POLYTECHNIC UNIVERSITY OF BUCHAREST

FACULTY OF APPLIED CHEMISTRY AND MATERIALS SCIENCE

PRODUCTION AND CONTENT ANALYSIS OF NATURAL EXTRACTS USED AS FOOD SUPPLEMENTS

Thesis summary

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Contents

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Note: The notations of the chapters, subchapters and of the figures, tables and graphs from this document are the same as those in the doctoral thesis.

OBJECTIVES AND STRUCTURE OF THE WORK

The purpose of this research program was to investigates the composition of six medicinal plants commonly used towards relieving inflammation (willow, rosemary and oregano), used towards the improvement of cardiovascular disease (motherwort and hawthorn), and with hepatoprotective effect (milk thistle), and extracts obtained from these plants by varying extraction parameters (extraction method, solvent concentration) to maximize the content of compounds of interest (certain flavones and polyphenols, anthocyanins, etc.).

The main objectives of the study are: obtaining and characterizing extracts of medicinal plants with anti-inflammatory effect (willow, rosemary and wild oregano), obtaining and characterizing extracts of medicinal plants used towards the improvement of cardiovascular diseases (goosefoot and hawthorn) and obtaining and characterizing extracts from milk thistle, a medicinal plant with hepatoprotective effect.

The paper is structured in two parts, namely: the first part consists of a literature study on the research topic, the second part consists of the original experimental part.

The study of literature is further structured in two chapters.

Chapter I contains the literature study on obtaining plant extracts containing information on the general principles for the extraction of biologically active substances from plant products, conventional methods of their extraction, as well as the stages and factors that influence it. Methods of characterisation and uses of plant extracts are also presented.

Chapter II contains the literature study on the six selected medicinal plants. It includes a description of plants with anti-inflammatory effect (willow, rosemary, oregano), plants used to alleviate cardiovascular disease (motherwort and hawthorn) and milk thistle, a plant with hepatoprotective effect.

The second part of the paper (the original part) is structured in four chapters.

Chapter III contains the research conducted for extraction and quantification of phenolic compounds from the six plants studied. This chapter is further divided into three subchapters in which the materials and equipment used are presented, how the extracts are obtained through conventional methods and spectrophotometric and chromatographic methods, which helped establish the phytochemical profile of biologically active extracts.

Chapter IV contains the research carried out on the characterization of the extracts obtained from medicinal plants with anti-inflammatory effect. This chapter provides the study of willow by components, of willow bark by varying the method of extraction, rosemary and oregano, but also the characterization of plant extracts with anti-inflammatory effect.

Chapter V contains the research conducted following the characterization of extracts obtained from studied medicinal plants. Next, we followed the extraction conditions of the phenolic compounds of interest by varying some extraction parameters: the concentration of ethyl alcohol and the extraction method, in order to find an adequate method of extraction of the classes of compounds of phytotherapeutic interest.

Chapter VI follows the influence exerted on the content of bioactive compounds in non-skimmed/skimmed milk thistle fruit, by the harvesting conditions, but also from aqueous extract and tincture obtained from skimmed milk thistle fruit.

INTRODUCTION

Products based on natural medicinal plants have been the main way of treating diseases around the globe, until the advent of scientific medicine, especially since the first human methods of treatment concerning diseases were directed towards the use of plants in the surrounding environment.

Medicinal plants, based on the growing knowledge of their chemical composition, of their pharmacodynamic properties, with the help of high-performance analytical methods of investigation, currently represent one of the main sources of raw materials used for the preparation of natural products necessary for maintaining a good state of health.

Also, the therapeutic value of medicinal plants is based on the relationship between the chemical structure of the active substances and their pharmacological action which they exert on the reactive elements of the human body.

The multiple pharmacodynamic properties of the same plant can be explained by the fact that most medicinal plants have a complex chemical composition, ranging from 2-3 compounds to 30-40 biologically active substances identified in some plants; biologically active substances, although in very low concentrations, combine their action and act through synergism. In this sense, we must marvel at the "miracle of nature" which achieved the ideal combinations of active substances in plants, with beneficial effects on human health.

Instead, the very long journey, which mankind went on, has been and is conditioned by the evolution of scientific thinking and methodology of investigation. Only now can we claim that we are truly on this path, but it will take many more years, decades, if not centuries, to unravel all the secrets that plants hide.

Modern phytotherapy takes into account all the rich human knowledge in this field, and greatly increases the requirements referring to: the quality control of plants by modern means and their chemical composition, the possible unwanted side effects and especially the development of formulas and pharmaceutical forms appropriate to the bioavailability and health efficacy.

All these aspects, of particular importance for the production of pharmaceutical products, were the basis of the research in this paper, used for obtaining new products with anti-inflammatory, hepatoprotective qualities, and some products used towards the relief of cardiovascular diseases.

CHAPTER III - MATERIALS AND METHODS

III. 1. Materials and equipment used

Materials

Tetrahydrofuran (Merck), methanol (Merck), acetonitrile (Merck), ethanol 96% (Merck), phosphoric acid (Merck), hydrochloric acid 97% (Merck), sodium hydroxide pellets (Merck)), D (-) salicin (Sigma-Aldrich), silymarin (Sigma-Aldrich), sodium acetate (VWR), aluminium chloride (VWR), rutin (Sigma-Aldrich), gallic acid monohydrate (Sigma-Aldrich), Folin-Ciocalteu reagent (Merck), anhydrous sodium carbonate (Merck), copper sulphate pentahydrate (Merck), neocuproine (Sigma-Aldrich), ammonium acetate (Merck), trolox (Sigma-Aldrich), skin powder -Aldrich), pyrogallol (Sigma-Aldrich) were of analytical quality and were used without further purification.

Ultrapure water (18.2 MΩ) was used for all experiments. Filtration of HPLC samples was performed using 0.2 μm diameter porous PA filters.

The bark, the tree branch and the leaves of young and mature white willow trees (*Salix alba*) were harvested from SC Hofigal Export-Import SA in June 2020. The samples were separated by hand, dried and ground.

Rosemary (*Rosmarinus officinalis* - herba) and oregano (*Origanum vulgare* - herba), motherwort (*Leonurus cardiaca* - herba) and hawthorn (*Crataegus monogyna* - fructus, flos and foliae) were harvested from SC Hofigal Export-Import SA during July -August 2019. The samples were separated by hand, dried and ground.

The milk thistle fruits (*Silybum marianum)* were harvested from SC Hofigal Export-Import SA between May and October 2019. The samples were separated by hand, dried and ground.

Equipment used

For the preparation of the raw materials, we used the following equipment: analytical balance Partner AS 310.R2; plant dryer Biovita-DEH600D; Retch GM 200 laboratory mill; Retch AS 200 Basic sifting machine.

For the preparation of the analysis samples we used the following equipment: Elmasonic P180 H ultrasound bath; Julabo TW8 water bath; centrifuge Ortoalresa 21 R; Buchi R-300 EL rotary evaporator, equipped with B-300 Base water bath, V-300 vacuum pump and F-308 recirculating chiller; thermobalance VWR MB 160; WTW CR 3200 thermoreactor; Milli-Q Direct water purification system 8.

HPLC analysis was performed using an Agilent EZChrom Elite system equipped with a DAD detector and a HiCHROM LiChrosorb 100 RP-18 column, 10 μm (4.6 x 250 mm) particles.

UV-Vis spectrophotometry was performed on a Jasco V-530 UV-Vis spectrophotometer with a resolution of 2.0 nm and a double beam configuration.

Gas chromatography coupled with mass spectrometry (GC-MS) was performed on a Termo-GC gas chromatograph with DSQ P 5000 mass spectrometer detector. A Macrogol 2000 column was used, and for the identification of the peaks of the respective analysed compound, the NIST spectrum library was used.

III.2. Obtaining extracts from the 6 investigated plants

After the application of the proposed extraction procedures, the following extracts with anti-inflammatory effect were obtained.

- Hydroalcoholic extract, aqueous extract, decoction and tincture of willow, rosemary and oregano;
- Extracts obtained by refluxing, by means of thermoreactor and ultrasounds from willow bark;
- Extracts obtained by means of ultrasound (at 110 °C, 120 °C and 150 °C) and in microwaves from willow bark waste;

After the application of the proposed extraction procedures, the following extracts have been obtained with a role in ameliorating cardiovascular diseases:

- Extracts obtained by maceration, percolation and reflux (extraction solvent: EtOH 30%, EtOH 50% and EtOH 70%) from hawthorn and motherwort;

After the application of the proposed extraction procedures, the following extracts with hepatoprotective effect were obtained:

- Aqueous extract and tincture from skimmed milk thistle fruit.

CHAPTER IV - CHARACTERIZATION OF MEDICINAL PLANTS AND EXTRACTS WITH ANTI-INFLAMMATORY EFFECT

IV.1. Obtaining extracts from willow, rosemary and oregano

a. Obtaining extracts by varying the extraction solvents

All the structures of the musculoskeletal system: bone, cartilage, synovial, ligaments, joint capsule, muscles can suffer from rheumatic diseases. Rheumatic diseases generally bring varying degrees of discomfort to the human body, from pain and difficulty moving, to unbearable pain and advanced immobilization. For these reasons, it is advisable to intervene in the early forms of the disease, mild forms of rheumatic manifestations, with natural products that are effective in these stages and which do not produce unwanted side effects.

Plants that have a significant content of salicylic derivatives have been used due to their analgesic and anti-inflammatory properties which have been known about, in folk medicine, since ancient times.

