Exploiting *Artemisia absinthium* L. extract as a source of bioactive molecules: Development of a functional food

María Sol Terisotto

Double degree agreement between the Polytechnic Institute of Bragança and the National Technological University - Córdoba Regional School to obtain the Master's Degree in Chemical Engineering.

Supervisors
Doctor Sandrina A. Heleno
Doctor Lillian Barros
Mgter. Ema Sabre

Bragança
2021

This work was financed by the FEDER-Interreg España-Portugal program through the project TRANSCoLAB 0612_TRANS_CO_LAB_2_P

ACKNOWLEDGEMENTS

I thank my parents first and foremost for all their love, teachings, values, education and all forms of support so that not only this but all the achievements of my life were possible. For accompanying me all this year away and holding me back as only they can.

In particular, I thank the teachers and counsellors **Dr. Sandrina Heleno** and **Mgter. Ema Sabre**, for its good predisposition and collaboration towards me, as well as for all the knowledge and experience transmitted. To **Dr. Lillian Barros**, for always accompany and assist the development of the work. To **Bruno Melgar** who helped with the optimization of the extraction process, to **Filipa Fernandes**, **Custódio Roriz** and **Virginie Xavier** for their patience and collaboration in the laboratory trials and to **Dr. Márcio Carocho** who helped in the analysis of texture and interpretation of data.

To my family and friends who accompanied me throughout this year, for their affection and support.

Finally, to the **National Technological University of Córdoba** and to the **Polytechnic Institute of Bragança** for having made this master's degree available and to the **Mountain Research Centre** for providing the workspaces that enabled me to achieve the proposed objectives of the work.

INDEX

FIGURE INDEX

TABLE INDEX

LIST OF ABBREVIATIONS

aW	Water activity
BHT	Butylated hydroxytoluene
CFU	Colony forming unit
CO_2	Carbon dioxide
DAD	Diode-Array Detector
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DPPH	2 ,2-difenil-1-picrylhydrazyl
Dw	Dry weight
EC_{20}	Minimum extract concentration with 20% of inhibition
EC_{50}	Minimum extract concentration with 50% of inhibition
EMM	Estimated Marginal Means
EtOH	Ethanol
FAME	Fatty acid methyl esters
FAO	Food and Agricultural Organization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FOSHU	Food for Specified Health Use
FUFOSE	Functional Food Science in Europe
GC-FID	Gas chromatography with flame ionization detection

HBSS Hanks' Balanced Salt Solution

HeLa Human Tumor Cell Line of cervical carcinoma

Hepg2 Human Tumor Cell Line of hepatocellular carcinoma

HPLC High performance liquid chromatography

HPLC-FL High performance liquid chromatography system coupled to a fluorescence detector

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

ILSI International Life Sciences Institute

kHz kilohertz

kV kilovolts

LPS Lipopolysaccharides

m/v mass by volume

m/z mass to charge ratio

MA Malt agar

MBC Minimum bactericidal concentration

MFC Minimum fungicidal concentration

MFC-7 58 Human Tumor Cell Line of breast carcinoma

MHB Mueller-Hinton agar

MIC Minimum inhibitory concentration

MS Mass spectrometry detector

MUFA Monounsaturated fatty acids

NCI-H460 Human Tumor Cell Line of Lung Carcinoma

NED N-(1-naphthyl) ethylenediamine hydrochloride

NO Nitric oxide

P	Power
pH	Hydrogen potential
PUFA	Polyunsaturated fatty acids
Rpm	Rotations per minute
RSM	Response surface methodology
S	Solvent
SEI	Source of electrospray ionization
SFA	Saturated fatty acids
SRB	Sulforhodamine **B**
S/L	Solid / liquid ratio
t	Time
TCA	Trichloroacetic acid
TPC	Total of polyphenolic compounds
TSB	Triptych soybean broth
UAE	Ultrasound assisted extraction
UFLC-PDA	Ultra-fast liquid chromatography coupled to a photo diode array detector
UPLC	Ultra Performance Liquid Chromatography
UV-Vis	Ultraviolet–visible spectroscopy
W	Watts

ABSTRACT

"Let food be your medicine and let medicine be your diet". More than 2500 years ago, Hippocrates, a Greek philosopher and the father of medicine, summed up in this significant phrase what is currently happening in terms of food. Global trends regarding the food industry show a marked interest of consumers in relation to certain foods that, in addition to nutritional value, bring health benefits. This is due to the concern of consumers to practice balanced eating habits, thus helping the body to stay as healthy as possible. Taking advantage of the knowledge acquired through traditional medicine, the food industry and the scientific community have developed a range of differentiated foods and, with the ability to provide beneficial health effects, based on natural matrices. These natural matrices, hold molecules with a strong bioactive potential, having been extensively studied in terms of their chemical composition and bioactivity, in order to be applied in the food industry, in different formulations, with bioactive capacity, providing the consumer with different benefits due to their antioxidant, antimicrobial, anti-inflammatory activity, among many others; thus, arising the functional foods.

In this way, the main objective of this work was to develop an innovative functional food, with improved biological properties. Therefore, *Artemisia absinthium* L., a medicinal plant was studied in relation to its nutritional, chemical composition and bioactivity. Also, the ultrasound assisted extraction (UAE) was applied and optimized in terms of yield and purity in bioactive molecules (phenolic compounds) through the Response surface methodology, in an attempt to obtain higher yields and purity than the infusion of this plant (the common consumed form). Furthermore, the richest extract in the target molecules and presenting the highest bioactivities were incorporated in a food product that is highly consumed, to functionalize this food and provide it with additional health benefits.

The developed food product was monitored in terms of its bioactivity and chemical composition, as also for the incorporation effect on the nutritional and physicochemical parameters, thus validating the incorporation and functionality of the developed extract. According to the obtained results, the UAE was efficient in extracting the target molecules, but, not as much as the infusion methodology, that can be due to the temperature inherent to the infusion procedure that is not considered in the UAE due to double energetic spending (heat + UAE) which would result in higher energetic bills.

It was possible to obtain a functionalized brownie with promising antioxidant, antimicrobial, anti-inflammatory and cytotoxic properties. Moreover, the incorporation of the extract did not cause significant alterations in the organoleptic brownie´s parameters. Overall, this work contributed to the knowledge of *A. absinthium* chemical composition and bioactivities and validated its potential to be applied in the food industry as a bioactive ingredient.

RESUMO

"Deixa que a alimentação seja a tua medicina e a medicina seja a tua alimentação". Há mais de 2500 anos que Hipocrates, filósofo grego e "pai" da medicina, resumiu nesta frase o que atualmente acontece em termos da alimentação. As tendências mundiais no que respeita à alimentação mostram um interesse marcante dos consumidores em relação a certos alimentos que, além do valor nutricional, aportam benefícios para a saúde. Este facto deve-se à preocupação dos consumidores em praticar hábitos alimentares equilibrados, ajudando desta forma o organismo a manter-se o mais saudável possível. Aproveitando o conhecimento adquirido através da medicina tradicional, a indústria alimentar e a comunidade científica têm desenvolvido um leque de alimentos diferenciados e, com capacidade de providenciar efeitos benéficos para a saúde, à base de matrizes naturais. Estas matrizes naturais, são detentoras de moléculas com um forte potencial bioativo, tendo sido extensivamente estudadas em termos da sua composição química e bioatividade, de forma a serem aplicadas na indústria alimentar, em formulações diferenciadas, com capacidade bioativa, proporcionando ao consumidor diferentes benefícios devido à sua atividade antioxidante, antimicrobiana, anti-inflamatória, entre muitas outras; surgindo assim os alimentos funcionais.

Assim, *Artemisia absinthium* L., uma planta medicinal, foi estudada relativamente ao seu valor nutricional, composição química e bioatividade. Além disso, a extração assistida por ultrassom (EAU) foi aplicada e otimizada em termos de rendimento e pureza em moléculas bioativas (compostos fenólicos) utilizando a metodologia de superfície de Resposta, na tentativa de obter rendimentos e pureza superiores à infusão desta planta (a forma consumida). Além disso, o extrato com a maior quantidade e pureza nas moléculas-alvo e ainda com o melhor potencial bioativo, foi incorporado numa matriz alimentar muito consumida (brownie), de forma a funcionalizar este alimento e proporcionar-lhe benefícios adicionais à saúde. O brownie desenvolvido foi monitorizado quanto à sua bioatividade e composição química, bem como quanto ao efeito da incorporação nos parâmetros nutricionais e físico-químicos, validando assim a incorporação e funcionalidade do extrato desenvolvido. De acordo com os resultados obtidos, a EAU revelou-se eficiente na extração das moléculas alvo. No entanto, não superou a metodologia de infusão, o que pode ser atribuído à temperatura inerente ao procedimento de infusão que não é considerada nos EAU devido ao duplo gasto energético (calor + EAU) que resultaria em faturas energéticas mais elevadas.. Foi possível obter um brownie

funcionalizado com promissoras propriedades antioxidantes, antimicrobianas, anti-inflamatórias e citotóxicas. Além disso, a incorporação do extrato não causou alterações significativas nos parâmetros organolépticos do brownie. Este trabalho contribuiu para o conhecimento da composição química e bioatividade de *A. absinthium*, e validou o seu potencial para aplicação na indústria alimentar como ingrediente bioativo.

1. INTRODUCTION

1.1 Functional foods

1.1.1 What they are and why they exist?

The consumption of foods that in addition to providing basic nutrition may help cure and prevent some diseases is one of the global trends in food in the last time. Foods that reduce cancer risk, reduce coronary heart disease risk, avoid osteoporosis, improve gastrointestinal function, regulate blood pressure, reduce cholesterol and even candy that prevents dental cavities can usually be found in many supermarkets around the world. Functional foods partially originated as answers to the increase of certain modern life-style diseases and have become an important alternative in improving world nutrition and public health.

Technological development and scientific advances have revealed the health benefits of consuming some foods or food components and have generated expectations for a higher quality of life. The resulting increase in the production and consumption of functional foods is creating an important market through which developing countries could increase their income and find an excellent alternative in their struggle to improve the nutrition and health of their populations (Sarmiento Rubiano, 2006).

Although a globally accepted definition of the term functional foods has not been achieved, the general concept is that they are foods or food components whose consumption, in addition to basic nutrition, generates health benefits and reduces the risk of disease. A functional food or food component can be a macro nutrient with a specific physiological effect or an essential micro-nutrient, but it can also be a food component that, although it doesn't have a high nutritional value or is not essential, its consumption achieves modulation of some function in the body that reduces the risk of disease, such as fiber and some viable microorganisms (Roberfroid, 2000).

A food in its natural state, or a food to which one or more of its components have been added, removed or modified, is considered functional (Roberfroid, 2002).

1.1.2 Global representation

Foods, food ingredients, and even bioactive molecules with health-promoting abilities present a large demand by worldwide consumers (Vieira da Silva, Barreira and Oliveira, 2016).

The global functional food market was valued at USD 161.49 billion in 2018 and is forecasted to grow with an annual growth rate of 7.9% for the next five years (GVR, 2019).

The term ‘‘functional food’’ itself was first used in Japan, in the 1980s, for food products fortified with special constituents that possess advantageous physiological effects (Hardy, 2000; Kwak and Jukes, 2001; Stanton, Ross, Fitzgerald and Van Sinderen, 2005).

Functional foods may improve the general conditions of the body (e.g. pre- and probiotics), decrease the risk of some diseases (e.g. cholesterol-lowering products), and could even be used for curing some illnesses. It was recognized that there is a demand for these products as different demographical studies revealed that the medical service of the aging population is rather expensive (Mark- Herbert, 2004; Menrad, 2003; Side, 2006).

In the Asian continent, in 1984, the Japanese Ministry of Education, Science and Culture (MESC) initiates a project of systematic analysis and development of functional foods, which relates the consumption of some foods or food components with beneficial effects on health, this being the first opportunity in which the term "functional foods" is officially used.

In 1991 Japan legalized the marketing of foods with healthy properties, placing them under the name of "FOSHU" (Food for Specified Health Use). Japanese legislation requires each FOSHU food to carry out a detailed scientific check of its physiological interactions and beneficial effects on health, which includes clinical tests, guarantee of safety of consumption and analytical determinations of the effectiveness of its components. The development of functional foods in Japan is currently based on four main points: 1) Technological innovation and scientific development, to create foods with proven health benefits; 2) Regularization and legalization by the state; 3) Industrial development and commercialization of new products and; 4) Adequate information and knowledge for consumers (Hirahara, 2004).

In the European Union (EU) during the 90s, a significant number of research projects were developed in the area of food and nutrition, topics such as dietary fibers, probiotics, prebiotics and more recently antioxidants, vitamins, and phytoestrogens, have been studied to assess the impact of regular consumption on human health (Weststrate, Van Poppel and Verschuren, 2002). The EU created a commission of concerted actions for research on functional foods in Europe FUFOSE (Functional Food Science in Europe), made up of researchers in related areas with nutrition and health under the coordination of the ILSI (International Life Sciences Institute). The commission's role is to define the

scientific development of functional foods, the creation of new products, and the scientific verification of their beneficial health effects (Roberfroid, 2002). In 1999, this commission published the first definition of functional foods, indicating that they are foods in which it has been satisfactorily demonstrated that in addition to adequate nutrition, they provide benefits in one or more functions of the organism, improving health or reducing the risk of disease when they are consumed in the expected amounts within a normal diet (Diplock, 1999).

Traditionally in North America there has been scientific interest in the relationship between diet and the prevention of certain diseases present in the population. Although the American legislation doesn't include a definition of "functional foods", for the entities in charge of food regulation the word "functional" implies a food that has properties that generate health benefits or reduce the risk of disease. The Food and Drug Administration (FDA) classifies some categories of foods with additional properties that include conventional foods, food additives, dietary supplements, medicated foods or foods for use in special diets, the category used to define a specific food or functional component, depends on its form of elaboration and the commercialization parameters (Ross, 2000).

Nearly 25,000 chemical compounds present in fruits and vegetables for human consumption have been related to healthy effects or decreased risk of disease, and more than 500 of them are directly associated with the prevention of carcinogenic processes (Hasler et al., 2004). There is an important knowledge potential about food and food components with functional properties, which at the same time with the advances in human and plant genomics, they will allow in the future to better understand the interactions between nutrients and body cells, even allowing the use of genetic manipulation for the benefit of health and the reduction of disease risk (Milner, 2002).

As far as Latin America is concerned, functional foods knowledge is relatively recent, in some cities health authorities legally recognize the healthy properties of foods. Only Brazil has a regulation in which a nutritional or non-nutritive food component is defined as functional, which can produce beneficial effects for health, different from basic nutrition when they are part of a normal diet without being a medicine (Lajolo, 2002).

Latin America is currently a potential producer and consumer of functional foods, it has great natural resources, a wide flora and fauna biodiversity associated with a wide variety of edible plants and fruits, with potential beneficial effects on health.

1.2. Functional ingredients

1.2.1. Main functions in foodstuff

Functional foods are complex mixtures that can intercept the compound modulate its release or inhibit its activity. In this way, the food matrix, both in its raw state and after cooking or storage, can have a significant influence on the activity or release of the main components. Thus, a crucial step towards the success of functional foods is the development of adequate food vehicles to maintain active form until the moment of consumption, and delivery to the desired target location in the body (Betoret et al., 2011).

There are different types of functional foods:

a) Naturally functional foods;

b) Foods to which some component added that provides functionality (e.g omega-3, vitamins, fiber, among others);

c) Food that have been subtracted from a component that can affect health (e.g, skim, reduced sodium, sugar free, lactose free, among others).

There are several natural matrices that have been studied and pointed out as excellent sources of bioactive compounds. The main bioactive compounds and functional ingredients added to foods are probiotics, prebiotics, dietary fibers, phytosterols, carotenoids, phenolic compounds and fatty acids (Salgado and Almeida, 2009).

Polyunsaturated fatty acids (PUFA), highlighting omega-3 and omega-6, are found in cold water fish, vegetable oils, flax seeds, nuts and some vegetables, and are considered to have effect of reducing hypercholesterolemia, anti-inflammatory, anti-coagulant, vasodilator and anti-aggregant (Rodríguez, Megías and Baena, 2003; Pimentel, Francki and Gollück , 2005; Ferreira, Cabral and Nardelli, 2009). Scientific evidence supports the beneficial effects of balanced diets on the omega 3 and omega 6, supplemented with antioxidants (Reglero et al., 2008).

The bioavailability of antioxidants plays an important role in food functional factors assessment, because it affects the biological activity of substances in the digestive tract and its absorption through the intestinal walls for blood circulation (Grajek, Olejnik and Sip, 2005). Among the most prominent antioxidant substances are vitamins C (Martí et al., 2009) and E (Miyazawa et al., 2009) carotenoids, zinc (Prasad, 2008; Prasad et al., 2009), polyphenols, among others (Cortés, Chiralt and Puente, 2005).

There is a growing interest in the development and use of extracts rich in antioxidant polyphenols, as functional ingredients. Its main sources are fruits, teas, coffee and red

wine. Vegetables, cereals, chocolates and dried vegetables also contribute to the total consumption of polyphenols. The main delaying factor in scientific evolution on the effects of polyphenols is considered the complexity and diversity of their chemical structures. Current evidence supports polyphenols contribution in cardiovascular diseases (Grassi et al., 2009) as well as cancer prevention (Koch et al., 2009), and suggest a role in the prevention of neurodegenerative diseases, diabetes mellitus (Scalbert et al., 2005) and in protecting the DNA (Ramful et al., 2010).

Carotenoids (β-carotene, β-cryptoxanthin, α-carotene, lycopene, lutein and zeaxanthin) are present in foods with yellow, red or orange pigmentation and are part of the antioxidant defense system in humans (Ferreira, Cabral and Nardelli, 2009; Maiani et al., 2009). β -carotene is a potent antioxidant with protective action against cardiovascular diseases, and both precursor vitamin A carotenes and non-precursors seem to have a preventive action against cancer, acting mainly on as a free radical scavenger (Grajek, Olejnik and Sip, 2005; Moraes and Colla, 2006; Della Lucia et al., 2008).

Studies reveal that foods enriched with vitamin D, become the best option in some countries, where the sun is weak in winter, promoting the production of this vitamin by the body itself. This vitamin has been studied for its action in reducing fractures by up to 20 percent (Daniells, 2009). Vitamin D is essential for the intestinal absorption of calcium, maintenance of calcium homeostasis and the health of the bone system. Still other beneficial effects can be associated with dietary supplementation and dermal production of vitamin D as, for example, prevention of autoimmune diseases, cancer and cardiovascular diseases (Holick, 2004).

Dietary fibers are not susceptible to hydrolysis by enzymes of the human intestine, and are classified as soluble and insoluble, the latter remaining intact throughout the gastrointestinal tract, favoring the increase of fecal bolus and intestinal motility, aiding in the bowel function (Roberfroid, 2002a; Maihara et al., 2006; Ferreira, Cabral and Nardelli, 2009). Fibers like inulin and fructo-oligosaccharides are recognized as prebiotics with functional effects in association with intestinal microflora and with potential effect about health, for example decrease in risks for some pathologies (intestinal infection, constipation, obesity, colon cancer, among others) (Roberfroid, 2002b).

Probiotic strains have been widely studied and commercially exploited in different products around the world (Soccol et al., 2010). According to the currently accepted definition, probiotics are preparations or products containing microorganisms viable,

well-defined and in sufficient quantity to change the intestinal microbiota, when administered in an adequate amount, which confer benefits to the health of the host (Reid et al., 2003; Picard et al., 2005). The most used probiotics involve the genera, among both yeasts, such as *Saccharomyces boulardii*, have also been used with probiotic potential (Szajewska and Mrukowicz, 2005).

Table 1 shows the different functional foods with their main bioactive compounds and its possible health benefits:

Table 1. Examples of functional foods and their health benefits.

Examples of natural functional foods			
Functional food	**Component**	**Potential health benefit**	**Ref.**
Carrot, broccoli, spinach, chard	Carotenoids	Antioxidant	Ferreira, Cabral and Nardelli, 2009; Maiani et al., 2009
Water fish, vegetable oils, flax seeds and nuts	Omega-3 fatty acids	Reduce hypercholesterolemia, anti-inflammatory, anti-coagulant, vasodilator and anti-aggregant	Rodríguez, Megías and Baena, 2003; Pimentel, Francki and Gollück , 2005; Ferreira, Cabral and Nardelli, 2009
Fatty fish, such as salmon, tuna, and mackerel	Vitamin D	Intestinal absorption of calcium, maintenance of calcium homeostasis and the health of the bone system	Holick, 2004
Whole-grainfoods	Bran / fiber	Reduced risk of some cancers. Gastrointestinal health benefits, including the prebiotic and the laxation effects	Aune et al., 2011. Hughes et al., 2007
Asparagus, onion, garlic, banana	Fiber (inuline)	Decrease in risks for some pathologies (intestinal infection, constipation, obesity, colon cancer)	Roberfroid, 2002b
Added or modified functional foods			

Yogurts	Prebiotics	Reduction of the blood LDL (low-density lipoprotein) level, stimulation of the immunological system, increased absorbability of calcium, maintenance of correct intestinal pH value, low caloric value, and alleviation of symptoms of peptic ulcers and vaginal mycosis	Socha P, Stolarczyk and Socha, J., 2002
Breads and cereals with added fiber	Fiber	Decreased risk of gastrointestinal cancers including colorectal, stomach and liver cancers	Bradbury, Appleby and Key, 2014
High calcium milk	Calcium	Reduces osteosporosis risk	Feskanich et al. 2018; Laird et al. 2017; Yoon et al. 2012; Matthews et al. 2011; Nieves et al. 2010
Margarine enriched with plant sterols	Plant sterols	Cholesteroll owering	Cater, Garcia-Garcia, Vega and Grundy, 2005
Wine	Flavonoids	Contribute to cardiovascular health	Fernandes, Perez-Gregorio, Soares, Mateus, de Freitas, 2017

1.2.2. Functional ingredient´s legislation

Approaching the issue of functional foods from the Codex Alimentarius is a difficult work. On one hand, the Codex Alimentarius itself responds to a complex cooperation structure established from the joint FAO/WHO Programme on Food Standars. On the other hand, there is no clear definition in the Codex of what we mean by functional foods. In the standard "Guidelines for the Use of Nutritional and Healthy Statements" (Codex Alimentarius Commission CAC-GL 23, 2004) established the scope of some definitions related to the topic. It also contains an annex that specifies recommendations on the scientific basis of health claims. Most States have not considered it necessary to recognize that this is a new category of food, nor is there consensus on the need to treat them as a specific issue.

The EU has set up a Concerted Action Commission on Functional Bromatology in Europe (Functional Food Science in Europe, FUFOSE). The program has been coordinated by the International Institute of Biological Sciences (International Life Sciences Institute Europe), and its goal is to develop and establish a scientific approach

to the evidence needed to support the development of foods products that may have a beneficial effect on a physiological function of the body, improve the health and well-being of an individual and/or reduce the risk of developing disease. In this regard, the Commission produced a consensus paper in 1999: "Scientific concepts on functional foods in Europe". Also, in December 2006, the EU approved Regulation (EC) N° 1924/2006 of the European Parliament and of the Council, nutrition and health claims in foods, which sets out definitions, specific criteria and conditions of use of these statements.

The list of denied and/or authorized health claims are then published through specific regulations specifying the nutrient, substance, food or food category, the type of declaration, the conditions and/or restrictions of use of the food, or a supplementary statement or warning.

According to the above-mentioned Regulation (EC) 1924/2006 with correction of errors of the European Parliament and of the Council, the following types of declarations can be differentiated: nutritional, health properties and reduction of disease risk reduction.

Nutritional statements

Any statement that affirms suggests or implies that a food has specific beneficial nutritional properties on the basis of:

- The energy contribution (caloric value): it provides, which provides a reduced or increased degree, that it does not provide and / or.
- Nutrients or other substances containing in reduced or increased proportions that it does not contain.

Healthy statements

Any statement that affirms suggests or implies that there is a relationship between a food category, a food or one of its constituents, and health.

Disease risk reduction statements and statements regarding children's development and health

Any health claim that affirms, suggests or implies that consuming a food category, a food or one of its constituents significantly reduces a risk factor for human disease on and off. As indicated in Regulation (EC) 1924/2006 with correction of errors of the European Parliament and of the Council, a statement must be scientifically substantiated by taking

into consideration all the available scientific data and weighing the evidence, hence the importance of the validation of scientific studies that prove a given statement.

1.3. Functional molecules

The most important groups of dietary phytochemicals are shown in **Figure 1.** The phytochemicals can be divided into general categories as phenolics, alkaloids, nitrogen-containing compounds, organosulfur compounds, phytosterols, and carotenoid.

