Universitatea "Lucian Blaga" din Sibiu

Interdisciplinary PhD school

PhD domain: Industrial Engineering

PHD THESIS SUMMARY

VALORIZATION OF BIOACTIVE COMPOUNDS FROM VEGETABLES WITH POTENTIAL FOR THE DEVELOPMENT OF INNOVATIVE NATURAL PRODUCTS

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SIBIU 2021

SUMMARY

This doctoral thesis is a personal approach in order to capitalize on the bioactive components of vegetables (pumpkin, parsnip root, red cabbage), with potential for the development of innovative natural products.Following the analysis in the literature, the 3 categories of compounds with immunostimulatory potential (anthocyanins, carotenes, inulin-type fructans) were established, and the sources rich in these compounds, selected based on sustainability criteria, based on the following aspects: native plant species, untapped for the purposes set out in this PhD thesis, economically accessible raw materials with a high content of bioactive compounds with a beneficial role, especially in the immune system.

To perform the extractions, different process parameters were tested, such as time, extraction temperature, solvent / solid ratio and various solvents, including environmentally friendly ones (water, ethanol of different concentrations, vegetable oil). Classical (maceration) and modern extraction methods (ultrasound-assisted extraction and enzyme-assisted extraction) and combinations of the two categories of methods were also tested. Among the unconventional methods, in the present study the extraction technique was applied for the first time using an enzymatic combination of pectinase and cellulase, on the selected plant materials, pre-treated by drying or lyophilization. Also, different solvent / solid ratios were tested as process parameters of ultrasonic maceration and extraction techniques, in order to use a minimum amount of solvent.

For the formulation of innovative products such as a food supplement with bioactive potential, the extracts obtained by applying the optimal extraction parameters determined in the first experimental part were used. In the second experimental part, two new emulsion products were developed, with practical application as antioxidant and anti-inflammatory food supplements. For their stabilization, natural additives (guar gum selected from complex preliminary tests) were used, with the main purpose of slowing down the kinetic destabilization. Thus, emulsion products were obtained, with a stability of up to 8 days, by the exclusive use of natural vegetable ingredients, approved by vegetarian consumers, whose anti-inflammatory action has been experimentally validated, being comparable to that of acetylsalicylic acid (aspirin).

The results obtained in this doctoral research bring original contributions of an applicative nature, being disseminated by publishing scientific articles in ISI-listed journals,

BDI, by presenting papers at national and international conferences in the field, by submitting an OSIM patent proposal.

KEY WORDS: antioxidants, anthocyanins, carotenes, cellulase, bioactive compounds, pumpkin pie, DPPH, emulsion, enzymes, FRAP, red cabbage leaves, immunostimulator, inulin, maceration, pectinase, polyphenols, rootstock, root.

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INTRODUCTION

The pharmaceutical and food supplements industry is in a continuous development, especially the phytotherapeutic branch that focuses on the development of products designed to improve health, herbal health but also the improvement of industrial production processes and quality control of these products. In this sense, numerous national and international researches are made on different species of plants, to identify the bioactive components in their composition, but also to improve the extractive processes and testing their biochemical properties and effects on the human body.

The idea of developing in this doctoral thesis an immunostimulatory food supplement started from the premise that, regardless of the lifestyle adopted, we currently face the problem of eating vegetables and fruits that are harvested prematurely, which means that their nutritional value is diminished, which leads to an imbalance in the body and, implicitly, an imbalance of the immune system. Because of this, there is a need to supplement the intake of vitamins, minerals and other biomolecules needed by the body. To achieve this goal, efficient extraction methods have been proposed which have been subsequently verified experimentally.

Food supplements are considered food products in the form of doses, which contain high concentrations of nutrients, the consumption of which aims to supplement the diet with various nutrients (Ministry of Public Health, 2007).

It has been scientifically proven that the immune system is sensitive to various factors, and stress is one of the elements that directly affects immunity. The mechanism by which it acts negatively on immunity is that under the influence of stress, the body produces a large amount of reactive oxygen species compared to enzymatic and non-enzymatic antioxidants that should fight them, leading to an imbalance. (Krishnaiah et al., 2011). Based on this theory, most studies on products with immunostimulatory properties have shown a beneficial effect on immunity through their antioxidant action and protective effect on immune cells against reactive oxygen species.

The present study aims to develop a plant-based product (vegetable), with a specific and unique composition, being effective and different from other products already on the market, through an innovative combination of biomolecules essential for the immune system. Moreover, the theme aims at the sustainable capitalization of some plants cultivated in the biodiversity of our country, other than the classic ones (medicinal plants, spices, fruits), the designed product being sustainable and suitable even for vegetarians.

This paper can be associated with the concepts of sustainability and sustainable development due to the fact that it is based on their principles. By using renewable raw materials and working methods with “environmentally friendly” reagents, access to safe food supplements for human consumption is ensured. Also, the selection of working methods is based on the principle of minimum reagent consumption, which is in line with the objective of the National Strategy for Sustainable Development of Romania Horizons 2013-2020-2030, which aims to use a small volume of energy and material resources.

The immune system is the body's defense system against infections. In the body, the body's protection is ensured by 3 main lines of defense: mucous membranes and skin secretions, leukocytes, antimicrobial proteins / peptides and the inflammatory response and the immune system, represented by lymphocytes and antibodies (Oancea, 2010). If these defense mechanisms are not sufficient, either preventive (with food supplements) or therapeutic (with specific drugs) intervention is needed.

Bioactive components in plants are bioelements in the composition of plant resources, with specific effects on the body. They can have catalytic properties of some chemical reactions inside the body, they can be connecting elements between different metabolic pathways or they can have a protective effect on cells. Knowing the pharmacological properties of biological compounds allows the development of appropriate extraction techniques, through which bioextracts can be obtained that are important ingredients in the industrial development of products of interest.

Among the bioactive components, those identified as necessary for the immune system are natural antioxidants such as anthocyanins, carotenes or micronutrients. These biomolecules have the property of protecting the immune system against the oxidative activity of free radicals. Prebiotics such as polyfructosaccharides have also been shown to have an indirect immunostimulatory effect by stimulating the growth of beneficial bacterial species in the gut and by increasing the concentration of immunoglobulin A (IgA) in tissues (Oancea, 2010).

In this context, the main purpose of this doctoral thesis is to formulate a new recipe for the development of a food supplement with immunostimulatory properties, obtained from bioactive extracts from carefully selected plants.

To achieve the proposed goal, the established research objectives are:

- Identification and careful selection of some raw materials, mainly local, with high content of biomolecules with immunostimulatory role (anthocyanins, inulin, carotenoids) suitable for achieving the purpose of the thesis.
- Extraction, physico-chemical characterization and evaluation of the stability of biologically active compounds in selected plants - use of specific classical and modern extraction methods and selection of that method to isolate high-yielding compounds and preserve bioactivity.
- Obtaining food supplement formulas based on a specific combination of plant extracts obtained (elaboration of the technological recipe, the form of presentation, the physico-chemical characteristics of the different compositions developed).
- Evaluation of the concentration of targeted bioactive components, both from extracts and from the final compositions developed.
- Validation of the properties of the compositions developed by *in vitro* testing of biological effects.

In order to accomplish this doctoral thesis and to go through these objectives, the thesis was elaborated in a number of 187 pages, being structured in 5 main chapters, organized in corresponding subchapters, containing 67 figures and 16 tables.

Chapter 1 (“Introduction”) presents the necessity and motivation of choosing the topic by exposing the main objectives of the doctoral thesis.

Chapter 2 (“Contributions on the use of indigenous plant resources with a high content of bioactive compounds of interest for the development of new products”) describes the characterization of selected plant resources of interest, as well as the development of appropriate methods for extraction of targeted bioactive compounds.

Chapter 3 (“Experimental research on obtaining and characterizing bioextracts rich in biologically active principles from selected raw materials)” presents the experimental results obtained from the extraction of bioactive compounds by various techniques.

Chapter 4 ("Experimental research to validate the immunostimulatory properties of new products based on prepared bioextracts") contains data on the development and development of two innovative food products, food supplement type, their physico-chemical characterization, as well as and validation of bioactive properties.

Chapter 5 ("Final conclusions, own contributions and future research directions") contains the main conclusions of this doctoral dissertation, own contributions in the field, both in the applied and theoretical sphere, and sets out new research directions derived from the topic proposed.

1. DOCUMENTARY STUDY - SUMMARY

IDENTIFICATION OF BIOACTIVE COMPONENTS ESSENTIAL FOR THE DEVELOPMENT OF A NATURAL IMMUNOSTIMULATING PRODUCT

The immune system needs many resources to function. In addition to vitamins and minerals essential to the immune system, which are now well known, there are other biomolecules that have proven a beneficial effect, sometimes even greater on immunity through their direct or indirect action on various components of the body's defense system.

Healthy nutrition is the key element of immune responses, with malnutrition being the most common cause of immunodeficiency worldwide. Through food, the body benefits from the necessary vitamins and minerals but also from biologically active compounds that have various beneficial effects on the biological processes in the body.

