 28 min, B = 75% and C=20%, we noticed that both the predicted and the experimental TP content were at 5 mg/g.



(a)

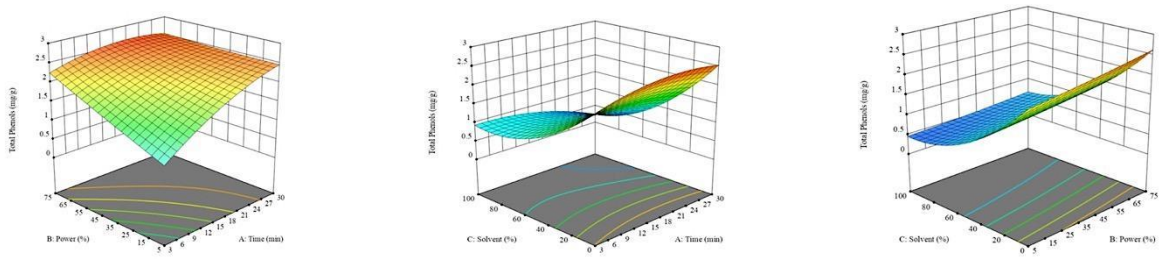


(b)

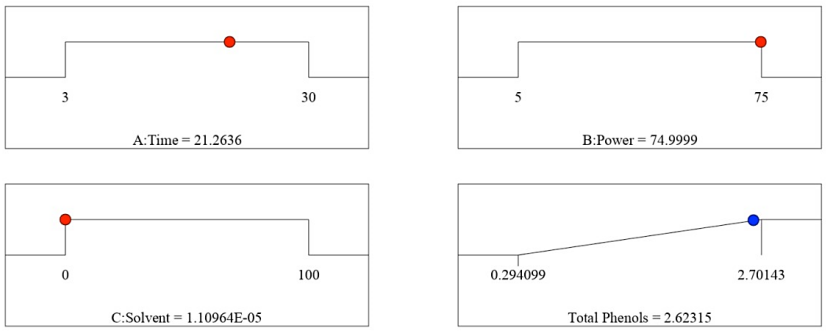
Figure 23: Graphical representation of the optimal points of **La** for the UAE extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

Referring to **Figure 24a**, for **Da**, the effect of time (A) and power (B) was emphasized. The red zone, which pointed to a higher yield is placed the maximum output of power and time. The time (A) was efficient between 18-21 min (precisely 21 min) while the power (B) was extended from 65 to 75% (precisely 75%). High extractability of TP was set at time (A) between 21 and 30 min and 0 to 20% of solvent (exactly at 0%), according to the AC plot (middle plot). The solvent (C) vs power (B) plot showed that ethanol at 0% with the highest power resulted in a significant amount of TP. Once evaluating the optimal conditions (A=21 min, B= 75% and

C= 0% ethanol), the predicted content of TP was slightly lower than the experimental one, as shown in **Figure 24b**.



(a)

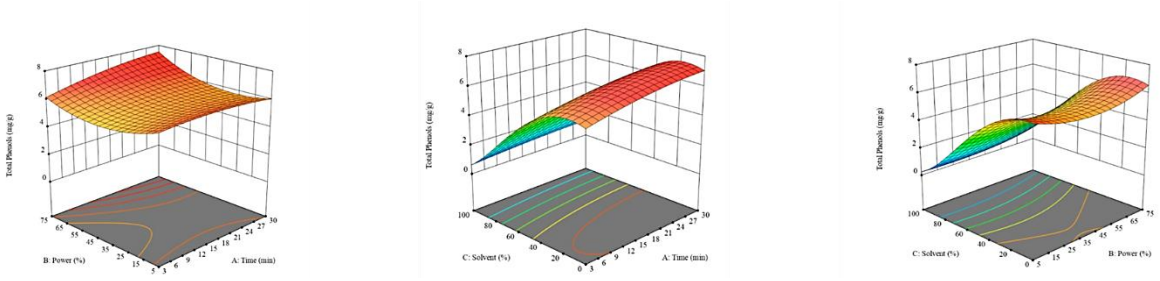


(b)

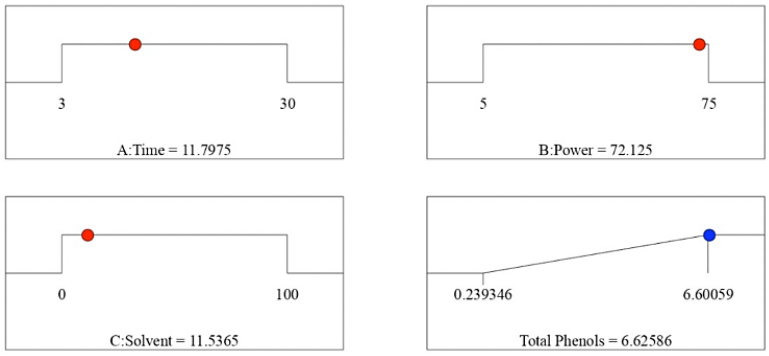
Figure 24: Graphical representation of the optimal points of **Da** for the UAE extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

The interaction between the various factors was shown through Bn's 3D plots. A broad red region with a high concentration of TP was seen at time (A) between 12 and 24 min (exactly at 12 min) and power (B) between 65% and 75 % (precisely at 72%).The maximum TP concentration was found at 12 min< A <30 min and 0%< C <10% while altering the time (A) and solvent (C). Regarding BC, the highest TP was displayed at 65%< B <75%, and 0%<C<10%. Overall, the solvent showed the highest influence on the yield of total phenols.

In fact, the estimated amount of TP was almost equal to the experimental amount (7 mg/g) when the optimal conditions (A=12 min, B=72% and C=11%) were applied.



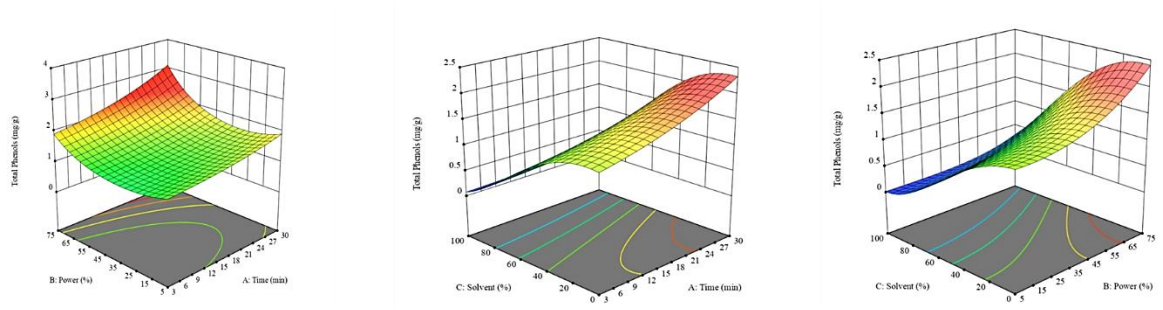
(a)