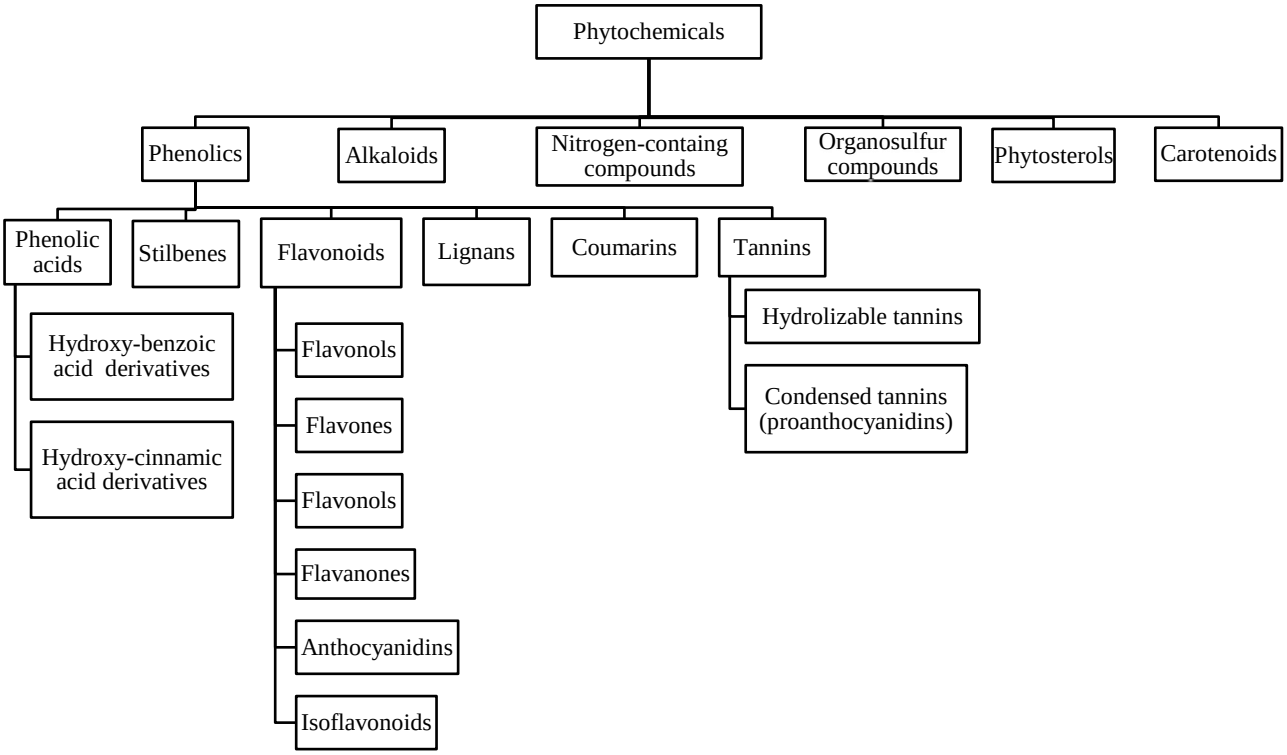


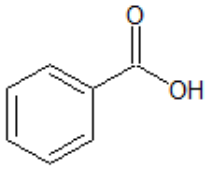
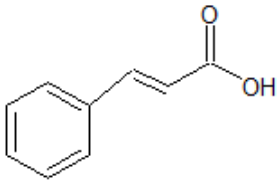
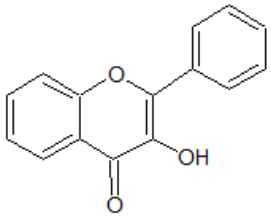
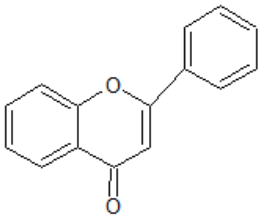
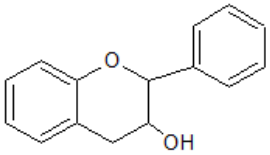
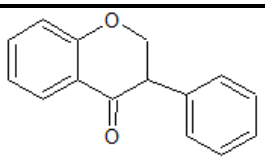
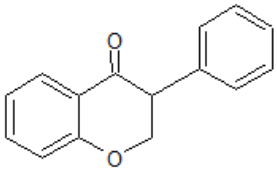
Figure 1. Classification of dietary phytochemicals (Liu, 2013).

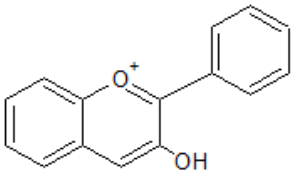
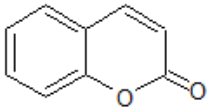
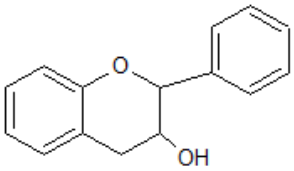
The most studied groups of dietary phytochemicals related to human health and well-being are phenolics and carotenoids (Liu, 2013).

1.3.1. Phenolic compounds

Table 2 shows the classification of phenolic compounds, examples of each category and foods where they can be found.

Table 2. Classification of phenolic compounds.

	Class	*Chemical structure*	*Examples*	*Source (food)*	*Ref.*
Acid phenols	Hydroxybenzoic acids		Protocatechuic acid Vanillic acid Gallic acid	Fruits, vegetables, and cereals	Lui, Wang, Chu, Cheng and Tseng, 2002
	Hydroxycinnamic acids		Curcumin Caffeic, ferulic and chlorogenic acids	Curries and mustard. Fruits, coffee seeds and soybeans	Drago Serrano, López López and Saínz Espuñes, 2006
Flavonoids	Flavonols		Quercetin. Kaempferol	Onion, coriander, grapes and apples	Hertog, Hollman and Venema, 1992
	Flavones		Apigenin	Honey and grapes	Chirinos, Betalleluz-Pallardel, Huamán, Arbizu, Pedeschi and Campos, 2009
	Flavanols		Taxifoline	Açaí, chocolate, wine, and berries	Pacheco-Palencia, Duncan and Talcott, 2009
	Isoflavones		Genistein. Daidzein	Beans, soybeans, and hops	Eldridge and Kwolek, 1983
	Flavanone		Naringenin	Citrus fruits, such as oranges, lemons, tangerines and grapefruits	Pacheco-Palencia, Duncan and Talcott, 2009

	Anthocyanins		Cyanidin Pelargonidin Peonidin Delphinidin Malvidin	Red berries, such as blackberries, blueberries, cherries, apples, pears, peaches, and plums	Vermerris, 2008
Coumarins			Umbelliferone	Fruits and vegetables	Peñarrieta, Tejeda, Mollinedo, Vila, Bravo, 2014
Tannnis			Tannic acid	Leaves, fruits and bark (Oak and Chestnut)	Vermerris and Nicholson, 2008

1.3.2. Carotenoids

Carotenoids are probably the most widely distributed pigments in nature (Hari, Patel and Martin, 1994). The most important role of carotenoid pigments in the diet of humans and other animals is their ability to function as precursors to vitamin A. Although carotenoid β-carotene has the highest pro-vitamin A activity due to its two β-rings. Ionone, other commonly consumed carotenoids, such as α-carotene and β-cryptoxanthin, also possess pro vitamin A activity. It is estimated that the provitamin A carotenoids present in fruits and vegetables provide 30-100% of the vitamin A needs of human populations (Fennema and Tannenbaum, 1992).

Orange and yellow vegetables and fruits, including carrots, spinach, pumpkins, papayas, sweet potatoes, winter squash, mangoes, cantaloupes, and red peppers, are rich sources of β-carotene. Dark green leafy vegetables, including spinach, kale, turnip greens, broccoli, Brussels sprouts, and collards, are rich sources of lutein and zeaxanthin. Tomatoes, watermelons, pink grapefruits, apricots, and pink guavas are the most common sources of lycopene. The most abundant carotenoids in potatoes are lutein and zeaxanthin, followed by β-carotene and β-cryptoxanthin (Liu, 2013).

Carotenoids belong to two structural groups: carotenes that are hydrocarbons and xanthophylls that are oxygenated. Oxygenated carotenoids (xanthophylls) form a group of derivatives that frequently contain hydroxyl, epoxy, aldehyde, and ketone groups. In addition, hydroxylated carotenoid esters with fatty acids are also widespread in nature.

Thus, some 560 carotenoid structures have been collected and identified (Fennema and Tannenbaum, 1992).

The basic structural frame of carotenoids consists of covalently linked isoprene units, either head-tail or tail-tail, creating symmetrical molecules (**Figure 2**).

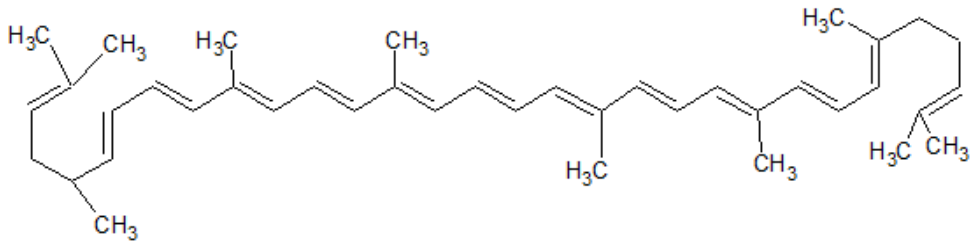


Figure 2. Carotenoid's basic structure.

The carotenoid most frequently found in plant tissues is β-carotene. β-carotene, α-carotene and β-cryptoxanthin (**Figure 3**) are precursors of vitamin A and the most nutritionally important forms.

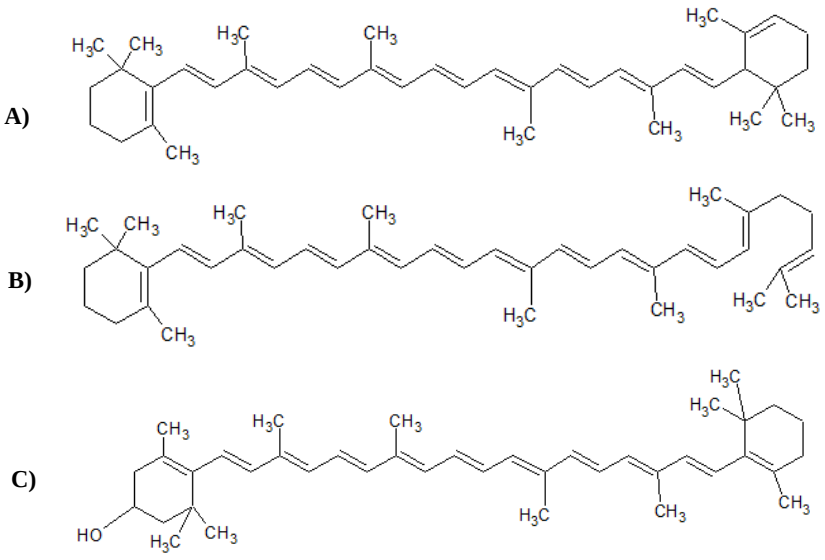


Figure 3. Carotenoids: A) β-carotene, B) α-carotene, C) β-cryptoxanthin.

Carotenoids are relatively stable during the storage and classic handling of almost all fruits and vegetables.

Freezing hardly changes in carotene content. However, scalding is known to influence t he decarotenoid level (Fennema and Tannenbaum, 1992).

Regarding the stability of the bioactive molecules described above, when choosing the optimal extraction technique and conditions, it must be taken into account the fact that

several external factors can lead to the degradation of the compounds, such as temperature, pH, the light and storage conditions.

Nowadays it is possible to protect bioactive compounds, increasing their stability, from encapsulation techniques. Over the decades, spray drying microencapsulation has been used successfully in the food industry, the first reference being the year 1930, which aimed to microencapsulate flavors using gum arabic as a matrix (Gouin, 2004).

Encapsulation is the technology by which small particles or droplets are involved by an agent encapsulant, in order to form microparticles with several beneficial properties. Thus, encapsulation provides a physical barrier between the material inside the microcapsule and the other components of the product.

The microcapsules help the food materials used to resist the processing and packaging conditions, improving the flavor, aroma, stability, nutritional value and appearance of their products (Yañez et al., 2002; Montes, De Paula and Ortega, 2007).

Choice of encapsulating material and encapsulation technique is one of the most important and decisive steps to ensure that the active agent is protected and only released at the defined target site, since this release must occur when different stimulation such as temperature and pH (Dias, Caleja, Ferreira and Barreiro, 2017).

1.4. Application of functional ingredients in pastry products

Likewise, consumers increasingly demand nutritious and healthy products that are delicious and attractive.

Several studies have been carried out with the replacement of part of the wheat flour by other sources of fiber or protein, in order to increase the nutritional value of cookies, such as the addition of cassava starch, sour starch and orange albedo (Santos et al., 2010, 2011), flour oat meal and parboiled rice flour (Assis et al.,2009), jackfruit and pumpkin seed flour (Moura et al., 2010; Borges et al., 2006), microalgae Spirulina platensis (Morais et al., 2006), amaranth (Capriles et al., 2006; Marcílio et al., 2005), oat flakes and β-glycans (Gutkoski et al., 2007a), bocaiuva flour (Kopper et al., 2009), jatobá-da-mata flour (Silva et al., 2001a), among other sources.

According to Mona et al., (2009), powdered chicory roots are ingredients alternatives to wheat flour and fat in the production of cracker cookies. The nutritional benefits of Chicory (*Cichoriumintybus*) as a source of dietary fiber, the presence of inulin in its composition and fructo-oligosaccharides, arouses a potential interest in the food industry regarding the use of this plant as a functional food ingredient.

According to Fasolin et al. (2007), other works carried out with different types of biscuits have shown a strong tendency of the industries and of research works in promoting the enrichment of cookies. In addition, Santucci et al. (2003), reported a mixture of non-conventional flours with wheat flour improves the nutritional quality of cookies and may even improve their palatability, making them more accepted by consumers.

1.5. *Artemisia absinthium* as a source of functional ingredients

Medicinal and aromatic plants have been in high demand in this area in the development of new functional foods due to the high amounts in phenolic compounds in their composition.

A. absinthium (**Figure 4)** commonly known as absinthe, wormwood, asensio, ajorizo, bitter artemisia or holy herb, is a medicinal herbaceous plant, of the genus Artemisia, native to the temperate regions of Europe, Asia and North Africa.

It is a perennial herbaceous plant characterized by having straight stems with a hard and woody rhizome. It grows between 80 to 120 cm (rarely 150 cm) and presents a silver-green color. The leaves, arranged spirally, are grayish green on the front and white on the reverse, covered with silver-white hairs, with oil-producing glands; the basal leaves are up to 25 cm long with smaller caulinal leaves (on the stem), 5 to 10 cm long, less divided. Its flowers are tubular and pale yellow. The fruit is a small achene[1] of 0.5 mm, more or less cylindrical, somewhat curved, with dark, glabrous and shiny nerves; absent vilano, being the dispersion of the seeds by gravity (Barkley, 2006).

Figure 4. *A. absinthium* (https://www.inaturalist.org)

[1] Dried fruit containing a single seed, the outer shell of which is not welded to it.

A. absinthium grows in temperate regions of Europe, Asia and North Africa. Absinthe grows without difficulty in poor sandy soils as well as in dry and sunny places. It grows spontaneously in uncultivated fields, on arid, rocky hills. It grows very well in fertile soils, not very heavy, preferably rich in nitrogen. It is propagated by segments between March and October in temperate climates or by seeds in planting beds.

The seeds of this plant are very small, so they should be placed in sites where they are not lifted by the wind or carried away by rain. For this reason, they are minimally covered with a little soil. This type of plant can be reproduced by cutting the roots. To care for the crops, the plant must be fertilized in small amounts at least once a year.

The plant contains 0.2 to 0.5 percent of an essence of greenish or bluish color (depending on its characteristics) and with a strong bitter taste, being thujone the main component, soluble in alcohol, but not in water. This plant presents another characteristic compound called absintine, an amorphous yellow substance, poorly soluble in alcohol, and soluble in water.

The herb has always been of a great botanical and pharmaceutical interest and has been employed in folk medicine against various diseases (Bora and Sharma, 2011). Wormwood essential oil has been widely used mainly for its neuroprotective (Bora and Sharma, 2010), antifungal (Kordali et al., 2005), antimicrobial (Juteau, Jerkovic, Masotti et al, 2003), insecticidal (Kordali et al., 2005), acaricidal (Chiasson, Belanger, Bostanian, Vincent and Poliquin, 2001), anthelmintic (Tariq, Chishti, Ahmad and Shawl, 2009), antimalarial (Irshad, Butt and Hira, 2011), hepatoprotective (Gilani and Janbaz, 1995), and antidepressant (Mahmoudi, Ebrahimzadeh, Ansaroudi, Nabavi and Nabavi, 2009) proprieties. In addition, it is used to make a tea for helping pregnant women during pain of labor and in treating leukemia and sclerosis (Canadanovic-Brunet, Djilas, Cetkovic, and Tumbas, 2005).

Wright, (2002) has reported that wormwood stimulant properties are caused by bitter substances as artabsin (sesquiterpene lactone) and absinthin (dimer of sesquiterpene lactone) present in plant extracts. In fact, Iranian wormwood essential oil was characterized by the predominance of β-pinene and β-thujone (Rezaeinodehi and Khangholi, 2008). Furthermore, Martín et al., (2011) showed that the major compounds of wormwood found in the supercritical fluid extraction (SFE) extracts as well as in the hydrodistilled essential oils were Z-epoxyocimene, chrysanthenol, and chrysanthenyl acetate.

1.6. The importance of the extraction process

The study of the use of plant extracts as a source of bioactive components is directly linked to the extraction process, in which it will influence the final result of the extract, that is, its composition and, consequently, the effect it will have on the product where it is used (Azmir et al., 2013).

Therefore, extraction techniques are of great importance to obtain the desired compounds from each plant properly and efficiently. A division that can be made between extraction techniques for the acquisition of phenolic compounds is in conventional (e.g., Soxhlet extraction, maceration, agitating maceration, etc.) and modern extracting methods (e.g., ultrasound-assisted, microwave and enzyme-assisted extraction, supercritical fluid extraction, among others). Conventional methods have disadvantages, such as longer extraction times, use of solvents high amounts of solvents, and the need for high purity, as well as can favor the decomposition of thermolable compounds at high temperatures (Azmir et al., 2013; Joavanović et al., 2017; Sik et al., 2020). However, it also has the advantages of being cheaper, safe and generally easy to climb (Oliveira et al., 2016).

Each plant usually has its own characteristics, with specific behavior through different stimuli, as well as the phenolic compounds present in these plants (Jovanović et al., 2017). Parameters such as time, temperature, solvent characteristics, solid-liquid ratio and pressure are important in the process and influence the final extraction result (Azmin et al., 2016).

Therefore, the choice of the appropriate extraction process with appropriate parameters should be studied, so that extracts can be obtained efficiently, satisfactorily and with quality of the desired components (Sik et al., 2020). The ideal extraction method should be performed in a short time, with less energy consumption, with the minimum possible consumption of solvents, preferably, solvents that are not harmful to human health, and that do not degrade the desired compounds (Sik et al., 2020). In order to provide a product with quality, concentrate and high recovery rate (Oliveira et al., 2016). So, optimization with the choice of appropriate parameters and factors to maximize component extraction is important, by means that it can be possible to obtain high yields and purity in the target molecules in an economic and sustainable way (Albuquerque et al., 2020).

1.6.1. Obtaining extracts by ultrasonic assisted extraction (UAE)

Phenolic compounds are usually extracted through the use of various solvents such as water, ethanol, methanol, organic acid solutions, or different mixtures of two or more solvents. Widely used and validated methodologies are applied, however, some of these solvents have disadvantages such as toxicity, volatility and flammability, reducing the performance and possible applicability of phenolic compounds obtained from extraction in food products (Dias et al., 2015).

The literature does not seem to define a specific solvent for the extraction of phenolic compounds and, points out the polarity of the compounds to be extracted as one of the main factors to take into account in the choice of solvent since that influences directly the extraction. The ideal solutions are formed with non-polar solutes in solvents also non-polar. Taking these characteristics into account, there are several extraction techniques that have been explored, such as ultrasound and microwave assisted extractions, Soxhlet extraction, maceration, among others.

Ultrasound assisted extraction (UAE) is usually performed in ultrasound baths or closed extractors equipped with probes and consists of the application of mechanical vibrations in liquid, solid and gaseous samples by sound waves with a frequency exceeding 20 kHz. Ultrasound promotes the phenomenon called cavitation that induces the formation of bubbles that rupture varying the pressure and temperature during the process. Therefore, the process variables allow the structural disruption of the sample and the reduction of the particles thus promoting a fast and efficient extraction.

UAE is a simple and efficient alternative compared to conventional extraction techniques. The main advantages of UAE in solid-liquid extraction include increased extraction performance and faster kinetics. Ultrasonic extraction is a technique frequently used for the extraction of plant materials and is proven for a faster and more complete extraction process compared to traditional methods. The solid and liquid phase is significantly higher due to cell disruption and particle dispersion.

Using sonication also reduces operating temperature, allowing the extraction of temperature-sensitive components. Compared to other innovative extraction techniques such as microwave-assisted extraction, the ultrasound device is more economical and easier to operate. In addition, UAE can be used with any solvent, such as Soxhlet extraction, to extract a wide variety of natural compounds (Wang et al. 2006).

A substantial advantage of ultrasound is the influence on the most important processing parameters: amplitude, time, temperature and pressure. In this way, the extraction process can be optimized to ensure that the structure of the extracts is not compromised.

It is for all the above mentioned that the UAE was chosen to carry out the extraction of phenolic compounds from the *A. absinthium* plant.

2. OBJECTIVES

The main objective of this work is the development of an innovative functional food, with improved biological properties from the extraction of molecules of the *A. absinthium* plant and their subsequent incorporation in a pastry product (brownie). In this sense, it is expected to obtain high yield and pure extract rich in phenolic compounds.

The work plan is shown in **Figure 5**.

The specific objectives of this work are:

1) Screening analysis of the extraction process of bioactive compounds, employing aqueous and hydroethanolic ultrasound-assisted extraction (UAE) for this and response surface methodology (RSM) to determinate extraction conditions ranges;
2) Chemical characterization of the extract obtained by liquid and gas chromatography techniques;
3) Bioactive characterization of the extract through its antioxidant, anti-inflammatory, cytotoxic and antimicrobial activities;
4) Incorporation of the most promising extract in a pastry product (brownie);
5) Evaluation of the physicochemical characteristics and bioactivity of the developed brownie;
6) Evaluation of the nutritional profile of the developed brownie over the shelf life.

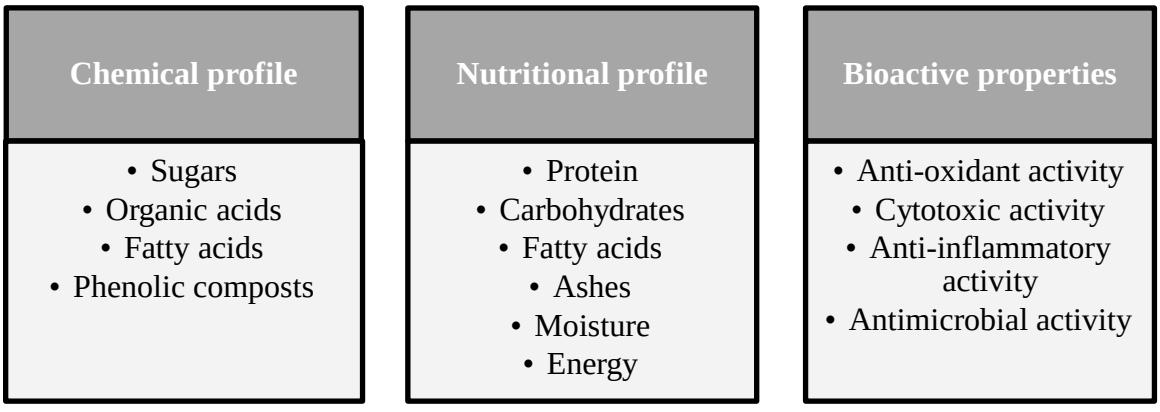


Figure 5. Schematic representation of the objectives of the present work.

The incorporation of bioactive ingredients from absinthe in highly consumed products makes this an innovative work, even if it does not reflect a considerable increase in health benefits.

3. MATERIALS AND METHODS

3.1 Samples

The sample of *A. absinthium* was provided by the company *Tea of the world*, in the form of strands as shown in **Figure 6**. After the acquisition, the plant was reduced to powder (20 mesh) and then stored protected from light and moisture.

Figure 6. Sample of *A. absinthium* in strands.

3.2 Extraction processes

3.2.1 Infusion extraction

In order to compare yields between techniques, an infusion extraction was performed, since this is the commonly consumed form of this plant.

The infusion of *A. absinthium* was prepared according to the method of traditional medicine: a beaker (200 mL) of boiling water (100 ºC) was added to a teaspoon of grass (1.0 g) and left to stand for 5 minutes. This infusion was heated on a magnetic stirrer plate (LBX H20D Labbox, 550W, Barcelona, Spain). Then, the mixture was filtered using filter paper and the supernatant was collected into jars, which were freeze-dried (-47 ºC, 0.045 bar; Freezone 4.5, Labconco, Kansas City, MO, USA) to determine the extraction yield.

3.2.2 Ultrasound-assisted extraction

Sample of crushed *A. absinthium* (1.75 g) was placed in a beaker with 50 mL of solvent and processed according to the experimental design matrix obtained by screening analysis mentioned above. The S/L remained constant (35 g/L), as well as T (30-35 °C; a cold-water bath was used to avoid heating the samples) (**Figure 7**).

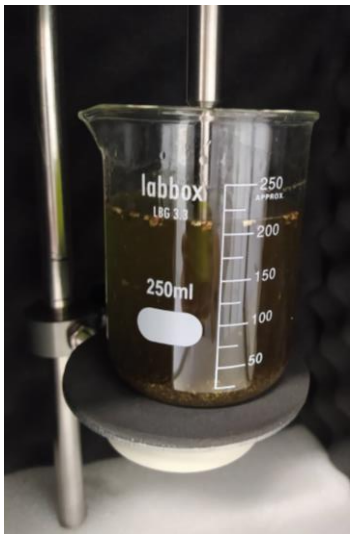


Figure 7. Ultrasound extraction illustration and positioning.

The extracts were obtained using the Ultrasonic Assisted-Extraction (UAE) (Sonicator QSonica, CL-33 model, 500 W, Newton, CT, USA) (**Figure 8**). After each extraction, the sample was centrifuged (5000 rpm; for 20 min at 10 °C), filtered through paper filters (Whatman Nº 4) and dried at 40 ºC with the rotary vacuum evaporator (Heidolph Hei-VAP Advantage), with an average rotation of 80 rpm. The aqueous fraction was lyophilized (-47 ºC, 0.045 bar; Freezone 4.5, Labconco, Kansas City, MO, USA) to obtain the extract powder. The extract was stored in a dry place protected from light.