For optimal functioning, the immune system especially needs vitamins A, B6, B9, B12, C, D, E, and the trace elements iron, zinc, copper and selenium, but there are phytocompounds that have proven immunomodulatory activities through direct action indirect. These include natural antioxidants in the category of carotenes and phenolic compounds that include the class of anthocyanins, with direct action, which have a protective effect on the cells of the immune system and which stimulate or inhibit the synthesis of compounds involved in cellular signaling in biological processes.

On the other hand, in addition to the beneficial effects on the intestinal microflora, prebiotics have immunostimulatory properties both directly and indirectly, being involved in increasing the production efficiency of some essential components of the immune system.

Knowledge of the pharmacological properties of these compounds allows the development of appropriate methods for their extraction from plant matrices. The bioextracts obtained can be important ingredients in the industrial development of some products of interest.

In order to develop a 100% natural product, sustainable and effective, with immunostimulatory effects, in this doctoral thesis we identified and verified experimentally 3 types of bioactive compounds concentrated in vegetables, being natural antioxidants such as carotenes, anthocyanins and fructooligosaccharides.

CAROTENS

Carotenes are a group of natural pigments in the category of pollen, which have various structures and functions (Landrum, 2010). They are substances with special properties that no other group of substances possesses. Their properties are determined by their molecular structure, and the link between their structure, properties and functions is necessary to understand their importance in different biological contexts (Britton et al., 2008).

Over time, the characteristics of carotenes have been extensively studied by researchers, but their properties in simple organic solutions do not fully characterize the biological activity they have *in vivo*. In living organisms, carotenes are part of complex systems and are often found in organized subcellular structures, in which their chemical properties are influenced by the presence of other molecules in the vicinity. They are much more stable *in vivo* than after isolation (Britton et al., 2008).

Carotenes are hydrophobic compounds, which is why they tend to associate with lipids, often being integrated into the complex structure of cell membranes. This association depends a lot on the physical peculiarities, especially on the size, shape and presence of functional groups (Britton et al., 2008).

Regarding the biological significance, it is important to remember their role as precursors of metabolites that have different functions in the body (Britton et al., 2008).

Carotenoids bring many benefits to the health of the human body, due to the properties they possess, especially their antioxidant capacity that protects against the action of reactive oxygen species.

Oxidative stress is one of the leading causes of chronic diseases today. According to statistics published in 2018 by the World Health Organization (WHO), it is estimated that 1 in 6 deaths worldwide were caused by cancer, and the number is constantly growing (World Health Organization, 2018).

Due to their antioxidant activity, carotenoids have a beneficial effect on the cells of the immune system, which they protect against oxidative stress. With age, there are some changes in the immune system that cause it to weaken. It has been observed that an adequate intake of carotenoids causes an increase in the number of natural-killer cells, especially in people over the age of 50 (Farges et al., 2012).

Carotenoids, especially β-carotene, are known to function as precursors of vitamin A. Due to the low efficiency of converting β-carotene to vitamin A, some organisms, including humans, have developed routes of absorption and transport of it. organism (Chew, 1993). This finding has led to further research that has shown that in the human body, carotenoids have many other functions including the ability to directly influence the immune system. The immunomodulatory effects of carotenoids have been observed especially on lymphocytes (Chew, 1993). Numerous studies have also shown the direct link between a high-carotenoid diet and the low incidence of various cancers (Etminan et al., 2004; Nishino et al., 2002; Tanaka et al., 2012).

Studies in young subjects have shown that dietary supplementation with β-carotene has the effect of increasing resistance to infections and developing thymus (Seifter et al., 2012). An increase in the number of lymphocytes in the thymus of mice was also observed, whose diet was enriched with an amount of 4.3 mg β-carotene / kg of food. On the other hand, retinoic acid, which is a metabolic product of vitamin A, has had the effect of decreasing the lymphocyte population in the thymus (Seifter et al., 2012). Supplementing the diet of Holstein cows with an amount of 300-600 mg β-carotene / day for 4 weeks before and after the birth of the fetus had the effect of increasing the lymphocyte population by stimulating the process of mitosis (Heirman et al., 1990). Stimulation of lymphocyte proliferation due to the diet rich in β-carotene has also been observed in pig species (Neacşu et al., 2003) and mice (Benedich and Shapiro, 1986).

Due to its antioxidant effect, β-carotene protects the DNA of lymphocyte cells against γ-rays (Konopacka and Rzeszowska-Wolny, 2001). *In vitro* studies on lymphocyte cultures subjected to oxidative stress with hydrogen peroxide (H2O2) have shown that a concentration of 10,000 μg / ml β-carotene is sufficient to protect DNA against degradation (Al-Shaban et al., 2016).

In the elderly, β-carotene together with zinc contributes to the increase of the T lymphocyte population by about 31% after 6 months of administration. After an additional 3 months of dieting with zinc and β-carotene, an increase in the percentage of natural-killer cells of up to 121% was observed (Steven et al., 2000). Following the supplementation of the diet with 180 mg β-carotene / day for 7 days, an increase in the number of T lymphocytes was observed in healthy adults (Alexander et al., 1985).

ANTHOCYAINS

Anthocyanins are a group of secondary metabolites synthesized by higher plants as pigments responsible for the colors pink, red, purple, blue and some shades of orange, mainly fruits and flowers (Castañeda-Ovando et al., 2009). The term anthocyanins comes from the Greek language, being composed of anthos = flower and kyanos = dark blue (Delgado-Vargas and Paredes-Lopez, 2003). To date, more than 635 types of anthocyanins have been identified.

Their role in the plant kingdom is to protect plants against damage caused by UV radiation, but also to attract animals that help in the processes of pollination and dispersal of seeds (Mazza and Miniati, 1993).

Anthocyanins are plant pigments that have demonstrated strong antioxidant activity because they have the ability to donate a free electron or hydrogen atoms to reactive free radicals (Castañeda-Ovando et al., 2009; Oancea et al., 2013).

Free radicals, peroxides or singlet oxygen are reactive oxygen species that are produced in the body as a result of biological reactions. Their role is important because they influence the immune system and cellular signaling, but also other processes that commonly take place in the body. If produced in large quantities, reactive oxygen species become harmful, leading to cell degradation, aging, inflammation, cancer, and cardiovascular disease (Allen and Tresini, 2000). In this sense, anthocyanins have a potential beneficial effect as they have proven antioxidant properties both *in vitro* and *in vivo*. They have the ability to capture free radicals and stop the chain reaction that leads to oxidative degradation.

The antioxidant capacity of several types of anthocyanins was measured *in vitro* by the widely accepted ORAC method "oxygen radical absorbance capacity", under conditions similar to pH in the human body. The results led to the conclusion that they have an antioxidant capacity equal to or greater than that of vitamin E (Tsuda et al., 1994; Wang et al., 1997). Anthocyanins may also have higher antioxidant activity than vitamin C and β-carotenes (Kowalczyk et al, 2003).

An *in vitro* study of human erythrocytes treated with hydrogen peroxide as an oxidizing agent and red wine with a high content of anthocyanins has been shown to significantly reduce reactive oxygen species in erythrocytes (Tedesco et al., 2001).

According to data obtained from *in vitro* experiments, anthocyanins have a protective effect against oxidative stress, which is then demonstrated by *in vivo* studies performed in mice (Tsuda et al., 2000). Animal studies have shown an increase in the total level of antioxidants and a decrease in the content of anthocyanin-induced thiobarbituric acid (TBARS) reactive substances (Kowalczyk et al., 2001).

The consumption of anthocyanins has been associated with the prevention of cardiovascular diseases, which are generally caused by the accumulation of large amounts of oxidized lipoproteins, low density (LDL), which are deposited on the wall of arteries. Anthocyanins have the effect of increasing the antioxidant capacity of serum, thus reducing the oxidation efficiency of cholesterol (Aviram et al., 2005).

Having the ability to capture free radicals through the hydrogen they donate, their antioxidant power is higher than that of vitamins C and E (Rice-Evans et al., 1996), being known for their anticancer effects. These effects are largely based on *in vitro* studies. In this regard, anthocyanins extracted from the petals of some flowers have shown a much stronger effect on malignant intestinal carcinoma, unlike other types of flavonoids (Kamei et al., 1995). Similar conclusions were drawn from the study of the effects of anthocyanins in red wine on gastric cancer cell lines (Kamei et al., 1998). Studies of cancer cells derived from colon cancer, which have been treated with anthocyanins extracted from different types of grapes, have shown the antiproliferative effect it possesses (Yi et al., 2005). Pure anthocyanins isolated from strawberries have an inhibitory effect on cancer cells derived from colon, prostate and bone cancer (Zhang et al., 2008).

The antiproliferative effect of anthocyanins depends largely on their chemical structure, which refers to the type of aglycone and the acylation and glycosylation model (Jing et al., 2008).

A study performed on mice demonstrated the effect of protective anthocyanins against γ-radiation but also their immunostimulatory potential against radiation-induced immunosuppression. Supplementing nutrition with anthocyanins for 14 days before irradiation significantly reduced damage to the thymus and spleen compared to the control group. At the same time, a reduction in the number of micronuclei in bone marrow erythrocytes was observed, which indicated the protective effect of anthocyanins against the degradation of genetic material (Fan et al., 2012).