White willow bark is the main source of salicin and other salicylic derivatives - salicortin, 2'-O-acetyl salicortin and tremulacin - similar in structure to aspirin (acetylsalicylic acid), with white willow often referred to as "vegetable aspirin". Salicin, by enzymatic hydrolysis induced by means of emulsin and diastase, breaks down into glucose and saligenin, also known as o-oxy benzyl alcohol or saligenol. Saligenin in turn is produced by oxidation, salicylic acid, with notable analgesic, antipyretic and antirheumatic properties, thus achieving a gradual, prolonged effect. Also, the tannins, present in willow bark, have a tonic, astringent, coagulant and slightly hemostatic action (Assessment Report on Salicis Cortex (Willow Bark) and Herbal Preparation (S) thereof with Well-Established Use and Traditional Use. EMEA / HMPC / 295337/2007, 2009).

Solvent extraction is the most widely used type of extraction for bioactive plant compounds. In the extraction operation, the choice of solvent is made, according to the nature of the substance which is to be extracted, and the nature of the raw material. In order to compare the results obtained and to continue to use the most efficient method of extracting the compounds of interest, several extraction methods were applied in parallel, thus obtaining tincture, decoction, hydroalcoholic extract and aqueous extract of willow, rosemary and wild oregano. (Tables 5-8).

Table 5. Composition of aqueous extract of willow, rosemary and oregano

Aqueous extract of willow, rosemary and oregano	**Quantity, g**
Willow bark *(Salix alba)*	167.5
Rosemary (*Rosmarinus officinalis*)	32.5
Oregano *(Origanum vulgare)*	32.5
Purified water	5500
Total (after concentration)	**100**

Table 6. Composition of the hydroalcoholic extract of willow, rosemary and oregano

Hydroalcoholic extract of willow, rosemary and oregano	**Quantity, g**
Willow bark *(Salix alba)*	20,00
Rosemary (*Rosmarinus officinalis*)	4,00
Oregano *(Origanum vulgare)*	4,00
Ethanol 96% (v/v)	20.00

Purified water	q.s.ad.100

Table 7. Composition of willow, rosemary and oregano decoction

Decoction of willow, rosemary and oregano	**Quantity, g**
Willow bark *(Salix alba)*	20.00
Rosemary (*Rosmarinus officinalis*)	4.00
Oregano *(Origanum vulgare)*	4.00
Purified water	75
Total (after cooling to 200 mL)	**100**

Table 8. Composition of willow, rosemary and oregano tincture

Tincture of willow, rosemary and oregano	**Quantity, g**
Willow bark *(Salix alba)*	28.00
Rosemary (*Rosmarinus officinalis*)	7,00
Oregano (*Origanum vulgare)*	7.00
Ethanol 96% (v/v)	210

b. Obtaining extracts by various extraction techniques

Salicylic derivatives were extracted from willow bark by means of various extraction techniques, such as water bath reflux, ultrasound and extraction using a thermoreactor. During the experiment, the ratio of 1:8 plant:solvent was kept constant. The extraction conditions were as follows:

- Extraction at reflux on a water bath was performed at a temperature of 60 °C for 60 minutes;
- The extraction at the thermoreactor was carried out at a temperature of 60 °C for 60 minutes;
- Ultrasound extraction was performed at room temperature for 30 minutes.

IV.2. Characterization of willow by components

The results in Figure 21 show a higher content of salicylic derivatives in willow bark than in the tree branch and in the leaves. Salicylic derivatives were indeterminate for both young and mature willow.

In the present study, the phytochemical profile of hydroethanolic extracts in the branch, leaves and bark of *Salix alba* was analyzed using UV-Vis spectrophotometry. The results of the qualitative and quantitative analysis are summarized in Figures 25-28.

According to the reported studies, the total polyphenolic content of *Salix alba* is between 20 and 50 mg / g, so the values obtained in our study, 53.2 mg / g and 63.1 m / g for young and mature willow bark, respectively are beyond this range (Ramos, et al., 2019) (Durak & U., 2014) (Sulaiman G., Hussien, Marzoog, & Awad, 2013) (Kenstavičienė, Nenortienė, Kiliuvienė, & Ževžikovas, 2009) (Toiu, Vlase, Oniga, Benedec, & Tămaș, 2011). Polyphenols have similar values in the bark and leaves, but much lower values in the tree branch; the results are also higher for mature willow.

In addition to the total polyphenolic content, spectrophotometric methods are also used to determine the total content of individual groups of phenolic compounds, such as total tannins and flavones. The determination of flavones shows a higher content in young willow, the highest value was obtained for the leaves (Piatczak, Dybowska, Płuciennik, & Kosla, 2020).

The lowest tannin content was obtained for the willow branch - an equal value for the young and mature willow - higher values were recorded, as expected, for the willow bark samples.

The results for the antioxidant activity of willow extracts indicate the influence of polyphenols and flavones present in each extract. For both the young and the mature willow branch, the antioxidant activity is very low compared to the other samples, also the highest values are recorded for the willow bark samples (Tyśkiewicz, et al., 2019) (Sulaiman GM, Hussien, Marzoog, & Awad, 2013).

Table 9. Summary of the characterization of young and mature willow

	Determination				
	Total salicylic derivatives, expr. as salicin, mg/g	**Total polyphenols expr. as gallic acid, mg/g**	**Total flavones expr. as rutin, mg/g**	**Total tannins expr. as pirogalol, mg/g**	**Antioxidant activity, mg equiv. Trolox/g sample**
Young willow - branches	0.5	10.9	0.7	1.9	29.8
Young willow - bark	16.0	53.2	4.3	16.6	288.5
Young willow - leaves	ND	53.2	8.1	9.1	201.5
Mature willow - branches	0.08	21.3	0.4	1.9	22.1
Mature willow - bark	19.3	63.1	2.7	18.8	343.0
Mature willow - leaves	ND	67.2	7.8	11.3	256.2

In the present study, the methods of extraction at reflux in a water bath, ultrasound extraction and extraction by means of thermoreactor were compared in order to optimize the extraction time and to decide on the most suitable method for a high yield of salicylic derivatives from willow bark.

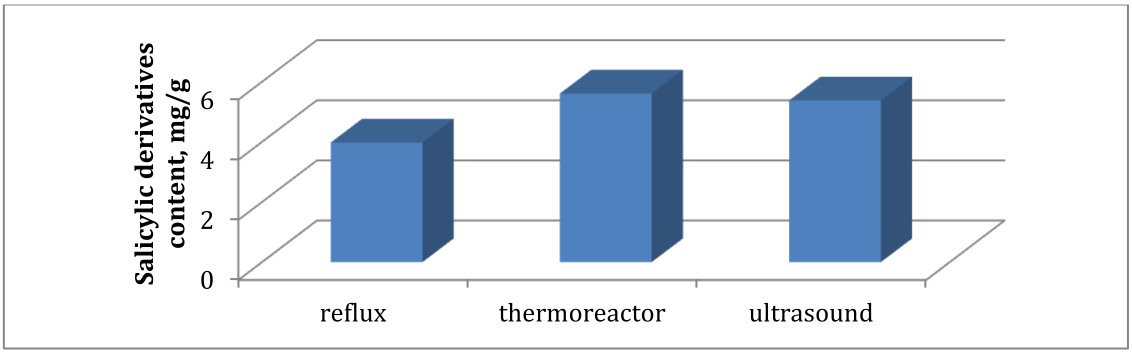

FIG. 32. Salicylic derivatives content (mg/g) of willow bark by different extraction techniques

The results obtained from the determination of salicylic derivatives from willow bark by different extraction methods are shown in Figure 32. These indicate similar values for the extraction from willow bark by means of ultrasound (5.38 mg/g), as well as for the extraction performed at the thermoreactor, with slightly higher values for the latter (5.61 mg/g). The difference obtained can be attributed to different extraction times, the extraction on the ultrasound bath taking place for 30 minutes, compared to the extraction at the thermoreactor which lasted one hour. Extraction by reflux on a water bath shows 3.98 mg/g salicylic derivatives expressed as salicin.

IV.5. Characterization of plant extracts with anti-inflammatory effect

Phytotherapy is based on the enhanced effect that can be obtained by the simultaneous action of the active principles of plants. In order for the active principles to be able to be used towards order to obtain a biological action on the human body, they must be concentrated, must be found in various phytotherapeutic forms (tinctures, syrups, extracts, etc.) that are easy to administer.

I set out to obtain a plant extract that has anti-inflammatory and analgesic properties and that acts effectively in relieving joint pain, headaches, rheumatism, neuralgia, menstrual cramps, muscle aches.

For this purpose, the following plants were selected: willow (*Salix alba* cortex), rosemary (*Rosmarinus officinalis* herba) and oregano (*Origanum vulgare* herba), after conducting preliminary documentary studies.

In terms of content in salicylic derivatives, we noticed that willow bark is the best source for salicin, along with rosemary and oregano, and phenolic compounds enhance their effect. Its analgesic and anti-inflammatory action is due to salicylic alcohol derivatives, also called salicylates, whose amounts vary between 1.5 and 11% in the bark harvested from the young willow branches.

In addition to the therapeutically relevant compounds - salicylic derivatives - dosed by an HPLC method, in the selected plant material were also determined spectrophotometrically:

- total polyphenols expressed as gallic acid - using the Folin-Ciocâlteu method - and caffeic acid;
- total flavones expressed as rutin;
- total tannins expressed as pirogalol.

The determination of the active ingredients content in plant materials was carried out after specific extractions for quantitative analysis, taking into account the specific solubility of the compounds of interest.

In order to optimize the content of salicylic derivatives in willow bark, it was necessary to conduct a study on their extractive conditions (aqueous, hydroalcoholic, decoction, tincture) from various samples of willow bark (Figure 35).

The obtained extracts were then subjected to analysis to identify the active principles using analytical methods based on high performance liquid chromatography techniques, UV-Vis spectrometry which led to their quantification.