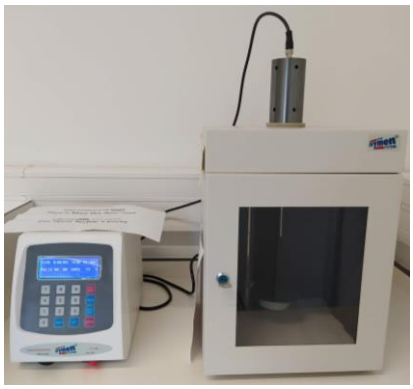


Figure 8. Ultrasonic homogenizer used for ultrasound-assisted extraction technique.

3.2.3 Pre-optimization Screening Analysis

Requested fix conditions on the extraction process of *A. absinthium* based on a comparison of UAE against the infusion methodology, and in order to improve energy and solvent efficiency, a twelve runs array matrix was designed and integrated randomly

in order to perform four screening analysis which was randomized and codify using statistical software Design Expert 12.0.1. software (Stat-Ease, Inc., Minneapolis, MN, USA), employing 3 factors X_1:Power [50-100%], X_2:Solvent percentage [0-50%], and X_3:Time [20-600s] and up to 5 levels depending on the analysis. Pre-selection of factor values was selected based on previous experimental designs and levels were adjusted in to minimized runs (**Table 3**).

Designs were conceptualized and explore individually using the Design Expert, and results were interpreted globally. Factorial effects alias was built as Intercept = Intercept + ABC, A = A + BC, B = B + AC and C = C + AB. Responses Y_1 and Y_2 were identified through HPLC as mention in section 3.3.1 and quantified in clusters Y_1:TPC - Total Phenolic Content and Y_2:TFC - Total Flavonoid Content, while Y_3:Yield was quantified gravimetrically. At factorial analysis, TBC – Total Bioactive Content was considered as the sum of TPC+TFC=TBC.

Table 3. Run row from table 1 letters correspond to their specific screening analysis, a) Factorial fractional design 2^{3-1} (Two levels with resolution III), b) Factorial design 2^2 of X_2 and X_3, and c) and d) simple unifactorial multi-level analysis of X_2 and X_3, respectively. Factors are represented as X_1(2 levels), X_2(3 levels), X_3(5 levels), and responses as Y_1-Y_3.

Run	***X_1: Power (%)***	***X_2: Solv (%EtOH)***	***X_3: Time (s)***	***Y_1: TPC mg/g extract***	***Y_2: TFC mg/g extract***	***Y_3: yield***	***Compounds***
Infusion				139.69	27.35		12
[d] 1	50	0	600	115.91	15.95	7.0	9
[d] 2	50	0	20	105.55	16.41	9.4	11
[a] 3	50	20	20	116.86	15.62	7.9	8
[b,c] 4	50	20	240	107.16	19.95	9.1	8
[d] 5	50	0	60	96.99	15.26	8.2	11
[c] 6	50	50	240	108.52	26.01	7.4	10
[a,b] 7	100	20	240	112.92	25.01	9.6	9
[b] 8	100	0	240	110.53	24.68	7.9	9
[d] 9	50	0	120	88.02	14.61	7.7	9
[a] 10	100	0	20	97.34	10.54	7.6	9
[a,b,c] 11	50	0	240	88.55	10.23	8.2	9
[d] 12	50	0	40	74.90	9.81	7.5	11

3.3 Determination of the chemical composition of *A. absinthium* plant

3.3.1 Phenolic compounds

For phenolic profile analysis, the extracts obtained by infusion and UAE were redissolved with a EtOH/H_2O (20:80 *v/v*) solution, reaching a concentration of 10 mg/mL.

Later, a Dionex Ultimate 3000 UPLC chromatograph equipped with a quaternary pump, an automatic injector (at 5 ºC), a degasser and a column compartment with automated thermostat was used for chromatographic analysis (Thermo Scientific, San Jose, CA, USA). The detection of the compounds was performed with Diode Detector (DAD), using the wavelengths of 280 nm, 330 nm and 370 nm and coupled to a mass spectrometry detector (MS).

A Waters Spherisorb S3 ODS-2 column was used to separate the compounds C18 reverse phase (4.6 x 150 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 established was isocratic: 10% to 15% B up to 5 min, 15-20% B up to 5 min, 20-25% B 10 min, 25-35% B 10 min, 35-50% B 10 min and column rebalancing for 10 min; a flow rate of 0.5 mL/min was defined. The HPLC system described was also connected to a mass spectrometer (MS).

MS detection was performed using an Ion Trap Linear LTQ mass spectrometer XL (Thermo Finnigan, San Jose, CA, USA), equipped with an SEI source (source of electrospray ionization). The drag 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 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). The data were collected and analyzed using the program Xcalibur® (Thermo Finnigan, San Jose, CA, USA).

To identify the compounds, the obtained data (retention times, UV-Vis spectra and mass spectra) were compared with data available in the literature and, when available, with standards. For quantitative analysis, calibration curves were obtained by injection of standard solutions, based on UV-Vis signals and using the maximum absorption wavelength of each standard compound. In cases where there was no availability of standards for the respective compounds, the quantification was made through calibration curves of compounds of the same phenolic group. The results were expressed as mg of compound per g of extract.

3.3.2 Organic acids

The determination of organic acids was portrayed by Barros et al. (2013) in previous studies. *A. absinthium* dried powder was weighed (1 g) for a beaker, 25 mL of metaphosphoric acid (4.5%) was added, and the beaker was covered with aluminum foil. The solution was placed under magnetic agitation at room temperature for 20 min. After this time, the samples were filtered through a paper filter into a test tube. With the aid of a syringe and a nylon filter, the samples were transferred to 1.5 mL amber Vials to proceed to the analysis by ultra-fast liquid chromatography coupled to a PDA detector (UFLC-PDA) (Shimadzu 20A series, Shimadzu Corporation, Quioto, Japan).

The identification and quantification of organic acids was performed by comparing the standard curves of each compound and the value of the area under the peaks. The results were expressed in mg of compound per 100 g of dry mass.

3.3.4 Fatty acids

Fatty acids were determined by gas chromatography with flame ionization detection (GC-FID), as previously described by Pereira et al. (2012).

To the residue obtained after Soxhlet extraction with petroleum ether, a solution of methanol/sulphuric acid/toluene in the ratio 2:1:1 (*v/v/v*) was added, and this mixture remained in a bath (Julabo, SW22; Seelbach, Germany) at 50 °C (with 160 rpm agitation) for approximately 12 h. After removing the tubes from the bath and in order to enhance phase separation, deionized water (3 mL) was added to the mixture and subsequently for recovery of fatty acid methyl esters (FAME) Diethyl ether (3 mL) was added, both steps with *vórtex* agitation.

After phase separation, the supernatant was transferred to a Vial, in which anhydrous sodium sulphate was previously added in order to dehydrate the supernatant. For 50 last, filtered the same through nylon filters (0.2 µm; Whatman) for a Vial, for later analysis in GC.

The fatty acid profile has been obtained through a GC (Model DANI GC 1000) system equipped with a *split/splitless* injector, a flame ionization detector (FID, 260 ºC) and one Zebron-Kame column (30 m 0.25 mm ID 0.20 µm df; Phenomenex, Lisbon, Portugal). The temperature program applied was as follows: initial temperature of 100 °C for 2 min; progressive temperature increases: 10 °C/min up to 140 °C; 3 °C/min up to 190 °C; 30 °C/min up to 260 °C which remained for 2 min. The carrier gas used was hydrogen with

a flow rate of 1.1 mL/min, measured at 100 °C. *Split* injection (1:50) was performed at 250 °C, where 1 µL of the sample was injected.

The identification of fatty acids was based on the relative retention times of the FAME peaks of the samples with known patterns. For the processing of the results, CSW 1,7 (Dataapex 1,7, Prague, Czech Republic) software was used and was expressed as a relative percentage (%) for each fatty acid detected.

3.3.5 Free Sugars

Free sugars were determined by high-efficiency liquid chromatography coupled to a refractive index detector (HPLC-RI), as described previously by Barros et al. (2013a).

The sample (1 g) was mixed with melezitose (used as internal standard, 25 mg/mL) and was extracted with 40 mL of ethanol (80/20 *v/v*) in an 80°C bath (Julabo, SW22; Seelbach, Germany), for 1 hour and 30 min, with agitation every 15 minutes. Subsequently, the supernatant obtained was centrifuged (K24OR refrigerated centrifuge, Centurion, West Sussex, UK) at 400 rpm for 10 minutes and transferred into a glass flask to evaporate the ethanol fraction using the evaporator rotary (Büchi rotary evaporator R-210, Flawil, Switzerland) (60 ºC, reduced pressure). The aqueous phase was washed 3 times with diethyl ether (10 mL) and subsequently evaporated. Water has been added to the dry residue obtained to make up a final volume of 5 mL and filtered 1.5 mL of it (nylon filters – 0.2 µm, Whatman) for a Vial, for further analysis of the sugar profile in the HPLC system.

The HPLC system is equipped with a pump (Knauer, Smartline System 1000, Berlin, Germany), a degassing system (Smartline manager 5000), an Automatic sampler (AS-2057 Jasco, Easton, Maryland, USA) and an index detector refractive separation (Knauer Smartline 2300). Chromatographic separation was obtained through a column Eurospher 100-5 NH2 (4.6 250 mm, 5 mm, Knauer), which operated on a temperature of 30 ºC (7971 R Grace).

As mobile phase was used Acetonitrile/deionized water (70:30; *v/v*) with a flow rate of 1 mL/min. Clarity 2.4 software was used to identify compounds Software (DataApex), from which relative retention times were compared of the sample peaks, with known patterns. The results were obtained by the internal standard method expressed in grams of compound per 100 g dry or fresh mass.

3.4 Determination of the nutritional composition of *A. absinthium*

Nutritional profile analyses (proteins, fats, moisture and ash) were performed according to the official methodology of AOAC, 17th edition (AOAC, 2016).

The humidity was analyzed by a moisture analyzer from Adam Equipment (model PBM 163, Oxford, USA) (**Figure 9**). About 2 g of the sample was placed on the metal plate and inserted into the equipment where was heated. Finally, the initial and final mass were obtained to calculate the moisture of the samples.

Figure 9. Equipment used to determine moisture content.

The ash content was determined by AOAC 923.03 method, which consists of the burning of organic matter at high temperatures. The sample (0.25 g) was weighed and added in porcelain crucibles, which were also weighed, and incinerated in the muffle (Optic Ivymen System, N-8L, Barcelona, Spain) (**Figure 10**) for 5 h at 550º C.

Figure 10. Muffle with crucibles inside.

The protein content was calculated by AOAC 920.87, which consists in the destruction of organic matter by a strong acid and is based on the amount of nitrogen (N) in the sample to quantify the proteins. The analysis was performed using the Macro-Kjedahl method, using conversion factor 6.25 (N x 6.25). Each sample (0.25 g) was added to the test tubes and digested with the catalyst agent, $K_2SO_4/CuSO_4$, and 15 mL of sulfuric acid for 70 min at 400 ºC. After cooling, the tubes were inserted into the Kjeldahl distiller (model Pro-nitro-A, JP Selecta, Barcelona) (**Figure 11**) and a stable alkaline distillation and titration occurred in the equipment, to inform the amount of nitrogen.

Figure 11. Kjeldahl distiller used for protein content analysis.

The fat content was determined by method AOAC 920.85, which is the fat extraction by Soxhlet of the samples by means of petroleum ether. Each sample (2 g) was weighed, placed in filter paper cartridges and covered with cotton. The cartridges were added to the Soxhlet equipment (**Figure 12)** along with the petroleum ether (solvent). The equipment was heated to approximately 80 ºC and after 4 hours, the solution containing the fat was removed, transferred to test tubes, previously weighed, and sent for

evaporation. After drying, the test tube was weighed again, and the fat content was quantified.

Figure 12. Soxhlet Extractor.

The carbohydrates were calculated by difference, while the total energy was determined according to the **equation 1** below.

$$Energy\ (Kcal) = 4\ x\ [protein\ (g) + carbohydrates\ (g)] + \ 9\ x\ [fat\ (g)] \qquad (1)$$

3.5 Bioactive characterization

3.5.1 Antioxidant activity

The methodologies for determining antioxidant activity are various and are subject to interferences, so it is currently recommended to use two or more techniques, since no single assay will accurately reflect the "total antioxidant capacity" of a sample (Huang, 2005; Prior, 2005).

Thus, in this work, two chemical methods were used to evaluate the antioxidant potential in the extract obtained: the DPPH scavenging activity and determination of the reducing power (RP); and also, a cellular based assay (CAA): the cellular antioxidant activity. Both chemical methods are based on the EC_{50} value, this being the minimum sample

concentration needed to provide 50 % antioxidant activity and the lower this value the more active the sample will be. Regarding the CAA, an inhibition concentration is determined meaning the percentage of cells that are protected from oxidation, as well as an IC_{50} value corresponding to the minimum concentration needed to avoid 50% of oxidation.

3.5.1.1 DPPH scavenging activity

Following the procedure proposed by Hatano et al. with some modifications, 0,3 mL of the various extract concentrations of each sample were mixed in test tubes with 2,7 mL of the methanolic solution containing free radicals of DPPH ($6x10^{-5}$ mol/L). After agitation in the vortex, the mixture was placed in the dark to rest for 60 minutes (**Figure 13**). Measurements were made in triplicate at 515 nm (Absorbance Microplate Reader ELx800, Bio-Tek) for each of the extracts.

Figure 13. Plate and equipment used for the measurement of absorbance by the DPPH method.

The decrease in absorbance indicates the reduction of the DPPH radical. The blocking effect of the DPPH radical was calculated as a percentage of the DPPH discoloration using the following **equation 2**:

$$DPPH - free\ radical\ scavenging\ effect\ (\%) = ((ADPPH - AA)/ADPPH) * 100 \quad (2)$$

where, ADPPH corresponds to the absorbance of the DPPH-free radical solution and AA the absorbance of the sample extract solution. The minimum extract concentration, which corresponds to 50 % inhibition, represented by EC_{50}, has been calculated from the graphic representation of the percentage of the blocking effect as a function of the extract concentration in mg/mL. The test was performed in triplicate. The EC_{50} values are expressed in terms of means and standard deviations.

3.5.1.2 Reducing power

The reducing power method is based on a reduction mechanism of the iron/ferricyanide complex [Fe^{3+}, $K_3Fe(CN)_6$] to a ferrous form [Fe^{2+},$K_4Fe(CN)_6$] in the presence of reducing agents (antioxidants) (Abreu, 2013). The product formed with reduced iron reacts with the ferric chloride solution forming a strongly colored complex and water insoluble, which can be spectrophotometrically measured in UV-VIS. As the absorbance of the sample increases it indicates a greater reducing power of the sample. Phenolic compounds have great ability to yield electrons so this mechanism is suitable to determine its antioxidant potential and can be correlated with other antioxidant or biological properties.

The reducing power method used was described by Oyaizu (1986), with some modifications. Thus, 5 mL of sodium phosphate buffer (0.2 M) was added to the sample at pH 6.6 and 0.5 mL of 1 % potassium ferricyanide. The mixture was vigorously agitated in the vortex and then incubated at 50 ºC for 20 minutes. After that period, 0.5 mL of trichloroacetic acid (TCA) was added to 10 % (m/v) and returned the mixture to the vortex. Subsequently, 0.6 mL of the supernatant was removed and mixed with 0.6 mL of distilled water and 0.12 mL of ferric chloride at 0.1 % (**Figure 14**). Measurements were made in triplicate at 690 nm.

The extract concentration corresponding to 0.5 of the absorbance is called EC_{50}, and the starting of the graphical representation of absorbance as a function of concentration in mg/mL of the corresponding extract has been calculated. The EC_{50} values are expressed in terms of means and standard deviations.

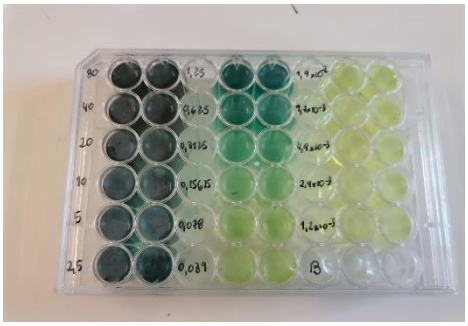


Figure 14. Plate used for the measurement of the absorbance by the reducing power method.

3.5.1.3 Cellular antioxidant activity

For determination of intracellular ROS, the cells were incubated with antioxidant compounds and AAPH, a compound that causes oxidative stress in order to promote the formation of free radicals. Thus, DCFH-DA was used as a fluorescent marker (Gomes et al, 2005; Wolfe et al., 2008), DCF-DA is a compound which, once in the cell medium is easily oxidized by peroxide radicals, to a fluorescent compound, resulting in DCFH-DA. The DCF-DA enters the cell and is targeted by esterase enzymes, the diacetate present in the molecule being cleaved. The molecule by the action of ROS is oxidized, thus forming DCFH-DA which has fluorescence. Oxidation can be inhibited by the action of antioxidant compounds that will reduce the antioxidant potential of ROS. The decrease in fluorescence emission compared to control cells indicates the antioxidant activity of the compounds tested.

Cellular antioxidant measurements were done following the method of Wolfe and Liu (2007) with modifications. After RAW cells reached confluence in the culture flasks they were washed twice with sterile HBSS (pH 7.4) and then separated from the surface using 0.05% trypsin-EDTA. Cells were plated (3.0×10^4) in 100 µl of cell / well culture medium in 96-well black background culture plates and incubated until confluency (24-48 h). The perimeter wells were left empty to reduce any variation due to the location of the plate. The growth medium was removed after confluence and the cells were then washed with HBSS. Then, 200 µl of different extracts concentrations was added to each well in triplicate with 50 µM DCFH-DA prepared in ethanol and diluted in HBSS. As a negative control 200 µl of DCFH-DA applied in triplicate was added. Cells were incubated for 1 h at 37ºC. Quercetin was used as a positive control. Thereafter the cells were washed 3x with HBSS to ensure that any antioxidant effects observed later in the assay were due exclusively to the compounds incorporated by the cells. Then 100 µl of AAPH was added. Cells were immediately placed on a microplate reader (FLX800 Biotek), where real-time fluorescence was read initially and every 5 min for 40 min. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Quantification of CAA

Regarding the quantification of CAA, the efficacy of the antioxidant treatments for both cell lines was quantified by examining the percentage reduction in fluorescence. Briefly, curves were generated by the 14 fluorescence response readings over a 40 min assay. The

area under each curve was calculated using the capabilities of Excel and Integral Calculator (https://www.integral-calculator.com/). As expected, the control wells exhibited the maximum fluorescence, since there was no inhibition of the DCFH-DA reaction. The percent reduction (or the CAA unit) was calculated from the formula described by Wolfe and Liu (2007), shown below:

$$CAA = \%\, reduction = 1 - \frac{\text{AUC sample}}{\text{AUC control}} \times 100$$

For quercetin, the percent reduction was determined by three independent assays described with mean.

Figure 15. Equipment used for fluorescence measurement to quantify cellular antioxidant activity.

3.5.2 Antimicrobial activity

The methodology described previously by Carocho et al. (2015) was used to evaluate antibacterial activity. Gram-negative bacteria were used: *Escherichia coli* (ATCC 35210), *Enterobacter cloacae* (clinical isolate), and Gram-positive bacteria: *Bacillus cereus* (isolated from food matrices), *Listeria monocytogenes* (NCTC 7973). These microorganisms were acquired in the Laboratory of the Department of Plant Physiology of the Institute of Biological Research "Siniša Stanković" of the University of Belgrade in Serbia. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the microdilution method.

The bacterial cultures were adjusted through the spectrophotometer with a concentration of $1x10^5$ CFU/mL, corresponding to a bacterial suspension determined in a spectrophotometer at 625 nm. The inoculum dilutions were grown in a solid medium to check the absence of contamination and to check validity of the inoculum. The different dilutions of the hydroethanolic extract were piped for wells containing 100 µL triptych soybean broth (TSB) and then 10 µL of inoculum were added. The microplates were incubated for 24 hours at 37 ºC.

For the determination of MIC (minimum inhibitory concentration; lowest concentration which has produced significant inhibition (around 50%) of bacteria growth compared to

the positive control) 40 µL of chloride have been added iodonittrazolium (INT) (0.2 mg/mL) and incubated at 37 ºC for 30 min. MIC obtained from the susceptibility test of various bacteria to the extract have been determined also by a colorimetric test of microbial viability based on the reduction of INT color and compared with a positive control for each bacterial strain. MBC (minimum bactericidal concentration) was determined by subculture in series, placing 10 µL of each well that did not present color change at 100 µL of TSB. The lowest concentration that did not present growth after this subculture was considered as MBC. Streptomycin and ampicillin were used as positive controls, while 5 % dimethylsulfoxide (DMSO) was used as negative control. The results of MIC and MBC were expressed in mg per mL of lyophilized hydroethanolic extract.

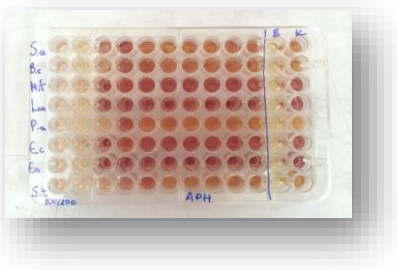


Figure 16. Microplate used in the determination of antimicrobial activity.

3.5.3 Cytotoxic activity

The effect of the extracts on the growth of human tumor cell lines was evaluated by the Sulforhodamine B assay (SRB) in order to determine cell growth inhibition. To this end, 4 tumor cell lines were used: MFC-7 58 (breast carcinoma), NCI-H460 (lung carcinoma), Hela (cervical carcinoma) and Hepg2 (hepatocellular carcinoma).

The cells were maintained as adherent cultures in RPMI-1640 medium containing 10% Heat-inactivated FBS (MFC-7 and NCI-H460) or in supplemented DMEM with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (Hela and HEPG2 cells) at 37°C in a humidified air and 5% CO_2 incubator.

Each cell line was prepared at the appropriate density (1,0 x10^4 cells/well) in 96-well plates and allowed to adhere for 24hs. After this time the cells were tested for 48 h with various concentrations of the extracts.

After this incubation period, the adherent cells were fixed by adding 10% cold trichloroacetic acid (TCA, 100 µL) and left to stand for 60 min at 4 ºC. The plates were then washed with deionized water and dried. Subsequently, 100 µL of the SRB solution (0.1% in 1% acetic acid) was added to each well and the plates were left to incubate at

room temperature for 30 min. Excess SRB was removed by washing the plates with 1% acetic acid and left to air dry. After the connected SRB was solubilized with 10 mM Tris (200 μL) and the absorbance was measured at 540 nm in a microplate reader (Biotek Elx800).

The dose-response curves were obtained for each extract and cell line tested. It was also calculated the value of GI_{50} that corresponds to the extract concentration that inhibits 50% of cellular growth (Vichai and Kirtikara, 2006; Abreu et al., 2011). Ellipticin was used as a positive control.

To evaluate the toxicity of the extracts for non-tumor cells, two cell lines were used: a cell culture prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to an established procedure and it was designed as PLP2 (porcine liver primary culture) and a cell line from monkey kidneys (Vero) (Abreu et al., 2011).

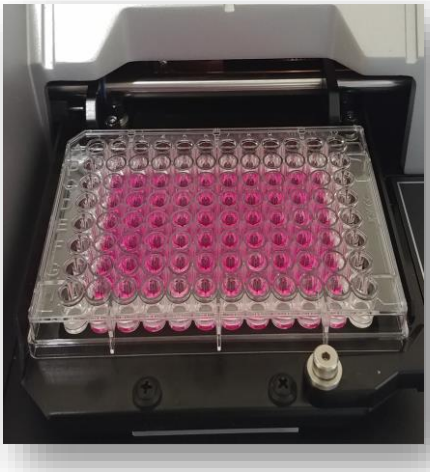


Figure 17. Microplate used in the determination of cytotoxic activity.

3.5.4 Anti-inflammatory activity

RAW 264.7 rat macrophage cells were used to evaluate anti-inflammatory activity according to the procedure of Jabeur et al. (2016). Cell culture was performed in DMEM medium, supplemented with 10% inactivated bovine serum by heat and L-glutamine at 37°C with 5% CO_2 in humidified air.

Cells with active growth were released with a cell scraper, the experimental cell density was established at $5x10^5$ cells/mL and the proportion of dead cells was less than 1%,

according to the Trypan Blue exclusion test. The cells were then distributed in a 96-well plate (150000 cells/well) and allowed to adhere to the microplate overnight.

Subsequently, the cells were treated with different concentrations of extracts for 1 hour, followed by stimulations with lipopolysaccharides (LPS) (1 µg/mL) over 18 hours. Controls were prepared without the addition of LPS to see if they induce changes in basal nitric oxide (NO) levels.

The presence of nitric oxide was determined using a Griess Reagent Kit (Promega) that contains sulfanilamide, N-(1-naphthyl) ethylenediamine hydrochloride (NED) and nitrated solutions. The supernatant of the cells (100 µL) has been transferred to the plate and mixed with sulfanilamide and NED solution, 5 to 10 minutes each at room temperature. The nitric oxide produced was determined by measuring the absorbance at 540 nm (ELX800 Biotek microplate reader) and compared with the curve Calibration (Jabeur et al., 2016).

Figure 18. Plate and equipment used for the measurement of anti-inflammatory activity.

3.6 Incorporation of the extract into the brownies

3.6.1 Preparation of the brownies

A traditional recipe was followed to prepare the brownies. The ingredients and their respective quantities were described in **Table 4**.

Table 4. Ingredients and quantities brownies.

Ingredients	Mass (g)
Flour	100
Eggs	100
Cocoa	150
Sugar	200
Butter	100

All the ingredients were weighed first. On the one hand, the chocolate and the butter were placed over a low heat for 5 minutes, homogenizing very well. The sugar was beaten with the eggs. Melted chocolate and butter were added and mixed until everything was well integrated. The sifted flour was added 2 times. The mixture was beaten vigorously before placing in the molds.