Inflammation is the body's response to tissue damage, being a process that promotes cancer, due to favorable environmental conditions for tumor development. For this reason, biological molecules with anti-inflammatory effect have the potential to prevent cancer progression and malignant conversion (Coussens and Werb, 2002).

The conversion of arachidonic acid into prostaglandins, which have the effect of stimulating the inflammatory process, takes place under the action of cyclooxygenase (Seeram et al., 2001). Studies have shown that cyanidin has inhibitory effects on cyclooxygenase, being stronger than those of anti-inflammatory drugs, such as aspirin (Wang et al., 1999), ibuprofen and naproxen, in a concentration of 10 μM (Seeram et al., 2001).

In vitro study of human colon cancer epithelial cell cultures (Caco-2) showed that anthocyanin extracts from currants with a concentration between 50 and 100 μg / ml reduce the activation process of the protein complex NF-κB (nuclear kappa-light-chain-enhancer factor of activated B cells) by up to 85.2%, having anti-inflammatory effect, thus reducing the process of uncontrolled division of cancer cells (Taverniti et al., 2014).

In addition to antioxidant activity, anthocyanins inhibit the expression of vascular cell adhesion protein (VCAM), thus reducing the process of leukocyte reaction with endothelial cells. On the other hand, they decrease the level of interleukin-2 (IL-2) and interferon γ (INF-γ) (Lin et al., 2002), inhibit mast cell degranulation (Middelton et al., 2000) and the production of tumor necrosis factor (TNF-α) (Lin et al, 2002).

INULIN-TYPE FRUCTANS

Fructans are part of the category of carbohydrates, consisting of 1 to 60 units of fructose, linked or not to a terminal molecule of sucrose. The linear structure characterized by β (2 → 6) fructosyl-fructose bonds is found in levan type fructans, while the β (2 → 1) bond is characteristic of inulin-type fructans (Carabin et al., 1999). The difference lies in the degree of polymerization, the inulin being characterized by a large variation in the number of monosaccharide units (Carabin et al., 1999).

Inulin is not metabolized in the human body, but can be hydrolyzed by the colon microbiota. Daily inulin consumption in Europe is estimated at 3-11 g / day (Bonnema et al., 2010), while in the American diet the values are between 1.3-3.5 g / day (Coussement, 1999).

In addition to the prebiotic function, inulin-type fructans have other functions in the body, acting both indirectly and directly. Their beneficial effects are often associated with the indirect mechanism of action that refers to the positive influence they have on the intestinal microflora (Gibson et al., 2004; Louis et al., 2016; Wopereis et al., 2018). Regular intake of fructans has been shown to have a positive effect on the overall well-being of the body, reducing the incidence of allergies and helping to increase resistance to pathogens in and out of the gut, lowering blood cholesterol levels, while facilitating mineral absorption. intestine (Delgrado et al., 2010; Scholoz-Ahrens et al., 2007).

Some research has suggested the potential beneficial effect of fructan use associated with reduced risk of colon cancer (Allsopp et al, 2013; Verma and Shukla, 2013).

Antioxidant capacity suggests a benefit in preventing diseases caused by reactive oxygen species and the positive effects in their treatment. In this sense, a link has been established between oxidative stress and the development of intestinal diseases (Bhattacharyya et al., 2014; Van den Ende et al., 2011).

In the intestine, fructans are subjected to the fermentation process under the action of the microbiota, which results in short-chain fatty acids. Fructans, like other carbohydrates, can bind to TLR2 (toll like receptor 2) and TLR4 (toll like receptor 4) receptors. This link has the effect of stimulating transcription factors, including NF-κB, which leads to the activation of different immune pathways depending on the cell type (Peshev and Van den Ende, 2014).On the other hand, fructans improve the rate of glucose absorption and stimulate protein kinase activity, which stops the inflammatory process either directly or by inhibiting the signal transmitted by NF-κB (Peshev and Van den Ende, 2014).

OTHER BIOMOLECULES WITH IMMUNOMODULATORY EFFECTS

Phenolic compounds are part of the category of phytochemicals with antioxidant properties, synthesized in all plants as a result of secondary metabolism. They react with lipid radicals, turning them into oxidatively stable compounds (Hurrel, 2003).

Quercetin, pelargonidine or kaempferol are flavonoids - a subclass of polyphenolic compounds, with proven immunomodulatory properties that inhibit the activation of the nuclear factor responsible for DNA transcription (NF-κB) by decreasing the production of tumor necrosis factor (TNF-α) and -β (IL-β), responsible in the process of lymphocyte activation (Cherng et al., 2008; Valles et al., 2010). *In vitro* studies performed on mononuclear cells in human peripheral blood have shown that flavonoids and coumarin in the composition of plants such as parsley or celery have the effect of activating lymphocytes and increasing the efficiency of IFN-γ synthesis (Cherng et al., 2008).

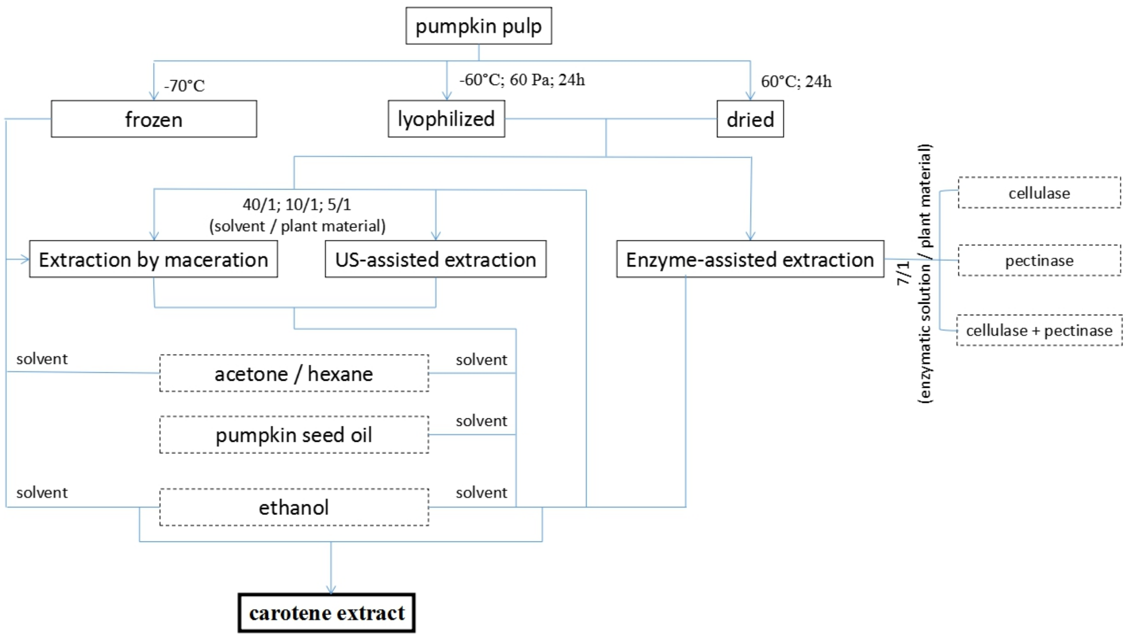
2. CONTRIBUTIONS REGARDING THE VALORIZATION OF INDIGENOUS VEGETABLE RESOURCES WITH HIGH CONTENT OF BIOACTIVE COMPOUNDS OF INTEREST FOR THE DEVELOPMENT OF NEW PRODUCTS - SUMMARY

RESEARCH OBJECTIVES

The objectives of this specific part of the doctoral thesis are:

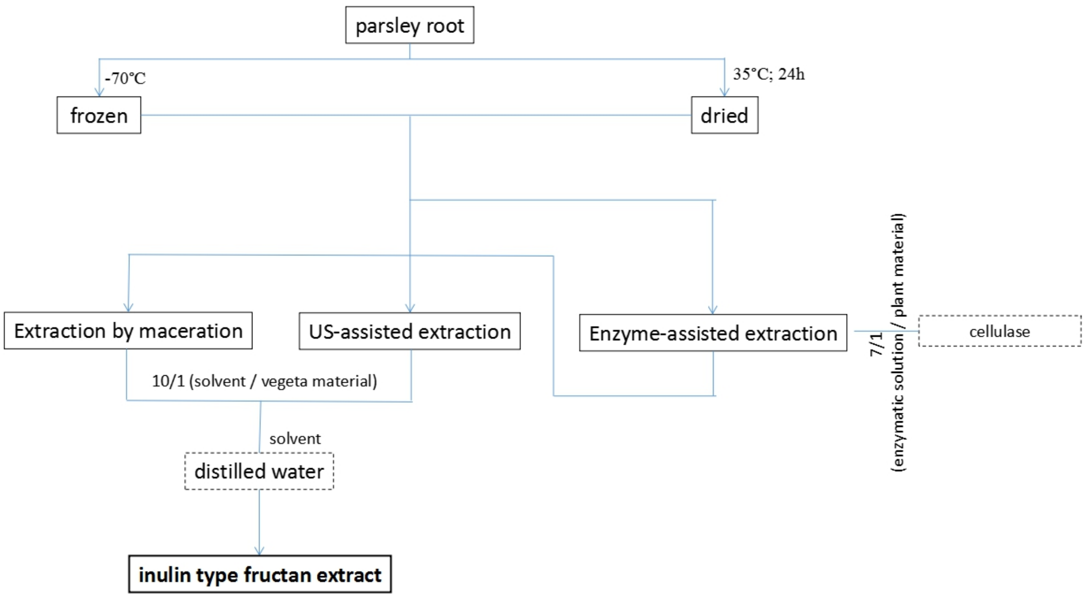
- identification and selection of sustainable local plant resources, other than medicinal plants that are often used for the development of food supplements, with a high content of biomolecules from the category of carotenes, anthocyanins and fructans
- elaboration of adequate methods for efficient extraction of the targeted biomolecules, in order to obtain bioactive extracts with high yield.