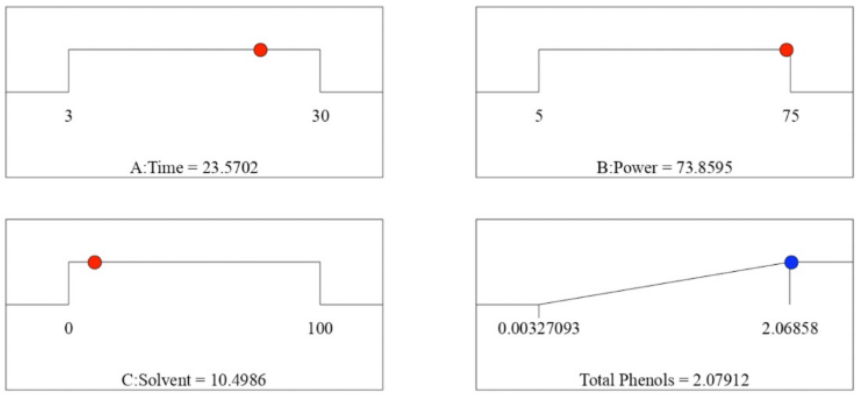
(b)

Figure 25: Graphical representation of the optimal points of **Bn** for the UAE extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

As mentioned in **Figure 26a**, for Ma the interaction between time (A) and power (B) was showcased. The plot AB showed that high TP content was obtained at high power and time (A) between 21-27 min (ideally 24 min). The higher placement of power and time promoted the yield. As for AC, a combination of 0 %< C <20 % and A>21 min resulted in maximum TP content, meaning that lower solvent values and high extraction time increased yields. While varying power (B) and solvent (C), we could notice that 65 %< power < 75% and percentage of solvent ranging between 0 and 20% revealed the highest amount of TP. The study of all the factors together set at their optimal conditions (A=26 min, B=74% and C=10%) displayed that both the estimated and the experimental amount of TP were equal 2.1 mg/g (**Figure 26b**).



(a)



(b)

Figure 26: Graphical representation of the optimal points of **Ma** for the UAE extraction – a) Representation of the 3D response surface plots. –b) Representation of the optimal points

The optimal conditions for UAE were found to be ranged between 12-28 min for time, 72-75% for power, and 0-20% for solvent to yield higher amounts of TP being between 2.1 mg/g (Ma) and 7 mg/g (Bn). Although DM revealed more red regions, thus implying the promotion of the extractability of total phenols, UAE was found to be cost-effective (less use of ethanol) and quicker (the highest time was 28 min versus 87 min for DM), thus using this technique. In addition to the ability to carry out extractions at a pilot scale which enabled us to stick to the deadlines as we worked under the scope of a project (100% figo).

4.3Bioactive analysis of the extracts at optimal points

After obtaining the optimal extraction points for UAE, each variety was extracted at its optimal conditions then the extracts were lyophilized before mixing them to study their bioactivities in order to confirm their incorporation as detailed in the following section.

4.3.1 Antioxidant activity

The antioxidant activity was performed through TBARS and CAA assays as shown in **Table 12** where the results were expressed in EC_{50}. Indeed, comparing the results of each variety on itself with the results obtained for the mixture, the antioxidant activity of the TBARS was higher when the leaves acted together with an EC_{50} of 120±10 µg/mL which was better

than the lowest EC_{50} obtained for Bn (230±10 µg/mL). While CAA of the mixture showed a percentage inhibition max tested of 65±11% which was the same recorded with the highest capacity to minimize the ROS by each variety.

Table 12: Antioxidant activity by thiobarbituric acid reactive substances inhibition assay method (TBARS) and cellular antioxidant activity (CAA) of mixture composed by 25% of different fig varieties leaf samples

	TBARS	**CAA**	
	EC_{50} (µg/mL)	max tested[] (µg/mL)	% inhibition max tested []
25% mix	120±10	2000	65±11
Positive Control	9.1±0.3 (Trolox µg/mL)	0.3 (Quercetin µg/mL)	95±5

4.3.2 Antimicrobial activity

The antimicrobial activity was analyzed through the microdilution method, allowing for the determination of the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) as shown in **Table 13**. The mixture of the four fig varieties revealed activity against all the bacteria and fungi strains. *E. coli*, *B. cereus*, *L. Monocytogenes and S.aureus* were found to be the most sensitive bacteria with MIC and MBC value of 5 mg/mL. *Aspergillus brasiliensis* was more sensitive to the extracts with MIC value of 2.5 mg/mL in comparison with *Aspergillus fumigatus* that presented a MIC value of 5 mg/mL. It is important to highlight that the varieties had an antibacterial activity against *Pseudomonas aeruginosa* when they acted together.

Table 13: Antimicrobial activity against food microorganisms, and antifungal activity of mixture composed by 25% of different fig varieties leaf samples.

			Positive Control					
	25% mix		Streptomicina 1 mg/mL		Methicillin 1 mg/mL		Ampicillin 20 mg/mL	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria								
Enterobacter Cloacae	10	10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	5	5	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	10	10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterocolitica	10	10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	10	10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria								
Bacillus cereus	5	5	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	5	5	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	5	5	0.007	0.007	0.007	0.007	0.15	0.15
Fungi					Ketoconazole			
	MIC	**MFC**		**MIC**			**MFC**	
Aspergillus brasiliensis	2.5	>10		0.06			0.125	
Aspergillus fumigatus	5	>10		0.5			1	

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MFC: Minimum fungicidal concentration, n.t: non tested

4.3.3 Cytotoxicity and anti-inflammatory activity

As presented in **Table 14**, a mix of 25% of varieties (La, Pa, Ma and Bn) was tested, excluding Da as it showed above inhibition of PLP2 proliferation in addition to the skin allergy it caused to me. The results obtained revealed that the mixture had the capacity to inhibit the proliferation of AGS and CaCO2 cell lines with GI_{50} of 360±5 µg/mL and 347±32 µg/mL respectively, while no activity was displayed for MCF-7. Further the mixture was safe as no activity was shown for PLP2 cells with a GI_{50}>400 µg/mL even with the use of LA. The mixture of the leaves revealed significant action against colorectal adenocarcinoma with GI_{50} of 347±32 µg/mL in comparison with the results recorded for each variety on itself (GI_{50} >400 µg/mL), however for MCF-7 cells each variety presented better activity than the mixture (GI_{50} >400 µg/mL).

As for the anti-inflammatory activity that was carried out to determine the capacity of a substance to inhibit the production of nitric oxide, the mixture did not reveal activity with a value of IC_{50}> 400 µg/mL (**Table 14**).

Table 14: Cytotoxicity and anti-inflammatory activity of leaves of the mixture

	25% mix	Ellipticine (positive control)
Cytotoxicity (GI_{50}, µg/mL)		
AGS	360±5	1.23±0.03
MCF-7	>400	1.02±0.02
CaCo2	347±32	1.21±0.02
Hepatotoxicity (GI_{50}, µg/mL)		
PLP2	>400	1.4±0.1
Anti-inflammatory activity (IC_{50}, µg/mL)		
RAW	>400	6.3±0.4

The results obtained revealed that the extracts could be safely incorporated as food preservatives in fig jam thus promoting circular economy and sustainability.

4.4Fig jam analysis

To the best of our knowledge, this was the first work of its kind to make a low sugar fig jam (only honey was used in some formulas) incorporated with natural preservatives from fig leaves. Indeed, the mixture of extracts obtained was added to different fig jam batches at a concentration of 10 mg/mL, a value determined by the results obtained on the antimicrobial activity assay. As mentioned in the methodology part, after cooking the jams at 100 °C the temperature on which the fig leaves extract was added varied between 80 °C and 50 °C in order to guarantee that at the temperature of 80 °C, the compounds present in the extracts did not undergo any degradation. The interpretation of the results was done using a two-way ANOVA as mentioned in the tables below.