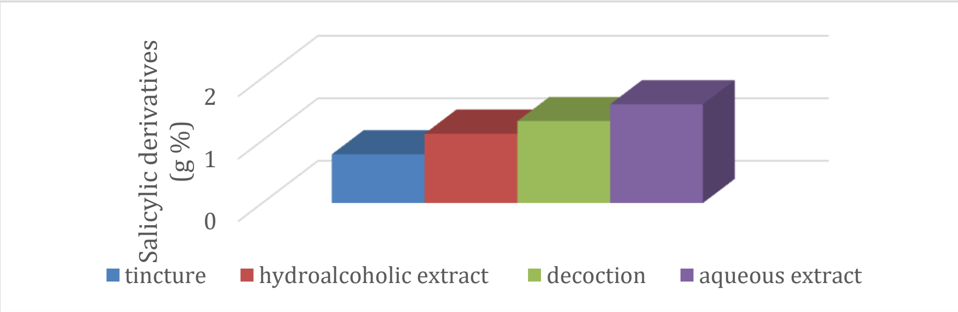

FIG. 35. Salicylic derivatives content (g%) for different extraction conditions

Table 12. Active principles from different extracts of willow, rosemary and oregano

Extract Type	**Determination**			
	Total salicylic derivatives, expr. as salicin, g%	**Total polyphenols expr. as gallic acid, g%**	**Total flavones expr. as rutin, g%**	**Total tannins expr. as pyrogallol, g%**
Tincture	0.7	3.4	2.2	0.4

Hydroalcoholic extract	1.1	5.2	3.8	0.5
Decoction	1.2	5.5	3.8	0.7
Aqueous extract (phytocomplex A)	1.45	7 , 0	5,2	1,0

We have identified that the most efficient extraction is in aqueous medium, because it dissolves and extracts most of the active components - especially glycosides, tannins - at a high yield. The extraction was made from a mixture of medicinal plants: willow, rosemary, oregano. The working parameters were as follows: plant material - brought to an advanced degree of fineness; extraction method - maceration; extraction solvent - purified water; extraction temperature - room temperature; extract concentration - 10% (g plant/mL water); extraction time - 24 hours.

The result was an opalescent, reddish-brown liquid with a characteristic odor and a strong bitter taste (phytocomplex A).

In order to make this new product, an intermediate in the form of an aqueous extract of willow bark and aerial parts of rosemary and oregano was used towards the first stage, with a composition rich in substances useful for the proposed purpose.

From the studies performed on the efficiency of phytocomplex A, it was found that it is necessary to increase the content in salicylic derivatives, with an important contribution for the purpose pursued, from about 1.4% to at least 2.5%.

In this regard, new research has been carried out on willow bark depending on the different areas of the country and the seasons.

For the selection of sources of willow bark rich in salicylic derivatives, several samples of willow bark were used, from different geographical areas of the country, in different stages of development: young (2-3 years) and mature, harvested throughout spring and summer (Figure 36).

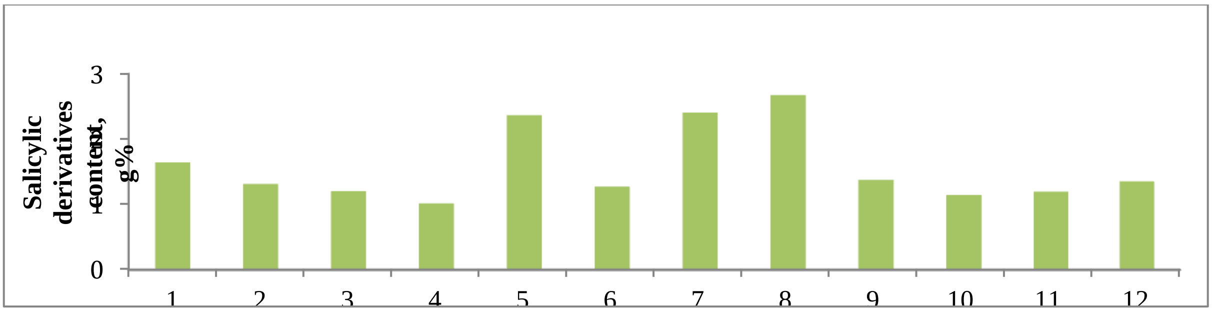

FIG. 36. Variation of content in salicylic derivatives (g%) for different samples of willow bark

Table 13. Description of the samples of willow bark

No. Crt.	Sample	Area of origin	Harvest period
1.	Ripe willow bark	Furculești, Teleorman	September 2017
2.	Ripe willow bark	Făgăraș, Brașov	July 2017
3.	Ripe willow bark	Brașov, Brașov	June 2017
4.	Ripe willow bark	Breaza, Prahova	June 2017
I. Crt.	**Sample**	**Area of origin**	**Harvest period**
5.	Young willow bark	Furculești, Teleorman	March 2018
6.	Young willow bark	Făgăraș, Brașov	March 2018
7.	Young willow bark	Furculești, Teleorman	April 2018
8.	Young willow bark	Furculești, Teleorman	May 2018

9	Young willow bark	Hofigal, Bucharest	May 2018
10.	Mature willow bark	Furculești, Teleorman	July 2018
11.	Mature willow bark	Făgăraș, Brașov	July 2018
12.	Young willow bark	Făgăraș, Brașov	June 2018

The studies were performed directly on the phytocomplex A acquired from the different samples of willow bark, described in Table 13.

From the presented data it is observed that the vegetal material corresponding to the composition necessary for phytocomplex A is the young willow bark (2-3 years), from the southern part of the country (Furculești-Teleorman), harvested in spring, with a content of min. 2.68% in salicylic derivatives expressed as salicin. As a result, the use of high quality willow bark in the preparation of the new phytocomplex led to an active phytocomplex B, in the form of an aqueous extract, improved, with a content of min. 2.5% salicylic derivatives expressed as salicin, which was analyzed and characterized from a physico-chemical point of view (Table 14).

The determination of the content of active ingredients in plant extracts was conducted after carrying out specific extractions for quantitative analysis, taking into account the specific solubility of the compounds of interest.

The obtained extract was then subjected to analysis to identify the active principles using analytical methods based on UV-Vis spectrometry and led to their quantification, thus dosing the following classes of compounds:

- total polyphenols, expressed as gallic acid - using the Folin-Ciocâlteu method - and caffeic acid;
- total flavones, expressed as rutin;
- total tannins, expressed as pirogalol.

At this stage we performed a study of salicylic derivatives expressed as salicin from natural plant extract using the HPLC method, adapting the dosing method of salicylic derivatives described in the European Pharmacopoeia 9th Edition. Thus, we identified and determined the concentration of salicylic derivatives, after acid hydrolysis of the methanolic extract in a basic medium prepared from plant materials.

Table 14. Physico-chemical characteristics of the plant extract

No. Crt.	Product	Characteristics	Results
1.	Aqueous extract of willow, rosemary and oregano	Total polyphenolic content expressed as gallic acid, %	8.00
		Total flavones content expressed as rutin, %	5.35
		Total tannins expressed as pyrogallol, %	1.75
		Salicylic derivatives, expressed as salicin, %	2.68

The obtained results confirm that the plant extracts are composed of chemicals - salicylic derivatives - which give them anti-inflammatory and analgesic properties.

CHAPTER V - CHARACTERIZATION OF MEDICINAL PLANTS AND EXTRACTS USED TOWARDS THE IMPROVEMENT OF CARDIOVASCULAR DISEASES

V.1. Obtaining extracts from motherwort and hawthorn

Extraction of phenolic compounds from motherwort (herba), hawthorn (fructus) and hawthorn (flos and foliae) was obtained by conventional extraction methods, namely maceration, percolation and reflux, the experimental conditions are summarized in Table 15.

Table 15. Experimental conditions for the three conventional methods used

Extraction technique

	Maceration	Percolation	Reflux
Solvent used	ethanolic solution 30% / 50% / 70%	ethanolic solution 30% / 50% / 70%	ethanolic solution 30% / 50% / 70%
Plant ratio: solvent	1: 7	1:15	1:10
Temperature	Temperature room	Room temperature	82-86 ° C
Applied pressure	Not applicable	Not applicable	Not applicable
Time	6 days	6 days	30 minutes
Solvent volume required	500 mL	500 mL	50 mL

V.2. Characterization of motherwort and hawthorn extracts

In this chapter we evaluated the correlation between the total polyphenolic constituents of *L. cardiaca* and *C. monogyna* and the bioactive properties of their hydroethanolic extracts.

In the present study, the phytochemical profile of the hydroethanolic extracts of motherwort (herba), hawthorn (fructus) and hawthorn (flos and foliae) was analysed using UV-Vis spectrophotometry. The results of the qualitative and quantitative analysis are summarized in Figures 41-50. Data are reported as the average of three analyses.