Development of methods for extracting bioactive compounds of interest from pumpkin pulp



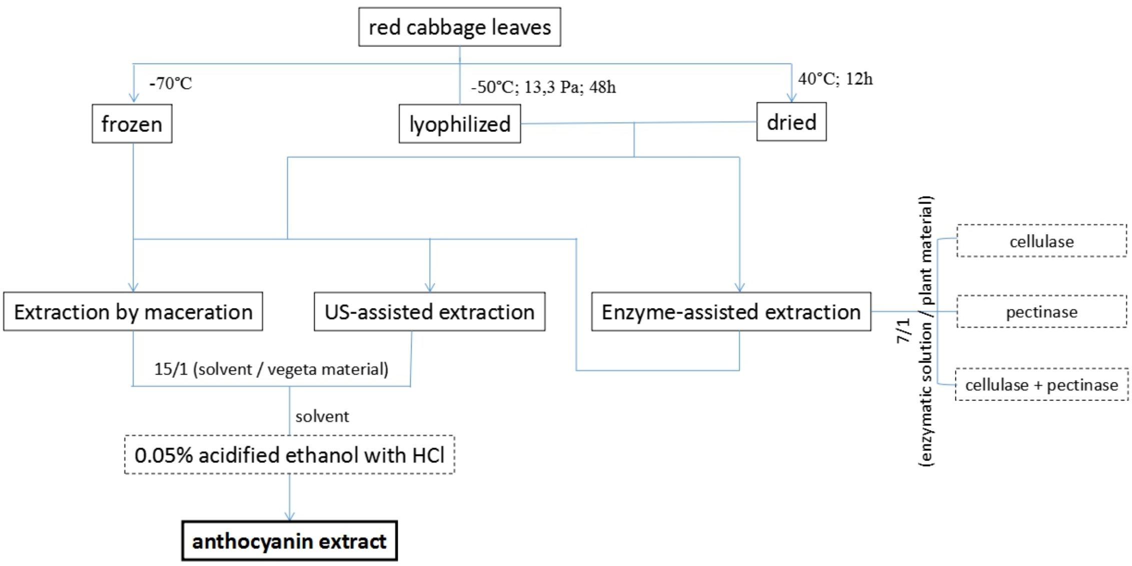
The extractive technologies of β-carotenes, applied to pumpkin pulp.

Development of methods for the extraction of bioactive compounds of interest from parsnip root



The extractive technologies of inulin, applied to parsnip root.

Development of methods for extracting bioactive compounds of interest from red cabbage



The extractive technologies of anthocyanins, applied to red cabbage leaves.

The plants identified as valuable raw materials for their bioactive potential and studied in this doctoral thesis were carefully selected, based on sustainability criteria.

In this specific part of the doctoral thesis were presented the criteria that formed the basis for the selection of raw materials and the development of efficient extraction methods of targeted bioactive compounds (β-carotene, inulin, anthocyanins). Thus, the raw materials were chosen on the basis of the following aspects: native plant species, unexploited or little exploited for the purposes set out in this thesis, economically accessible raw materials with a high content of bioactive compounds with beneficial role especially at the system level. immune. Natural antioxidants such as β-carotenes and polyphenolic compounds (eg anthocyanins) have a protective effect on the cells of the immune system, and prebiotics (inulin) help stimulate the immune system through direct or indirect action.

Due to the high content of carotenoid compounds, pumpkin pie (*Cucurbita maxima* local population var. Tudor) is a rich source of natural antioxidants, especially β-carotene.

Parsley root (*Pastinaca sativa*) is a rich source of inulin-type fructans, prebiotics with immunostimulatory effects. Also, polyphenolic compounds present in large quantities in aqueous extracts indicate a strong antioxidant effect.

Red cabbage (*Brassica oleracea* var. *Capitata* f. *Rubra*) is rich in anthocyanins, pigments that have a protective effect on oxidative degradation and immunostimulatory effect. In addition, due to its chemical composition, this raw material contributes to the proper intake of essential vitamins and minerals, being a rich source of vitamin C and other antioxidants.

Extractive techniques were selected taking into account both the possibility of extracting as many bioactive compounds as possible, but also the principles of sustainability regarding the minimum consumption of reagents and the consumption of those environmentally friendly solvents.

Maceration was tested as a classic method of extraction of bioactive molecules of interest from the three plant matrices, following the use of a solvent / solid ratio as low as possible, and of some solvents approved in the food industry (water, ethanol, vegetable oil).

Ultrasonic-assisted extraction was also tested as an unconventional technique, used to reduce the time and amount of solvent used for the three plant resources, the method being controlled in terms of process temperature, a high value can negatively influence the thermolabile compounds. (β-carotenes, anthocyanins).

To optimize the extraction of bioactive compounds of interest, a third combined extractive variant (biotechnological) was tested by which the three plant raw materials were subjected to an enzymatic treatment using cellulase and / or pectinase enzymes, followed by the extraction of bioactive compounds targeted by the classical method of maceration and respectively by modern ultrasound-assisted extraction technique.

3. EXPERIMENTAL RESEARCH ON OBTAINING AND CHARACTERIZING BIOEXTRACTS RICH IN BIOLOGICAL ACTIVE PRINCIPLES IN SELECTED RAW MATERIALS - SUMMARY

This chapter includes the experimental results obtained from analyzes performed in order to obtain extracts rich in bioactive compounds of interest, from selected plant resources (carotenes - from the pulp of *Cucurbita maxima*, inulin-type fructans - from the root of *Pastinaca sativa*, native population Bielas, anthocyanins - from the leaves of *Brassica oleracea* var. capitata f. rubra). In this sense, different process parameters were tested, such as time, extraction temperature, solvent / solid ratio and various solvents, including environmentally friendly ones (water, ethanol of different concentrations, vegetable oil). Classical (maceration) and modern extraction methods (ultrasound-assisted extraction and enzyme-assisted extraction) and combinations of the two categories of methods were also tested.

Among the unconventional methods, in the present study the extraction technique was applied for the first time using an enzymatic combination of pectinase and cellulase, on the selected plant materials, pre-treated by drying or lyophilization. Also, different solvent / solid ratios were tested as process parameters of ultrasonic maceration and extraction techniques, in order to use a minimum amount of solvent.

The experimental results obtained from the analysis of the content of bioactive molecules of interest from the extracts made by the 3 extractive techniques, were analyzed comparatively, in order to identify those that are most efficient, while respecting the principles of sustainability regarding minimum energy and reagents.

Comparing the obtained values, it was demonstrated that each of the 3 selected local plant resources has its peculiarities regarding the efficiency of the extraction technique applied and the process parameters used to obtain samples rich in active principles. Thus, the extraction of carotenes from pumpkin pulp was most efficiently achieved by the classical method of maceration, inulin from parsnip root was efficiently extracted by the enzyme-assisted extraction technique combined with the ultrasound-assisted extraction technique, and anthocyanins from red cabbage leaves were -they extracted efficiently by the enzyme-assisted technique combined with the classical method of maceration.

Following the comparative analysis of the experimental results obtained, the optimal extraction conditions were determined, further used for the optimal formulation of the newly developed food supplement, consisting of two aqueous extracts - anthocyanin extract and inulin-type fructan extract and an oily extract, made in oil obtained from sunflower seeds, enriched with β-carotene extracted from pumpkin pulp.

The studies aimed to determine the optimal conditions for the extraction of bioactive compounds from vegetables of local origin, mainly β-carotene from pumpkin pie, parsnip from parsley root and anthocyanins from red cabbage leaves. These bioactive compounds, along with other compounds such as polyphenols have immune-stimulating properties, being used in the following chapters for the development of an innovative food product.

The total content of bioactive compounds was determined under the influence of various extraction parameters, such as solvent, extraction temperature, solvent / solid ratio, time and amplitude of sound waves, using 3 extraction techniques, maceration, ultrasound-assisted extraction and enzyme-assisted extraction, as well as pre-treatments applied to the raw material (freezing, drying, freeze-drying).