4.4.1 Physical parameters

In **Table 15**, the color coordinates (L* (lightness), a* (red-greeness) and b* (blue-yellowness), pH values and water activity were represented, pertaining to the samples cooled to 80 ºC. For all samples, a significant interaction was sought, and thus no concrete differences could be described for the samples.

Table 15: Color coordinates, pH and water activity of the jams cooled to 80 ºC

80 ºC		L*	a*	b*	pH	aw
Storage Time	0 Days	35±1	10±2	16±3	3.7±0.1	0.92±0.02
(ST)	15 Days	33±1	10±1	17±2	3.7±0.1	0.91±0.01
	30 Days	34±2	10±1	17±2	3.71±0.05	0.93±0.02
***p*-value (n=5)**	Tukey's test	<0.001	<0.001	0.006	<0.001	<0.001
Incorporation (I)	Control	35±1	12±1	19±1	3.61±0.02	0.90±0.01
	Honey	35±1	10±1	16±2	3.70±0.04	0.92±0.01
	Extract	34±2	10±1	16±1	3.8±0.1	0.93±0.01
	Honey+Extract	32±1	9±1	15±2	3.76±0.01	0.94±0.01
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001

For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

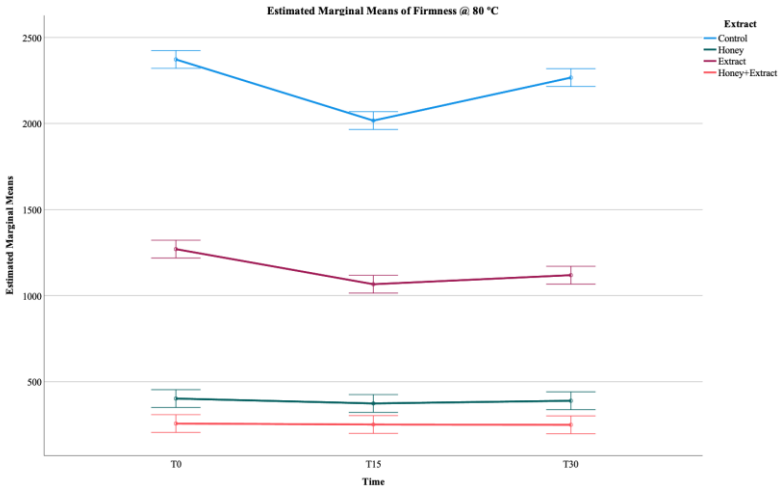
Table 16 showed the different dimensions of texture, obtained through a backward extrusion test, as well as the centesimal composition of the jams. Firmness and cohesiveness showed a significant interaction between ST and I, and thus some tendencies could be extracted from the EMM plots for firmness. For consistency, the way an object keeps together, both factors could be quantified. Thus, it seemed that over time there was a statistically significant decrease in consistency until 15 days after manufacture, and then a slight but significant increase from the fifteenth to the thirtieth day. For the incorporation type, the two samples incorporated with honey showed the least significant consistency, while the control sample was the most consistent. Contrarily, for the work of cohesion, defined as the work needed to separate a liquid, the I could be classified, in which the least work was needed for the control sample, while the samples with honey needed more work. These results were in line with what was sought for consistency. It is logical that a substance that has higher consistency needs lower work of cohesion.

Table 16: Texture dimensions and centesimal composition of the samples cooled to 80 ºC

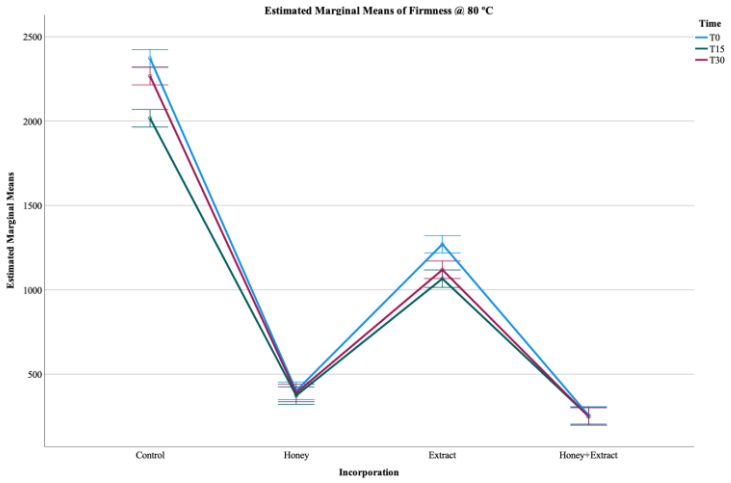
80°C		Firmness g	Consistency g.sec	Cohesiveness g	Work of Cohesion g.sec
Storage Time (ST)	0 Days	1075±868	1097±510[b]	-914±585	-386±198
	15 Days	927±720	884±339[a]	-921±635	-374±249
	30 Days	1006±822	963±311[a, b]	-964±668	-344±163
***p*-value (n=5)**	Tukey's test	<0.001	0.009	0.569	0.397
Incorporation (I)	Control	2219±186	1422±338[d]	-1671±343	-637±176[a]
	Honey	388±13	785±73[b]	-431±28	-248±19[c]
	Extract	1152±92	1138±322[c]	-1347±103	-411±95[b]
	Honey+Extract	253±4	581±60[a]	-283±8	-176±10[c]
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	0.063	0.004	0.169

For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Figure 27 showed the EMM plots for firmness, in which a) showed how this dimension of texture changed over time for each jam. Overall, the control sample was the firmest, namely the reliability of withstanding pressure, while the honey and honey with extract jams were the least firm due to their higher water content. Overall, the incorporated samples showed a very low or nonexistent variation over time. This was quite interesting, due to jams being appreciated for their soft, almost fluid-like texture. In the b) section of **Figure 27**, the softness of the jam was easily perceptible, showing that the jams with extracts and honey reduce the firmness of the jam.



(a)



(b)

Figure 27: EMM plots for jams at 80 ºC showing a) firmness by plotting the EMM's with time and b) EMM's with incorporation type.

Table 17 showed the sample parameters as **Table 15**, but for the samples cooled down to 50 ºC. For these samples a significant interaction was found for ST x I, and thus no definitive conclusions were found. Furthermore, no tendencies could be found in the EMM plots, meaning that the effect of the incorporations did not have influence on the jams. This is quite interesting and desirable, due to the fact that any incorporation with a technological effect (preserve, color), like food additives, should not change any aspect of the food they are added to.

Table 17: Color coordinates, pH and water activity of the jams cooled to 50 ºC.

50ºC		**L***	**a***	**b***	**pH**	**aw**
Storage Time (ST)	0 Days	34±2	9±2	15±3	3.7±0.1	0.92±0.01
	15 Days	33±1	10±1	17±2	3.7±0.1	0.91±0.01
	30 Days	35±2	10±1	16±2	3.7±0.1	0.91±0.01
***p*-value (n=5)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001
Incorporation (I)	Control	35±1	12±1	19±1	3.61±0.02	0.90±0.01
	Honey	35±1	10±1	16±2	3.70±0.04	0.92±0.01
	Extract	33±2	9±1	15.1±1	3.69±0.04	0.91±0.01
	Honey+Extract	33±1	9±1	14±2	3.81±0.01	0.92±0.01
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001

For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Table 18 showed the same parameters of texture as **Table 16**. As for **table 18**, a significant interaction was found for firmness and cohesiveness, so some tendencies were extracted from the EMM plots. For consistency, in the case of the 50 ºC jams, the incorporations could be classified, showing a higher value of consistency for the jam incorporated with the extract, while the samples with honey were the lowest. Overall, once again the honey seemed to reduce the consistency of the jams, even at a lower temperature. For the work of cohesion, the incorporations could be classified, with a higher work needed for the samples with lower consistency, as was expected.