Two brownies of 150 g each one were prepared and baked for 20 min at 220 ºC (**Figure 19**): a) Brownie with no extract serving as control; b) Brownie incorporated with 10 mg of the infusion extract as it revealed the highest content in phenolic compounds and stronger bioactivities/g of brownie corresponding to the active doses obtained in the bioactivities evaluation. The extract was added at the end of the remaining ingredients and was well homogenized before the baking process.

Figure 19. Process **A)** weighing and **B)** addition of extract to the mass.

Each sample was analyzed in triplicate, immediately after its preparation (zero time) and after 3 days of storage (at room temperature and packed in aluminum foil).

The samples were referenced according to their content and the time of analysis, as shown in **Table 5.** Codes of the elaborated brownies

Table 5. Codes of the elaborated brownies.

Brownie´s codes	Descripcion
BC0	Brownie control (no extract) at zero time
BE0	Brownie with extract at zero time
BC3	Brownie control - 3 days of shelf life
BE3	Brownie with extract - 3 days of shelf life

For the analysis of the developed brownies, the physicochemical characteristics, monitoring of phenolic compounds, nutritional value and bioactivity were performed.
After the physical analysis of pH, color index, water content, moisture and texture, the remaining samples were lyophilized for further analysis.
The chemical, nutritional composition and bioactive evaluation was performed according to the described in sections: 3.3, 3.4 and 3.5, respectively.

3.6.2 Physicochemical analysis

3.6.2.1 pH

The pH measurements were made on the inside and on top of the brownies, using a digital pH-meter Hanna as shown in **Figure 20** .

Figure 20. Measurement of pH.

3.6.2.2 Available water content

Water activity (*aw*) indicates the state of water energy in a system, which is the fraction of the total moisture content that is free and is therefore available for the growth of micro-organisms and for various chemical reactions affecting their stability.

Knowing the value of water activity achieves a measure of product quality quickly, easily and accurately.

The measurement of this parameter was carried out with the equipment AquaLab S4TE from the company Lab-Ferrer (**Figure 21**).

Figure 21. Measurement of aw using the AquaLab S4TE equipment.

3.6.2.3 Color index

The top and interior colors of the 2 brownies were analyzed (3 repeats and at different points) with the aid of the portable colorimeter CR 400 from the company Konica Minolta (Tokyo, Japan) described by Carocho et al. (2020)) and shown in **Figure 22**. The standard of the International Lighting Commission (CIE), D65 illuminating, with 8 mm aperture and 10º observation was used. According to the measurement space of CIE L* a* b*, L* represents the luminosity (L = 0 black, L = 100 white), a* represents redness (-a = 0 green, +a = redness) and b* represents yellowing (-b = bluish; +b = yellowish). The reference of the blank (L*=79.95; a*=13.92; b*=1.33) was used to calibrate the equipment.

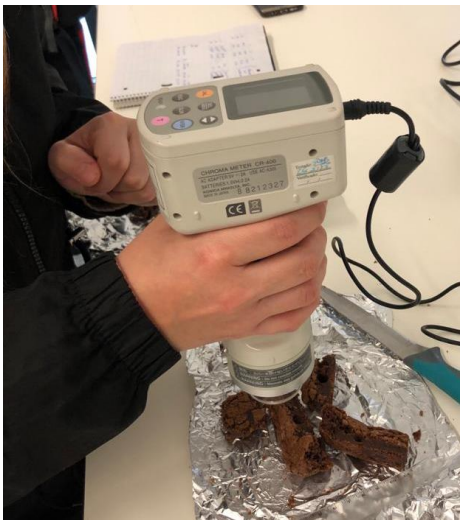


Figure 22. Color measurement using the CR 400 portable colorimeter.

3.6.2.4 Texture

In order to understand the effects that different extracts can have on the texture profile of brownies, they were subjected to an analysis in a *TA.XT* Plus texturometer from Stable Micro Systems (Vienna Court, Godalming, UK) with a 30 kg load cell (**Figure 23**). The analysis used was a "*Texture profile analysis*", that is, a texture analysis that mimics human chewing by making two compressions in the same food, managing to extract several parameters through the use of macros in fundamental parameters. In this way it was possible to analyze the hardness, adhesiveness, resilience, elasticity, gomosity and mastigability. Using the 35 mm (P/35) metal cylinder as the probe, some tests speed of 5 mm/s, 3 mm/s and 10 mm/s were performed and a muffin strain stress of 25% from a force of 10 g as the trigger for the analysis. The brownies were placed whole in the equipment for the study and the process was done with three repetitions. The results were studied by the Exponent program.

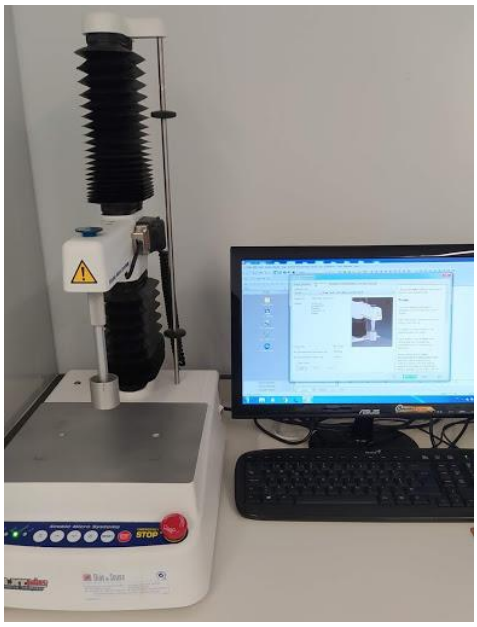


Figure 23. Texturometer with 30 kg load cell and cylindrical P/35 aluminum probe.

3.7 Statistical analysis

Throughout the whole document, all data was expressed as mean ± standard deviation. Furthermore, to better understand the effects of the incorporation of the absinth extract (I) and the storage time (ST), a two-way ANOVA with type III sums of squares using the SPSS Software, version 25 was used for the analysis. This multivariate general linear model treats the two factors, I and ST 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 (p<0.05) was recorded among the two factors (I×ST), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM) when possible. If there was no significant interaction (p>0.05), each factor was evaluated independently using a student's T-test. All analyses were carried out using a p-value of 0.05.

For the general factorial design, a comparison was made between the different samples through simple analysis of variance (ANOVA) using F-test, together with the Fisher's Least Significant Difference test, using the Design expert and Statgraphics Centurion XVI software (StatPoint Technologies, Inc. Warrenton, VA, USA) software.

4. RESULTS

4.1 Screening array

From the results of **Table 3**, a triple-section of data analysis of screening was made, using the fractional factorial design as the primary one, the unifactorial assays as secondary (time and solvent), and the factorial design as tertiary. **Figure 24**, **25** and **26** represents graphically runs 3,7,10 and 11, from which a double graph representation was embedded, the outer cube represents the 4 points tested (circle corners) and the 4 aliased points (regular corners), and at the further X-axis the Pareto graph was embed were larger effects exhibits greater t-Values. For the bioactive compound, clear indications of a big magnitude from the solvent factor are shown and even though Factor time is shown as the biggest magnitude from the Pareto, so it was decided to ignore based on the secondary results. Therefore, as X_1, and X_2 are displayed as significant factors with positive magnitude could point at A+, B+ and C+ as the vertices with maximum extraction as shown in the green rectangle of **Figure 24**, which also happen to be tested points.

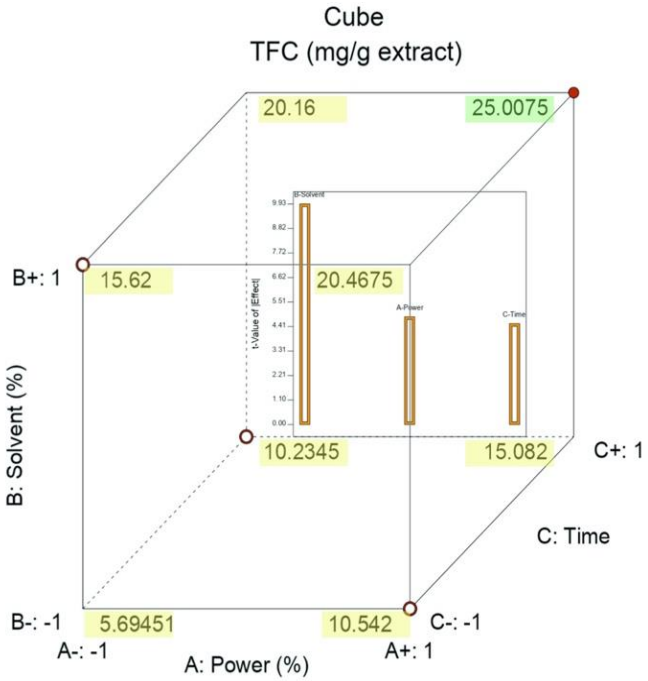


Figure 24. Double embedded graphical representation of fractional factorial design points and their Pareto representation from Y_2 response. TFC - Total Flavonoid Content.

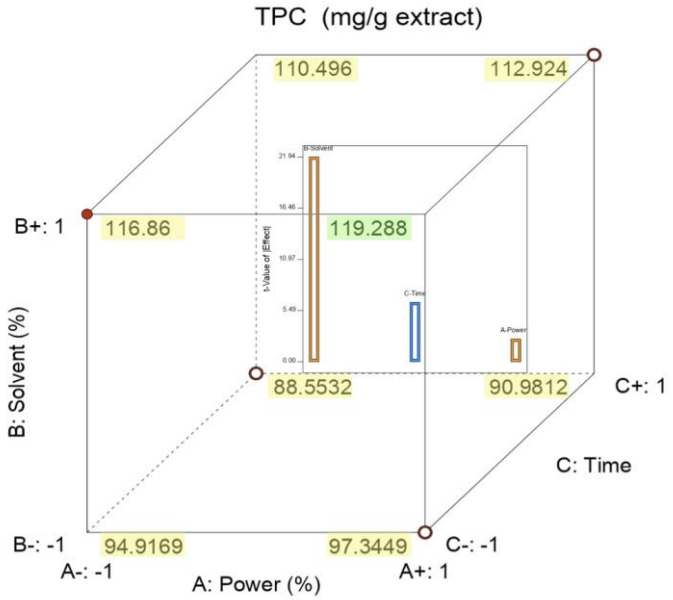


Figure 25. Double embedded graphical representation of fractional factorial design points and their Pareto representation from Y1 response. TPC - Total Phenolics Content.

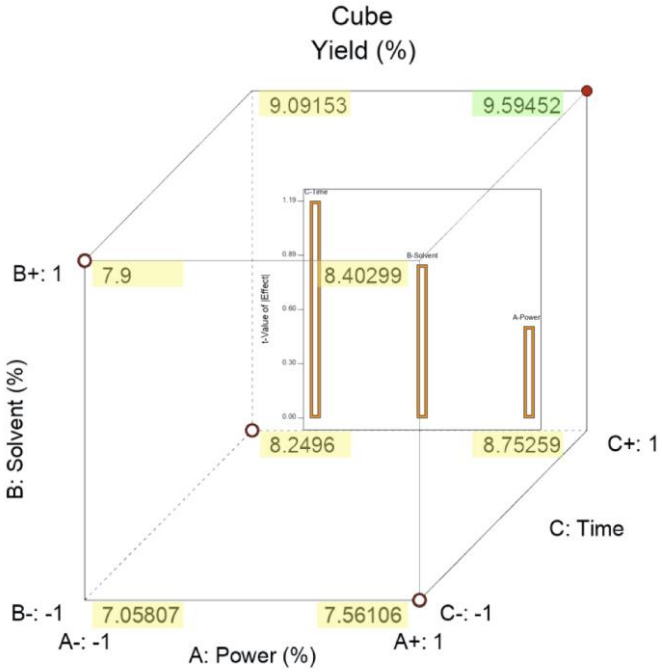


Figure 26. Double embedded graphical representation of fractional factorial design points and their Pareto representation from Y3 response.

The secondary section shown in **Figure 27** was considered in order to expand and interpolate data on X_2 and X_3 (**Table 3**). For unifactorial designs related to X_2 and X_3, the three and six tested variations respectively shown the data tendency fixing power at 50% and solvent at 0% when analyzing time, and fixing power to 50% and time to 240 s when the solvent was monitored. Time results did not show clear patterns, and as well as was

stated in the work of Pedrosa et al., (2021), time did not show a significant effect and variability could be linked to cavitation effects, but since longer times do not increase steadily the extraction, lower times are recommended in order to improve energy efficiency. This could also be explained due to the high energy of the equipment testing small volumes, therefore, at tertiary section power is also analyzed.

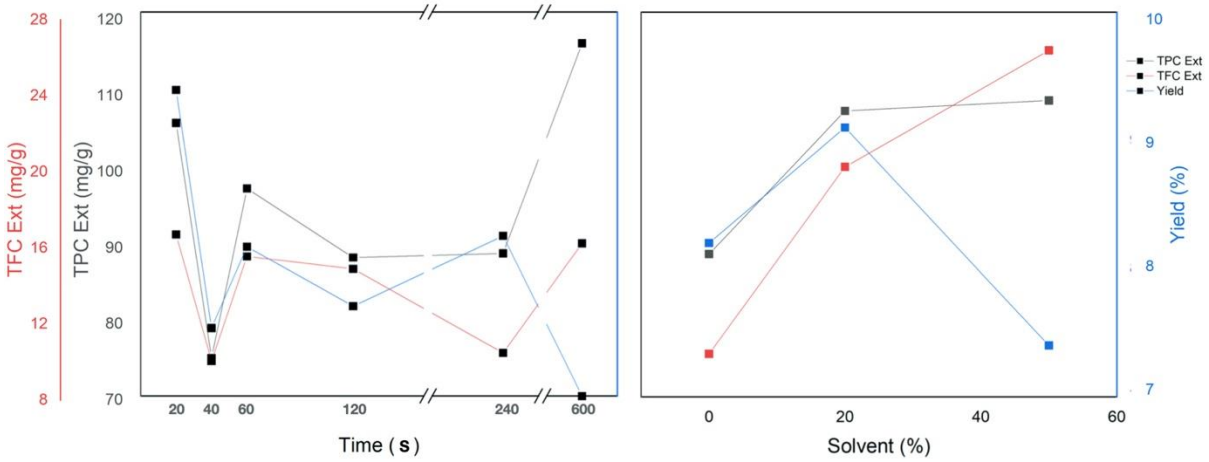


Figure 27. Unifactorial graphic representation of Factors X_3: Time (left) and X_2: Solvent (right).

Solvent results displayed a clear pattern for the bioactive compounds TPC and TFC, where the addition of ethanolic solvent increases the extractability of these compounds which is explained due to the polarity of the bioactive compounds as has been shown by the work of Şahin et al., (2013). On the other hand, Y_3 displayed and inversed V shape, due to the quantification by gravimetric methodology, this test includes not only bioactive compounds but also other polar compounds like sugars, which at higher concentration of ethanol become harder to extract, although, comparing extractability of bioactive compound and overall yield, higher concentrations of solvents will extract cleaner compounds mixtures.

Finally, for the tertiary section of the screening analysis, the 2^2 factorial design (runs 4,7,8, and 11) included factors X_1 and X_2 with a fixed time of 240 s. **Figure 28**, added a new terminology for the response surface, the Total Bioactive Content (TBC) is the result of the addition of TPC and TFC, which behave in the same way, therefore, the analysis of both of them combined seems to give better information, and since Y_3 at 0% concentration of ethanol extract other compounds with less interest in this assay, **Figure 29** is added, but the main focus is given to **Figure 28**.

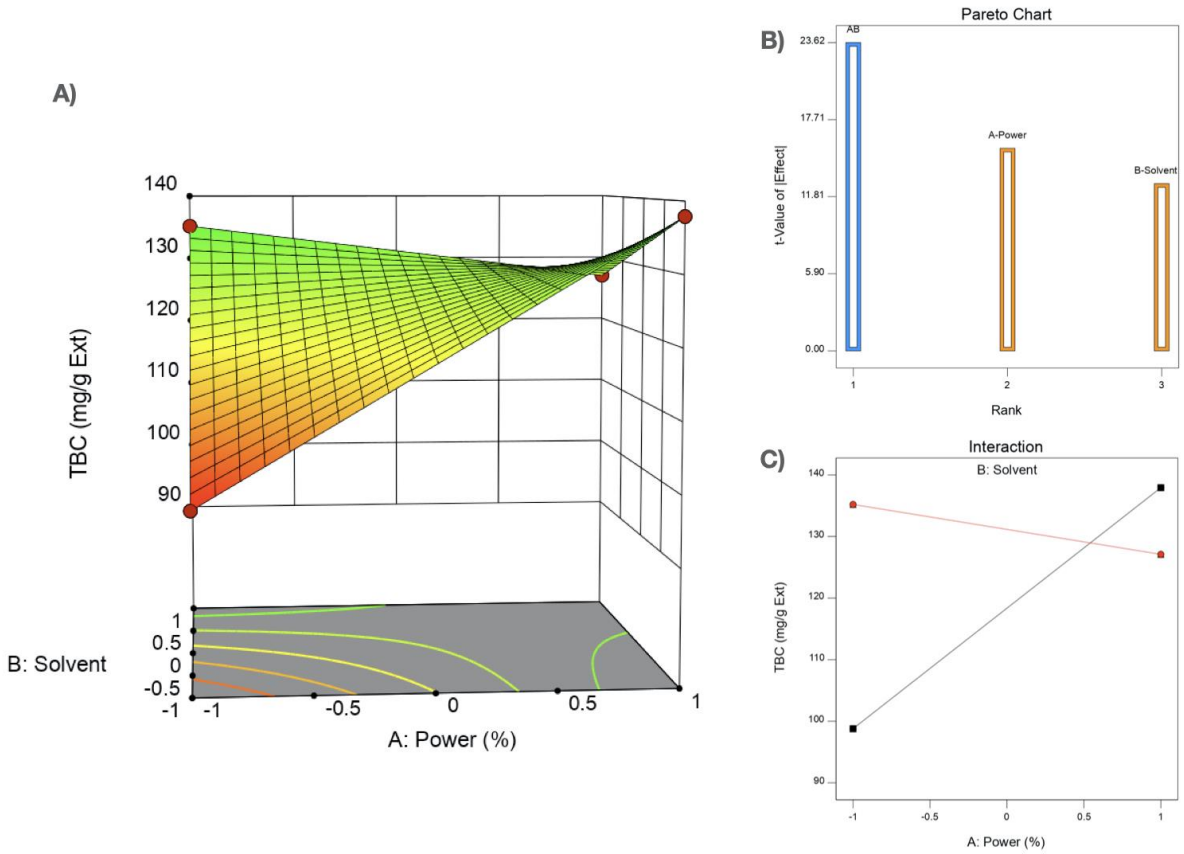


Figure 28. Factorial results Y1+Y2 from X1 and X2 with a fixed time of 240 s. **A)** tridimensional surface-like graph representation, **B)** Factors magnitude according to Pareto plot, and **C)** Factors interaction graph.

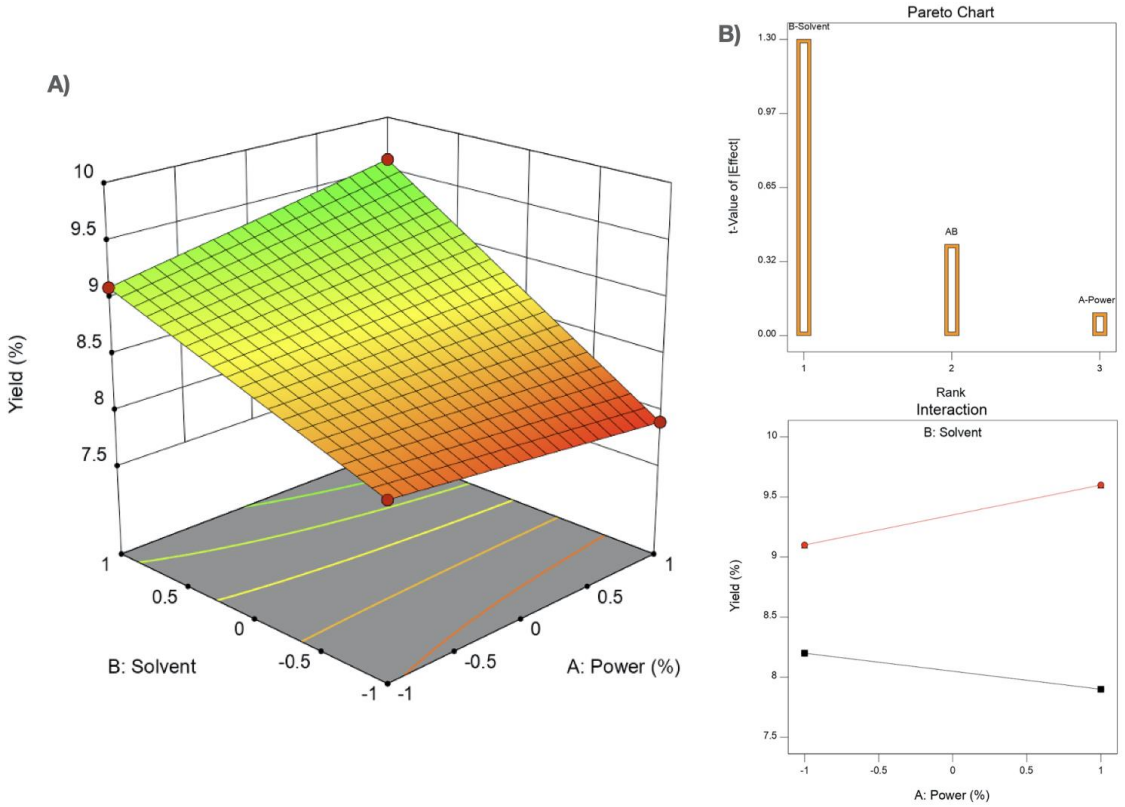


Figure 29. Factorial results of Y3 from X1 and X2 with a fixed time of 240 s. **A)** tridimensional surface like graph representation, **B)** Factors magnitude according to Pareto plot and **C)** Factors interaction graph.

The most important attribute shown in **Figure 28** is the higher negative magnitude that displayed the factor interaction, at this point is worth noting that the higher extraction point was found at a lower solvent concentration (100% water) and higher power supplied (100%), and although, low X_1 and high X_2 displayed also a high TBC response is worth nothing the inflection points generate provided by high X_1 and high X_2, and even worst extractability at low X_1 and low X_2.

In the secondary section observed a higher extractability with 50% solvent rather than 0%, the same is observed in **Figure 28** where it also shows the combined effect between a high power with a low quantity of solvent in the extraction process. In concordance with the results shown in secondary section, observed a higher extractability with 50% of solvent rather than 0%. Therefore, it can be concluded that the combined effect between high potency and low solvent concentration is very important in the extraction process.

Even though the next phase of this assay was to perform a face-centered design couple to an RSM to provide precise optimization of extraction, it has been found that all the values provided by the screening analysis were lower to the infusion methodology. One of the factors to which could be attributed this effect is temperature, although, considering the stated at section 3.2.3, reducing solvent and minimizing energetic toll, the combination

of heat UAE extraction would require higher energy. Even though, UAE equipment required 500 W of power (compared to the 550 W of the heating plate) in order to increase extractability with a combined UAE-heated system, the energetic tall could rise up to the double amounts exhibited here. In this sense, in order to reduce the energetic demand in the extraction process, infusion is presented as the appropriate technique compared to the UAE. Therefore, if UAE extraction with the parameters selected: combination of higher X1 (100% W), lower X2 (0% ethanol) is used, it is suggested to perform it for short periods of time, in the range of 20-40 s.

4.2 Chemical and nutritional characterization of *A. absinthium*

4.2.1. Phenolic compounds

Regarding the phenolic compound's profile, **Table 6** presents the chromatographic characteristics (obtained by HPLC-DAD/ESI-MS) and tentative identification of the phenolic compounds present in the extract of *A. absinthium* obtained from the infusion. The quantification of each identified compound is presented in **Table 6**. Thirteen compounds were tentatively identified in the samples, seven phenolic acids (hydroxycinnamic acid derivatives) and six flavonoids (*C*-glycosylated apigenin derivatives and *O*-glycosylated quercetin and isorhamnetin derivatives).

Regarding the phenolic acids found, peaks 1, 2, 3, 8, 9, 11, and 13 presented an UV spectra with λ_{max} = 321–334 nm (characteristic of hydroxycinnamic acids) and pseudomolecular ions $[M-H]^-$ at *m/z* 353 (peaks 1, 2, and 3) and *m/z* 515 (peaks 8, 9, 11, and 13). Besides that, all of them produced a fragment ion at *m/z* 191, corresponding to the deprotonated quinic acid moiety, being for that manner identified as quinic acid derivatives that contained one or two caffeic acid units, *O*-caffeoylquinic acid or *O*-dicaffeoylquinic acid, respectively. Peak 3 was identified as 5-*O*-caffeoylquinic acid, also known as chlorogenic acid, by comparison their retention time and UV spectra with the available standard compound. The assignment of the reaming isomers, with one or two caffeic acid units, was performed following the hierarchal keys developed by Clifford et al., (2003, 2006), and following the numbering system recommended by IUPAC (Abrankó & Clifford, 2017).

As for the flavonoid family of compounds, three *C*-glycosylated apigenin derivatives were identified. Peaks 6 ($[M-H]^-$ at *m/z* 431) and 12 ($[M-H]^-$ at *m/z* 593) were tentatively assigned as apigenin-8-*C*-hexoside and apigenin-6,8-*C*-dihexoside, respectively, by comparing their fragmentation pattern with the previously described by Tahir et al.,

(2012). Peak 5 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 737, and in the MS^2 data a fragment ion signal at *m/z* 563, that corresponds to the loss of 174 u, dehydrated quinic acid. This MS^2 fragment of *m/z* 563 also suggest apigenin as the aglycone linked to carbon. Hypothesis confirmed by the presence of the MS^2 fragments at *m/z* 473, 443, 383, and 353, being for that manner tentatively identified as apigenin 8-*C*-(6"-*O*-quinoyl)-6-*C*-glucoside. The tentative identification of this compound as also is numbering was performed based on the previously described by Benayad, Z., Gómez-Cordovés, C., & Es-Safi (2014).