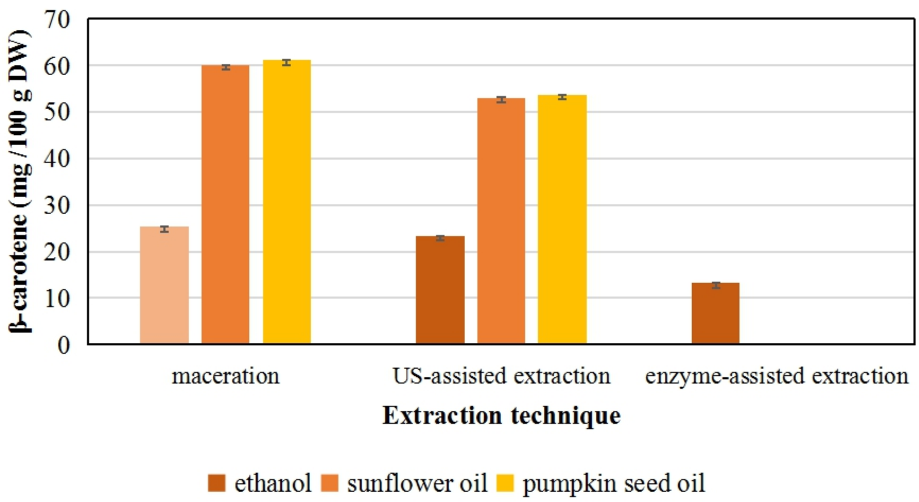
Regarding the extraction of β-carotene from pumpkin pulp, following the application of the 3 extraction techniques, using different combinations of parameters that could influence their amount the final extract, the highest content (62.6 ± 1.1 mg β -carotene / 100 g d.w.) was obtained in the lyophilized vegetable plant material, by applying the classical method of maceration, for 2 hours in the reference solvent, acetone / hexane (1/1), at room temperature, in solvent / solid ratio of 40/1. An appreciable carotene content of 61.13 mg β-carotene / 100 g d.w., was obtained in the extract made from the same type of plant material (lyophilized), using pumpkin seed oil obtained by cold pressing, as an alternative to organic solvent , environmentally friendly, in a solid solvent ratio of 10/1. Analyzing comparatively the experimental results obtained, it was observed that, regardless of the extraction technique applied and the solvent used, in the extracts from the lyophilized plant material were identified the highest contents of bioactive molecules of interest.

By applying ultrasound-assisted extraction, using the solvent / solid ratio of 10/1, the extraction time of 30 minutes, at the amplitude of the sound waves of 70%, lower values of β-carotene content were obtained in the pumpkin samples lyophilized, compared to those obtained by the classical method, these being mainly influenced by the extraction temperature.

By enzyme-assisted extraction technique, combined with maceration, after incubation of the lyophilised plant material with cellulase (122.5 U / g) and pectinase (100 U / g), for 120 minutes at 40 ° C, followed of conventional extraction by maceration in 70% ethanol, a β-carotene content was reduced by half compared to the values obtained by using the respective maceration techniques with US extraction, in the same solvent, the results being probably influenced by long exposure of thermolabile molecules of carotene at the action of higher temperatures during incubation with the enzyme solution.

To make the extract used to develop the new food supplement, the extraction by the classic method of maceration was applied, in pumpkin seed oil, using lyophilized vegetable material in a solvent / solid ratio of 10/1, for 30 minutes in this extract being determined a polyphenol content of 110.7 ± 3.5 mg GAE / 100g d.w..

Following the analysis of the antioxidant activity, determined by the FRAP method, in the extracts made in 70% ethanol, from the lyophilized pumpkin pulp, by applying the optimal parameters of the 3 extractive techniques, the highest content of 55.25 ± 1.5 mg acid ascorbic acid / 100 g d.w., was identified in the macerated sample for 30 minutes in a solvent / solid ratio of 10/1, this being 9% higher than the antioxidant activity of the extract obtained by assisted enzyme extraction combined with maceration, respectively with 12% higher than the one identified in the extract obtained by the modern US-assisted extraction technique.

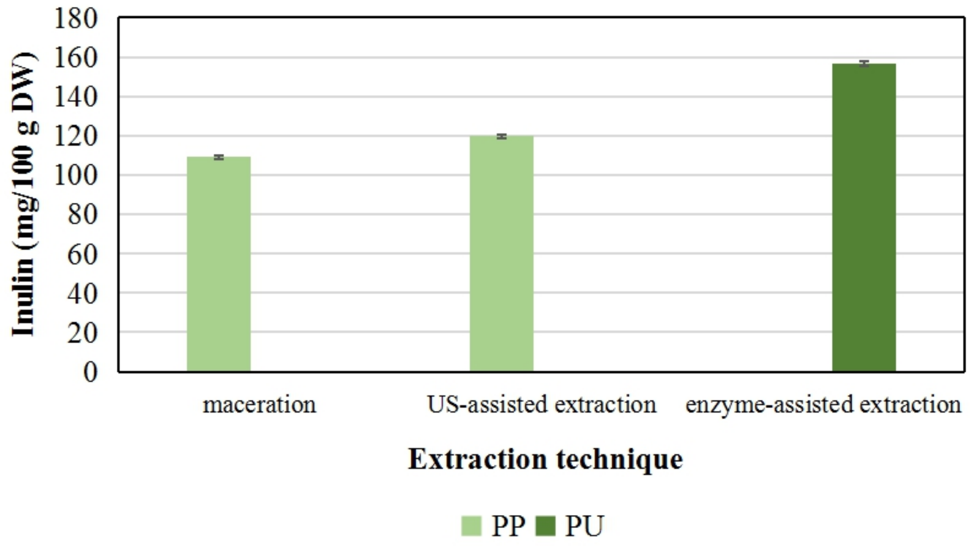


The β-carotene content of the DL sample, depending on the extraction technique applied and the type of solvent used (solvent / solid ratio = 10/1).

Regarding the extraction of inulin-type fructans from parsley root samples in distilled water, in a solvent / solid ratio of 10/1, using the classical method of extraction by maceration at 80 ° C, for 2 hours, in two successive stages, in the fresh sample, a content about 2 times higher was obtained compared to that obtained in the dry sample.

In the extracts made by the modern US extraction technique, for 5 minutes, at the amplitude of the acoustic waves of 70%, a content was obtained by approximately 9% higher compared to that obtained in the macerated extract for 2 hours, in two successive stages, in the fresh parsnip sample, a result indicating that the use of the unconventional extraction technique is a fast and efficient method of extracting inulin-type fructans from the parsnip root, supported by the theory and the results obtained in the dried parsnip samples, in which also an increase of about 20%.

Following the application of the two unconventional techniques (enzyme-assisted extraction and ultrasonic-assisted extraction) simultaneously, there was a dramatic increase in the content of inulin-type fructans, especially in the dried parsnip sample, in which a content of 156 was obtained. , 67 ± 3,1 mg inulin / 100 g d.w., which is approximately 60% higher than the values obtained in the other samples.



The content of inulin-type fructans obtained from the extraction of parsnip root by various extraction techniques, at the optimal parameters identified.

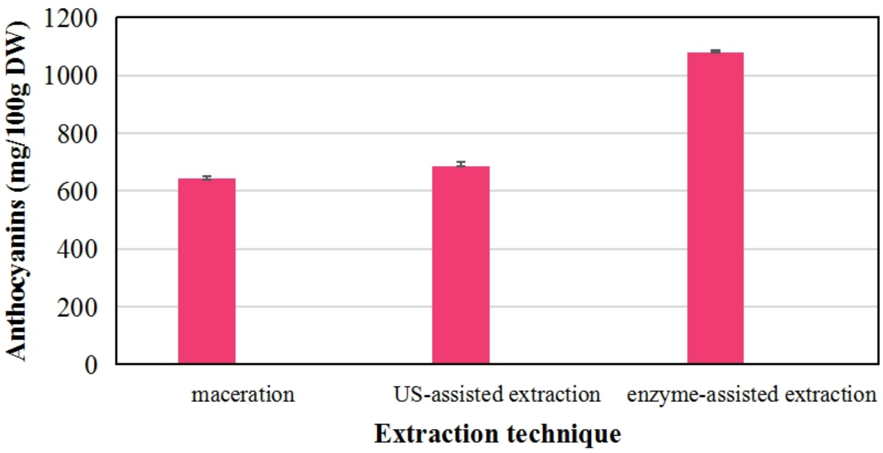
Analyzing the experimental results obtained, for the production of the final extract, rich in inulin-type fructans, from the composition of the newly developed food supplement, the combined extraction technique (enzyme-assisted extraction + US-assisted extraction) was used, using dried parsley root, incubated with cellulase (122.5 U / g), in enzyme solution / sample ratio of 7/1, for 180 minutes at 40 ° C, followed by extraction in distilled water by US assisted technique, for 5 minutes , at a solvent / solid ratio of 10/1.

The highest content of polyphenolic compounds in parsnip root (432.60 mg GAE / 100 g d.w.), was obtained in the dried plant material, incubated with cellulase at 40 ° C for 180 minutes, treatment followed by a US-assisted extraction in distilled water, in a solid solvent ratio of 10/1, for 5 minutes, at an amplitude of 70%, this value being about 20% higher than that obtained in the same type of plant material, following extraction with US for 5 minutes, without prior application of enzyme treatment.