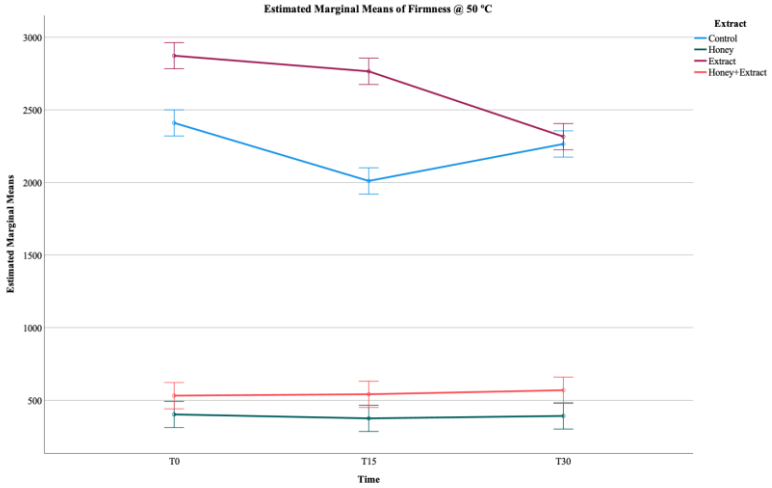
Table 18: Texture dimensions and centesimal composition of the samples cooled to 50 ºC

50ºC		**Firmness**	**Consistency**	**Cohesiveness**	**Work of Cohesion**
		g	**g.sec**	**g**	**g.sec**
Storage Time	0 Days	1554±1136	1494±712	-1310±926	-549±303
(ST)	15 Days	1423±1031	1316±614	-1279±832	-528±275
	30 Days	1385±935	1467±838	-1286±802	-553±311
***p*-value (n=5)**	Tukey's test	<0.001	0.410	0.898	0.878
Incorporation (I)	Control	2229±196	1421±325^{b}	-1659±363	-619±151^{b}
	Honey	389±13	786±73^{a}	-431±27	-249±21^{d}
	Extract	2652±296	2328±829^{c}	-2418±343	-902±280^{a}
	Honey + Extract	547±18	1168±20$^{a, b}$	-658 ±9	-403±11^{c}
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	0.541	0.004	0.669

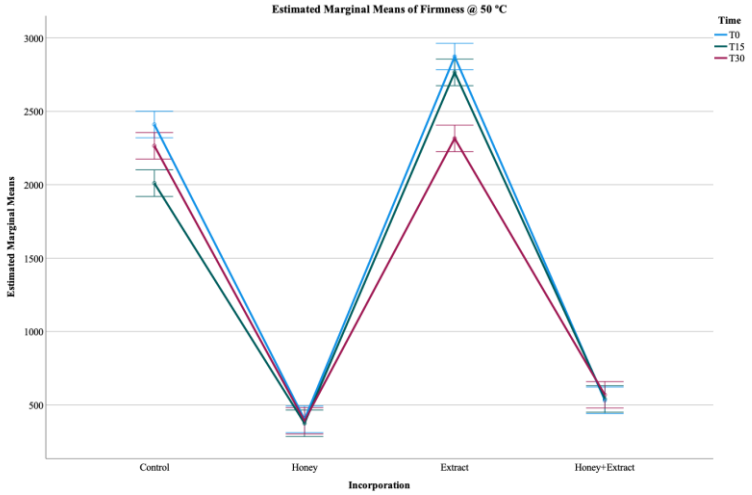
For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Figure 28 showed the EMM plot for firmness where it was clear that in a) the control with honey and the jam with extracts and honey were lower when compared to the other samples, which could be explained by the fact that the addition of honey allowed to reach more quickly the value of 40 Brix, therefore their water content was higher which changed the texture profile, making the jam more fluid, independently of the cooling temperature. Still, overtime, the control sample and the jam with extract tended to show the same values, while the samples

with honey did not show any change. In **Figure 28b**, the difference in firmness was easily perceived.



(a)



(b)

Figure 28: EMM plot of firmness for jams at 50 ºC, plotting a) EMM vs. time and b) EMM vs incorporation.

Figure 29 showed the EMM plot for cohesiveness over time, where the jams with honey showed a higher cohesiveness and no changes over time. The control sample tended to reduce its cohesiveness while the extract increased.

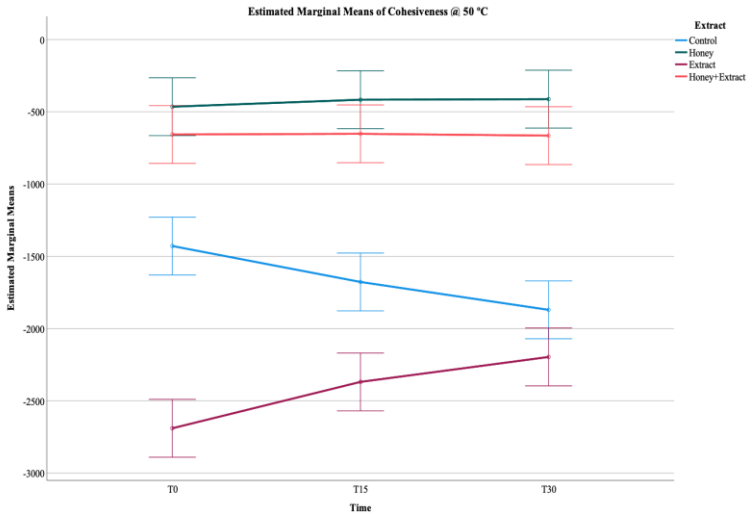


Figure 29: EMM plot of cohesiveness over time for jams at 50 ºC

4.4.2 Nutritional and chemical characterization

Table 19 represented the nutritional value of the samples where the fig leaves extract was added at 80 ºC. As anticipated, total carbohydrates and moisture were the two nutrients that were most prevalent, whereas fat and protein were only identified in very little amounts. As for those results, there was a significant interaction among the two factors (ST and I). There were no differences among the EMM plots for the different nutrients (results not shown), and thus, the temperature of 80 °C did not seem to affect the centesimal composition of the jams.

Table 19: The nutritional value of the samples cooled down to 80 ºC

80ºC		Moisture (%)	Fat (g/100g)	Protein (g/100g)	Ash (g/100g)	Carbohydrates (g/100g)	Energy (Kcal)
Storage Time (ST)	0 Days	49±8	0.22±0.02	1.3±0.6	1.1±0.3	48±8	198±33
	15 Days	49±9	0.22±0.01	1.2±0.5	1.1±0.3	49±9	202±35
	30 Days	49±9	0.21±0.02	1.3±0.4	1.1±0.3	48±9	199±36
***p*-value (n=5)**	Tukey's test	<0.001	<0.001	0.021	<0.001	<0.001	<0.001
Incorporation (I)	Control	43±1	0.235±0.003	1.8±0.3	1.3±0.1	54±1	224±3
	Honey	39±2	0.203±0.004	0.9±0.1	0.70±0.03	59±2	241±7
	Extract	55.7±0.6	0.231±0.003	1.7±0.1	1.52±0.03	41±1	172±2
	Honey+Extract	59±1	0.20±0.01	0.7±0.1	0.95±0.02	39±1	162±4
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

The soluble sugars identified in the jams where the extracts were added at 80 °C were depicted in **Table 20**. The jams contained only fructose and glucose, both in nearly the same amount. A significant interaction was sought for both ST and I, hence an EMM plot of fructose was employed to analyze some general tendencies.