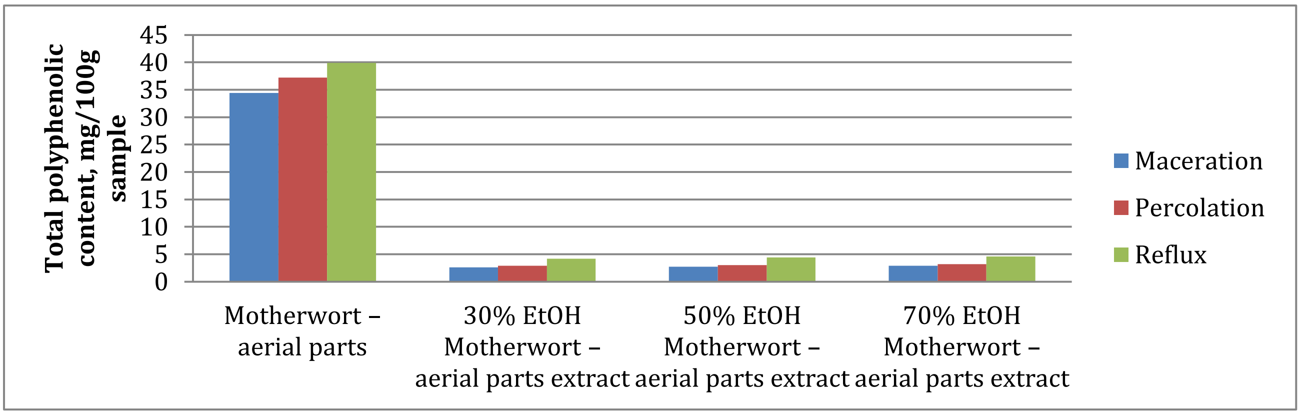

FIG. 41. The total polyphenolic content of motherwort - herba and motherwort - extract, expressed as gallic acid

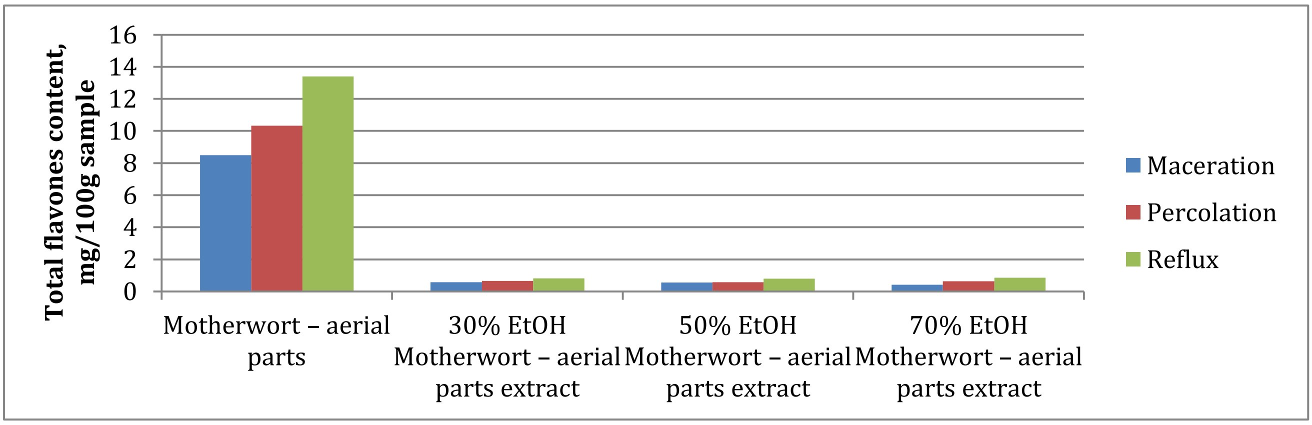

Fig. 42. Total flavones content of motherwort - herba and motherwort - extract, expressed as rutin

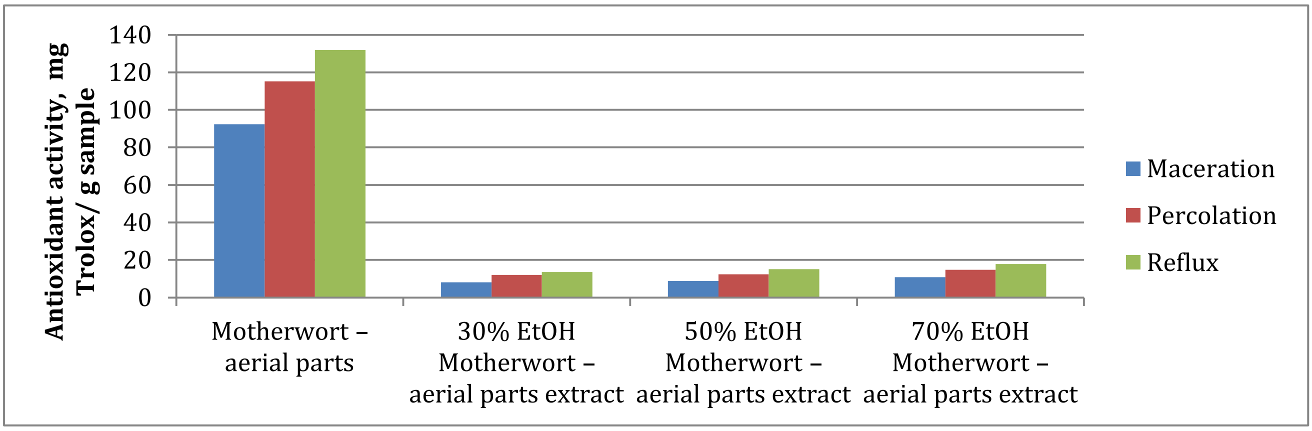

FIG. 43. Antioxidant activity of motherwort - herba and motherwort - extract, expressed as equiv. Trolox

Figures 41-43 describe the results for motherwort - herba and motherwort extracts for total polyphenols, total flavones and antioxidant activity. According to the reported studies, the total polyphenolic content of motherwort extracts is between 0.4 and 7.3 mg/100 g of sample, so that the values obtained in our study are above this range (Fierascu, et al., 2019). The best results were obtained for 70% ethanol extracts (total polyphenolic content - about 5 mg/100 g sample, total flavonoid content - about 1 mg/100 g sample). Reflux with 70% ethanol is the most suitable extraction method for motherwort - herba as the highest contents of total polyphenols, total flavonoids, major active compounds, and the strongest antioxidant activity were obtained.

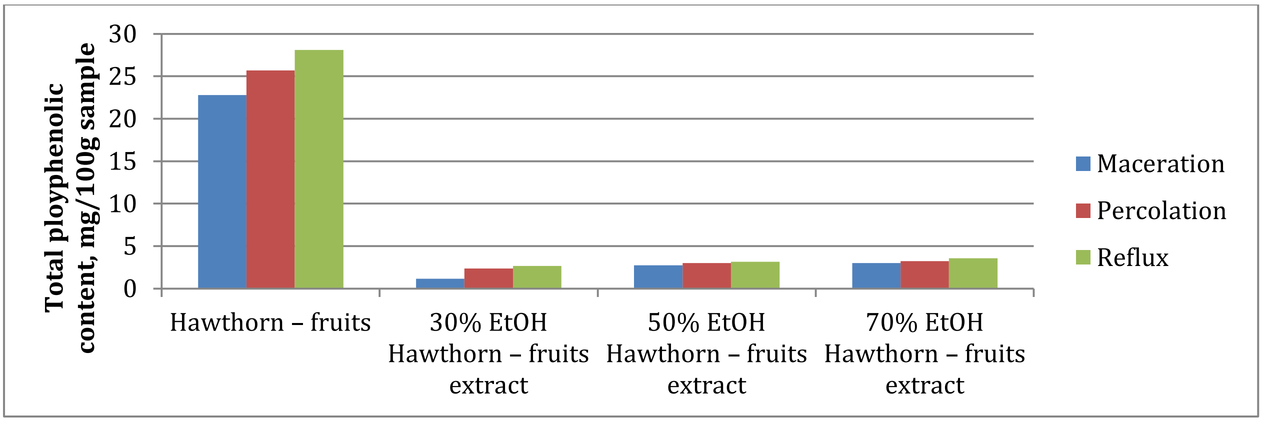

FIG. 44. The total polyphenolic content of hawthorn - fructus and hawthorn - extract, expressed as gallic acid

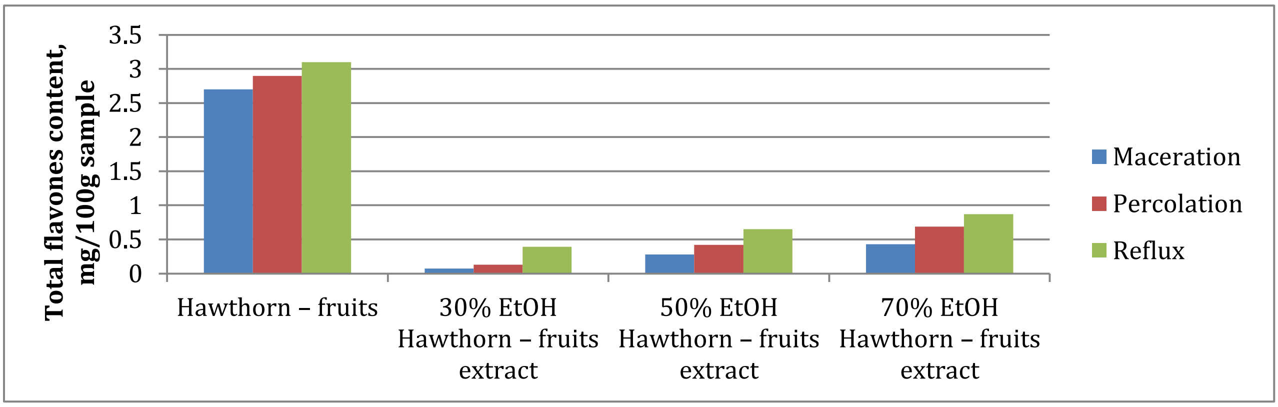

Fig. 45. Total flavones content of hawthorn - fruit and hawthorn - extract, expressed as rutin