Regarding the antioxidant activity of parsnip extracts, the best results were obtained in samples performed by combining the two unconventional techniques (enzyme-assisted extraction and ultrasound-assisted extraction), thus obtaining a content of 25.44 mg acid. ascorbic / 100 g d.w. in the dried parsnip sample.

By applying the classical method of extracting anthocyanins from red cabbage leaves, the highest content of 642,655 ± 8,2 mg / 100 g d.w.was obtained in the lyophilized plant material, macerated for 20 minutes at room temperature, in 50% ethanol, acidified 0.05% with HCl, solvent / solid ratio of 15/1.

By applying the modern US-assisted extraction technique, the highest anthocyanin content (687.34 ± 4.2 mg / 100g d.w.) was obtained in lyophilized red cabbage leaves at -50 ° C, after extraction for 20 minutes, at an amplitude of 70% sound waves, in 50% ethanol acidified with 0.05% HCl, in a solid solvent ratio of 15/1, thus obtaining an anthocyanin content of approximately 4.2% higher than of the one in the macerated sample.



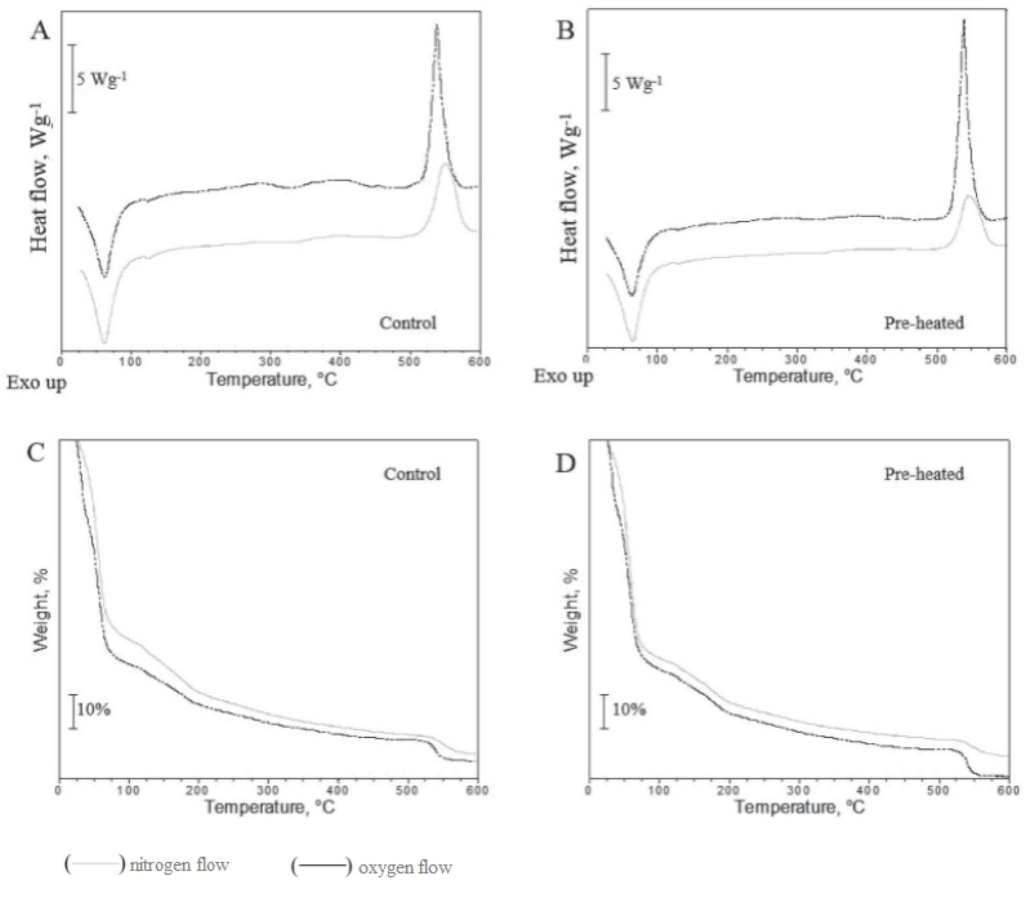
The anthocyanin contents obtained after extraction from lyophilized red cabbage leaves (VL), by different extraction techniques, using the optimal parameters.

The simultaneous action of cellulase and pectinase causes an increase in the content of anthocyanins in the extract, because both cellulase and pectinase contribute to the degradation of cell wall components by hydrolysis of glycosidic bonds of complex polysaccharides in its structure (cellulose, pectin, hemicellulite) anthocyanins in vacuoles, but also phenolic compounds bound to the cell wall. Thus, the highest content, 1078,834 ± 8.2 mg anthocyanins / 100 g d.w., was obtained in the lyophilized plant material, following the application of the enzymatic treatment at the incubation temperature of 47 ° C, using a solution consisting of cellulase and pectinase. , method combined with classical maceration extraction, in 50% ethanol acidified with 0.05% HCl, for 20 minutes, in 3 successive steps, at a solvent / solid ratio of 15/1, thus obtaining a content of about 40% higher than the other samples.

To obtain the extract from the composition of the newly developed food supplement, the two combined extraction methods (enzyme-assisted extraction + 3 x maceration extraction) were applied, using the parameters described above. In the sample thus obtained, the highest content of phenolic compounds was identified, of 1637.2 ± 8.3 mg GAE / 100g d.w. respectively the strongest antioxidant activity, of 135.91 ± 3.3 mg AAE / 100g d.w.).

The thermal stability of anthocyanins in red cabbage anthocyanin extract was studied under pH 3.5, at temperatures of 50 ° C and 80 ° C, respectively. The thermal degradation of anthocyanins followed a first order kinetic model, the determined kinetic parameters being the velocity constant, k, of 1.7 10-3 min-1 and the half-life, t1 / 2, of 405 minutes (6.7 hours).

Thermo-oxidative degradation of anthocyanins, determined by thermal analysis DSC and TGA highlights several stages of degradation of anthocyanins in red cabbage leaves, most of the events in the DSC analysis being correlated with those in the TG analysis.



The DSC (A, B) and TG (C, D) curves of the control and heat-treated samples, analyzed under nitrogen and oxygen flow, respectively.

4.EXPERIMENTAL RESEARCH TO VALIDATE THE IMMUNOSTIMULATORY PROPERTIES OF NEW FORMULATIONS OF PRODUCTS BASED ON PREPARED BIOEXTRACTS - SUMMARY

This chapter contains original data on the formulation of a new type of food supplement developed as an emulsion in 2 experimental variants obtained by combining the three categories of extracts rich in biologically active compounds - carotenes, inulin-like fructans and anthocyanins - extracted by efficient techniques from local plant resources selected for this doctoral thesis, namely pumpkin pie (*Cucurbita maxima* var. Tudor), parsnip root (*Pastinaca sativa*, variety Bielas) and red cabbage leaves (*Brassica oleracea* var. *capitata* f. rubra).

In order to make the extracts, the extraction techniques were used, which proved to be the most efficient, determined on the basis of the preliminary studies performed for each plant material, presented in the previous chapter. In this regard, classical extraction techniques (maceration) as well as modern techniques (US ultrasound-assisted extraction and enzyme-assisted extraction combined with maceration or US) were tested by combining different experimental parameters in terms of time and extraction temperature, solvent and solvent / solid ratio.

Since the bioactive compounds concerned belong to two different categories in terms of solubility, carotenes being lipophilic compounds, anthocyanins and inulin-type fructans being hydrophilic compounds, in order to obtain a homogeneous mixture, it was decided that the newly developed product be presented as a emulsions.

In this chapter, two new emulsion products have been developed, with practical application as antioxidant and anti-inflammatory food supplements. To stabilize the developed emulsions, natural additives or stabilizers (guar gum selected from complex preliminary tests) were used, with the main purpose of slowing down the kinetic destabilization. Thus, in order to obtain a homogeneous mixture of the oily extract of β-carotene, the hydroalcoholic extract of anthocyanins and the aqueous extract of fructooligosaccharides (inulin), several variants of concentrations and combinations between natural food additives acting as emulsifiers (lecithin from soy, gum arabic and guar gum).

These additives are approved by the European Union for use in food, and of these, only lecithin has a maximum permissible dose of up to 5000 mg / l.

Following the preliminary tests, which followed the stability over time of the emulsions in which different percentages of natural additives were added, the emulsion with the addition of 0.5% guar gum, a percentage relative to the total mass of the solution, was determined as the optimal variant.

For the development of a new, innovative bioactive emulsion product based on the 3 investigated plant ingredients, two recipes were established, different in terms of bioactive molecule content, based on studies on the effects of the interaction between these biomolecules.