Table 20: Soluble sugars profile of the jams cooled to 80°C

80ºC		Fructose (g/100g Fw)	Glucose (g/100g Fw)	Total sugars (g/100g Fw)
Storage Time (ST)	0 Days	25±2	24±1	49±4
	15 Days	23±1	22±1	46±2
	30 Days	21±1	20.3±0.5	41±2
***p*-value (n=5)**	Tukey's test	<0.001	<0.001	<0.001
Incorporation (I)	Control	21±1	22±1	43±2
	Honey	24±2	23±1	47±2
	Extract	23±2	23±2	46±4
	Honey+Extract	25±3	22±3	47±5
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001

For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values. Fw: Fresh weight

Figure 30 plotted the EMM means with the incorporation time for fructose. The sample with honey + extract showed the highest values while the control samples showed the least amount. It was also clear that in the jam samples fructose reduced over time (colored lines).

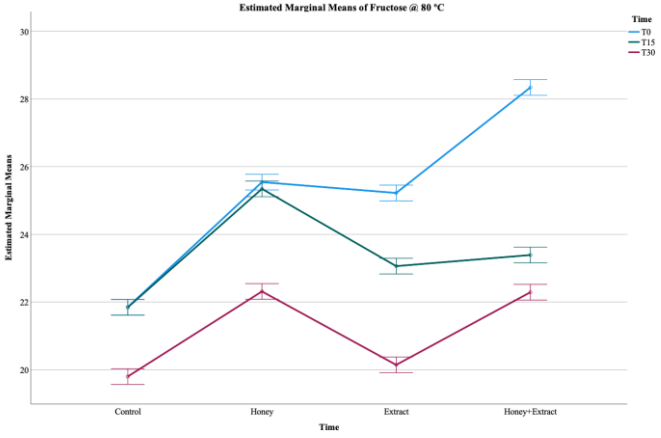


Figure 30: EMM plot of fructose for the samples cooled to 80 ºC, although not plotting the EMM vs. time, but EMM vs. incorporation

Table 21 showed the individual fatty acids (only the ones with more than 2%) and saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) cooled to 80 °C. SFA were predominant and the highest individual fatty acid was palmitic acid, a constituent fatty acid contained in almost all cellular membranes, that also functions as a fuel by generating 106 moles of adenosine triphosphate energy through beta oxidation and known

as a significant component of storage lipids (German., 2011). A significant interaction was sought for ST x I and thus no general conclusion could be made. Furthermore, no tendencies could be extracted from the EMM meaning that both factors were relevant for the changes of the fatty acids.

Table 21: Fatty acids profile of the jams cooled to 80 °C

80ºC		C14:1	C16:0	C17:0	C17:1	C18:0	C18:1n 9c	C18:2 n6c	C18:3n 3	C20:0	C22:0	C24:0	SFA	MUFA	PUFA
Storage Time (ST)	0 days	2±1	31±6	2.5±1.6	2±1	11±2	25±14	6±2	3±1	2.6±0.4	4±1	2±1	61±14	29±13	10±2
	15 Days	1.5±0.3	34±4	2.4±0.4	2.3±0.5	10±1	19±1	6.6±1.5	4±2	1±1	4±1	2.8±0.3	64±3	23±1	13±3
	30 Days	1±1	32±6	2±1	2±1	12±3	20±4	7±2	4±1	2±1	5±1	3±1	65±3	23±3	12±2
***p*-value (n=5)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Incorporation (I)	Control	2±1	31±2	3.4±0.5	3±0.2	12.4±0.3	16±2	4±1	2.8±0.3	2.6±0.1	6.1±0.4	3.1±0.1	70±2	22±2	8±1
	Honey	2±1	32±4	3±1	2±1	9.8±0.2	19±4	6±1	5±1	3.1±0.2	5±1	2.1±0.2	65±3	23±3	11±2
	Extract	1.5±0.3	29±7	2±1	1±1	10±1	30±13	7.9±0.5	3±1	2±1	3±1	3±1	54±12	33±13	13±1
	Honey+ Extract	1±1	38±5	1±1	2±0.2	13±3	19±2	8±1	4±1	1±1	3.9±0.4	2.6±0.2	64±2	22±3	13±3
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

C14:0 myrestoleic acid, C16:0 palmitic acid, C17:0 margaric acid, C17:1 heptadecenoic acid, C18:0 stearic acid, C18:1n9c oleic acid, C18:2n6c linoleic acid, C183n3 α-linolenic acid, C20: arachidic acid, C22:0 Behenic acid, C23:0 tricocyclic acid, C24:0 lignoceric acid, SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

Table 22 showed the nutritional profile of the jams with the fig leaves extracts incorporated at 50 ºC, which followed the same patterns in terms of quantity of the samples submitted to 80 ºC. As expected moisture followed by total carbohydrates were the most abundant nutrients, while fat and proteins were found in very low quantities. Again, a significant interaction was found between ST and I and thus some general conclusions could be drawn from the EMM plots. In **Figure 31**, moisture was plotted showing that the variation in moisture was low over time and that the jams with extract and honey showed the highest moisture value which can be explained by the high water content of this jam as detailed in the methodology part, and followed by the jam with extracts. **Figure 32** showed the EMM plots for energy, in which it was clear that the samples with higher energy values were the control and honey incorporated samples, while the other two samples showed lower values. Overall the variations of energetic value did not change much over time, except for the jam incorporated with honey.

Table 22: The nutritional value of the samples cooled down to 50 ºC

50ºC		**Moisture (%)**	**Fat (g/100g)**	**Protein (g/100g)**	**Ash (g/100g)**	**Carbohydrates (g/100g)**	**Energy (Kcal)**
Storage Time (ST)	0 Days	50±7	0.22±0.02	1.4±0.5	1.1±0.3	48±7	202±30
	15 Days	48±8	0.22±0.01	1.4±0.6	1.1±0.3	49±8	204±33
	30 Days	48±8	0.22±0.02	1.3±0.3	1.1±0.3	49±8	203±32
***p*-value (n=5)**	Tukey's test	0.037	0.093	0.49	<0.001	<0.001	0.011
Incorporation (I)	Control	43±1	0.235±0.003	1.8±0.2	1.3±0.1	54±1	225±3
	Honey	39±2	0.203±0.004	0.9±0.1	0.69±0.03	59±2	241±7
	Extract	54±1	0.23±0.01	1.9±0.1	1.45±0.04	43±1	181±3
	Honey+Extract	58±1	0.21±0.01	1.1 ±0.1	0.94±0.02	40.1±0.5	166±2
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

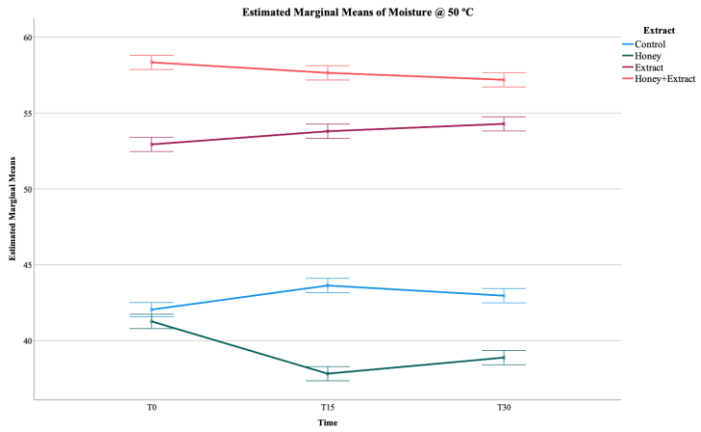


Figure 31 EMM plots for moisture regarding the samples cooled to 50 ºC.