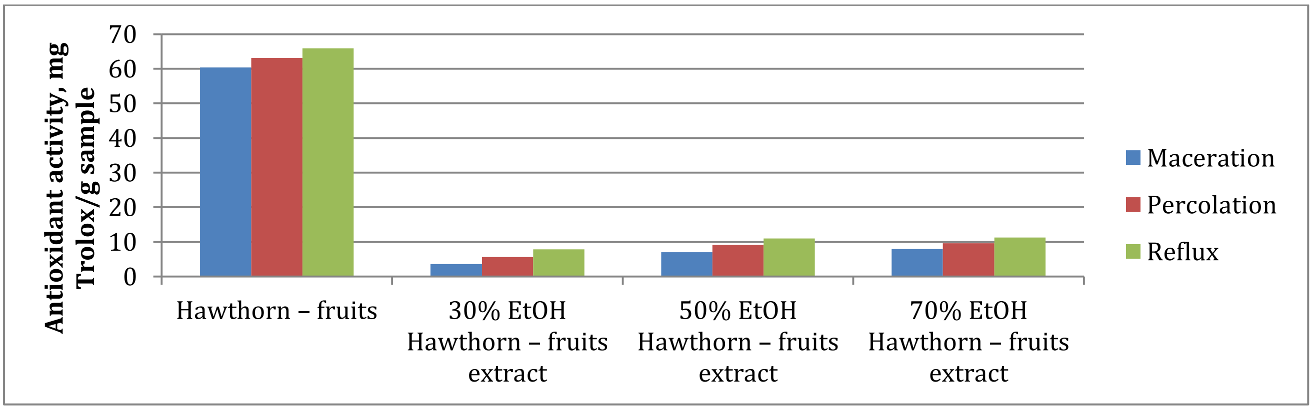

FIG. 46. The antioxidant activity of hawthorn - fructus and hawthorn - extract, expressed as equiv. Trolox

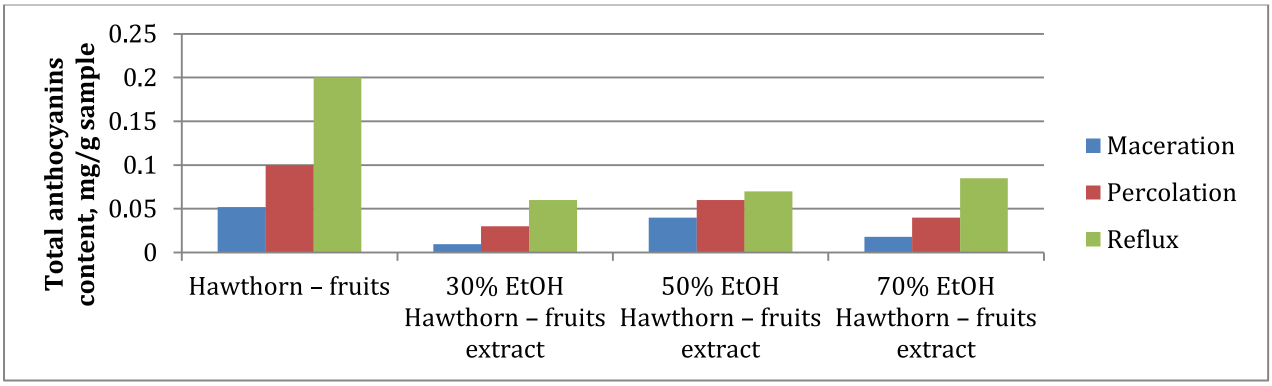

Fig. 47. Total anthocyanin content of hawthorn - fructus and hawthorn - extract, expressed as cyanidin 3-*O*-glucoside

Figures 44-47 show the results obtained for hawthorn - fruit and hawthorn - extract for total polyphenols, total flavones, total anthocyanins and antioxidant activity. Polyphenols have similar values in 50% and 70% ethanol extracts, but much lower values in 30% ethanol extracts, the results are also higher for reflux extracts. In addition to the total polyphenolic content, spectrophotometric methods are also used to determine the total content of individual groups of phenolic compounds, such as total flavones and the total anthocyanin content expressed as cyanidin 3-glucoside chloride. The determination of flavones shows better results for extracts obtained by reflux, ranging from 0.4 to 0.8 mg/100 g sample. As for the total anthocyanin content of hawthorn - fructus extracts, we can notice a better extraction by percolation in 50% ethanol solution (0.06 mg/g sample) and by reflux in 70% ethanol solution (0.09 sample mg/g).

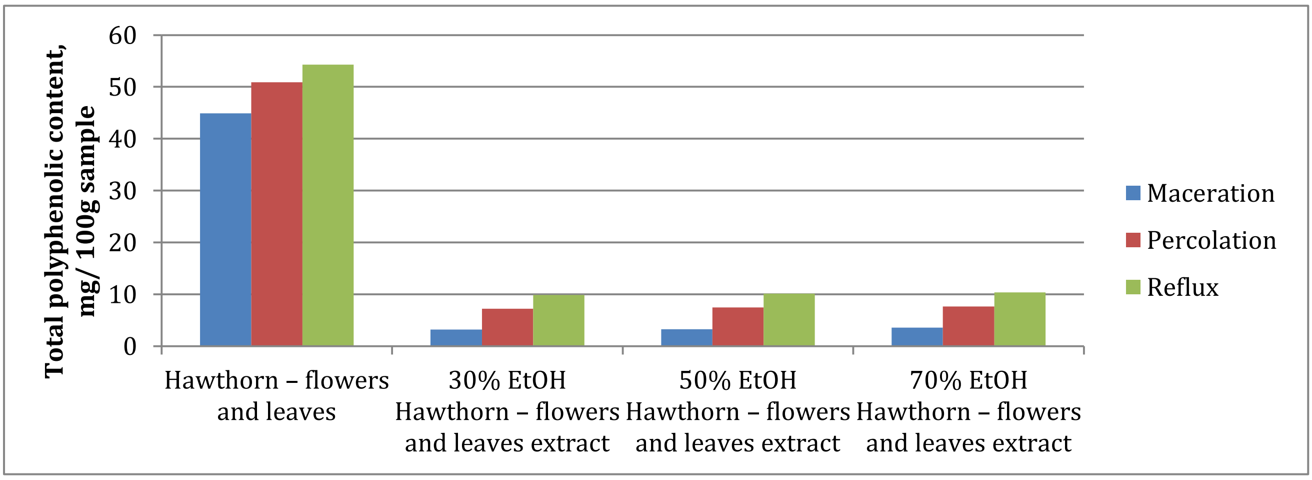

FIG. 48. The total polyphenolic content of hawthorn - flos and foliae and hawthorn - extract, expressed as gallic acid

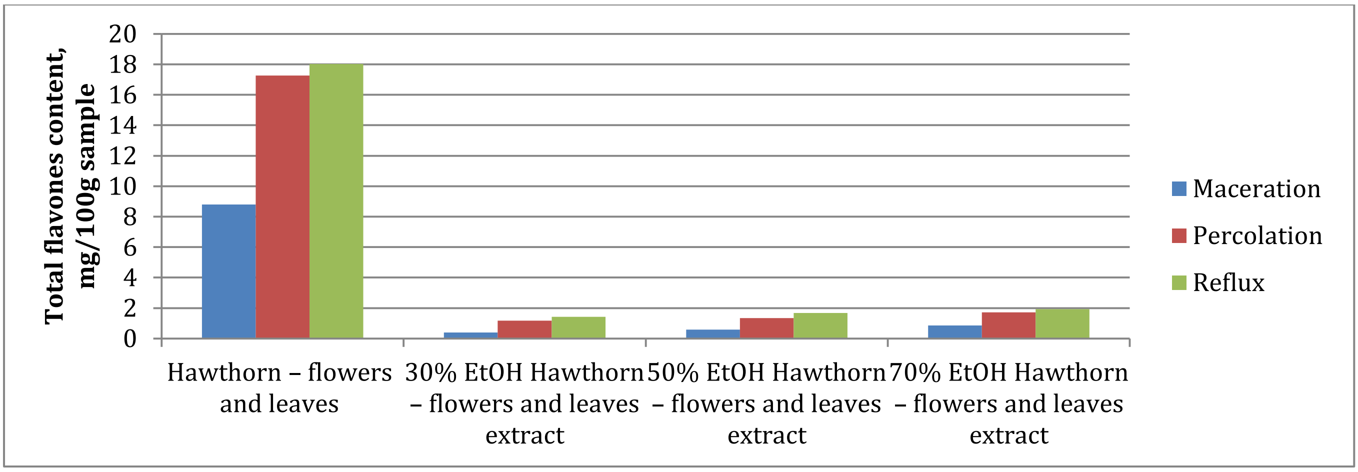

Fig. 49. The total flavone content of hawthorn - flos and foliae and hawthorn - extract, expressed as a rutin

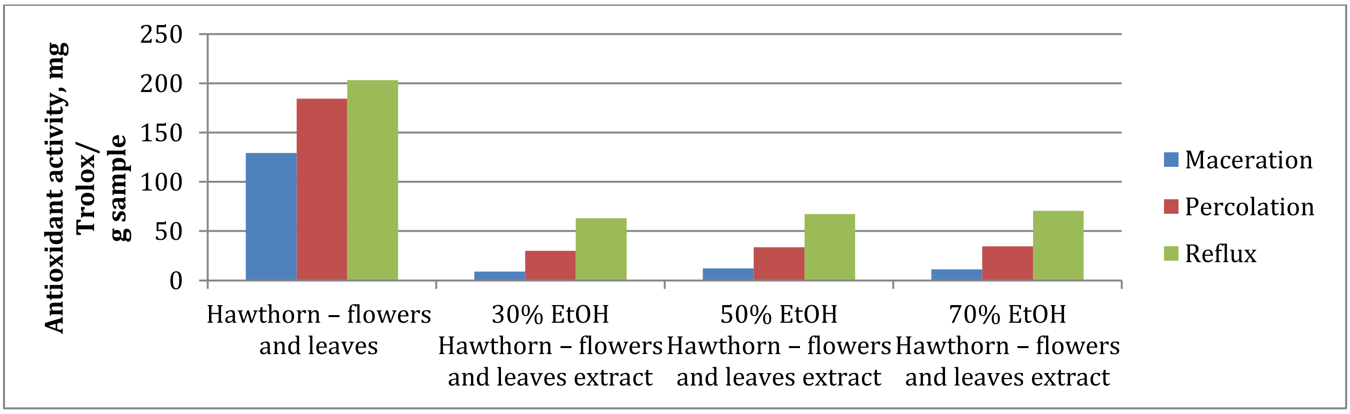

Fig. 50. The antioxidant activity of hawthorn - flos and foliae and hawthorn - extract, expressed as equiv. Trolox

Figures 48-50 describe the results obtained for hawthorn - flos and foliae and extracts from hawthorn - flos and foliae for total polyphenols, total flavones and for antioxidant activity. They have similar values regardless of the extraction method applied, with higher values for the total polyphenolic content of about 10 mg / 100g sample.