The emulsions were analyzed both in terms of stability over time and in terms of content and biological activity. The stability over time determined by optical analysis of the newly developed emulsions was 7-8 days for both proposed variants. Analyzing the viscosity of the emulsion at temperatures between 4° and 40° C, a decrease was observed with increasing temperature, which indicates a negative influence of increased temperature on stability over time, as an emulsion with a low viscosity is prone to destabilization. Based on this analysis, it is recommended to store at a temperature of 4° C (refrigeration temperature), a temperature at which the surface tension of the two component liquids is high, which contributes to increasing stability over time.

Following the analysis of the content of bioactive compounds in the composition of the two newly developed emulsions, differences were observed, these being due to the different ratio between the 3 types of extracts from the analyzed formulas. Thus, the highest contents of anthocyanins (523.3 ± 4.3 mg / 100 g) and β-carotenes (2933 ± 6.2 mg / 100 ml) were determined in the E1 emulsion, while in the E2 emulsion determined the highest content of inulin-type fructans (1589 ± 6.6 mg / 100 ml).

In order to validate the bioactive properties of the newly developed products, the antioxidant, anti-inflammatory and antibacterial activity was analyzed. Thus, the results on the antioxidant activity of the newly developed extracts and emulsions, determined by the FRAP and DPPH methods, showed that the anthocyanin extract has the most intense antioxidant action (41.67 ± 0.6 mg AAE / 100 ml extract, 18, 91 ± 0.2% DPPH), followed by β-carotene extract (40.41 ± 0.1 mg AAE / 100 ml extract, 16.83 ± 0.1% DPPH). This was also reflected in the more intense antioxidant activity of the first variant of emulsions, E1, which contains 50% β-carotene extract and 25% anthocyanin extract.

The results obtained after testing the anti-inflammatory properties of the extracts and the two newly developed emulsions led to the conclusion that the anthocyanin extract has a strong anti-inflammatory activity (30.09%), close to that of acetylsalicylic acid used as a control (30.67%), followed by β-carotene extract (26.05). Among the emulsions, the first formulated variant, E1, having a composition of 25% anthocyanins and 50% β-carotenes, has a more accentuated anti-inflammatory activity by 4.21% compared to the emulsion which has a content of 15% anthocyanins and 25% carotenes. However, both emulsion formulations showed a stronger anti-inflammatory effect than acetylsalicylic acid.

Regarding the antibacterial activity tested on the strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterobacter* sp. ATCC 23355 and *Streptococcus haemolyticus*, the results obtained showed a slight inhibition of anthocyanin extract, inulin and E1 emulsion on the development of Streptococcus haemolyticus strain, indicating that these extracts / emulsions may have a potential antibacterial effect, bigger.

Obtaining an emulsion-type product, with a stability of up to 8 days, by the exclusive use of natural vegetable ingredients, approved by vegetarians, whose anti-inflammatory action is comparable to that of acetylsalicylic acid (aspirin), is a notable result, as for which a Patent Application was filed and registered at OSIM (Oancea S., Tecucianu AC, Dulf FV., 2020).

5. FINAL CONCLUSIONS, OWN CONTRIBUTIONS AND FUTURE RESEARCH DIRECTIONS

1. To achieve the proposed purpose of developing an innovative food supplement product with a unique composition, the following types of biological compounds have been selected: natural antioxidants such as β-carotenes and polyphenolic compounds (anthocyanins) that have a protective effect on the cells of the immune system, and prebiotics (inulin), which contribute to the stimulation of the immune system through direct or indirect action.
2. The raw materials, valuable for their bioactive potential and studied in this doctoral thesis, have been carefully selected, based on sustainability criteria, based on the following aspects: native plant species, unexploited or little exploited for the purposes established in this thesis, economically accessible raw materials with a high content of bioactive compounds with a beneficial role especially in the immune system.
3. To achieve the proposed goal, the following plant resources were selected: pumpkin pie (*Cucurbita maxima* local population var. Tudor), which is a rich source of natural antioxidants, especially β-carotene, parsnip root (*Pastinaca sativa*), which is a source rich in fructans such as inulin, prebiotics with immunostimulatory effects, and red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*) which is rich in anthocyanins, pigments that show a protective effect on oxidative degradation and immunostimulatory effect.
4. Extractive techniques were selected taking into account both the need to extract as many bioactive compounds as possible, but also the principles of sustainability regarding the minimum consumption of reagents and the use of those environmentally friendly solvents.
5. Maceration was tested as a classic method of extraction of bioactive molecules of interest from the three plant matrices, following the use of a solvent / solid ratio as low as possible, and solvents approved in the food industry (water, ethanol, vegetable oil). Ultrasonic-assisted extraction was also tested as an unconventional technique, used to reduce the time and amount of solvent used for the three plant resources, the method being controlled in terms of process temperature, a high value can negatively influence the thermolabile compounds. (β-carotenes, anthocyanins). To optimize the extraction of bioactive compounds of interest, a third combined extractive variant (biotechnological) was tested by which the three plant raw materials were subjected to an enzymatic treatment using cellulase and / or

pectinase enzymes, followed by the extraction of bioactive compounds targeted by the classical method of maceration and respectively by modern ultrasound-assisted extraction technique.

6. The total content of bioactive compounds was determined under the influence of various extraction parameters, such as solvent, extraction temperature, solvent / solid ratio, time and amplitude of sound waves, using 3 extraction techniques, maceration, ultrasonic assisted extraction and assisted extraction. enzymes, as well as pre-treatments applied to the raw material (freezing, drying, freeze-drying).

7. For the extraction of β-carotene from pumpkin pulp, following the application of the 3 extraction techniques, using different combinations of parameters that could influence their quantity in the final extract, the highest content (62.6 ± 1, 1 mg β-carotene / 100 g d.w.) was obtained in the lyophilized vegetable plant material, by applying the classical method of maceration, for 2 hours in acetone / hexane (1/1), at room temperature, in solvent / solid ratio of 40 /1. An appreciable β-carotene content of 61.13 mg β-carotene / 100 g d.w., was obtained in the extract made from the same type of plant material (lyophilized), using pumpkin seed oil obtained by cold pressing, as an alternative to organic solvents, being environmentally friendly, in a solid solvent ratio of 10/1. Analyzing comparatively the experimental results obtained, it was observed that, regardless of the extraction technique applied and the solvent used, in the extracts from the lyophilized plant material were identified the highest contents of bioactive molecules of interest.

8. By applying ultrasound-assisted extraction, using a solvent / solid ratio of 10/1, for 30 minutes, at a sound amplitude of 70%, lower β-carotene values were obtained in the lyophilized pumpkin samples , compared to those obtained by the classical method, these being mainly influenced by the extraction temperature.

9. By enzyme-assisted extraction technique, combined with maceration, after incubation of the lyophilised plant material with cellulase (122.5 U / g) and pectinase (100 U / g), for 120 minutes at 40 ° C , followed by conventional extraction by maceration in 70% ethanol, the β-carotene content was reduced by half compared to the values obtained by using the respective maceration techniques with US extraction in the same solvent, the results being probably influenced by prolonged exposure to thermolabile molecules of β-carotene at the action of high temperatures during incubation with the enzyme solution.

10. To make the extract used in the development of the new food supplement, the extraction by the classic method of maceration was applied, in pumpkin seed oil, using the lyophilized vegetable material in a solvent / solid ratio of 10/1, for 30 minutes.

11. Following the analysis of the antioxidant activity, determined by the FRAP method, in the extracts made in 70% ethanol, from the lyophilized pumpkin pulp, by applying the optimal parameters of the 3 extractive techniques, the highest content of 55.25 ± 1.5 mg ascorbic acid / 100 g d.w., was identified in the macerated sample for 30 minutes in a solvent / solid ratio of 10/1, which is 9% higher than the antioxidant activity of the extract obtained by assisted enzyme extraction combined with maceration, respectively 12% higher than the one identified in the extract obtained by the modern US-assisted extraction technique.

12. As regards the extraction of inulin-type fructans from the parsley root samples by the maceration method, for 2 hours, in two successive stages, the fresh sample obtained a content approximately 2 times higher than that obtained in dry sample.

13. In the extracts made by the modern US extraction technique, for 5 minutes, at the amplitude of the acoustic waves of 70%, a content was obtained approximately 9% higher compared to that obtained in the macerated extract for 2 hours in two successive stages, in the fresh parsnip sample, a result indicating that the use of the unconventional extraction technique is a fast and efficient method of extracting inulin-type fructans from parsnip root, a theory supported by the results obtained in dried parsnip samples, in which also recorded an increase of about 20%.

14. Following the application of the two unconventional techniques (enzyme-assisted extraction and ultrasound-assisted extraction) simultaneously, there was a dramatic increase in the content of inulin-type fructans, especially in the dried parsnip sample, in which a of 156.67 ± 3.1 mg inulin / 100 g su, which is approximately 60% higher compared to the values obtained in the other samples.

15. Analyzing the experimental results obtained for the production of the final extract, rich in inulin-type fructans, from the composition of the newly developed food supplement, the combined extraction technique (enzyme-assisted extraction + US-assisted extraction) was used, using dried parsley root, incubated with cellulase (122.5 U / g), in an enzyme solution / sample ratio of 7/1, for 180 minutes at 40 ° C, followed by extraction into distilled water by US assisted technique, for 5 minutes, at a solvent / solid ratio of 10/1.