Two more flavonoids were found in the samples, peaks 7 ($[M-H]^-$ at *m/z* 609) and 10 ($[M-H]^-$ at *m/z* 623), that presented a unique MS^2 fragment at *m/z* 301 (quercetin aglycone) and *m/z* 315 (isorhamnetin aglycone), respectively, that corresponded, in both cases to the loss of a deoxyhexosyl group linked to a hexose, being for that manner tentatively identified as quercetin-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside, respectively, positively identified with the commercial standards.

Table 6. Phenolic compounds identified and quantified in *A. absinthium* infusion extract.

Pic	Rt (min)	Uvmáx	[M-H]- (m/z)	MS2	Tentative identification	Quantification (mg/g extract)
1a	5.840	323	353	191(100),179(23),173(10),135(5)	3-*O*-Caffeoylquinic acid	0.32±0.01
2a	6.551	326	353	191(10),179(7),173(100),135(5)	4-*O*-Caffeoylquinic acid	1.93±0.05
3a	8.543	323	353	191(100),179(12),173(5),135(5)	5-*O*-Caffeoylquinic acid	0.43±0.01
5b	14.014	321	737	563 (20),473(42),443(100),383(5),353(5)	Apigenin 8-C-(6"-*O*-quinoyl)-6-C-glucoside	0.20±0.01
6b	15.362	340	431	341(24),311(100)	Apigenin-8-*C*-hexoside	0.18±0.01
7c	16.821	342	609	301(100)	Quercetin-3-*O*-rutinoside	0.56±0.01
8a	17.682	334	515	353(10),191(20),179(35),173(100),161(5),135(8)	*cis* 3,4-*O*-Dicaffeyolquinic acid	062±0.02
9a	20.193	328	515	353(11),191(6),179(25),173(100),161(5),135(5)	*trans* 3,4-*O*-Dicaffeyolquinic acid	2.85±0.02
10c	20.427	341	623	315(100)	Isorhamnetin-3-*O*-rutinoside	0.749±0.003
11a	21.584	329	515	353(11),191(69),179(65),161(5),135(5)	*cis* 3,5 -*O*-Dicaffeyolquinic acid	0.65±0.01
12b	22.210	345	593	473(97),383(27),353(15)	Apigenin-6,8-C-dihexoside	0.32±0.01
13a	22.528	329	515	353(12),191(49),179(73),161(5),135(7)	*trans* 3,5 -*O*-Dicaffeyolquinic acid	0.53±0.03
					TPA	7.32±0.02
					TF	2.00±0.01
					TPC	9.33±0.01

n.d.- not detected. Calibration curve standards: Calibration curve standards: a – chlorogenic acid (y = 312503x - 199432, R^2 = 0.999, LOD = 0.20 µg/mL and LOQ = 0.68 µg/mL); b - Apigenin-6-C-glucoside (y = 107025x + 61531; R2 = 0.998; LOD = 0.19 µg/mL and LOQ = 0.63 µg/mL); c – Quercetin-3-O-glucoside (y = 34843x – 160173, R^2 = 0.999, LOD = 0.21 µg/mL and LOQ = 0.71 µg/mL).

4.2.2. Nutritional value, soluble sugars and organic acids profiles of A. absinthium

The results of the nutritional characterization, soluble sugars and organic acids of the *A. absinthium* plant are present in **Table 7.** After the evaluation of the nutritional parameters of absinthe, it was evident that carbohydrates were the macronutrients in greater quantity, with 85.2 ± 0.2 grams per 100 grams of dry weight of the plant, followed by the protein content 6.1 ± 0.4 g/100 g dw and ash 5.8 ± 0.5 g/100 g dw. The result respect of the quantity of fat in the plant indicates a value of 2.9 ± 0.4 g/100 g dw, it is low compared with the values of the other components. Regarding energy, it is observed that absinthe provides a value of 392 ± 3 Kcal/100 g of dry weight. There are several studies found in the literature regarding the characterization of absinthe, however there are few studies that contemplate the nutritional characterization of the plant, so it is difficult to compare the values obtained. In that sense, Akzhigitova et al., (2018) studied the chemical characterization of *A. absinthium* and presented values of the biological constituents of the aerial parts of the plant, however the nutritional level only makes reference to the value of the ash (6.4%), however the nutritional level only refers to the value of the ash and it is verified that the value of the ash content of the plant under study goes against the value reported by the authors. Pereira et al., (2020) presented a review article on the nutritional value of edible wild plants from northern Spain, including the *A. absinthium*, however, in the nutritional characterization they report the characteristics of sweet absinthe (*Artemisia annua* L.) presenting the results in g/100g of fresh weight, making it impossible to compare the results. Beigh & Ganai, (2017) have reviewed the potential of wormwood (*A. absinthium*) and report several scientifically proven beneficial effects of absinthe, however the nutritional and chemical composition is not addressed. Liu et al., (2019) conducted a study on absinthe, and make a chemical characterization, however their research was directed to the content in phenolic compounds, not addressing the nutritional and chemical characterization carried out in this dissertation.

Furthermore, the **Table 7** contains the identification and quantification of soluble sugars and organic acids of the absinthe plant. Four sugar molecules were identified, fructose, glucose, sucrose and trehalose, with the following contents respectively: 0.7 ± 0.1 g/100 g dw, 0.71 ± 0.06 g/100 g dw, 0.12 ± 0.02 g/100 g dw and 0.13 ± 0.02 g/100 g dw.

These results are similar with those reported by Szparaga et al., (2021), even if the comparison of contents of these sugars is difficult because they report them in other units. Six organic acids have been identified: oxalic, quinic, malic, shikimic, citric and fumaric acid. The fumaric and shikimic acids are found in very residual concentrations amount less than 0.01 g/100 g dw, citric, malic and oxalic acid with 0.97 ± 0.01 g/100 g dw, 0.75 ± 0.02 g/100 g dw and 0.56 ± 0.01g/100 g respectively and the quinic acid was present with the higher quantity, 9.2 ± 0.2 g/100 g dw. Kim et al., (2015) carried out the nutritional characterization of two plants of the same species of absinthe (*Artemisia princeps* Pamp and *Artemisia argyi* H. Lév. & Vaniot) where they quantified the content in vitamin C (ascorbic acid), reporting values of 100.6 ± 2.2 mg/100 g dw and 209.1 ± 3.2 mg/100g dw, respectively, in this work it was not possible to determine the ascorbic acid. Akzhigitova et al., (2018) in their research on the chemical composition of absinthe, quantified the content of organic acids with a value of 1.08%, in our study it is evident that absinthe has a higher content in organic acids (11.5 ± 0.2 g/100 g dw) than reported by the authors, this significant difference may be related to the extraction method applied. The organic acids quantified and identified in this work have already been described as containing several bioactive functions, namely quinic acid (major organic acid - 9.2 ± 0.2 g/100 g dw), Jang et al., (2017) described quinic acid as a compound that has radioprotective, antidiabetic and anti-neuroinflammatory activities and also, Marrubini et al., (2015) described the quinic acid as an antioxidant agent.

Table 7. Nutritional profile, soluble sugars and organic acids of the *A. absinthium*. The results are expressed in g/100 g of dry weight.

Nutritional profile	Amount	Soluble sugars	Amount (g/100 g dw)	Organic acids	Amount (g/100 g dw)
Fat (g/100 g)	2.9 ± 0.4	Fructose	0.7 ± 0.1	Oxalic	0.56 ± 0.01
Proteins (g/100 g)	6.1 ± 0.4	Glucose	0.71 ± 0.06	Quinic	9.2 ± 0.2
Ash (g/100 g)	5.8 ± 0.5	Sacarose	0.12 ± 0.02	Malic	0.75 ± 0.02
Carbohydrates (g/100 g)	85.2 ± 0.2	Trehalose	0.13 ± 0.02	Shikimic	0.009 ± 0.001
Energy (kcal)	392 ± 3	Total	1.6 ± 0.2	Citric	0.97 ± 0.01
				Fumaric	0.003 ± 0.002
				Total	11.5 ± 0.2

4.2.3. Fatty acids profile of A. absinthium

To complete the chemical characterization of absinthe, fatty acids were identified and quantified, and the results expressed in relative percentage are shown in **Table 8**. Fourteen fatty acid molecules were identified in *A. absinthium* plant, however eight of these compounds are in amounts less than 1% (caproic acid - C6:0, caprylic acid - C8:0, lauric acid - C12:0, myristic acid - C14:0, pentadecanoic acid - C15:0, palmitoleic acid - C16:1, heptadecanoic acid - C17:0, *cis*-13-16-docosadienoic acid - C22:2). Thus, the major compound in the absinthe, representing more than 50% of fatty acids, is linoleic acid (C18:2n6c), followed by oleic acid (C18:1n9c; 15.76 ± 0.02 %), palmitic acid (C16:0; 14.9 ± 0.2%), lignoceric acid (C24:0; 5.6 ± 0.2 %), α-linolenic acid (C18:3n3; 3.06 ± 0.03%) and stearic acid (C18:0; 1.78 ± 0.02%). These results translate into a higher percentage of polyunsaturated fatty acids (59.76 ± 0.01 %), followed by saturated fatty acids (23.90 ± 0.04 %) and monounsaturated fatty acids (16.34 ± 0.03 %). Akzhigitova et al., (2018) reported eight fatty acids in *A. absinthium*, where the linolenic acid was present in higher amount, in concordance with the results shown in **Table 8**. Kim et al., (2015) characterized two species of the genus Artemisia identifying and quantifying nine fatty acids in the plants. The authors observed significant differences in the concentration of fatty acids between the two species, for example in *A. princeps* oleic acid is the major compound (C18:1; 34.91 ± 0.06 %) and in *A. argyi* the gamma-linolenic acid is the majority compound (C18:3(ω−6); 36.36 ± 0.20%). This suggests that the acid content in plants depends on their species. The high concentration of linoleic acid present in absinthe makes it an even more interesting plant, because it an essential nutrient for human life and is beneficial to health because it reduces of cholesterol levels in the blood. (Marangoni et al., 2020).

Table 8. Fatty acid profile of the *A. absinthium*, expressed in relative percentage.

Fatty acid	Amount (%)
C6:0	0.111 ± 0.004
C8:0	0.054 ± 004
C12:0	0.07 ± 0.02
C14:0	0.75 ± 0.02
C15:0	0.24 ± 0.02
C16:0	14.9 ± 0.2
C16:1	0.58 ± 0.01
C17:0	0.41 ± 0.01
C18:0	1.78 ± 0,02
C18:1n9c	15.76 ± 0.02
C18:2n6c	56.50 ± 0.04
C18:3n3	3.06 ± 0.03
C22:2	0.198 ± 0.001
C24:0	5.6 ± 0.2
SFA	23.90 ± 0.04
MUFA	16.34 ± 0.03
PUFA	59.76 ± 0.01

Caproic acid (C6:0); Caprylic acid (C8:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); *Cis*-13-16-docosadienoic acid (C22:2); Lignoceric acid (C24:0). SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids.

4.3 Bioactive properties of the developed extract of *A. absinthium*

As the infusion revealed the highest content in phenolic compounds, this extract was selected to proceed the studies of bioactive evaluation. The antioxidant activity was evaluated using three *in vitro* assays (DPPH, RP and CAA), being the results shown in **Table 9**. It was evident that the studied extract has a strong antioxidant power, once that the extract revealed a strong activity for all the assays. *A. absinthium* was also able to protect about 76% of cells from oxidation which reveals its promising activity.

Craciunescu et al., (2012) evaluated the antioxidant and cytoprotective activity of ethanolic extracts from two plants, including *A. absinthium*, and found that the extract obtained a high antioxidant power, showing an DPPH radical scavenging assay activity

IC_{50} = 0.57 ± 0.05 mg/mL. Wake et al. (2000) screened a series of plant extracts used to improve or restore mental functions, including A. *absinthium*, which had IC_{50} concentrations of <1 mg/mL.

The difference between the values IC_{50} and EC_{50} (showing above) can be explained by the use of different solvents for the extraction, since the hydroethanolic extract contains a greater number of molecules with higher power antioxidant than the ethanolic extract.

Table 9. Antioxidant activity of the infusion extract of *A. absinthium*.

Antioxidant activity		
DPPH radical capturing activity	EC_{50} values (mg/mL)	7.49 ± 0.90
Reducing Power		0.307 ± 0.003
Cellular based assay	Oxidation inhibition at 2000 µg/mL (%)	76%
	GI_{50} value (µg/mL)	798±77

*EC_{50}: Concentration of extract corresponding to 50% of antioxidant activity or 0.5 of the absorbance in the test of reducing power. Trolox (positive control) EC_{50} values: 42 µg/mL (DPPH radical capturing activity), 41 µg/mL (reducing power). Cellular based assay: Quercetin: % oxidation inhibition: 0,3 ug/ml inhibits 95 %; GI50 = 0.08 µg/mL.

The antibacterial activity of the infusion extract of *A. absinthium* is presented in **Table *10***. The extract was tested against a panel of pathogenic bacteria, Gram-positive and Gram-negative, and showed the ability to inhibit bacterial growth against the studied strains, however, at the maximum tested concentration (20mg/mL), it was not possible to observe the bactericidal action of the extract. The best results in inhibiting bacterial growth were obtained for *Escherichia coli* (2.5 mg/mL) and *Methicillin-resistant Staphylococcus aureus* (2.5 mg/mL). When comparing the results obtained by the absinthe extract with the results of the positive controls, in most of the tested bacteria the MIC values were higher, representing less antimicrobial activity of the extract compared to the controls, however the extract shows greater inhibitory capacity in inhibiting the growth of *Pseudomonas aeruginosa* than ampicillin (20 mg/mL; >20 mg/mL, respectively). Stanković et al., (2016) studied the antibacterial activity of traditional medicinal plants from the Balkan Peninsula, one of which *A. absinthium* methanolic extract and calculated the minimum inhibitory concentration and minimum bacterial concentration from a concentration of 100 mg/mL using the micro-well dilution assay.

Contrary to the results presented in this dissertation, the authors were able to present values for MIC and MBC for all the studied bacteria, this is explained by the high concentration of extract used and for most bacteria the MBC values are the maximum concentration of tested extract (100 mg/mL). Also, MIC values are higher than those presented in this dissertation.

Riahi et al., (2015) corroborated previous reports on the antibacterial and antifungal potential of wormwood essential oils that validate the traditional medicinal use of the species studied as an antiseptic. For Gram-negative bacteria strains,comparable levels of antibacterial activities were observed among the studied oil and the highest activity was recorded against the bacteria *P. aeruginosa*.

Joshi (2013) investigated the composition and antimicrobial activity of the essential oil *A. Absinthium* from the region of India, not observing activity against microorganisms *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis* and *Pseudomonas aeruginosa*, at a range of 250– 259 µg/mL.

According to Wan et al. (1998), the majority of the essential oils assayed for their antibacterial properties showed a more pronounced effect against the Gram-positive bacteria. The resistance of Gram-negative bacteria to essential oil has been ascribed to their hydrophilic outer membrane, which can block the penetration of hydrophobic compounds into target cell membrane (Inouye et al., 2001; Joshi, 2010). These results are about the essential oils of *A. absinthium*, thus as expected the results are not comparable with the ones obtained in this work.

Table 10. Antibacterial activity of the infusion extract of *A. absinthium*.

Antibacterial activity						
		Gram-negative bacteria				
		Escherichia coli	*Klebsiella pneumoniae*	*Morganella morganii*	*Proteus mirabilis*	*Pseudomonas aeruginosa*
Infusion extract (maximum concentration of 20mg/mL)	MIC	2.5	10	20	20	20
	BMC	>20	>20	>20	>20	>20

Ampicillin (maximum concentration of 20mg/mL)	MIC	<0.15	10	20	<015	>20
	BMC	<0.15	20	>20	<0.15	>20
Imipenem (maximum concentration of 1 mg/mL)	MIC	<0.0078	<0.0078	<0.0078	<0.0078	0.5
	BMC	<0.0078	<0.0078	<0.0078	<0.0078	1

		Gram-positive bacteria		
		Enterococcus faecalis	*Listeria monocytogenes*	*Methicillin-resistant Staphylococcus aureus*
Infusion extract (maximum concentration of 20 mg/mL)	MIC	10	10	2.5
	BMC	>20	>20	>20
Ampicillin (maximum concentration of 20 mg/mL)	MIC	<0.15	<0.15	<0.15
	BMC	<0.15	<0.15	<0.15
Imipenem (maximum concentration of 1 mg/mL)	MIC	n.t	<0.0078	n.t
	BMC	n.t	<0.0078	n.t
Vancomycin (maximum concentration of 1 mg/mL)	MIC	<0.0078	n.t	0.25
	BMC	<0.0078	n.t	0.5

n.t: not tested.

The results of cytotoxic, hepatotoxic, and anti-inflammatory activity of the hydroethanolic extract of *A. absinthium* shown in **Table 11**. Regarding cytotoxic activity, it was evident that the infusion extract (80:20 v/v), only showed anti-proliferative capacity against AGS (gastric adenocarcinoma) and $CaCo_2$ (colorectal adenocarcinoma), with GI_{50} values of 249 ± 5 μg/mL and 240 ± 8 μg/mL, respectively. In the remaining tumor lines

tested, it was not possible to determine the GI_{50} value with the maximum extract concentration used (400 μg/mL).

When assessing hepatotoxicity using a primary non-tumor cell culture (PLP2) and a monkey non-tumor cell culture (VERO), the results showed that the extract present no toxicity against these cells with GI_{50} value < 400 μg/mL, allowing to have an idea about the security of this extract for application in the food industry.

Mughees et al., (2020) analyzed the cytotoxic potential of polymeric nanoparticles loaded with *A. absinthium* extract. The cytotoxicity of the different part extracts of *A. absinthium* (root, leaf and aerial extact) was evaluated on breast cancer cell line, MCF-7, by MTT assay. The results obtained showed that whole plant extract possesses more cytotoxicity in comparison to other part extracts (with least IC_{50} values i.e. 307.16 μg/mL compared to 440.25 μg/mL in the roots, 439.49 μg/mL in the leaf and 422.73 μg/mL in the aerial parts of the plant) against the cell line.

Shafi et al., (2012) studied methanolic extract of *A. absinthium* as a potential new alternative medicine for breast cancer through MTT assays, fluorescence microscopy after propidium iodide staining, western blotting and cell cycle analysis. In this study the methanol extract of absinthe showed high antiproliferative activity, found that 25 μg/mL of extract causes almost 50% inhibition of cell proliferation in MCF-7 cells compared to controls.

In relation to the anti-inflammatory activity tested in a murine macrophage cell line (RAW 264.7), it was not possible to calculate the GI_{50} with the maximum tested concentration of extract (400 μg/mL).

Jeong et al., (2018) investigated the anti-inflammatory activity of an ethanolic extract from a plant of the same genus as absinthe, *Artemisia montana* L.. The authors performed this test on RAW 264.7 macrophage cells, the same ones used in this dissertation, and proved that the studied extract has anti-inflammatory activity, suggesting its use as a potential therapeutic for the treatment of inflammatory diseases.

Table 11. Cytotoxic, hepatotoxic, and anti-inflammatory activity of the infusion extract of *A. absinthium* (maximum concentration of 400 µg/mL).

Cytotoxic activity (GI_{50} values, µg/mL)	
	Concentration
AGS (gastric adenocarcinoma)	249 ± 5
CaCo2 (colorectal adenocarcinoma)	240 ±8
MCF-7 (breast carcinoma)	>400
NCI-H460 (non-small cell lung carcinoma)	>400
Toxicity in non-tumor cells (GI_{50} values, µg/mL)	
PLP2 (non-tumour porcine liver primary culture)	>400
VERO (non-tumour culture from African green monkey)	>400
Anti-inflammatory activity (GI50 values, µg/mL)	
RAW 264.7 (murine macrophage cell line)	>400

GI_{50} - concentration that inhibited 50% of cell growth. GI_{50} values of ellipticine (positive control): 1.23 ± 0.03 µg/mL (AGS), 1.21 ± 0.02 µg/mL (CaCo2), 1.02 ± 0.02 µg/mL (MCF-7), 1.01 ± 0.01 µg/mL (NCI), 1.4 ± 0.1 (PLP2), 1.4 ± 0.1 (VERO). GI_{50} values of dexamethasone (positive control): 6.3 ± 0.4 ug/mL.

4.4 Chemical and nutritional characterization of the developed brownies

4.4.1 Phenolic compounds

After the incorporation of the infusion extract of *A. absinthium*, the brownies were monitored for the presence of the phenolic compounds, to evaluate the stability of the compounds after the incorporation in this food product.

Table 12 presents the phenolic compounds identification in the brownies at time zero and after three days of storage. From the perform analysis, it was possible to verify that some of the compounds that were present in the extract, were not detected in the brownies, a fact that may be due to the bakery process with high temperatures. Nevertheless, the presence of quercetin-3-*O*-rutinoside, Isorhamnetin-3-*O*-rutinoside, Apigenin-8,6-C-dihexoside, Apigenin-8-C-hexoside, and Apigenin 8-C-(6"-*O*-quinoyl)-6-C-glucoside was detected. At this moment, the quantification of these molecules in the brownies is ongoing. Besides the compounds belonging to the *A. absinthium* extract, other compounds were also identified such as β-type (epi)catechin dimer, (-)-epicatequin, (epi)catechin-gallate-glucuronide isomer I, and (epi)catechin-gallate-glucuronide isomer II. The presence of these compounds is attributed to the use of cocoa in the brownie´s recipe.

Table 12. Phenolic compounds identification in the developed brownies.

Pic	Rt (min)	Uvmáx	$[M-H]^-$ (m/z)	MS^2	Tentative identification	Quantification (mg/g extract)			
						Brownie control		Brownie with extract	
						T0	T3	T0	T3
1a	5.840	323	353	191(100),179(23),173(10),135(5)	3-*O*-Caffeoylquinic acid	n.d.	n.d.	n.d.	n.d
2a	6.551	326	353	191(10),179(7),173(100),135(5)	4-*O*-Caffeoylquinic acid	n.d	n.d.	n.d.	n.d.
3a	8.543	323	353	191(100),179(12),173(5),135(5)	5-*O*-Caffeoylquinic acid	n.d.	n.d.	n.d.	n.d.
5b	14.014	321	737	563 (20),473(42),443(100),383(5),353(5)	Apigenin 8-C-(6"-*O*-quinoyl)-6-C-glucoside	n.d	n.d.	**det**	**det**
6b	15.362	340	431	341(24),311(100)	Apigenin-8-*C*-hexoside	n.d.	n.d.	**det**	**det**
7c	16.821	342	609	301(100)	Quercetin-3-*O*-rutinoside	n.d.	n.d.	**det**	**det**
8a	17.682	334	515	353(10),191(20),179(35),173(100),161(5),135(8)	*cis* 3,4-*O*-Dicaffeyolquinic acid	n.d.	n.d.	n.d.	n.d.
9a	20.193	328	515	353(11),191(6),179(25),173(100),161(5),135(5)	*trans* 3,4-*O*-Dicaffeyolquinic acid	n.d.	n.d.	n.d.	n.d.
10c	20.427	341	623	315(100)	Isorhamnetin-3-*O*-rutinoside	n.d	n.d	**det**	**det**
11a	21.584	329	515	353(11),191(69),179(65),161(5),135(5)	*cis* 3,5 -*O*-Dicaffeyolquinic acid	n.d.	n.d.	n.d.	n.d.
12b	22.210	345	593	473(97),383(27),353(15)	Apigenin-8,6-C-dihexoside	n.d.	n.d.	**det**	**det**
13a	22.528	329	515	353(12),191(49),179(73),161(5),135(7)	*trans* 3,5 -*O*-Dicaffeyolquinic acid	n.d.	n.d.	n.d.	n.d.
14d	4.67	280	577	451(100), 575(40),425(5),407(5),289(5),287(10)	*β*-type (Epi)catechin dimer	**det**	**det**	**det**	**det**
15d	9.91	280	289	245(100),205(16),179(10)	(-)-Epicatequin	**det**	**det**	**det**	**det**
16d	43.26	279	617	465(70),435(65),327(10),289(5)	(Epi)catechin-gallate-glucuronide isomer I	**det**	**det**	**det**	**det**
17d	44.09	279	617	465(32),435(13),327(5),289(5)	(Epi)catechin-gallate-glucuronide isomer II	**det**	**det**	**det**	**det**

n.d.- not detected, det – detected.

4.4.1 Nutritional profile and sugars present in the developed brownies

For a better interpretation of the results, the following tables result of a two-way ANOVA. **Table 13** is divided in two sections, the upper represents the incorporation (control and absinth) (I) but, included in the standard deviation of each incorporation are both the storage times (0 and 3 days), and, in the bottom section for each storage time (ST) both incorporations are included. Using this statistical tool, each factor can be analysed independently (p-value I × ST > 0.05) and a post-hoc classification can be applied, namely a student's T-test. However, when p-value I × ST < 0.05, then, a significant interaction between both factors hinders an independent classification, and thus, for some cases, only general trends can be obtained through the estimated marginal means plots (EMM). **Table 13** shows the nutritional value and the only soluble sugar, sucrose. Regarding the nutritional profile, the nutrient with the highest prevalence were the carbohydrates, reaching 68 g/100 g of fresh weight (FW), followed by the crude fat with an average of 20 g/100 g FW. Overall, a significant interaction was found for all nutrients and sucrose, not allowing for tendencies from the estimated marginal means. Still, from the *partial eta squares*, it is apparent that for moisture, the impact of I was double when compared to the ST, while for sucrose, ST had a nine-fold higher influence than I, probably due to the breakdown of sucrose into fructose and glucose over time. Its amount and breakdown were not influenced by the addition of absinth extract.

Table 13. Nutritional profile and sucrose of the developed brownies at different storage times. The results are expressed in g/100 g of dry weight.