Hydroethanolic extracts of hawthorn (fructus), hawthorn (flos and foliae) and motherwort (herba) have a promising antioxidant potential, evaluated by the CUPRAC test, the order of potentials being hawthorn (floss and leaves) > motherwort (herba) > hawthorn (fructus) and values between 3.6 and 70.5 mg Trolox/g sample. The results obtained for the antioxidant activity of the studied extracts indicate the influence of polyphenols and flavones present in each extract.

In general, it can be clearly stated that the pharmacological activities of the examined medicinal plants traditionally used cannot be attributed to a single active compound, but must be considered as the effect of the full spectrum of their phenolic constituents.

CHAPTER VI - CHARACTERIZATION OF MEDICINAL PLANTS AND EXTRACTS WITH HEPAT

VI.1. Obtaining extracts from the milk thistle

Silymarin is a hepatoprotective polyphenolic compound that is isolated from the milk thistle. Silymarin is a mixture of flavonolignans that includes isomers: silibinin, silicristin, silydianin, and has the role of protecting cells from free radical damage.

The content and composition of silymarin in milk thistle plants are influenced by the depth of sowing, the distance between plants, fertilizers, harvesting and post-harvest treatment. In order to compare the results obtained in the extraction of the compounds of interest, two extraction methods were applied in parallel, thus obtaining a tincture and aqueous extract from the fruits of the ripening fruit at different stages of maturity. Milk thistle fruits were harvested monthly between May and October 2019 (Tables 16, 17).

The antioxidant activity of the raw material may depend on the chemical structure of the biologically active compounds in the plants, as well as on the preparation of the extract, i.e. the method of extraction, including the solvents used. The chemical composition and polarity of the solvent used towards extraction are important factors in determining the antioxidant capacity as they may affect the mechanism of hydrogen atom transfer (HAT) (Nowak, Florkowska, Zielonka-Brzezicka, Duchnik, Muzykiewicz, & Klimowicz, 2021).

Table 16. Composition of milk thistle aqueous extract

Milk thistle aqueous extract	**Quantity, g**
Grease skimmed fruit *(Silybum marianum L.)*	300
Purified water	2400
Total (by concentration)	**120**

Table 17. Composition of milk thistle tincture

Milk thistle tincture	**Quantity, g**
Grease skimmed fruit *(Silybum marianum L.)*	20.00
Ethanol 96% (v/v) diluted to 70% with purified water	q.s.ad. 100.00

VI.2. Characterization of milk thistle extracts

In the present study, the phytochemical profile of non-skimmed/skimmed fruits and extracts obtained from skimmed fruits of *Silybum marianum* was analyzed using both UV-Vis spectrophotometry to determine total polyphenols expressed as gallic acid and total flavones expressed as rutin, as well as HPLC analysis of the total silymarin content expressed as silibinin. The results of the qualitative and quantitative analysis are summarized in Figures 51-54.

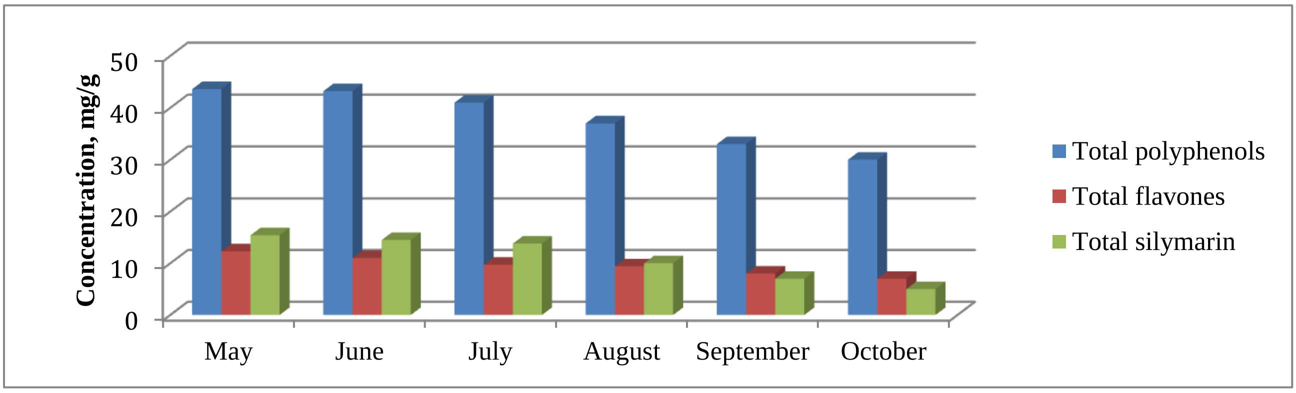

FIG. 51. Content in polyphenolic compounds from non-skimmed fruit of milk thistle, mg/g

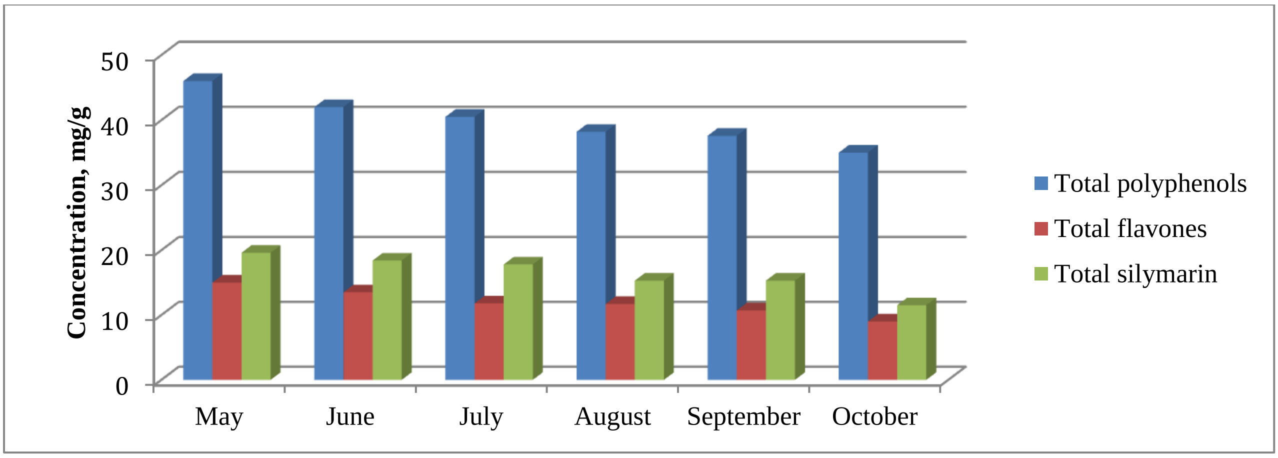

Fig. 52. Content in polyphenolic compounds of skimmed fruit of milk thistle, mg/g

The antioxidant activity of this plant depends on the maturity of the raw material, where the highest level was observed in immature fruits (harvested in May-June), then in intermediate ones (harvested in July-August), while the lowest was observed in those with full maturity (harvested between September and October). Polyphenols are components of plants with high antioxidant capacity and could play an important role in hepatoprotective activity. In addition, the concentration of polyphenols can be highly correlated with antioxidant activity, as demonstrated in the fruits of *Silybum marianum*. In the present study, in most cases, significant connections were found between the determined antioxidant capacity and the content of polyphenolic compounds.

As shown in Figures 51-52, the results obtained from spectrophotometric and HPLC determinations are higher for skimmed fruit than non-skimmed fruit, as expected, in terms of total polyphenols and total silymarin content; in the case of total flavones, the results obtained for the samples of milk thistle fruit are similar, having values in the range of 7-12.3 mg / g - non-skimmed fruit and 9-15 mg/g - skimmed fruit. The maximum concentration in polyphenolic compounds is reached in May-June, this being the most suitable extraction period for the milk thistle fruits.