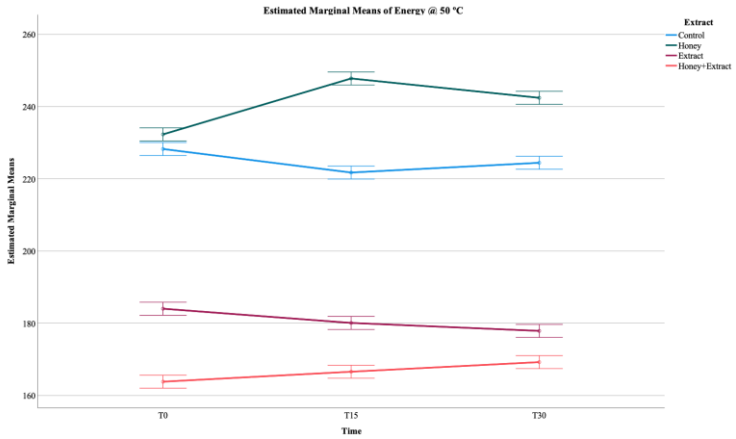


Figure 32: EMM plots for energy of the samples cooled to 50 ºC.

Table 23 showed the soluble sugars detected in the samples with the fig leaves extracts added at 50 ºC. Only fructose and glucose were found in the samples with very similar quantities. There was a significant interaction among ST and I, and thus an EMM plot of glucose was used to interpret some general tendencies.

Table 23: Soluble sugars profile of the jams cooled to 50°C

50ºC		Fructose (g/100g Fw)	Glucose (g/100g) Fw)	Total sugars (g/100g Fw)
Storage Time (ST)	0 Days	25±3	24±2	50±4
	15 Days	24±2	23.0±0.5	47±2
	30 Days	22±1	21±1	44±3
***p*-value (n=5)**	Tukey's test	<0.001	<0.001	<0.001
Incorporation (I)	Control	21±1	22±1	43±2
	Honey	24±1	23±1	47±3
	Extract	23±1	24±1	47±2
	Honey+Extract	26±2	24±2	50±4
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001

For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values. Fw: Fresh weight.

In **Figure 33** the EMM plot revealed the time incorporation for glucose, where it was clear that glucose showed a tendency to reduce over time. Still, the highest value for this soluble sugar was found in the sample incorporated with honey and extract.

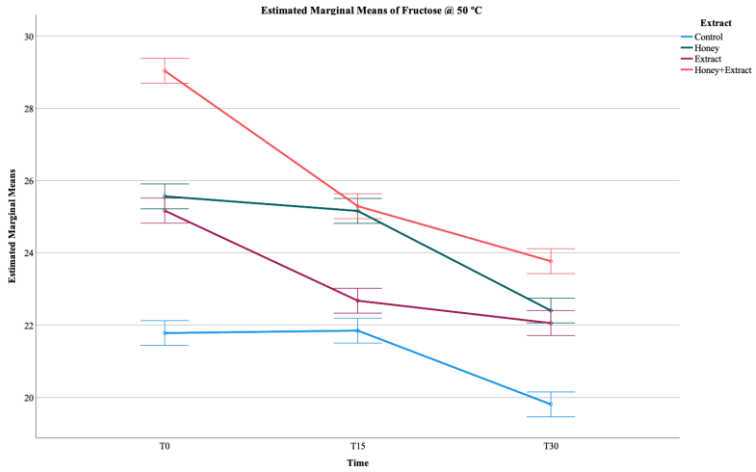


Figure 33: EMM plot of glucose for the samples cooled to 50 ºC.

Table 24 showed the fatty acid profile and the SFA, MUFA and PUFA for the samples where the fig leaves extracts were added at 50 ºC. Overall, the SFA was the most prevalent type, followed by MUFA. In terms of the most abundant individual fatty acid, C16:0 (palmitic

acid). As with the nutritional profile, the fatty acids for samples with the extracts added at 50 ºC had a similar profile to the 80 ºC. Overall, a significant interaction was sought for all samples and thus no general conclusions could be drawn, meaning that both ST and I contributed for the variation in the fatty acids profile.

Table 24: Fatty acids profile of the jams cooled to 50 °C

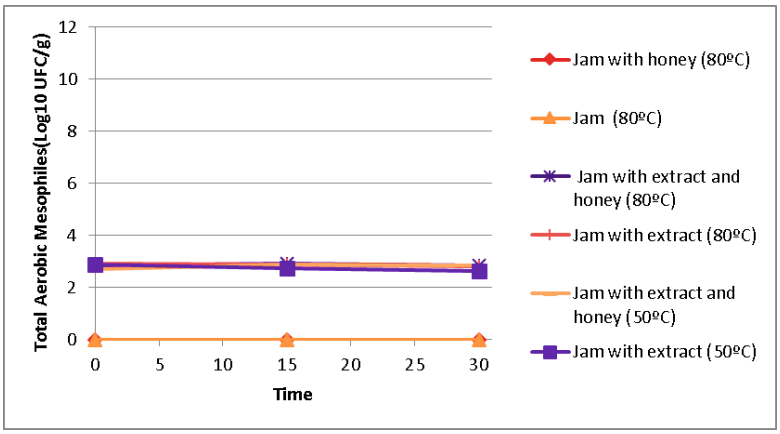
50ºC		C14:1	C16:0	C17:0	C17:1	C18:0	C18:1n9c	C18:2n6c	C18:3n3	C20:0	C22:0	C23:0	C24:0	SFA	MUFA	PUFA
Storage Time (ST)	0 days	3±1	34±2	2±2	2±1	12±1	19±5	5±2	3.0±0.5	2.7±0.2	4±2	4.5±0.5	2±1	66±6	25±6	9±2
	15 Days	2±1	32±2	2.9±0.2	2±1	11±1	18±3	8±3	4±2	2.8±0.4	4±1	5±1	2±1	65±3	23±3	12±3
	30 Days	2±1	31±2	3±1	2±1	11±1	17±5	6±1	4±1	2.9±0.2	5±1	4±3	3±1	66±4	21±4	12±4
***p*-value (n=5)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.375	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Incorporation (I)	Control	2±1	31±2	3.4±0.5	3.2±0.2	12.4±0.3	16±2	4±1	3±1	2.6±0.1	6.1±0.4	5±1	3.1±0.1	70±2	22±2	8±1
	Honey	2±1	32±4	3±1	2±1	9.8±0.2	19±4	6±1	5±1	3.1±0.2	5±1	4±1	2.1±0.2	65±3	23±3	11±2
	Extract	3±1	32.7±0.5	2.9±0.3	2.5±0.2	12±1	18±7	7±4	3±1	2.6±0.3	3±1	5±1	2±2	65±5	24±8	11±4
	Honey+ Extract	3±1	33±2	2±1	1±1	12±1	19±4	8±1	5±1	2.9±0.3	4±1	3±2	2.3±0.3	62±1	23±4	14±3
***p*-value (n=15)**	Tukey's test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.401	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST×I (n=60)	*p*-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.436	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

C14:0 myrestoleic acid, C16:0 palmitic acid, C17:0 margaric acid, C17:1 heptadecenoic acid, C18:0 stearic acid, C18:1n9c oleic acid, C18:2n6c linoleic acid, C183n3 α-linolenic acid, C20: arachidic acid, C22:0 Behenic acid, C23:0 tricocyclic acid, C24:0 lignoceric acid, SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. For the storage time (ST) and incorporation (I), different letters represent statistically significant differences using a p-value of 0.05. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values.