		Moisture (g/100 g fw)	Crude Fat (g/100 g fw)	Proteins (g/100 g fw)	Ash (g/100 g fw)	Carbohydrates (g/100 g fw)	Energy (Kcal)	Energy (Kj)	Sucrose (g/100 g fw)
Incorporation (I)	Control	6.9±0.6	20.0±0.7	5.0±0.3	0.53±0.04	68±2	470±3	1967±14	243±8
	Absinth	6.1±0.7	19.8±0.5	5.0±0.2	0.68±0.09	68.4±0.5	472±5	1975±21	236±12
p-value (n=6)	T-test	0.268	0.551	0.225	0.957	0.397	0.799	0.799	0.874
Storage Time (ST)	0 Days	6.7±0.7	20.0±0.4	4.9±0.3	0.6±0.1	67.8±0.7	471±4	1970±17	240±12
	3 Days	6.3±0.8	19.8±0.8	5.06±0.03	0.6±0.1	68±1	471±5	1972±19	239±9
p-value (n=6)	T-test	0.061	0.707	0.965	0.126	0.179	0.428	0.428	0.338
ST×I (n=36)	*p*-value	0.345	0.234	0.839	0.965	0.822	0.143	0.143	0.320

In each row, an asterisk (*) means significant statistical differences, with an overall p-value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. T-test stands for Student's T-test.

4.4.2 Organic acids profile of the developed brownies

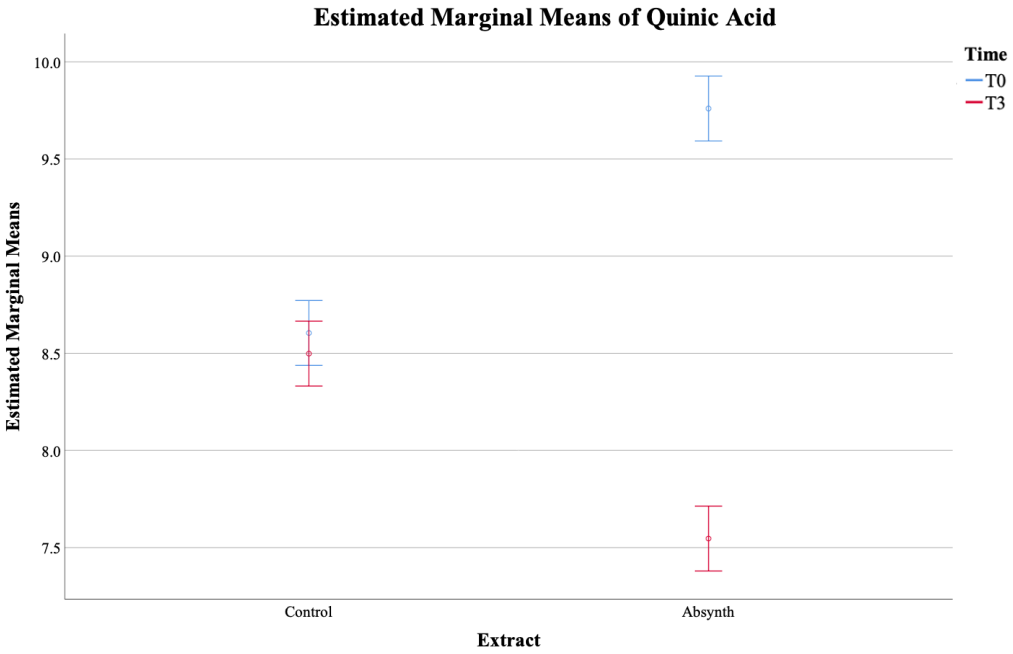
Table 14 shows the different detected organic acids, namely oxalic, quinic, malic, shikimic citric and fumaric acid. Of the six, quinic acid was by far the most abundant, with values averaging 9 mg/mL, being the major contributor to the 11 mg/mL found for the sum of all organic acids. For all, a significant interaction was found, allowing for some tendencies to be extracted from the EMM plots. In **Figure 30 A)** quinic acid showed a higher abundance in the brownies incorporated with absinth at T0, but then tended to reduce in a drastic way in these same brownies at T3, while citric acid (a known antioxidant) was found in higher values in the brownies with absinth, and, although it did decrease from T0 to T3, its amount was always higher in the brownies with absinth. From the partial eta squares it could be concluded that for shikimic acid, the impact of the addition of absinth was much higher than the storage time, while the effect of the three days had a higher impact (0.960) on the total organic acids, that the addition of the absinth extract.

Table 14. Organic acids profile of the developed brownies at different storage times.

		Oxalic Acid	Quinic Acid	Malic Acid	Shikimic Acid	Citric Acid	Fumaric Acid	Total Organic Acids
Incorporation	Control	0.34±0.02	8.5±0.1	0.69±0.01	0.009±0.001	0.91±0.001	0.004±0.001	10.5±0.2
(I)	Absinth	0.34±0.03	9±1	0.67±0.05	0.009±0.001	0.97±0.002	0.005±0.001	11±1
p-value (n=6)	T-test	0.889	0.197	0.133	0.101	<0.001	0.290	0.159
Storage Time	0 Days	0.34±0.02	9.2±0.6	0.70±0.03	0.010±0.001	0.95±0.04	0.004±0.001	11.2±0.7
(ST)	3 Days	0.34±0.03	8.0±0.5	0.66±0.01	0.009±0.002	0.93±0.03	0.004±0.001	10.0±0.5
p-value (n=6)	T-test	0.945	<0.001	0.085	0.949	<0.001	0.301	<0.001
ST×I (n=36)	*p*-value	0.099	<0.001	0.181	0.745	0.031	0.037	<0.001

In each row, an asterisk (*) means significant statistical differences, with an overall p-value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. T-test stands for Student's T-test.

A)



B)

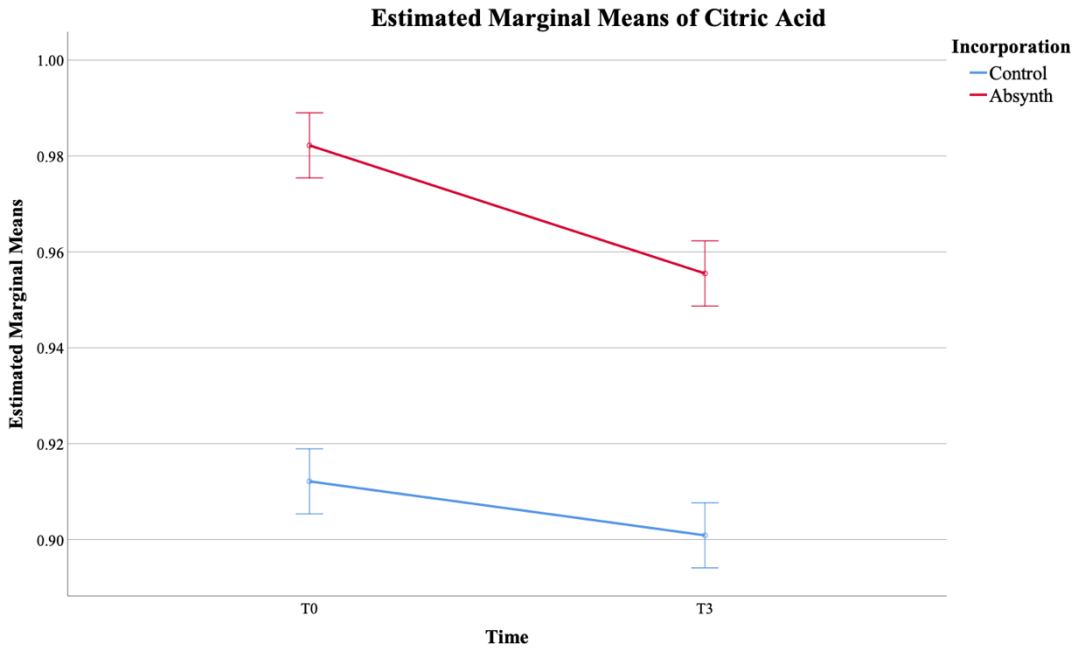


Figure 30. EMM plots of **A)** quinic and **B)** citric acid.

4.4.3 Fatty acids profile of the developed brownies

Table 15 shows the detected fatty acids in the brownie samples, represented in relative percentage. A total of 15 individual fatty acids were detected in the samples, with most of them being saturated fatty acids (SFA). Overall, in terms of the relative amount of each, SFA were the most abundant, representing about 70% of the total fat, and monounsaturated fatty acids (MUFA) representing about 26% and PUFA only amounting to 3 to 4%. The individual fatty acid with the highest relative percentage was C16:0 (palmitic acid) a SFA, followed by C18:1 (oleic acid) a MUFA. In terms of the contribution of each factor, all but C10:0, C16:1, C18:0 and C20:0 showed significative interaction and thus, due the slight variations, no tendencies could be extracted from the EMM plots. For C10:0 (capric acid), a significant increase was found from T0 to T3. An increase was also found for palmitoleic acid. Oleic acid was found in a significant higher amount in the control brownies, and over the three days it decreased significantly. Finally, arachidonic acid (C20:0) also decreased over the three days and was found in significantly higher amounts at T0. Overall, even considering the statistical differences, the variation in the fatty acids was very slight, revealing that the absinth extract, as expected from a preservative, did not significantly change the profile.

Table 15. Fatty acid profile of the developed brownies at different storage times., expressed in relative percentage.

	Incorporation (I)			**Storage Time (ST)**			
	Control	**Absinth**	***p*-value (n=6)**	**0 Days**	**3 Days**	***p*-value (n=6)**	**ST×I (n=36)**
C4:0	4.3±0.2	3.3±0.6	<0.001	3.4±0.8	4.1±0.3	<0.001	<0.001
C6:0	2.4±0.1	2.4±0.4	0.549	2.6±0.3	2.3±0.3	<0.001	<0.001
C8:0	1.17±0.09	1.21±0.09	0.241	1.2±0.1	1.19±0.08	0.903	<0.001
C10:0	2.2±0.2	2.1±0.1	0.184	2.10±0.03*	2.3±0.1	0.022	0.526
C12:0	2.81±0.09	2.5±0.3	0.001	2.5±0.3	2.8±0.1	0.002	0.005
C14:0	7.3±0.1	6.8±0.5	0.001	6.7±0.6	7.4±0.1	<0.001	<0.001
C16:0	30.1±0.4	30.2±0.6	0.497	30.2±0.6	30.2±0.5	0.978	0.011
C16:1	1.13±0.2	1.15±0.3	0.139	1.13±0.2*	1.16±0.01	0.031	0.969
C18:0	18.9±0.1*	19.4±0.2	0.022	19.4±0.2*	18.9±0.1	0.040	0.072
C18:1	23.8±0.2	25±1	0.002	25±1	23.8±0.2	0.003	0.007
C18:2	3.54±0.05	3.6±0.2	0.017	3.7±0.2	3.52±0.06	0.003	<0.001
C20:0	0.46±0.02*	0.48±0.03	0.043	0.49±0.02*	0.46±0.01	0.002	0.224
C23:0	0.43±0.04	0.55±0.02	<0.001	0.51±0.05	0.47±0.09	0.005	0.012
SFA	70.7±0.2	70±1	0.002	70±1	70.7±0.2	0.002	0.003
MUFA	25.7±0.2	26.7±0.9	0.002	26.6±0.9	25.7±0.2	0.002	0.006
PUFA	3.54±0.05	3.6±0.2	0.017	3.7±0.2	3.52±0.07	0.003	<0.001

In each row, an asterisk (*) means significant statistical differences, with an overall p-value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. T-test stands for Student's T-test.

4.5 Bioactive properties of the developed brownies

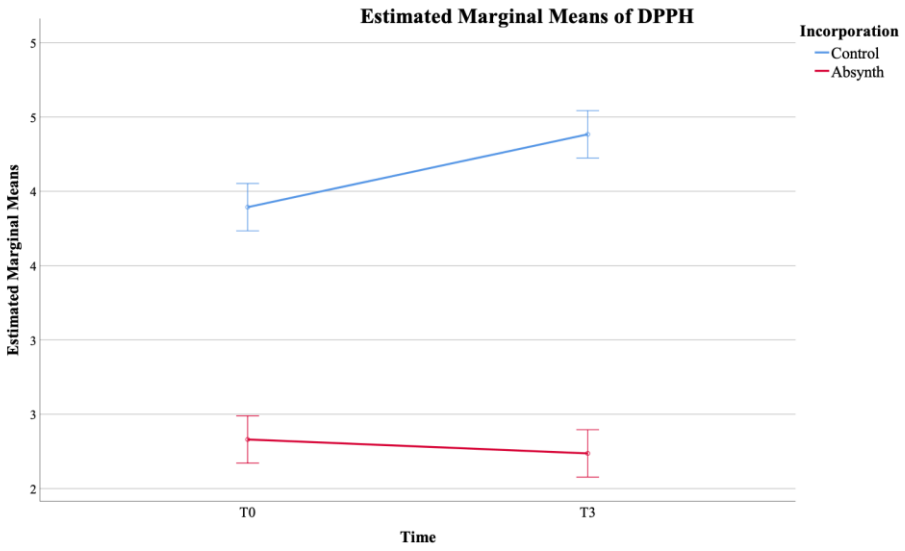
The antioxidant activity, cytotoxicity and anti-inflammatory activity of the different brownies is presented in **Table 16.** Cytotoxic, antioxidant and anti-inflammatory activity of the infusion extract of the brownies at different storage times. Considering the antioxidant activity, from the two analyzed assays that use synthetic oxidants DPPH and RP, a significant interaction was sought for both (*p*-value < 0.05), meaning that one factor did not show a significant influence over the other for the result obtained. Thus, some tendencies were extracted from the EMM, and shown in **Figure 31, A) and B)**. Both for the DPPH and RP, the control sample shows much higher EC_{50} values, meaning a lower antioxidant activity, which tends to increase over the 3 days, while the brownies with absinth show higher antioxidant activity and this activity increases over the three days. In the measurement of the cellular antioxidant activity, the use living cells contribute to obtain much more accurate values, which increases the reliability. There was a significant difference between the absinth incorporated brownie, which showed a higher antioxidant activity when compared to the control brownie, and also an increase in antioxidant activity from T0 to T3, probably due to dehydration, that increased the concentration of the antioxidants in the brownie. Still, absinth is an interesting antioxidant that could be used in foods, pending its lack of toxicity. Thus, concerning cytotoxicity, it is divided in tumoral cell lines (AGS, CaCo2, MCF-7 and NCI-H460), and primary non tumor cell lines (PLP2 and Vero). For the tumoral cell lines, no toxicity against CaCo2 cell lines was sought, and for the other cell lines, very slight toxicity was detected for either brownie. Still, it was not expected that a brownie extract could have any effect on tumor cell lines. Most importantly, a lack of toxicity against non-tumor cell lines which was detected for PLP2 (primary porcine liver cell line) and Vero (monkey liver cell line) rules out toxicity of the absinth extract. Finally, a significant interaction was detected for the RAW 264.7 (anti-inflammatory) cell line, and no general conclusions could be extracted from the EMM plots (**Figure 31C)**), proving no difference between using the absinth extract in the brownies on the anti-inflammatory activity.

Table 16. Cytotoxic, antioxidant and anti-inflammatory activity of the infusion extract of the brownies at different storage times.

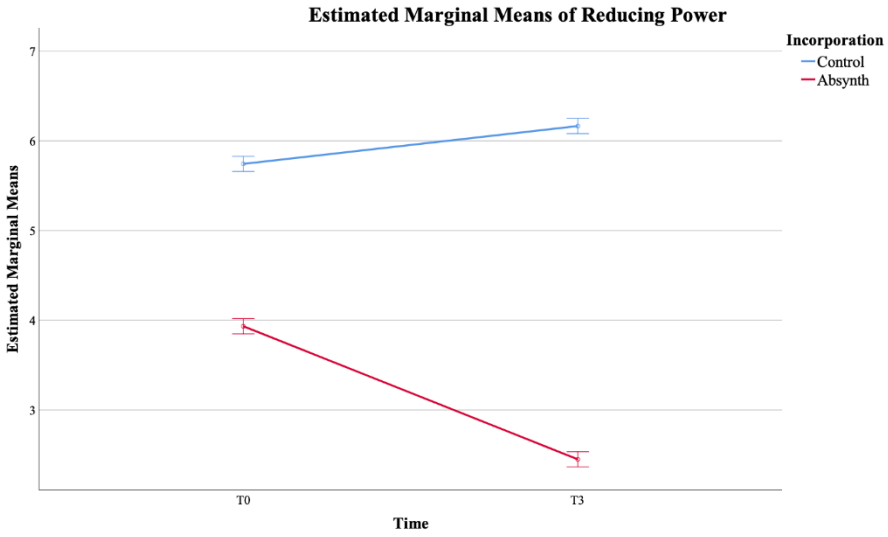
		DPPH	RP	Inhibition	AGS	$CaCo_2$	MCF-7	NCI-H460	PLP2	Vero	RAW 264.7
Incorporation (I)	Control	4.1±0.3	5.9±0.2	19±5*	205±23	-	235±24	117±66	-	-	21±3
	Absinth	2.28±0.06	3.2±0.8	41±5	65±30	-	191±52	38±5	-	-	17±1
p-value (n=6)	T-test	0.021	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	-	<0.001
Storage Time (ST)	0 Days	3.1±0.6	4±1	34±6*	159±73	-	247±13	50±8	-	-	17±2
	3 Days	3.2±0.8	4±2	25±4	111±81	-	179±39	105±78	-	-	21±3
p-value (n=6)	T-test	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	-	<0.001
ST×I (n=36)	*p*-value	0.003	<0.001	0.572	0.034	-	0.003	<0.001	-	-	0.018

In each row, an asterisk (*) means significant statistical differences, with an overall p-value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. *t*-test stands for Student's *t*-test.

A)


Estimated Marginal Means of DPPH
Incorporation
Control
Absynth
Estimated Marginal Means
5
5
4
4
3
3
2
T0
T3
Time

B)


Estimated Marginal Means of Reducing Power
Incorporation
Control
Absynth
Estimated Marginal Means
7
6
5
4
3
T0
T3
Time

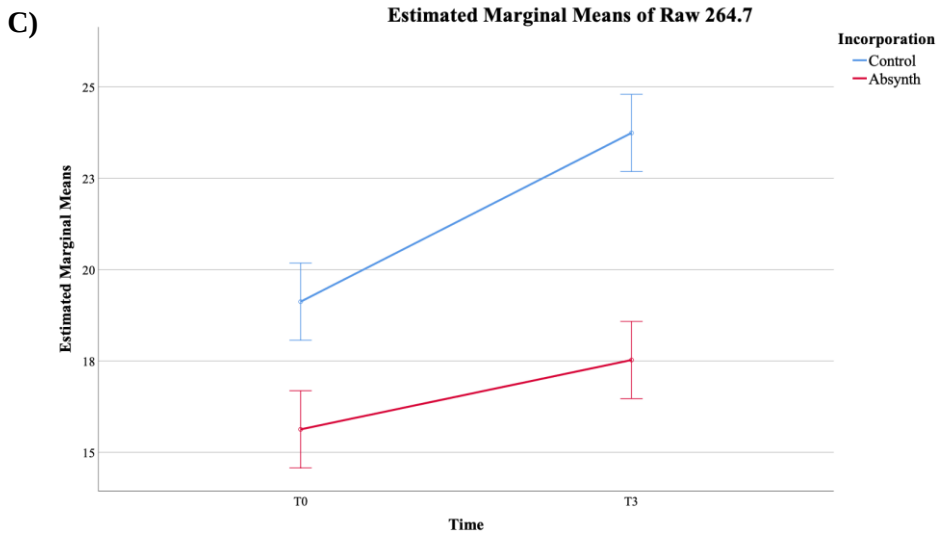


Figure 31. EMM plots of the a) DPPH antioxidant activity, b) reducing power, and c) anti-inflammatory activity.

Table 17 exhibits the antimicrobial potential of the developed brownies and comparatively with the infusion extract of *A. absinthium*, the MIC increased one concentration. Nevertheless, the exhibited activity cannot be attributed to the presence of the extract, since the control brownies also revealed the same inhibition concentrations than the brownies with the extract incorporation. Therefore, in terms of antimicrobial activity, the extract incorporation did not increase the antimicrobial effect of the brownie ate the maximum tested concentration of 20 mg/mL.

Table 17. Antimicrobial activity of the developed brownies.

	BC0 (20mg/mL)		BC3 (20mg/mL)		BE0 (20mg/mL)		BE3 (20mg/mL)		Ampicillin (20mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria														
Escherichia coli	**5**	>20	**5**	>20	**5**	>20	**5**	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	**20**	>20	**20**	>20	**20**	>20	**20**	>20	10	20	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	>20	>20	>20	>20	>20	>20	>20	>20	20	>20	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	>20	>20	>20	>20	>20	>20	>20	>20	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.5	1	n.t.	n.t.
Gram-positive bacteria														
Enterococcus faecalis	**20**	>20	**20**	>20	**20**	>20	**20**	>20	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	**20**	>20	**20**	>20	**20**	>20	**20**	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Methicillin-resistant Staphylococcus aureus	**5**	>20	**5**	>20	**5**	>20	**5**	>20	<0.15	<0.15	n.t.	n.t.	0.25	0.5

n.t: not tested

4.6 Physical parameters of the developed brownies

The textural analysis included several dimensions, hardness, adhesiveness, fracturability, springiness, cohesiveness and chewiness, calculated following a Texture Profile Analysis (TPA), which imitates the human chewing process. The results shown in **Table 18**. Hardness, defined as the force the teeth apply on food, and reached between 4016 and 4973 g. Hardness did not have significant differences among samples, and although no tendencies could be extracted from the EMM plots, from the partial eta squared it was possible to determine that the time showed double the influence the incorporation of absinth extract. Adhesiveness, defined as the capacity for food to adhere to the teeth, was also analysed. A significant interaction was found for both factors, and no tendencies could be extracted from the EMM plots, and from the *partial eta squared*, the contribution of both factors was equal. Fracturability is the ability of a food to break into pieces when being chewed by the incisor teeth. In the brownies' case, a significant interaction was

detected, but some tendencies could be sought from the EMM plot (**Figure 32**), in which at T0 none of the brownies showed fracturability, meaning that a total deformation of the cake took place rather than its fracture. However, the fracturability of the control brownie at T3 was higher than the one incorporated with absinth meaning the control samples had lower tendency to deform before breaking, so the brownies with absinth improved this tendency for small cakes to become brittle over the course of a few days, allowing for a softer and more deformable texture. Springiness is defined by the rate at which a deformed food to revert to its undeformed state after removing the deforming force. Springiness showed differences between samples, with the storage time having a higher influence than the incorporation of absinth reducing its value from T0 to T3, which was expected due to the drying of the bread (staling). Cohesiveness is the ability for a food to resist a second deformation relative to a first deformation. In the case of the brownies, a significant interaction was found, and thus concrete conclusions could not be drawn, neither were there EMM plots enough for some general conclusions, and thus, the partial eta square allowed to conclude that time showed twice the effect on this dimension of the texture when compared to the incorporation. Finally, chewiness, which is defined as the energy necessary to chew food, resulting from the combination of hardness, cohesiveness and springiness. This dimension showed a significantly higher value at T0 when compared to T3, showing that time had a higher influence on the reduction of the chewiness of the brownies. Overall, it seems that storage time had more effect than the addition of absinth extract on the brownies, although the extract modified the fracturability, thus reducing the brittleness of the cake after the 3 days of storage which was quite appropriate.

Table 18. The textural analysis of the developed brownies at different storage times.

		Hardness (g)	**Adhesiveness (g.sec)**	**Fracturability (g/s)**	**Springiness (%)**	**Cohesiveness (%)**	**Chewiness**
Incorporation	Control	4754±1216	-44±47	1575±1723	0.18±0.04	0.15±0.03	127±42
(I)	Absinth	4235±1036	-7±1	565±620	0.16±0.05	0.15±0.03	107±75
p-value (n=6)	T-test	0.407	0.006	<0.001	0.390	0.857	0.351
Storage Time	0 Days	4973±973	-45±47	-	0.21±0.02*	0.16±0.02	160±32*
(ST)	3 Days	4016±1103	-7±1	2140±1109	0.14±0.04	0.13±0.03	74±47
p-value (n=6)	T-test	0.145	0.005	<0.001	0.011	0.165	0.003
ST×I (n=36)	*p*-value	0.253	0.005	<0.001	0.560	0.986	0.084

In each row, an asterisk (*) means significant statistical differences, with an overall p-value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. T-test stands for Student's T-test.

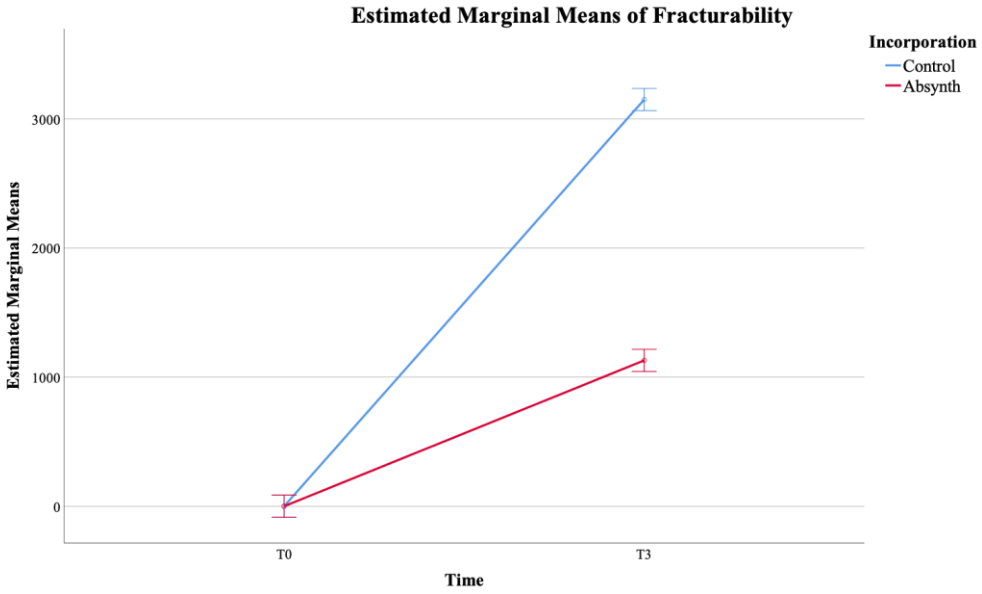


Figure 32. EMM plots of fracturability.