16. The highest content of polyphenolic compounds in parsnip root (432.60 mg GAE / 100 g d.w.), was obtained in the dried plant material, incubated with cellulase at 40 ° C for 180 minutes, treatment followed by a US-assisted extraction in distilled water, in a solid solvent ratio of 10/1, for 5 minutes, at an amplitude of 70%, this value being about 20% higher than that obtained in the same type of plant material, following extraction with US for 5 minutes, without prior application of enzyme treatment.

17. Regarding the antioxidant activity of parsnip extracts, determined by the FRAP method, the best results were obtained in samples performed by combining the two unconventional techniques (enzyme-assisted extraction and ultrasound-assisted extraction), thus obtaining a content of 25.44 mg ascorbic acid / 100 g d.w. in the dried parsnip sample.

18. By applying the classical method of extracting anthocyanins from red cabbage leaves, the highest content of 642,655 ± 8,2 mg / 100 g d.w.was obtained in the lyophilized plant material, soaked for 20 minutes at room temperature, in 50% ethanol, acidified 0.05% with HCl, solvent / solid ratio of 15/1.

19. By applying the modern US-assisted extraction technique, the highest anthocyanin content (687.34 ± 4.2 mg / 100g d.w.) was obtained in lyophilized red cabbage leaves at -50 ° C, after extraction. for 20 minutes, at an amplitude of 70% sound waves, in 50% ethanol acidified 0.05% with HCl, in a solid solvent ratio of 15/1, thus obtaining an anthocyanin content of approximately 4.2% higher large compared to the one in the macerated sample.

20. The simultaneous action of cellulase and pectinase causes an increase in the content of anthocyanins in the extract, because both cellulase and pectinase contribute to the degradation of cell wall components by hydrolysis of glycosidic bonds of complex polysaccharides in its structure (cellulose, pectin, hemicell facilitates the release of anthocyanins from vacuoles, but also of phenolic compounds bound to the cell wall. Thus, the highest content, 1078,834 ± 8.2 mg anthocyanins / 100 g d.w., was obtained in the lyophilized plant material, following the application of the enzymatic treatment at the incubation temperature of 47 ° C, using a solution consisting of cellulase and pectinase, method combined with classical maceration extraction, in 50% ethanol acidified with 0.05% HCl, for 20 minutes, in 3 successive steps, at a solvent / solid ratio of 15/1, thus obtaining a content of about 40% higher than the other samples.

21. To obtain the extract from the composition of the newly developed food supplement, the two combined extraction methods (enzyme-assisted extraction + 3 successive extractions by maceration) were applied, using the parameters described above. In the sample thus obtained, the highest content of phenolic compounds was identified, of 1637.2 ± 8.3 mg GAE / 100g d.w. respectively the strongest antioxidant activity, of 135.91 ± 3.3 mg AAE / 100g d.w..

22. The thermal stability of anthocyanins in anthocyanin extract of red cabbage was studied under pH 3.5, at temperatures of 50 ° C and 80 ° C, respectively. The thermal degradation of anthocyanins followed a first order kinetic model, the determined kinetic parameters being the velocity constant, k, of 1.7 10-3 min^{-1} and the half-life, $t_{1/2}$, of 405 minutes (6.7 hours).

23. Thermo-oxidative degradation of anthocyanins, determined by thermal analysis DSC and TGA highlighted several stages of degradation of anthocyanins in red cabbage leaves, most of the events in the DSC analysis being correlated with those in the TG analysis.

24. For the capitalization of the obtained extracts, two new emulsion products were developed, with practical application as antioxidant and anti-inflammatory food supplements, the E1 emulsion being composed of 50% β-carotene extract, 25% anthocyanin-rich extract and 25% extract rich in fructans of the inulin type, and the E2 emulsion being composed of 25% extract of β-carotene, 15% extract of anthocyanins and 60% extract of fructans of the inulin type.

25. To obtain a homogeneous mixture of the oily extract of β-carotenes, the hydroalcoholic extract of anthocyanins and the aqueous extract of fructooligosaccharides (inulin), several variants of concentrations and combinations between natural food additives as emulsifiers (soy lecithin) were tested. , gum arabic and guar gum).

26. The test for slowing down the kinetic destabilization of newly developed emulsions led to the conclusion that guar gum at a concentration of 0,5% has a stabilizing effect, being selected following complex preliminary tests.

27. The time stability of the newly developed emulsions, determined by analysis under an optical microscope, was 7-8 days for both proposed variants.

28. Analysis of the viscosity of the emulsion at temperatures between 4 ° and 40 ° C showed a decrease with increasing temperature, which indicates a negative influence of increased temperature on stability over time, as an emulsion with a low viscosity is prone to destabilization phenomena. Based on this analysis, it is recommended to store at a temperature of 4 ° C (refrigeration temperature), a temperature at which the surface tension of the two component liquids is high, which contributes to increasing stability over time.

29. Following the analysis of the content of bioactive compounds in the composition of the two newly developed emulsions, differences were observed, these being due to the different ratio between the 3 types of extracts in the analyzed formulas. Thus, the highest contents of anthocyanins (523.3 ± 4.3 mg / 100 ml) and β-carotenes (2933 ± 6.2 mg / 100 ml) were determined in E1, while in E2 the higher content of inulin-type fructans (1589 ± 6.6 mg / 100 ml).

30. In order to validate the bioactive properties of the newly developed products, the antioxidant, anti-inflammatory and antibacterial activity was analyzed, the results obtained being the following:

- The results on the antioxidant activity of the newly developed extracts and emulsions, determined by the FRAP and DPPH methods, showed that the anthocyanin extract has the most intense antioxidant action (41.67 ± 0.6 mg AAE / 100 ml extract, 18.91 ± 0.2% DPPH), followed by β-carotene extract (40.41 ± 0.1 mg AAE / 100 ml, 16.83 ± 0.1% DPPH). This was also reflected in the more intense antioxidant activity of the first variant of emulsions, E1, which contains 50% β-carotene extract, 25% anthocyanin extract and 25% fructans.

- The results obtained after testing the anti-inflammatory properties of the extracts and the two newly developed emulsions led to the conclusion that the anthocyanin extract has a strong anti-inflammatory activity (30.09%), close to that of acetylsalicylic acid used as a control (30.67 %), followed by β-carotene extract (26.05%). Among the emulsions, the first variant, E1, having a composition of 25% anthocyanins, 50% β-carotenes and 25% fructans, has a higher anti-inflammatory activity by 4.21% compared to the emulsion which has a content of 15% anthocyanins and 25 % carotenes 60% fructans. However, both emulsion formulations showed a stronger anti-inflammatory effect than acetylsalicylic acid.
- Results obtained regarding the antibacterial activity tested on standard strains of gram-positive and gram-negative bacteria, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterobacter* sp. ATCC 23355 and *Streptococcus haemolyticus* showed a slight inhibition of their growth demonstrated by the anthocyanin extract, inulin and E1 emulsion, respectively, on the development of *Streptococcus haemolyticus* strain, indicating that these extracts / emulsions may have a potential antibacterial effect. higher concentrations.

Original contributions

Through this research paper, important theoretical, experimental and applied contributions have been made in the field of extracts of selected bioactive compounds of interest - anthocyanins, β-carotenes, inulin-type fructans, but also other compounds of interest, such as polyphenolic compounds. , with the potential to lead to the production of valuable, innovative products, developed for the benefit of biological activities, and applications in the food sector:

- Contributions to the enrichment of the theoretical basis through the synthesis of the potential benefits, especially on the immune system, of selected bioactive compounds of interest, study materialized by publishing two scientific articles on the immunostimulatory potential of certain categories of compounds extracted from plants, respectively an approach historically the use of antioxidants in medicine and food.

- Studies on the application of classical (maceration) and modern extractive techniques (ultrasound-assisted extraction, enzyme-assisted extraction), by using different experimental parameters.
- Experimental research to establish the optimal conditions for the extraction of bioactive compounds targeted from extracts prepared and proposed as natural ingredients of innovative bioactive products
- Physico-chemical and biological characterization of the obtained bioextarcts
- Design and development of two innovative food products with acceptable stability, through the exclusive use of natural plant ingredients (the 3 bioextracts prepared), approved by vegetarians, the bioactive potential being validated by testing antioxidant, anti-inflammatory and antimicrobial properties.

Recommendations and future research directions

The notable experimental results obtained in this doctoral thesis are intended to be continued in terms of standardization of the extracts obtained, because, as demonstrated by other studies conducted in this regard, the content of bioactive molecules of interest varies, within the same species, from a variety to another and even from one plant to another, being strongly influenced by environmental factors and the geographical area in which it developed (brightness, temperature, altitude, etc.).

Another recommendation consists in the research from the point of view of the activity manifested *in vitro* and *in vivo* of the synergistic action of the categories of bioactive molecules in the composition of the newly developed products, of food supplement type.

Also, future research can be directed towards interdisciplinary approaches, by conducting clinical studies, by category of patients, to identify the benefits to the human body, in addition to laboratory testing completed in this doctoral thesis.

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