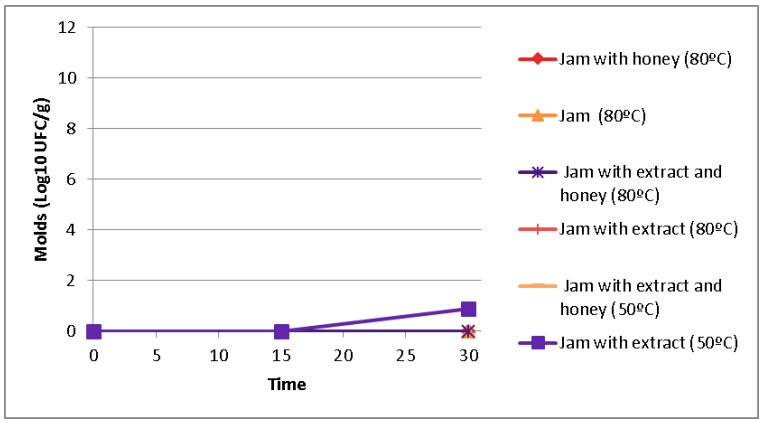
4.4.3 Microbial load

The microbial load of the different fig jams prepared was sought in **Figure 34**, including total aerobic mesophiles and molds while *E.coli*, *Clostridium* and yeasts were not detected. Considering the total aerobic mesophiles, the two controls (jam with honey at 80 °C and jam without honey at 80°C) did not show any growth during the storage period, whereas all the incorporated jams revealed bacterial growth with no statistical significant changes from T_0 to T_{30} (**Figure 34 a**). This could be explained by the contamination of the extracts. Although the incorporated jams showed some bacterial growth the pH did not present significant changes from T_0 to T_{30} along with the nutritional and chemical composition, thus implying that the microorganisms did not alter the quality of the jams.

As for the molds, all the batches presented the same aspect with no growth during the whole storage period except for jam with the fig leaves extract incorporated at 50 °C that presented an increase from T=15d to T=30d (**Figure 34 b**).



(a)



(b)

Figure 34: Microbial load over the 30 days: a) Aerobic mesophilic microorganisms; b) Molds

5. Swot analysis

To evaluate the present work a SWOT analysis was done which pointed the strengths, the weaknesses, the opportunities and the threats

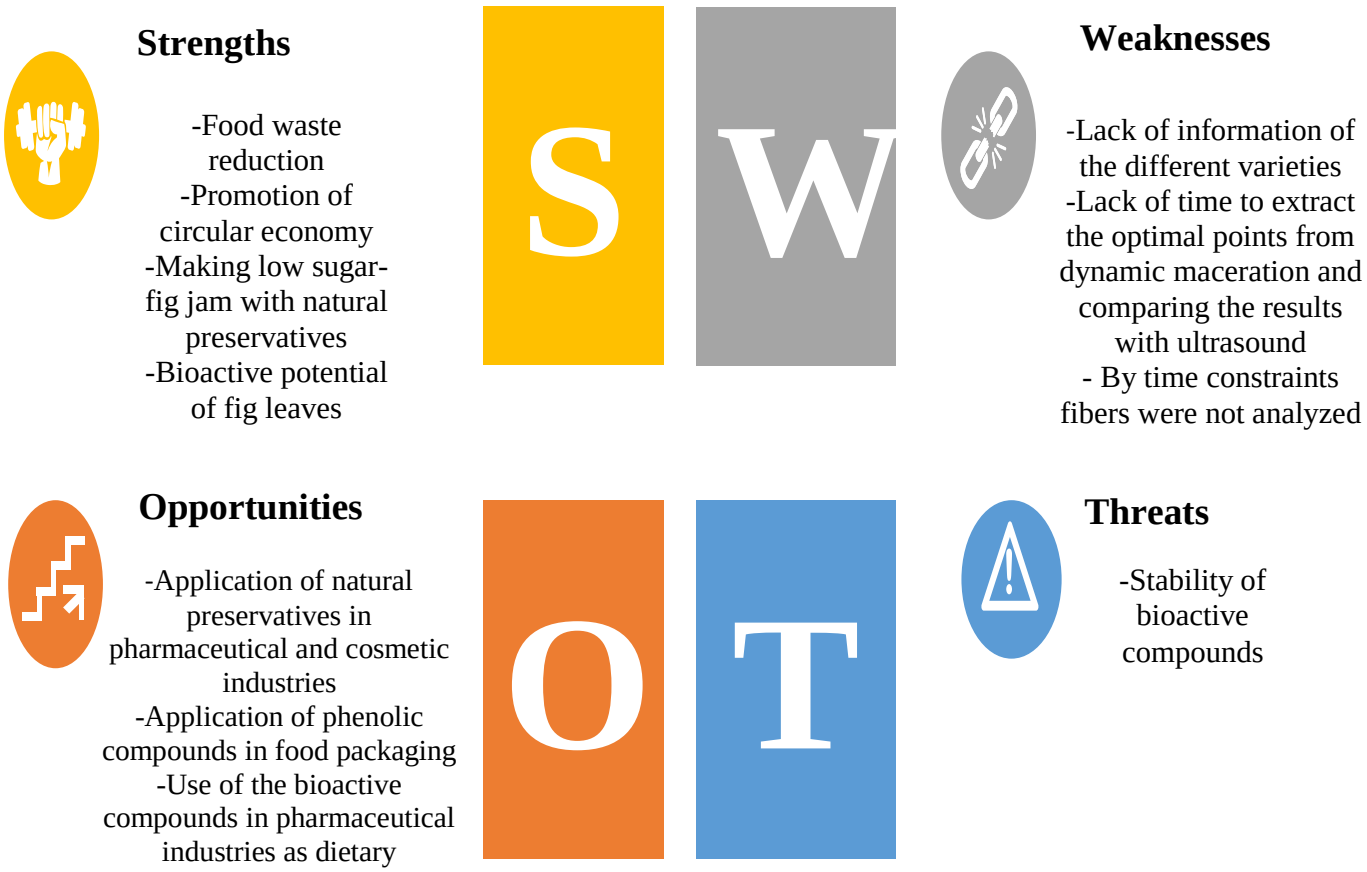


Figure 35: SWOT analysis

Conclusion

6. Conclusion

Food preservatives are synthetic or natural substances added to foods to preserve their quality also by fighting spoilage caused by microorganisms. Indeed, synthetic preservatives can be harmful to human health while ingested. That's why food industries are eagerly looking to find natural alternatives which can be derived from several sources. Food bio-residues particularly fruit and vegetable by-products, for instance, revealed to be a potential source of bioactive compounds according to different studies. Among these by-products fig leaves usually discarded by the fig industries have shown to be rich in bioactive molecules such as phenolic compounds that can be used as natural preservatives in a fig-based product thus enhancing circular economy and sustainability. For this reason, fig leaves from five varieties namely Bourjassote noire (Bn), Dauphine (Da), Longue d'Aout (La), Marseille (Ma) and Pastillière (Pa) were valorized and exploited as alternatives to synthetic preservatives.