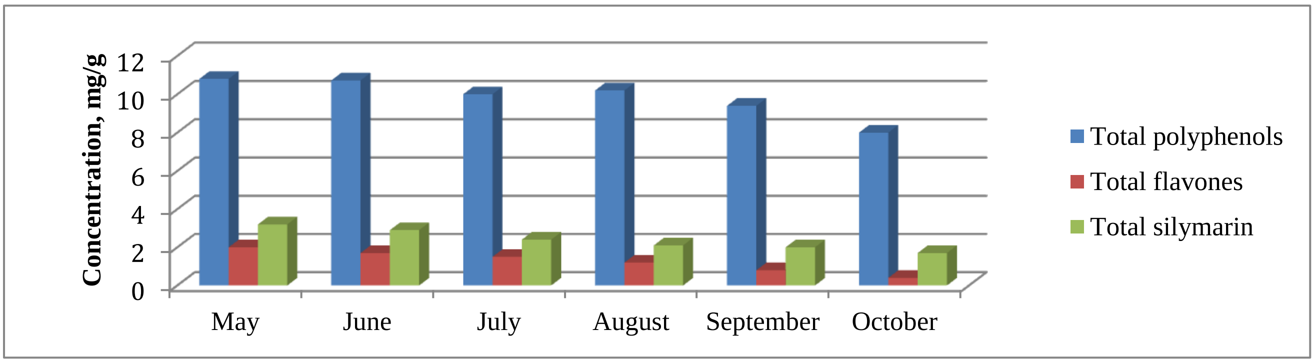

FIG. 53. Content of polyphenolic compounds in milk thistle aqueous extract, mg/g

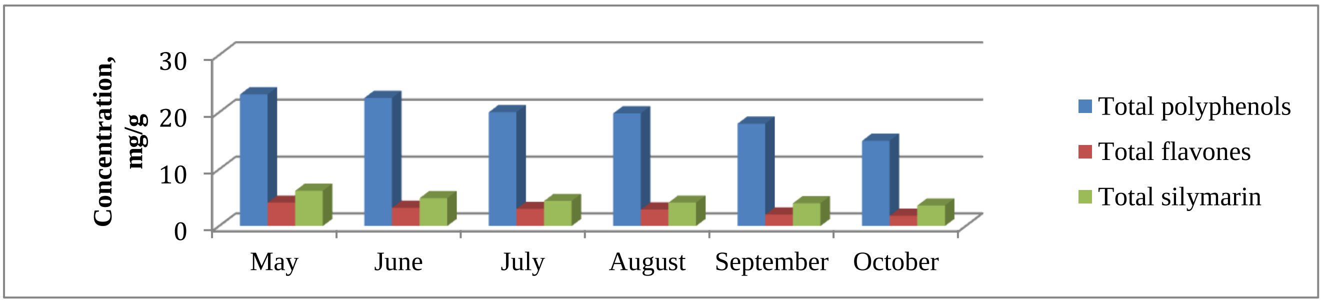

Fig. 54. Content of polyphenolic compounds in milk thistle tincture, mg/g

Figures 53-54 describe the content of polyphenolic compounds of milk thistle aqueous extract and tincture. Thus, the results obtained for the two types of extracts show a higher content in total polyphenols for tincture than aqueous extract, having values in the range of 15-23.1 mg/g, respectively 8-10, 8 mg/g. HPLC analysis shows approximately twice the content of total silymarin expressed as silibinin in the tincture of milk thistle compared to the aqueous extract from the milk thistle fruit, which confirms the increased efficiency of extraction with hydroalcoholic solutions of polyphenolic compounds. Also, the highest content in total flavones was observed in the case of tincture and aqueous extract from skimmed fruit harvested in May, respectively 4.1 and 2 mg/g.

The antioxidant activity of milk thistle fruit extracts has been confirmed by many authors (Chambers, et al., 2017) (Lucini, et al., 2016) (Malinowska, 2017). The extraction of the raw material can be done both by classical methods and by newly developed methods. The extraction method selected for this study was Soxhlet extraction, which has been a standard technique used for decades, often used as a reference to evaluate the effectiveness of other methods. Soxhlet extraction is considered to be efficient, however, its disadvantages are relatively high solvent consumption and quite long extraction time.

The aim of this study was to evaluate the antioxidant capacity of ripe fruit at different stages of maturity and the extracts obtained from them: aqueous extract and tincture. The results obtained for the antioxidant activity of the studied extracts indicate the influence of polyphenols and flavones present in each extract. Thus, the results in Figures 59-62 show an increased value of antioxidant activity for the skimmed milk thistle fruit, with a maximum of 258 mg Trolox/g sample for fruit harvested in May; non-skimmed fruits have an antioxidant activity with values in the range of 156-230 mg Trolox/g sample, lower values than in the case of skimmed fruit. We can observe a trend of significant decrease in antioxidant capacity over time in the case of both types of samples, which indicates May as the most suitable month for harvesting the milk thistle fruits.

Extraction using ethanol as the extraction solvent proved to be more effective than extraction in aqueous medium, the results regarding the antioxidant activity for the milk thistle tincture being in the range 74-115 mg Trolox/g sample, and those for the aqueous extract 30-49 mg Trolox/g sample.

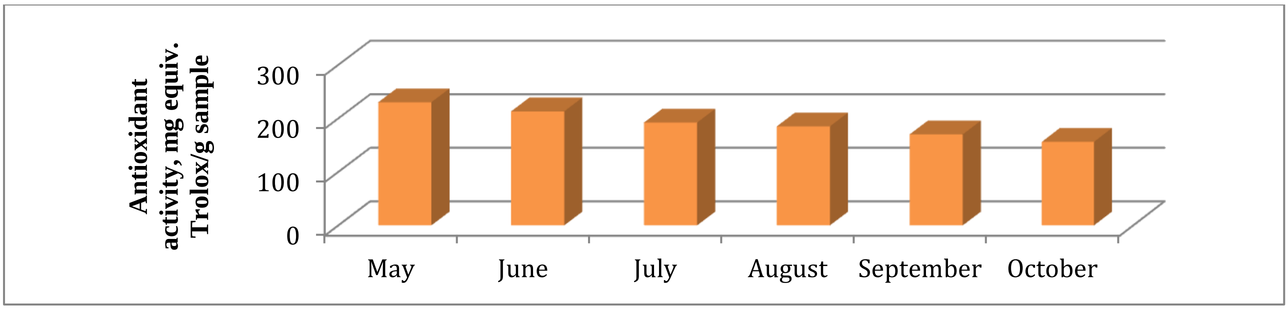

FIG. 59. Antioxidant activity of non-skimmed milk thistle fruits, mg Trolox/g sample

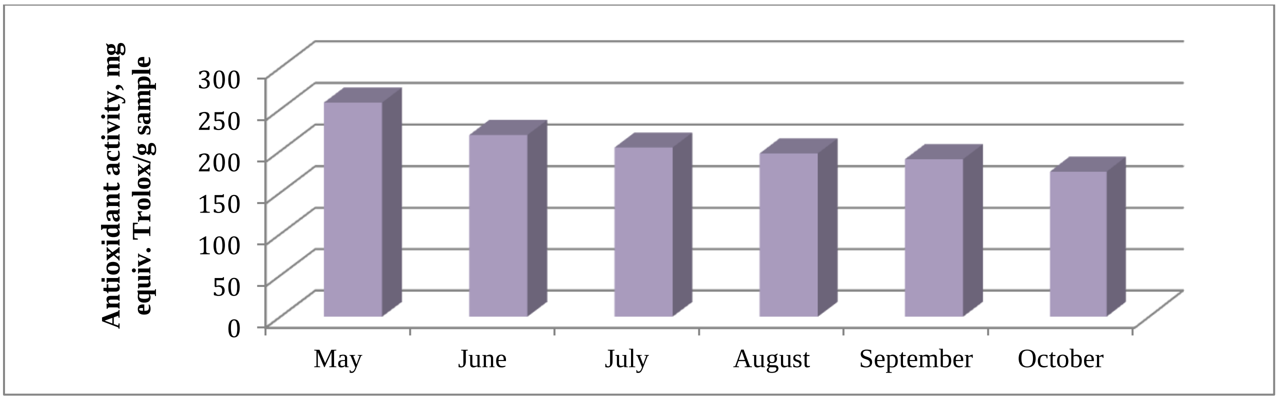

Fig. 60. Antioxidant activity of skimmed milk thistle fruits, mg Trolox/g sample

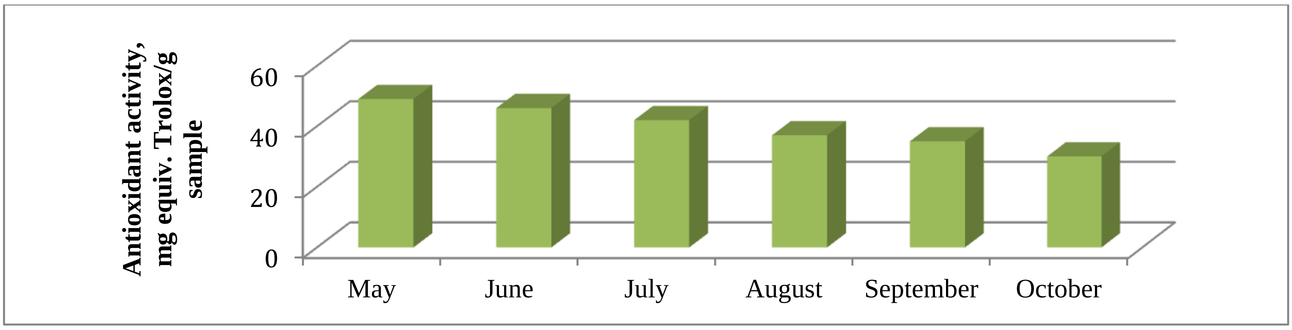

Fig. 61. Antioxidant activity of aqueous milk thistle aqueous extract, mg Trolox/g sample

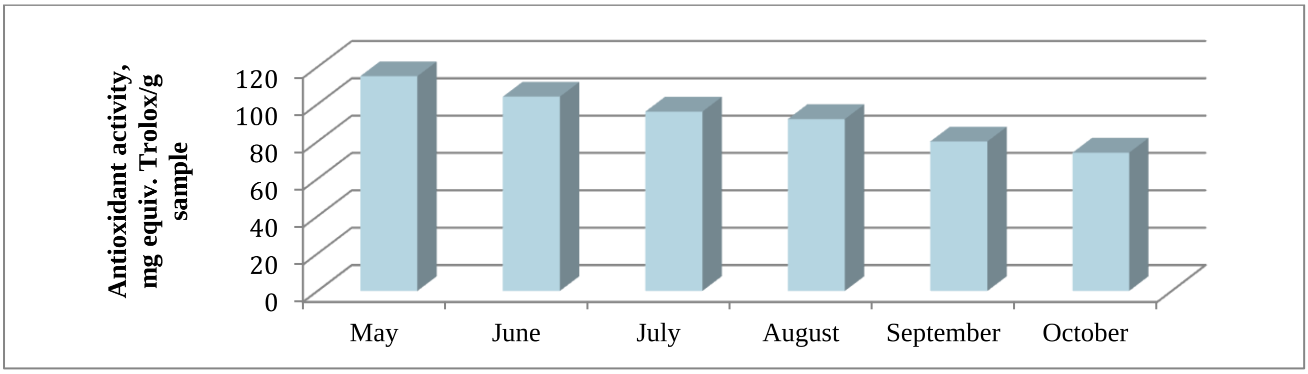

Fig. 62. Antioxidant activity of milk thistle tincture, mg Trolox/g sample

GENERAL CONCLUSIONS

The present research study involved investigation of the composition of six medicinal plants commonly used towards the relief of inflammation, in the relief of cardiovascular and hepatoprotective diseases, and extracts obtained from these plants by varying extraction parameters (extraction method, solvent concentration) to maximize the content of compounds of interest (certain flavones and polyphenols, anthocyanins, etc.).