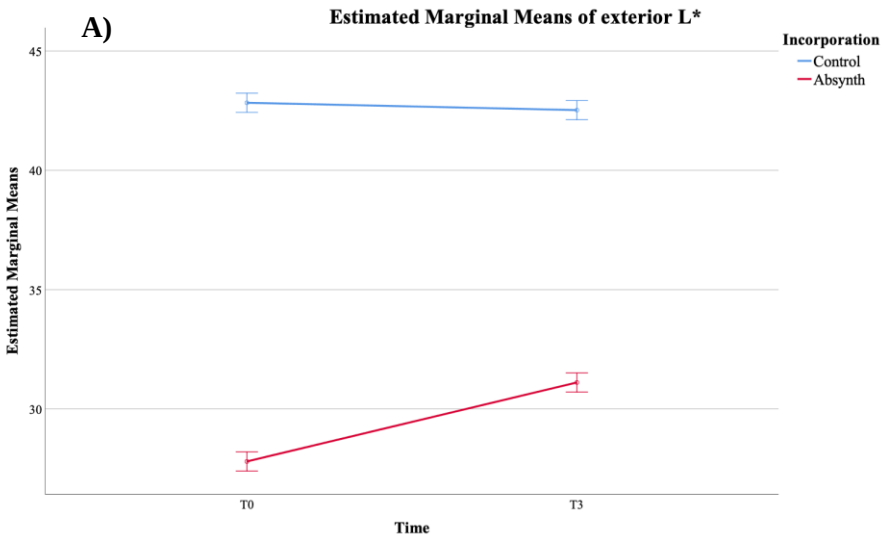
Table 19 shows the colors of the brownies, both from the interior crumb and the outer crust, the water activity, and the pH values. Regarding the color, it is decomposed in the L*, a* b* coordinates in which L* represents lightness, a* the redness-greenness, and finally b* the yellowness-blueness. Regarding the exterior colors, a significant interaction was sought for the three coordinates L*, a* and b*, and thus only some minor tendencies were extracted from the EMM plots (**Figure 33**). In **Figure 33A)**, it is clear that the control brownies showed higher lightness than the ones incorporated with absinth extract in the crust, while also showing a slight tendency for yellow, showed in **Figure 33B).** However, over the three storage days, the samples with absinth extract did show a tendency to develop a yellow color becoming similar to the control brownies. Regarding the interior colors, both L* and a* showed significant interactions among ST and I, and only b* did show statistical differences, in which the brownies on the day of production were yellower on the inside and tended to decrease this yellow. There was also a significant difference between the two incorporation brownies, with the control showing yellower tones. This migration of a yellow color from the crumb to the crust overtime could be explained by the staling of the flour. The L*, obtained from the EMM plots (**Figure 33C)**) shows that the crumb of the control brownies showed lighter tones, but over time these tended to decrease. This lowering of L* was also found for the absinth incorporated brownies, always at lower lightness values than the control samples. Overall, the color was only slightly changed, with significant differences only being sought for

one coordinate (internal b*), with variations of about 10 units in a spectrum that comprehends 200. For the aW, the storage time showed a higher influence over the incorporation, with significant differences between the two samples, revealing that a higher availability of water was obtained at T3. Finally, not observed a significant variation in the pH values in the brownie with the extract, between the beginning of the test and after the 3 days of storage. Therefore, these two parameters (aW and pH) were not influenced by the addition of absinth extract and the three days of storage.

Table 19. Profile of the colorimetric coordenates (L*, a*, b*) of the inside and top of the brownies, the water activity and pH at different storage times.

		Exterior L*	Exterior a*	Exterior b*	Interior L*	Interior a*	Interior b*	aW	pH
Incorporation	Control	42.7±0.4	12.9±0.7	19.8±0.6	36±5	12±1	18±1*	0.65±0.06	6.2±0.2
(I)	Absinth	29±2	8.9±0.6	12±4	28±2	10.4±0.6	12±1	0.62±0.06	5.94±0.05
p-value (n=6)	T-test	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.123	<0.001
Storage Time	0 Days	35±8	11±3	14±7	35±6	12±1	16.0±0.3*	0.58±0.02*	5.96±0.07
(ST)	3 Days	37±6	11±2	17±2	29±2	10.6±0.8	14.1±0.2	0.69±0.06	6.1±0.2
p-value (n=6)	T-test	<0.001	0.746	<0.001	<0.001	<0.001	0.001	<0.001	0.001
ST×I (n=36)	*p*-value	<0.001	0.001	<0.001	0.001	<0.001	0.518	0.263	0.002

In each row, an asterisk (*) means significant statistical differences, with an overall p-value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values. T-test stands for Student's T-test.



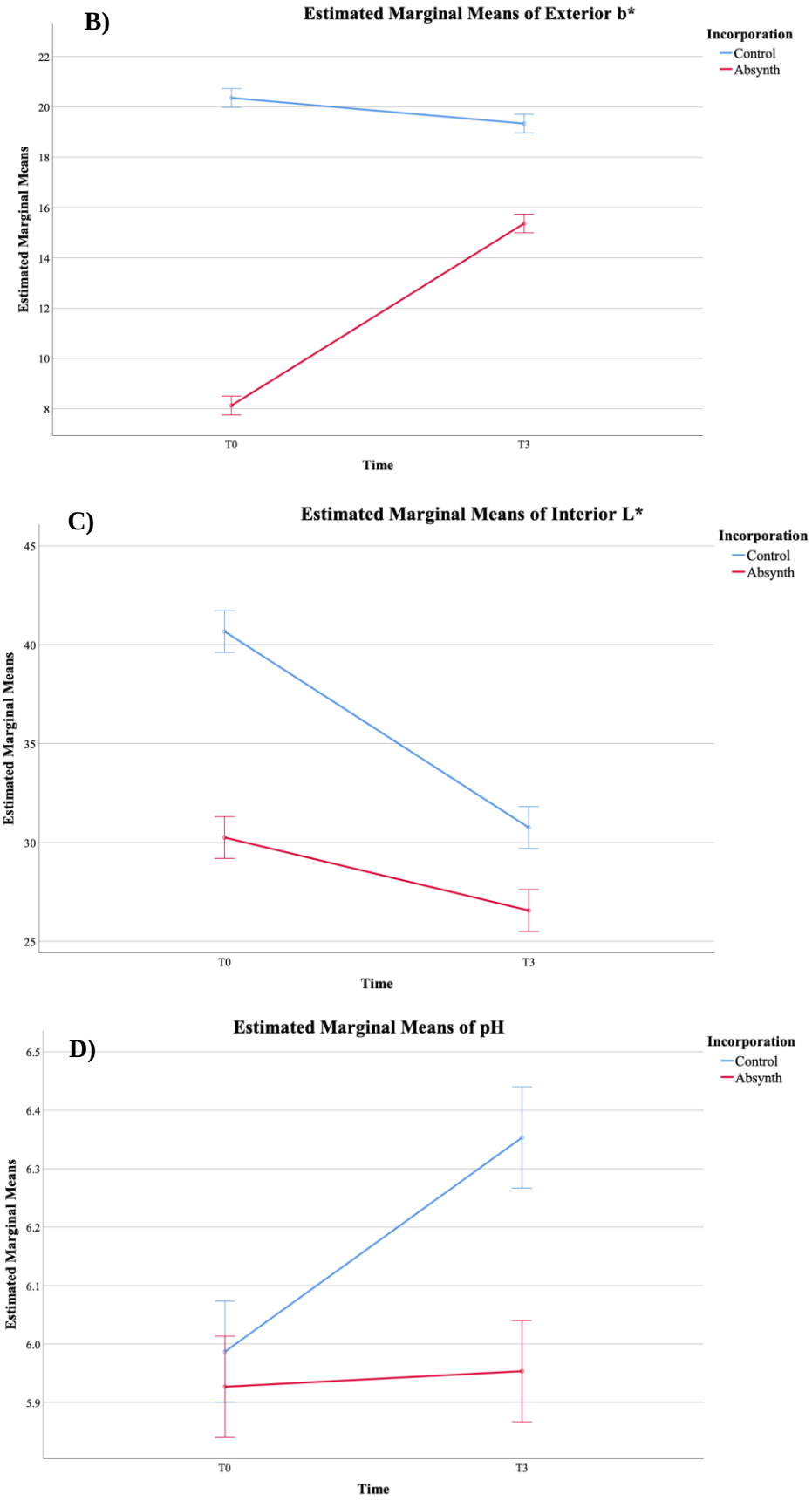


Figure 33. EMM plots of the color, **A)** exterior L*, **B)** exterior b*, **C)** interior L* and **D)** pH.

5. CONCLUSION AND PERSPECTIVES

The results of this work show that it was possible to develop a functional food with the incorporation of *A. absinthium*, with improved biological properties.

The screening test methodology and factor design proved to be an essential tool for the study of the pre-optimization of extractions, to avoid high numbers of extractive trials and to promote resource savings.

Based on the energetic efficiency request, infusion methodology proved to be the extract with the higher amount of Total Bioactive Compounds (TPC and TFC) due to usage of higher temperature and also due to the higher hydrophilic affinity of the compounds. The provided results from the UAE, highlighted again the shorter times needed in order to extract the bioactive compounds from the organic matrixes, which due to the low energy consumption of the machine, could benefit in the extraction of thermolabile bioactive molecules.

The incorporation of the extract into the brownies increased the biological activity of the brownies, namely the antioxidant and cytotoxic ones, that was increased over the days, so it was shown that absinthe was able to provide beneficial added value to this food and make it functional.

Furthermore, the addition of the extract in the brownie had no significant impact on the nutritional profile, soluble sugars and fatty acids, which reveals that, according to what is expected of this ingredient, it is a beneficial result since it does not alter the organoleptic parameters. However, the extract slightly influenced the texture of the brownies, specifically its incorporation modified the fracture, giving the brownies a softer and deformable texture over the course of the days. On the other hand, the color, water activity and pH were not modified.

For future work, it is expected to be able to compare the extraction process yield, between infusion and UAE, using the same solid/liquid ratio. With regard to the study of the behavior of the brownies with the incorporated extract, it would be appropriate to continue to perform the analysis for longer periods, using modified atmospheres to avoid hardening of them, as well as evaluating the possibility of encapsulating the extract for reduce the bitter taste it generates.

Overall, this work corroborated the use of *A. absinthium* in the traditional medicine as a bioactive plant since this extract was able to functionalize a food product providing the consumers additional biological properties while they are enjoying this “cake”.

6. BIBLIOGRAPHY

Abrankó, L., & Clifford, M. N. An unambiguous nomenclature for the acyl-quinic acids commonly known as chlorogenic acids. Journal of agricultural and food chemistry 2017, 65(18), 3602-3608.

Abreu, R.M.V., Ferreira, I.C.F.R., Calhelha, R.C., Lima, R.T., Vasconcelos, M.H., Adega, F., Chaves, R. and Queiroz, MJ.R.P. (2011). Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-b]pyridine-2-carboxylate derivatives: In vitro evaluation, cell cycle analysis and QSAR studies. *European Journal of Medicinal Chemistry*, 46, 5800-5806.

Abreu, L., 2013. "Estudo do poder antioxidante em infusões de ervas utilizadas como chás". Tese de mestrado em Ciência e Tecnologia dos Alimentos. Universidade Federal de Santa Maria (UFSM, RS) do Brasil.

Akzhigitova, Z. Z., Baiseitova, A. M., Ye, Y., & Jenis, J. (2018). Investigation of chemical constituients of *Artesimia absinthium*. *International Journal of Biology and Chemistry*, 11, 169.

Albuquerque, B.R., Pinela, J., Barros, L., Oliveira, M.B.P.P., Ferreira, I.C.F.R (2020). Anthocyanin-rich extract of jabuticaba epicarp as a natural colorant: Optimization of heat- and ultrasound-assisted extractions and application in a bakery product. *Food Chemistry*. Vol. 316, p. 126364.

AOAC. (2016). Official methods of analysis of AOAC International, 20th edition. Association of Official Analytical Chemists International; Arlington; USA.

Assis, L.M.; Zavareze, E.R.; Radünz, A.L.; Dias, A.R.G.; Gutkoski, L.C.; Elias, M.C. (2009). Nutritional, technological and sensory properties of biscuits with substitution of wheat flour for oatmeal or parboiled rice flour. *Food and Nutrition*, Araraquara, 20 (1), 15-24.

Aune D, Chan D.S, Lau R, Vieira R, Greenwood D.C, Kampman E, et al (2011). Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response metaanalysis of prospective studies. *BMJ, 343*. d6617. doi: 10.1136/bmj.d6617.

Azmin, S. N. H. M., Manan, Z. A., Alwi, S. R. W., Chua, L. S., Mustaffa, A. A., & Yunus, N. A., 2016. Herbal processing and extraction technologies. *Separation and Purification Reviews*. Vol. 45, no. 3, 305–320.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N., & Omar, A. K. M., 2013. Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering*. Vol. 117, no. 4, 426–436.

Barkley, Theodore. (2006). *Flora of North America, 19* (20, 21), 503-519.

Barros, L., Pereira, C., and Ferreira, I.C.F.R. (2013a). Optimized Analysis of Organic Acids in Edible Mushrooms from Portugal by Ultra Fast Liquid 82 Chromatography and Photodiode Array Detection. *Food Analytical Methods*, 6, 309–316.

Barros, L., Pereira, E., Calhelha, R.C., Dueñas, M., Carvalho, A.M., SantosBuelga, C., and Ferreira, I.C.F.R. (2013b). Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L. *Journal of Functional Foods*, 5, 1732–1740.

Beigh, Y. A., & Ganai, A. M. (2017). Potential of Wormwood (Artemisia absinthium Linn.) herb for use as additive in livestock feeding: A review. *The Pharma Innovation Journal*, 6, 176–187.

Benayad, Z., Gómez-Cordovés, C., & Es-Safi, N. E. (2014). Identification and quantification of flavonoid glycosides from fenugreek (Trigonella foenum-graecum) germinated seeds by LC–DAD–ESI/MS analysis. *Journal of Food Composition and Analysis*, *35*(1), 21-29.

Betoret, E., Betoret, N., Vidal, D. and Fito, P. (2011). Functional foods development: Trends and technologies. *Trends in Food Science & Technology, 22*, 498-508.

Bora K.S. and Sharma A. (2010). "Phytochemical and pharmacological potential of *Artemisia absinthium* Linn. and *Artemisia asiatica* Nakai: a review". *Journal of Pharmacy Research, 3* (2), 325–328.

Bora K.S. and Sharma A. (2011). "The genus *Artemisia:* a comprehensive review". *Pharmaceutical Biology, 49* (1), 101–109.

Borges, S.V.; Bonilha, C.C.; Mancini, M.C. (2006). Jackfruit (*Artocapus integrifolia*) and pumpkin (*Curcubita moschata*) seeds dehydrated at different temperatures and used as ingredients in cookie-type cookies. *Food and Nutrition*, Araraquara, 17 (3), 317-321.

Bradbury K.E, Appleby P.N and Key T.J. (2014). Fruit, vegetable, and fiber intake in relation to cancer risk: findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). *Am J Clin Nutr, 100* (1), 394S-398S.

Canadanovic-Brunet J.M., Djilas S.M., Cetkovic G.S. and Tumbas V.T. (2005). "Free-radical scavenging activity of wormwood (*Artemisia absinthium* L) extracts". *Journal of the Science of Food and Agriculture, 85* (2), 265–272.

Capriles, V.D.; Coelho, K.D.; Matias, C.G.; Arêas, J.A.G. (2006). Effect of the addition of amaranth on the composition and acceptability of the cookie and loaf. *Food and Nutrition*, Araraquara, 17 (3), 269-274.

Carocho, M., Morales, P., Ciudad-Mulero, Fernández-Ruiz, V., Ferreira, E., Heleno, S., Rodrigues, P., Barros, L., Ferreira, I.C.F.R. (2020). Comparison of diferent bread types: Chemical and physical parameters. *Food Chemistry*. Vol. 310, p. 125954.

Cater, N.B., Garcia-Garcia, A.B, Vega, G.L, Grundy, S.M. (2005). Responsiveness of Plasma Lipids and Lipoproteinsto Plant Stanol Esters. *The American Journal of Cardiology, 96* (1A).

Chiasson H., Belanger A., Bostanian N., Vincent C. and Poliquin A. (2001). "Acaricidal properties of Artemisia absinthium and Tanacetum vulgare (Asteraceae) essential oils obtained by three methods of extraction". *Journal of Economic Entomology, 94* (1), 167–171.

Chirinos, R., Betalleluz-Pallardel, I., Huamán, A., Arbizu, C., Pedeschi, R. and Campos D. (2009). HPLC-DAD characterisation of phenolic compounds from Andean oca (*Oxalis tuberosa* Mol.) tubers and their contribution to the antioxidant capacity. *Food Chemistry, 113,* 1243-1251.

Choi, C. W., Kim, S. C., Hwang, S. S., Choi, B. K., Ahn, H. J., Lee, M. Y., et al. (2002). Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science, 163,* 1161–1168.

Clifford, M.N.; Zheng, W.; Kuhnert, N. Profiling the chlorogenic acids of aster by HPLC–MSn. Phytochem. Anal. 2006, 17, 384–393.

Clifford, M.N.; Johnston, K.L.; Knight, S.; Kuhnert, N. Hierarchical scheme for LC-MSnidentification of chlorogenic acids. J. Agric. Food Chem. 2003, 51, 2900–2911.

Codex Alimentarius Commission. (1997). CAC-GL 23, rev. 2004.

Codex Alimentarius. (1999). Proposed Draft Recommendations for the Use of Health Claims. *Geneva: WHO*. Alinorm 01/22, Apendix VIII. Vol. 99/22A Apendix VII.

Cortés R., Chiralt B. y Puente D. (2005). Alimentos funcionales: una historia con mucho presente y futuro. *Vitae, revista de la facultad de química farmacéutica, 12* (1).

Craciunescu, O., Constantin, D., Gaspar, A., Toma, L., Utoiu, E., & Moldovan, L. (2012). Evaluation of antioxidant and cytoprotective activities of Arnica montana L. and Artemisia absinthium L. ethanolic extracts. *Chemistry Central Journal*, 6(1), 1–11.

Daniells, S. (2009). Science supports vitamin D to fewer broken bones. https://www.foodnavigator.com/

Della Lucia, C.M., Campos, F.M., Mata, G.M.S.C. and Sant'ana, H.M.P. (2008). Controle de perdas de carotenóides em hortaliças preparadas em unidade de alimentação e nutrição hospitalar. *Ciência Saúde Coletiva, Viçosa, 13* (5), 1627-1636.

Dias, M.I., Caleja, C., Ferreira, I.C.F.R., & Barreiro, M.F. (2017). The use of encapsulation to guarantee the stability of phenolic compounds. Chapter 6. In New Polymers for Encapsulation of Nutraceutical Compounds. Wiley-Blackwell. Ed: Jorge Carlos Ruiz Ruiz and Maira Rubi Segura Campos. ISBN: 978-1-119-22879-0.

Diplock, A.T., Aggett, P.J., Ashwell, M., Bornet, F., Fern, E.B., and Roberfroid, M.B. (1999). Scientific concepts of functional foods in Europe: Concensus document. *British Journal of Nutrition, 81* (1), S1–S27.

Drago Serrano, M.E, López López, M, Sainz Espuñes, Teresita del Rosario (2006). Componentes bioactivos de alimentos funcionales de origen vegetal. *Revista Mexicana de Ciencias Farmacéuticas, 37*, (4).

Eldridge, A and Kwolek W. (1983). Soybean isoflavone: Effect of enviroment and variety on composition. *J. Agric. Food Chem*. 31, 394-396.

Fasolin, L.H.; Almeida, G.C.; Castanho, P.S.; Nettooliveira, E.R. (2007). Cookies made with banana flour: chemical, physical and sensory evaluations. *Food Science and Technology*, Campinas, 27 (3), 524-529. http:// dx.doi.org/10.1590/S0101-20612007000300016

Fennema, O. R. y Tannenbaum S. R. (1992). Introducción a la ciencia de los alimentos. En O. R. Fennema, *Química de los alimentos* (pp. 3-27). España, Acribia.

Ferreira, E.H.R., Cabral, J.R.A. and Nardelli, P.M. (2009). Alimentos funcionais: mercado, regulamentação e benefícios à saúde. *Leites e Derivado, São Paulo, 113*.

Feskanich, D., Meyer H.E., Fung T.T., Bischoff-Ferrari H.A. and Willett W.C. (2018). Milk and other dairy foods and risk of hip fracture in men and women. *Osteoporosis International, 29* (2), 385–396. doi: 10.1007/s00198-017-4285-8.

Gilani A.U.H. and Janbaz K.H. (1995). "Preventive and curative effects of *Artemisia absinthium* on acetaminophen and CCl4- induced hepatotoxicity". *General Pharmacology, 26* (2), 309–315.

Gomes, A., E. Fernandes, and J.L.F.C. Lima, Fluorescence probes used for detection of reactive oxygen species. *Journal of Biochemical and Biophysical Methods*, 2005. 65(2): p. 45-80.

Gouin, S. (2004). Micro-encapsulation: Industrial appraisal of existing technologies and trends. *Trends in Food Science and Technology,* 15, 330–347.

Grajek, W., Olejnik, A. and Sip, A. (2005). Probiotics, prebiotics and antioxidants as functional foods. *Acta Biochimica Polonica, Poznań*, 52 (3), 665-671.

Grassi, D., Desideri, G., Croce, G., Tiberti, S., Aggio, A. and Ferri, C. (2009). Flavonoids, vascular function and cardiovascular protection. *Current Phamaceutical Design, Coppito, 15* (10), 1072-1084.

Gutkoski, L.C.; Ianiski, F.; Damo, T.V.; Pedó, I. (2007a). Oatmeal "cookies" enriched with β-glycans concentrate. *Brazilian Journal of Food Technology*, Campinas, 10 (2), 104-110.

GVR. Grand View Research. (2019). Functional Foods Market Size, Share & Trends Analysis Report By Ingredient (Carotenoids, Prebiotics & Probiotics, Fatty Acids, Dietary Fibers), By Product, By Application, And Segment Forecasts, 2019 – 2025. https://www.grandviewresearch.com/industry-analysis/functional-food-market.

Hardy, G. (2000). Nutraceuticals and functional foods: Introduction and meaning. *Nutrition, 16*, 688–697.

Hari, R.K., Patel T.R. and Martin A.M. (1994). An overview of pigment production in biological systems: functions, biosynthesis, and applications in food industry. *Food Reviews International, 10,* 49-70.

Hasler, C. M., Bloch, A. S., Thomson, C. A., Enrione, E., and Manning, C. (2004). Position of the merican Dietetic Association: Functional foods. *J Am Diet Assoc, 104*, 814-826.

Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T., 1988. "Two new flavonoids". *Chemical and Pharmaceutical Bulletin.* 36, 2090-2097.

Hertog M.G.L., Hollman P.C.H. and Venema D. P. (1992). Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *Journal of Agricultural and Food Chemistry, 40,* 1591- 1598.

Hirahara, T. (2004). Key factors for the success of functional foods. *Biofactors, (22),* 289-294.

Holick, M.F. (2004). Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers and cardiovascular disease. *The American Journal of Clinical Nutrition, Boston, 80* (6), 1678-1688.

Huang, D.; Ou, B.; Prior, R.L., 2005. "The chemistry behind antioxidant capacity assays". *Journal of Agricultural and Food Chemistry.* 53, 1841-1856.

Hughes SA, Shewry PR, Li L, Gibson GR, Sanz ML and Rastall RA. (2007). In vitro fermentation by human fecal microflora of wheat arabinoxylans. *J Agric Food Chem, 55*.

ILSI - International Life Sciences Institute (Europe). (1999). FUFOSE : scientific concepts of functional foods in Europe. Consensus Document. *Br J Nutr, 81,* 1-27.

Inouye S, Yamaguchi H, Takizawa T. (2001). Screening of the antibacterial effects of variety of essential oils on respiratory tract pathogens, using a modified dilution assay method. *J Infect Chemother* 7:251–4.

Irshad S., Butt M. and Hira Y. (2011). "In-vitro antibacterial activity of two medicinal plants neem (*Azadirachta indica*) and peppermint". *International Research Journal of Pharmaceuticals, 1* (1), 9–14.

Jabeur, I., Tobaldini, F., Martins, N., Barros, L., Martins, I., Calhelha, R. C., Henriques, M., Silva, S., Achour, L., Santos-Buelga, C. and Ferreira, I.C.F.R. (2016). Bioactive properties and functional constituents of *Hypericum androsaemum* L.: A focus on the phenolic profile. *Food Research International*, 89, 422-431.

Jang, S. A., Park, D. W., Kwon, J. E., Song, H. S., Park, B., Jeon, H., Sohn, E. H., Koo, H. J., & Kang, S. C. (2017). Quinic acid inhibits vascular inflammation in TNF-α-stimulated vascular smooth muscle cells. *Biomedicine and Pharmacotherapy*, 96, 563–571.

Jeong, S. H., Kim, J., & Min, H. (2018). In vitro anti-inflammatory activity of the Artemisia Montana leaf ethanol extract in macrophage RAW 264.7 cells. *Food and Agricultural Immunology,* 29(1), 688–698.

Joshi RK, Mujawar MHK, Kholkute SD. (2010). Antimicrobial activity of the extracts of Craniotome furcata (Lamiaceae). *J Ethnopharmacol* 128:703–4.