The leaves were screened to determine their phenolic profile and around 12 phenolic compounds were found, being La the variety that showed highest contents in these molecules. The principal phenolic acid was caffeic acid while the Apigenin-*C*-hexoside-*C*-pentoside was the major flavonoid. The richness in these molecules impacted the bioactive potential of the leaves that revealed promising results in terms of antioxidant, antimicrobial by being more efficient on Gram negative than Gram positive bacteria, cytotoxicity and anti-inflammatory activity.

After screening the varieties, the total phenols were extracted using dynamic maceration and ultrasound assisted extraction, analyzed by the Folin ciocalteu method and optimized by response surface methodology. Although dynamic maceration showed a high extracting amount of total phenols, ultrasound was used because it's quicker and cost effective. The fig leaves were then extracted at their optimal conditions to recover the highest amount of total phenols.

After lyophilization, the extracts were mixed together and analyzed in terms of bioactivities. When they acted together, the mixture revealed better antioxidant activity with an EC_{50} of 120±10 µg/mL better than the lowest EC_{50} recorded for Bn (230±10 µg/mL). They were also able to act against all the bacteria and fungi strains in addition to presenting promising results against colorectal adenocarcinoma with GI_{50} of 347 µg/mL ±32 in comparison with the GI_{50} of each variety (>400 µg/mL). Besides, the mixture did not show activity against PLP2 cells thus involving its safety.

After incorporating the extracts in different batches of fig jams and varying the cooling temperature (50 °C and 80 °C) to study their efficiency, the monitoring of the physical, nutritional and chemical properties as well as the microbial load after opening, was carried out for 30 days. The low-sugar fig jam incorporated with natural preservatives from fig leaves revealed that overall the incorporation did not change the aspect of two batches of jam presented at T=80 °C and T=50 °C, whereas some differences regarding the consistency and firmness was sought because of the addition of honey, as the jams with honey had higher water content than the ones without honey due to the Brix value. Furthermore, the different cooling temperatures did not present obvious changes over time, concluding that the extracts were not deteriorated.

Overall this study enabled the presentation of innovative potentials of fig leaves enriched extracts, optimized and used as natural preservatives in a fig jam. It was quite important to highlight that these additives did not change the aspect of the jams to which they were added which is desirable. Furthermore, this work contributed to the promotion of the circular economy by valorizing this promising source.

This work reinforces a promising area of research based on the exploitation of fig leaves rich in bioactive compounds that can be used in pharmaceutical and cosmetic industries not only as natural preservatives but also as enriched ingredients in antioxidant antimicrobial agents and vitamins among others. In addition, the phenolic compounds known as antioxidant and antimicrobial agents can also be applied in food packaging.

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Annex

1. Book chapter

Hashed, H., Cassani, L., Zbiss, Y., Fraga-Corral, M., Oliveira, I., Pereira, A.G., Prieto, M.A., Heleno, S., Carocho, Marcio. (2022). Non-Alkaloid Nitrogen Containing Compounds from Fungi. In Carocho,M., Heleno,S., Barros,L (Eds), *Natural Secondary Metabolites: From Nature, Through Science, to Industry*. New York. Springer Nature International Publishing. ISBN-13: 9783031185861

2. Article

Yosra Zbiss, Carlos S. H. Shiraishi, Custódio Lobo Roriz, Marcio Carocho, Sara Domingos, Feriel Rezouga, Sandrina A. Heleno and Lillian Barros. 2022. Optimization of phenolic compounds extraction through ultrasound assisted extraction and dynamic maceration from Ficus carica.L leaves using Response Surface Methodology. (Almost finished to submit).

3. Conference participation

Carlos S. H. Shiraishi, Yosra Zbiss,, Custódio L. Roriz , Marcio Carocho , Sara Domingos , Ricardo C. Calhelha , Maria José Alves , Rui M. V. Abreu , Miguel A. Prieto , Sandrina Heleno and Lillian Barros.FIG (FICUS CARICA L.) BIORESIDUES AS SOURCES OF BIOACTIVE COMPOUNDS. Transcolab Submmit. Bragança, Portugal. March 23-25th, 2022.

Carlos S. H. Shiraishi, Yosra Zbiss,, Custódio L. Roriz , Marcio Carocho, Sara Domingos, Ricardo C. Calhelha, Maria José Alves, Rui M. V. Abreu, Miguel A. Prieto, Sandrina Heleno and Lillian Barros*. FIG (FICUS CARICA L.) FRUIT BIORESIDUES AS SOURCES OF BIOACTIVE COMPOUNDS FOR THE FOOD INDUSTRY. Beijing, China, August 05-13, 2022.

Carlos Shirashi, Yosra Zbiss, Custódio Lobo Roriz, Marcio Carocho*, Sara Domingos, Marta Evangelista , Sandrina Heleno , Lillian Barros. Fig Tree Leaves as Natural Food Preservatives. Retaste – Rethink food waste 2022. Herakeion, Greece, October 20-21, 2022

Carlos S. H. Shiraishi , Yosra Zbiss, Custódio L. Roriz , Márcio Carocho, Vasco da Cunha Mendes , Rui M. V. Abreu , Miguel A. Prieto, Sandrina Heleno and Lillian Barros *. “FIG (FICUS CARICA L.) BIOWASTE AS A SOURCE OF ORGANIC ACIDS FOR THE FOOD INDUSTRY. 1st International Congress on Food, Nutrition & Public Health - Towards a sustainable future. Lisboa, Portugal, 17 November 2022.

Carlos S. H. Shiraishi, Yosra Zbiss, Custódio L. Roriz, Marcio Carocho, Sara Domingos, Ricardo C. Calhelha , Maria José Alves, Rui M. V. Abreu, Miguel A. Prieto, Sandrina Heleno and Lillian Barros. Chromatographic profile of free sugars in fruits from five different varieties of Ficus carica L.. XIV – Encontro Nacional de Cromatografia. Aveiro, Portugal, December 06-08, 2022.

Yosra Zbiss, Carlos S. H. Shiraishi, Custódio Lobo Roriz, Marcio Carocho, Sara Domingos, Rui M. V. Abreu, Sandrina A. Heleno and Lillian Barros. Optimization of Total Phenolic Content of Fig Leaves Obtained Through Response Surface Methodology. At the "International Conference "INNOVATION IN MEDITERRANEAN TRADITIONAL FOODS: novel products and processes (IMTF)". Bragança, Portugal, October 13-14, 2022.

Yosra Zbiss, Carlos S. H. Shiraishi, Custódio Lobo Roriz, Marcio Carocho, Sara Domingos, Rui M. V. Abreu, Sandrina A. Heleno and Lillian Barros. *Ficus carica* L. leaves as promising sources of phenolic compounds and strong bioactivities. At the "I Encontro Consórcio Montanha para o Conhecimento de Jovens Investigadores – STEAM VII Encontro Jovens Investigadores ". Bragança, Portugal, November 22-24, 2022.