Juteau F., Jerkovic I., Masotti V. et al. (2003). "Composition and antimicrobial activity of the essential oil of *Artemisia absinthium* from Croatia and France". *Planta Medica, 69* (2), 158–161.

Jovanović, A. A., Đorđević, V. B., Zdunić, G. M., Pljevljakušić, D. S., Šavikin, K. P., Gođevac, D. M., & Bugarski, B. M., 2017. Optimization of the extraction process of polyphenols from Thymus serpyllum L. herb using maceration, heat- and ultrasound-assisted techniques. *Separation and Purification Technology*. Vol. 179, 369–380.

Kern, Ken. (2012) Plants for a Future: Edible and Useful Plants for a Healthier World ISBN 1-85623-011-2.

Kim, J. K., Shin, E. C., Lim, H. J., Choi, S. J., Kim, C. R., Suh, S. H., Kim, C. J., Park, G. G., Park, C. S., Kim, H. K., Choi, J. H., Song, S. W., & Shin, D. H. (2015). Characterization of Nutritional Composition, Antioxidative Capacity, and Sensory Attributes of Seomae Mugwort, a Native Korean Variety of Artemisia argyi H. Lév. & Vaniot. *Journal of Analytical Methods in Chemistry*, 1–9.

Koch, T. C., Briviba, K., Watzl, B., Fähndrich, C., Bub, A., Rechkemmer, G., Barth, S. W. (2009). Prevention of colon carcinogenesis by apple juice in vivo: impact of juice constituents and obesity. *Molecular Nutrition & Food Research, Weinheim,* 53 (10), 1289-1302.

Kopper, A.C.; Saravia, A.P.K.; Ribani, R.H.; Lorenzi, G.M.A.C. (2009). Technological use of bocaiuva flour in the preparation of cookies. *Food and Nutrition*, Araraquara, 20 (3), 463-469.

Kordali S., Kotan R., Mavi A., Cakir A., Ala A. and Yildirim A. (2005). "Determination of the chemical composition and antioxidant activity of the essential oil of *Artemisia dracunculus* and of the antifungal and antibacterial activities of Turkish *Artemisia absinthium, A. dracunculus, Artemisia santonicum,* and *Artemisia spicigera* essential oils," *Journal of Agricultural and Food Chemistry*, *53* (24), 9452–9458.

Kwak, N.S. and Jukes, D.J. (2001). Functional foods. Part 1: the development of a regulatory concept. *Food Control, 12,* 99-107. https://doi.org/10.1016/S0956-7135(00)00028-1.

Laird, E., Molloy A.M., McNulty H., Ward M., McCarroll K., Hoey L., Hughes C. F., Cunningham C., Strain J.J. and Casey M. C. (2017). Greater yogurt consumption is

associated with increased bone mineral density and physical function in older adults. *Osteoporosis International, 28* (8), 2409–2419. doi: 10.1007/s00198-017-4049-5.

Lajolo, F. M. (2002). Functional foods: Latin American perspectives. *Br J Nutr, 88* (2), 145-150.

Liu R.H. (2013). Health-promoting components of fruits and vegetables in the diet. Advances in nutrition (Bethesda, Md.), 4 (3), 384S–92S. https://doi.org/10.3945/an.112.003517.

Lui C.L., Wang J.M., Chu C.Y., Cheng, M.T. and Tseng T.H. (2002). In vivo protective effect of protocatechuic acid on tertbutylhydroperoxide-induced rat hepatotoxicity. *Food Chemistry and Toxicology, 40,* 635-641.

Liu, T. ting, Wu, H. bin, Wu, H. bo, & Zhang, J. (2019). Wormwood (Artemisia absinthium L.) as a promising nematicidal and antifungal agent: Chemical composition, comparison of extraction techniques and bioassay-guided isolation. *Industrial Crops and Products*, 133, 295–303.

Mahmoudi M., Ebrahimzadeh M.A., Ansaroudi F., Nabavi S.F. and Nabavi S.M., "Antidepressant and antioxidant activities of *Artemisia absinthium* L. at flowering stage". *African Journal of Biotechnology, 8* (24), 7170–7175.

Maiani, G., Castón, M.J.P., Catasta, G., Toti, E., Cambrodón, I.G., Bysted, A., Granadolorencio, F., Olmedilla-Alonso, B., Knuthsen, P., Valoti, M., Böhm, V., Mayermiebach, E., Behsnilian, D. and Schlemmer, U. (2009). Carotenoids: actual knowledge on food sources,intakes, stability and bioavailability and their protective role in humans. *Molecular Nutrition and Food Research, Weinheim, 53* (2), 194-218.

Maihara, V.A., Silva, M.G., Baldini, V.L.S., Miguel, A.M.R. and Fávaro, D.I.T. (2006). Avaliação nutricional de dietas de trabalhadores em relação a proteínas, lipídeos, carboidratos, fibras alimentares e vitaminas. *Ciência e Tecnologia de Alimentos, São Paulo, 26* (3), 672-677.

Marangoni, F., Agostoni, C., Borghi, C., Catapano, A. L., Cena, H., Ghiselli, A., La Vecchia, C., Lercker, G., Manzato, E., Pirillo, A., Riccardi, G., Risé, P., Visioli, F., & Poli, A. (2020). Dietary linoleic acid and human health: Focus on cardiovascular and cardiometabolic effects. In *Atherosclerosis*, 292, 90–98.

Marcílio, R.; Amaya-Farfan, J.; Silva, M.A.A.P. (2005). Evaluation of amaranth flour in the preparation of gluten-free cookie. *Brazilian Journal of Food Technology*, Campinas, 8 (2), 75-181.

Mark-Herbert, C. (2004). Innovation of a new product category—Functional foods. *Technovation, 24,* 713–719.

Marrubini, G., Appelblad, P., Gazzani, G., & Papetti, A. (2015). Determination of free quinic acid in food matrices by Hydrophilic Interaction Liquid Chromatography with UV detection. *Journal of Food Composition and Analysis, 44,* 80–85.

Martí, N., Mena, P., Cánovas, J.A.; Micol, V. and Saura, D. (2009). Vitamin C and the role of citrus juices as functional food. *Natural Products Communications, Alicante, 4* (5), 677-700.

Martín L., Mainar A.M., Gonzalez-Coloma A., Burillo J. and Urieta J.S. (2011). "Supercritical fluid extraction of wormwood (*Artemisia absinthium* L.)". *Journal of Supercritical Fluids, 56* (1), 64–71.

Matthews, V.L., Knutsen S.F., Beeson W.L. and Fraser G.E. (2011). Soy milk and dairy consumption is independently associated with ultrasound attenuation of the heel bone among postmenopausal women: the Adventist health study-2. *Nutrition Research, 31* (10), 766–75. doi: 10.1016/j.nutres.2011.09.016.

Meng, J., Leung, K. S. Y., Jiang, Z., Dong, X., Zhao, Z., & Xu, L. J. (2005). Establishment of HPLC-DAD-MS fingerprint of fresh Houttuynia cordata. *Chemical and Pharmaceutical Bulletin, 53,* 1604–1609.

Menrad, K. (2003). Market and marketing of functional food in Europe. *Journal of Food Engineering, 56,* 181–188.

Milner, J. A. (2002). Functional foods and health: a US perspective. *Br J Nutr, 88* (2), 151-158.

Miyazawa, T., Shibata, A., Sookwong, P., Kawakami, Y., Eitsuka, T., Asai, A., Oikawa, S. and Nakagawa, K. (2009). Antiangiogenic and anticancer potential of unsaturated vitamin E (tocotrienol). *Journal of Nutritional Biochemistry, Okayama, 20* (2), 79- 86.

Mona, I.M., Wafaa, A.A. and Elgindy, A.A. (2009). Chemical and Technological Studies on Chicory (*Cichorium Intybus* L) and Its Applications in Some Functional Food. J. Adv. Agric. Res. (Fac. Ag. Saba Basha) v. 14 (3).

Montes, E., C. De Paula y F. Ortega. 2007. Determinación de las condiciones óptimas de encapsulamiento por co-cristalización de jugo de maracuya (*Passiflora edulis*). Revista Temas Agrarios 12: 5-12.

Moraes, F.P. and Colla, L.M. (2006). Alimentos funcionais e nutracêuticos: definições, legislação e benefícios à saúde. *Revista Eletrônica de Farmácia, Passo Fundo, 3* (2), 109-122.

Morais, M.G.; Miranda, M.Z.; Costa, J.A.V. (2006). Chocolate cookies enriched with *Spirulina platensis*: physical-chemical, sensory and digestibility characteristics. *Food and Nutrition*, Araraquara, 17 (3), 323-328.

Nieves, J. W., Melsop K., Curtis M., Kelsey J.L., Bachrach L.K., Greendale G., Sowers M.F., and Sainani K.L. (2010). Nutritional factors that influence change in bone density and stress fracture risk among young female cross-country runners. *Pm R ,2* (8), 740–50. Quiz 794. doi: 10.1016/j.pmrj.2010.04.020.

Moura, F.A.; Spier, F.; Zavareze, E.R.; Dias, A.R.G.; Elias, M.C. (2010). Cookies type "cookies" made with different fractions of pumpkin seed (*Curcubita maxi*ma). *Food and Nutri*tion, Araraquara, v. 21 (4), 579-585.

Mugheesa Mohd, Wajida Saima and Samimb Mohd. (2020). Cytotoxic potential of Artemisia absinthium extract loaded polymeric nanoparticles against breast cancer cells: Insight into the protein target. *International Journal of Pharmaceutics*, India, 586.

Nuengchamnong, N., Krittasilp, K., and Ingkaninan, K. (2009). Rapid screening and identification of antioxidants in aqueous extracts of Houttuynia cordata using LC-ESI-MS coupled with DPPH assay. *Food Chemistry, 117*, 750–756.

Oliveira, G. D. A. R., De Oliveira, A. E., Da Conceição, E. C., and Leles, M. I. G. (2016). Multiresponse optimization of an extraction procedure of carnosol and rosmarinic and carnosic acids from rosemary. *Food Chemistry*. Vol. 211, 465–473.

Oyaizu, M. (1986). "Studies of products of browning reactions: Antioxidative activities of products of browning reaction propagated from glucosamine". *Japanese Journal of Nutrition*. 44, 307-315.

Pacheco-Palencia L.A., Duncan C.E. and Talcott S.T. (2009). Phytochemical composition and thermal stability of two commercial acai species, *Euterpe oleracea* and *Euterpe precatoria*. *Food Chemistry, 115*, 1199-1205.

Pedrosa, M. C., Ueda, J. M., Melgar, B., In, M., Pinela, J., Calhelha, R. C., Ivanov, M., Sokovi, M., Heleno, S., Bruna, A., & Barros, L. (2021). *Preservation of Chocolate Muffins with Lemon Balm, Oregano, and Rosemary Extracts*. 1–18.

Peñarrieta J. M., Alvarado J. A., Bergenståhl. B and Åkesson B. (2014). Total antioxidant capacity and content of phenolic compounds in wild Strawberries (Fragaria vesca) collected in Bolivia. *International Journal of Fruit Science, 9*, 344-359.

Peñarrieta J.M, Tejeda L., Mollinedo P., Vila J.L., Bravo J.A. (2014). Compuestos fenólicos y su presencia en alimentos. *Revista Boliviana de Química*, 31 (2), 68-81.

Pereira, E., Barros, L., Martins, A., & Ferreira, I. C. F. R. (2012). Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats. *Food Chemistry*, 130, 394–403.

Pereira, A. G., Fraga-Corral, M., Garciá-Oliveira, P., Jimenez-Lopez, C., Lourenço-Lopes, C., Carpena, M., Otero, P., Gullón, P., Prieto, M. A., & Simal-Gandara, J. (2020). Culinary and nutritional value of edible wild plants from northern Spain rich in phenolic compounds with potential health benefits. In *Food and Function, 11*, 8493–8515.

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F. and Matuchansky, C. (2005). Bifidobacteria as a probiotic agent - physiological effects and clinical benefits. *Alimentary Pharmacology & Therapeutics, Palaiseau, 22* (6), 495-512.

Pimentel, B. M. V.; Francki, M.; Gollücke, B. P. (2005). Alimentos funcionais: introdução as principais substâncias bioativas em alimentos. *São Paulo: Ed. Varella.*

Prasad, A.S. (2008). Clinical, immunological, antiinflammatory and antioxidant roles of zinc. *Experimental Gerontology, Detroid, 43* (5), 370-377.

Prasad, A.S., Beck, F.W.J., Snell, D.C. and Kucuk, O. (2009). Zinc in cancer prevention. *Nutrition and Cancer, Netherlands, 61* (6), 879-887.

Prior, R.L.; Wu, X.L.; Schaich, K, 2005. "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements". *Journal of Agricultural and Food Chemistry*. 53, 4290-4320.

Ramful, D., Bahorun, T., Bourdon, E., Tarnus, E. and Aruoma, O.I. (2010). Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: potential prophylactic ingredients for functional foods application. *Toxicology, Réduit, 28* (278), 75-87.

Reglero, G., Frial, P., Cifuentes, A., García-Risco, M.R., Jaime, L., Marin, F.R., Palanca, V., Ruiz-Rodríguez, A., Santoyo, S., Señoráns, F.J., Soler-Rivas, C., Torres, C., Ibañez, E. (2008). Meat-based functional foods for dietary equilibrium omega-6/omega-3. *Molecular Nutrition and Food Research, Madrid, 52* (10), 1153-1161.

Reid, G., Sanders, M.E., Gaskins, H.R., Gibson, G.R., Mercenier, A., Rastall, R., Roberfroid, M., Rowland, I., Cherbut, C. and Klaenhammer, T. R. (2003). New scientific paradigms for probiotics and prebiotics. *Journal of Clinical Gastroenterology, Ontario, 37* (2), 105-118.

Rezaeinodehi A. and Khangholi S. (2008). "Chemical composition of the essential oil of *Artemisia absinthium* growing wild in Iran". *Pakistan Journal of Biological Sciences, 11* (6), 946–949.

RHS - Royal Horticultural Society, UK. (2018). https://www.rhs.org.uk/Plants/90526/i-Houttuynia-cordata-i-Chameleon-(v)/Details.

Roberfroid MB. (2000). Concepts and strategy of functional food science: the European perspective. *Am J Clin Nutr, 71* (6), 1660S-5S. doi:10.1093/ajcn/71.6.1660S.

Roberfroid MB. (2002). Global view on functional foods: European perspectives. *Br J Nutr.*; *88* (2), S133-S138. doi:10.1079/BJN2002677.

Roberfroid, M. B. (2002a). Functional food concept and its application to prebiotics. *Digestive and Liver Disease, Louvain, 34* (2), 105-110.

Roberfroid, M. B. (2002b). Functional foods: concepts and application to inulin and oligofructose. *British Journal of Nutrition, Brussels, 87* (2), 139-143.

Rodríguez, M. B. S.; Megías, S. M.; Baena, B. M. (2003). Alimentos funcionales y nutrición óptima. *Revista da Espanha de Salut Pública, Madrid, 77* (3), 317- 331.

Ross, S. (2000). Functional foods: the Food and Drug Administration perspective. *Am J Clin Nutr, 71*, 1735-1738.

Şahin, S., Aybastier, Ö., & Işik, E. (2013). Optimisation of ultrasonic-assisted extraction of antioxidant compounds from Artemisia absinthium using response surface methodology. *Food Chemistry*, 141(2), 1361–1368. https://doi.org/10.1016/j.foodchem.2013.04.003

Salgado, J. M.; Almeida, M. A. (2009). Mercado de alimentos funcionais: desafios e tendências. Sociedade Brasileira de Alimentos Funcionais (SBAF).

Santos, A.A.O.; Santos, A.J.A.O.; Silva, I.C.V.; Leite, M.L.C.; Soares, S.M.; Marcellini, P.S. (2010). Development of chocolate chip cookies based on the incorporation of cassava starch and orange albedo. *Food and Nutrition,* Araraquara, 21 (3). 469-480.

Santos, A.A.O.; Silva, I.V.C.; Santos, J.P.A.; Santana, D.G.; Almeida, M.L.; Marcellini, P.S. (2011). Elaboration of chocolate cookies with partial replacement of

wheat flour with sour flour and orange albedo flour. *Ciência Rural*, Santa Maria, 41(3), 531-536. http://dx.doi. org/10.1590/S0103-84782011000300028.

Santucci, M.C.C. (2003). Effect of enriching water and salt cookies with yeast extract (*Saccharomyces sp*.). *Food Science and Technology*, 23 (3), 441-446.

Sarmiento Rubiano, L.A. (2006). Alimentos funcionales, una nueva alternativa de alimentación. O*rinoquia*, *10 (1)*, 16-23. ISSN: 0121-3709.

Scalbert A., Johnson I.T., Saltmarsh M. (2005). Polyphenols: antioxidants and beyond. *Am J Clin Nutr, 81 (1)*, 215–217.

Side, C. (2006). Overview on marketing functional foods in Europe. Functional food network general meeting. (Cited from Siro, I., Kapolna, E., Kapolna, B., and Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance) - A review. *Appetite* 51(3): 456-467.

Silva, M.R.; Borges, S.; Martins, K.A. (2001a). Chemical, physical and sensory evaluation of biscuits enriched with jatobá-do-cerrado flour and jatobá-da-mata as a source of dietary fiber. *Brazilian Journal of Food Technology*, Campinas, 4, 173-170.

Silva A.R., Pinela, J., Dias, M.I., Calhelha, R.C., Alves, M.J., Mocan, A., García, P.A., Barros, L., Fereira, I.C.F.R. (2020). Exploring the phytochemical profile of *Cytinus hypocistis* (L.) L. as a source of health-promoting biomolecules behind its in vitro bioactive and enzyme inhibitory properties. *Food and Chemical Toxicology*. Vol. 136, p. 111071.

Sik, B., Hanczné, E. L., Kapcsándi, V., & Ajtony, Z. (2020). Conventional and nonconventional extraction techniques for optimal extraction processes of rosmarinic acid from six Lamiaceae plants as determined by HPLC-DAD measurement. *Journal of Pharmaceutical and Biomedical Analysis*. Vol. 184.

Soccol, C.R., Vandenberghe, L.P.D.S., Medeiros, A.B.P., Yamaguishi, C.T., De Dea lindner, J., Pandey, A. and Thomaz-Soccol, V. (2010). The potential of the probiotics: a review. *Food Technology and Biotechnology, Curitiba, 48* (4), 413-434.

Socha, P., Stolarczyk, M. And Socha, J. (2002). Wpływ probiotyków i prebiotyków na gospodark, e lipidow, a. Pediatr. Współcz. Gastroenterol. Hepatol. Zyw. *Dziecka, 4*, 85–88.

Stanton C, Ross R.P, Fitzgerald G.F and Van Sinderen D. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. *Curr Opin Biotechnol, 16(2),* 198-203. doi:10.1016/j.copbio.2005.02.008.

State Pharmacopoeia Commission of People's Republic of China. (2005). Pharmacopoeia of the People's Republic of China (p. 155). Beijing, China: Chemical Industry Press.

Szajewska, H. and Mrukowicz, J. (2005). Meta-analysis: non-pathogenic yeast Saccharomices boulardii in the prevention of antibiotic - associated diarrhoea. *Alimentary Pharmacology & Therapeutics, Varsóvia*, *22* (3), 365-372.

Jang, S. A., Park, D. W., Kwon, J. E., Song, H. S., Park, B., Jeon, H., Sohn, E. H., Koo, H. J., & Kang, S. C. (2017). Quinic acid inhibits vascular inflammation in TNF-α-stimulated vascular smooth muscle cells. *Biomedicine and Pharmacotherapy*, 96, 563–571.

Kim, J. K., Shin, E. C., Lim, H. J., Choi, S. J., Kim, C. R., Suh, S. H., Kim, C. J., Park, G. G., Park, C. S., Kim, H. K., Choi, J. H., Song, S. W., & Shin, D. H. (2015). Characterization of Nutritional Composition, Antioxidative Capacity, and Sensory Attributes of Seomae Mugwort, a Native Korean Variety of Artemisia argyi H. Lév. & Vaniot. *Journal of Analytical Methods in Chemistry*, 1–9.

Liu, T. ting, Wu, H. bin, Wu, H. bo, & Zhang, J. (2019). Wormwood (Artemisia absinthium L.) as a promising nematicidal and antifungal agent: Chemical composition, comparison of extraction techniques and bioassay-guided isolation. *Industrial Crops and Products*, 133, 295–303.

Marangoni, F., Agostoni, C., Borghi, C., Catapano, A. L., Cena, H., Ghiselli, A., La Vecchia, C., Lercker, G., Manzato, E., Pirillo, A., Riccardi, G., Risé, P., Visioli, F., & Poli, A. (2020). Dietary linoleic acid and human health: Focus on cardiovascular and cardiometabolic effects. In *Atherosclerosis*, 292, 90–98.

Marrubini, G., Appelblad, P., Gazzani, G., & Papetti, A. (2015). Determination of free quinic acid in food matrices by Hydrophilic Interaction Liquid Chromatography with UV detection. *Journal of Food Composition and Analysis*, 44, 80–85.

Pereira, A. G., Fraga-Corral, M., Garciá-Oliveira, P., Jimenez-Lopez, C., Lourenço-Lopes, C., Carpena, M., Otero, P., Gullón, P., Prieto, M. A., and Simal-Gandara, J. (2020). Culinary and nutritional value of edible wild plants from northern Spain rich in phenolic compounds with potential health benefits. In *Food and Function*, 11, 8493–8515.

Rajesh Kumar Joshi. (2013). Volatile composition and antimicrobial activity of the essential oil of Artemisia absinthium growing in Western Ghats region of North West Karnataka, India, *Pharmaceutical Biology*, 51:7, 888-892.

Shafi, G., Hasan, T. N., Syed, N. A., Al-Hazzani, A. A., Alshatwi, A. A., Jyothi, A., & Munshi, A. (2012). Artemisia absinthium (AA): A novel potential complementary and alternative medicine for breast cancer. *Molecular Biology Reports*, 39(7), 7373–7379.

Stanković, N., Mihajilov-Krstev, T., Zlatković, B., Stankov-Jovanović, V., Mitić, V., Jović, J., Čomić, L., Kocić, B., & Bernstein, N. (2016). Antibacterial and Antioxidant Activity of Traditional Medicinal Plants from the Balkan Peninsula. *NJAS - Wageningen Journal of Life Sciences, 78*, 21–28.

Szparaga, A., Kocira, S., Kapusta, I., & Zaguła, G. (2021). Prototyping extracts from Artemisia absinthium L. for their biostimulating properties yield-enhancing, and farmer income-increasing properties. *Industrial Crops and Products, 160*, 113125.

Tahir, N.I.; Shaari, K.; Abas, F.; Parveez, G.K.A.; Ishak, Z.; Ramli, U.S. Characterization of apigenin and luteolin derivatives from oil palm (Elaeis guineensis Jacq.) Leaf using LC-ESI-MS/MS. *J. Agric. Food Chem*. 2012, 60, 11201–11210, doi:10.1021/jf303267e.

Tariq K.A., Chishti M.Z., Ahmad F. and Shawl A.S. (2009). "Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes". *Veterinary Parasitology, 160* (1-2), 83–88.

Tejeda L, Alvarado J, Dębiec M, Peñarrieta J.M, Cardenas O, Alvarez M.T, Chawade A, Bergenståhl B. (2014). Relating genes in the biosynthesis of the polyphenol composition of Andean colored potato collection. *Food Science and Nutrition, 2*, 46-57.

Vermerris W. and Nicholson, R. (2008). Phenolic compound biochemistry, Springer Science+Business Media B.V., Berlin. ISBN 978-1-4020-9289-3.

Vichai, V. and Kirtikara, K. (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols*, 1, 1112-1116.

Vieira da Silva B.; Barreira JCM and Oliveira MBPP. (2016). Natural phytochemicals and probiotics as bioactive ingredients for functional foods: extraction, biochemistry and protected-delivery technologies. *Trends Food Sci Technol, 50*, 144-158.

Wake, G., Court, J., Pickering, A., Lewis, R., Wilkins, R., Perry, E., 2000. CNS acetylcholine receptor activity in European medicinal plants traditionally used to improve failing memory. *J. Ethnopharmacol.* 69, 105–114.

Wan J, Wilcock A, Coventry MJ. (1998). The effect of essential oils of basil on the growth of Aeromonas hydrophila and Pseudomonas fluorescens. *Appl Microbiol* 84:152–8.

Weststrate JA., Van Poppel G. and Verschuren PM. (2002). Functional foods, trends and future. *Br J Nutr.*, 88 (2), 233-235. doi:10.1079/BJN2002688.

Wolfe, K.L., et al., Cellular antioxidant activity of common fruits. *J Agric Food Chem*, 2008. 56(18): p. 8418-26.

Wright C. W., (2002). *Artemisia*, Taylor & Francis, New York, NY, USA. Doi: org/10.1201/9780203303061.

Yañez, J., J. Salazar, L. Chaires, J. Jimenez, M. Marquez y E. Ramos. 2002. Aplicaciones biotecnológicas de la microencapsulación. *Revista Avance y Perspectiva*, 21, 313-319.

Yoon, E.H., Noh H., Lee H.M., Hwang H.S., Park H.K. and Park Y.S. (2012). Bone mineral density and food-frequency in Korean adults: The 2008 and 2009 Korea national health and nutrition examination survey. *Korean Journal of Family Medicine, 33* (5), 287–295. doi: 10.4082/kjfm.2012.33.5.287.

Ziani, B.E.C., Heleno, S.A., Bachari, K., Dias, M.I., Alves, M.J., Barros, L., Ferreira, I.C.F.R.,9 (2019). Phenolic compounds characterization by LC-DAD-ESI/MSn and bioactive properties of *Thymus algeriensis* Boiss. & Reut. and *Ephedra alata* Decne. *Food Research International*. Vol. 116, p. 312-319.