The main objectives of the study were:

- obtaining and characterizing extracts from medicinal plants with anti-inflammatory effect: willow (*Salix alba*), rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*);
- obtaining and characterizing extracts from medicinal plants used towards the improvement of cardiovascular diseases: motherwort (*Leonurus cardiaca*) and hawthorn (*Crataegus monogyna*);
- obtaining and characterizing milk thistle extracts (*Silybum marianum*), a medicinal plant with hepatoprotective effect.

Thus, considering the experimental results obtained we can conclude that:

- Biologically active substances obtained by extraction have been identified and quantified by modern methods of analysis including high performance liquid chromatography (HPLC), UV-Vis spectrophotometry and gas chromatography (GC) coupled with mass spectrometry (MS).
- UV-Vis spectrophotometric methods have been developed for the determination of total polyphenols expressed as gallic acid, total flavones expressed as routine, anthocyanins expressed as cyanidin-3-glucoside chloride, tannins expressed as pyrogallol and the determination of antioxidant activity of selected plants and extracts. obtained from them.
- Various extraction methods and extractive conditions of various components of willow (branches, leaves and bark) were compared to obtain extracts rich in salicylic derivatives, but also other polyphenolic compounds, with an effect in improving discomfort in the joints and muscles. Extraction in aqueous medium proved to be the most efficient. A good source of salicylic derivatives turned out to be the young willow bark, with the highest concentration in the compounds of interest.

- The willow bark residue resulting from the extraction was further used for the additional extraction of active ingredients; the microwave treatment was more efficient than the ultrasound one - at 150 ° C an additional 8.7% salicin was extracted (vs. the amount extracted from the plant material not subjected to extraction).

- The efficacy of US or MW treatment in the additional extraction of active ingredients was also tested in terms of the stability of the active compounds (salicin derivatives) under the treatment conditions. These compounds have been shown to be stable under the experimental conditions used.
- Two plants with a high content of chemical compounds were selected that have a beneficial effect in ameliorating the symptoms and unpleasant effects of cardiovascular diseases: motherwort (aerial parts) and hawthorn (leaves and flowers; fruit).
- Extracts were made from the two plants mentioned above by maceration and percolation, using as solvent alcohol solutions: water (30:70, 50:50, respectively 70:30 *V/V*), to maximize the content of compounds of interest. (certain flavones and polyphenols, anthocyanins, etc.).
- Extraction by percolation proved to be more efficient than extraction by maceration, the content of polyphenolic compounds being higher for the extracts thus obtained. Hawthorn fruits have a low anthocyanin content.
- A comparative assessment was made of the content of polyphenolic compounds and the antioxidant activity of non-defatted/defatted milk thistle fruits and of the extracts (aqueous extract and tincture) obtained from the defatted fruits, harvested in May-October.
- Following this evaluation we can conclude that the optimal harvest time for the milk thistle fruit is May-June, thus favouring the efficient use of the plant.
- The high content of silymarin and other phenolic compounds, the antioxidant activity, as well as the beneficial effects on health recommend the use of the extract from skimmed milk thistle fruit in the formulation of food supplements with hepatoprotective effect.

ORIGINAL CONTRIBUTIONS

Through this study, important contributions were made in the field of natural extracts from medicinal plants selected from a theoretical, experimental and applied point of view, with the potential to be valuable products for the development of food supplements.

This research aims to investigate the composition of the various components of white willow (*Salix alba*), which is commonly used towards the treatment of painful mobility disorders, such as back pain and arthritis, due to the content of salicylic derivatives, tannins and other phenols. Total polyphenols, total flavones, total tannins and antioxidant activity were determined by UV-Vis spectrophotometric methods. Salicylic derivatives were determined by the HPLC method. The results show a higher concentration of salicin in the mature shell sample of 20 mg/g. Polyphenols have similar values in bark and leaves with higher results for mature willow.

We followed the extraction conditions of polyphenols and flavones from two medicinal plants known for their role in ameliorating cardiovascular disease by varying some extraction parameters: ethanol concentration and extraction method, aiming to identify an appropriate method of extraction of the two classes of compounds of phytotherapeutic interest. The extracts were prepared from hawthorn (fruit), hawthorn (leaves and flowers) and motherwort (aerial parts) by maceration, percolation and reflux using ethanol solutions: water (30:70, 50:50, respectively 70:30 *V/V*), to maximize the content of compounds of interest (certain flavones and polyphenols, anthocyanins, etc.). Knowing that both classes of compounds also have antioxidant activity, we correlated the results obtained for polyphenols and flavones with antioxidant activity.

We conducted a study on the optimal harvesting time of the fruit of the tree and the influence of the extraction solvent used on the extraction of the compounds of interest (distilled water or hydroalcoholic solution), by identifying and quantifying silymarin and other bioactive compounds present in the fruit of the tree and also by correlating them with antioxidant activity.

FUTURE RESEARCH RECOMMENDATIONS AND DIRECTIONS

The main areas of study for further research are:

- Extending the applied research of natural extracts on other raw plant materials.
- Demonstration of pharmacological effects (anti-inflammatory, cardiovascular disease-relieving and hepatoprotective) by testing on cell lines the obtained extracts within doctoral research.
- Use of biologically active compounds which were obtained by means of extraction from selected plant materials by encapsulation in modern lipid-based vehicles to increase bioavailability.

LIST OF PUBLICATIONS RESULTING FROM DOCTORAL RESEARCH

Articles which have been published in ISI listed journals:

1. **A.M. Neagu**, C.M. Codreanu, V. Staicu, R. Stan. Bioactive polyphenolic compounds from Motherwort and Hawthorn hydroethanolic extracts. *Studia UBB Chemia*. **2021,** *LXVI*, 4, p. 123-132. **(IF – 0,495)**

DOI:10.24193/subbchem.2021.4.09

2. V. Staicu, C. Luntraru, I. Călinescu, C.G. Chisega-Negrilă, M. Vânătoru, **M. Neagu**, A.I. Gavrilă, I. Popa. Ultrasonic and Microwave Cascade Treatment of Medicinal Plant Waste. *Sustainability*. **2021**, *13*, 12849. **(IF – 3,251)**

https://doi.org/10.3390/su132212849

Articles which have been published in ISI indexed journals:

1. **M. Neagu**, C. Luntraru, A. Bîra (Popescu), J. Tomescu, N. Ionescu (Bordei), R. Stan. Bioactive Phenolic Compounds from White Willow (*Salix alba*) Bark, Leaves and Branches. *U.P.B. Scientific Bulletin*, Series B. **2021**, *83*, 3.

Communications at international scientific events:

1. International Symposium of Chemical Engineering and Materials – SICHEM 2018, 6-7 septembrie 2018, București

· *A new phytotherapeutic product with health promoting effects in rheumatic ailments* Autori: V. Carabela, V. Tamaş, G.C. Ivopol, **A.M. Neagu**, published in Bulletin of Romanian Chemical Engineering Society, Vol 5, No. 2, 2018

2. International Symposium *Prioritățile chimiei pentru o dezvoltare durabilă* – PRIOCHEM, Ed. a XIV-a, 17-19 octombrie 2018, București

· *Research on various sources of Willow Bark in order to obtain plant extracts rich in salicylic derivatives* Autori: V. Carabela, V. Tamas, A. Suciu, **A.M. Neagu** - POSTER

3. 21st Romanian International Conference on Chemistry and Chemical Engineering, 4-7 septembrie 2019, Constanța – Mamaia

· *Natural intermediates for obtaining phytotherapeutic products beneficial in cardiovascular diseases* Autori: **A.M. Neagu**, V. Staicu, C. Luntraru, A. Popescu, M. Neagu – POSTER

4. International Symposium of Chemical Engineering and Materials – SICHEM 2020, 17-18 septembrie 2020, București

· *Characterization of white willow by HPLC and spectrophotometric methods* Autori: **A.M. Neagu**, C.M. Luntraru, A.F. Bîra (Popescu), J.A. Tomescu, N. Ionescu (Bordei) – POSTER

5. 8th International Conference on Materials Science and Technologies – RoMat 2020, 26-27 noiembrie 2020, București

· *Spectrophotometric and HPLC Determination of Phenolic Compounds from White Willow (Salix alba)* Autori: **A.M. Neagu**, C.M. Luntraru, A.F. Bîra (Popescu), J.A. Tomescu, N. Ionescu (Bordei) – POSTER

Gratitude towards:

1. Project POCU 380/6/13 "Sisteme de învățare bazate pe muncă prin burse antreprenor pentru doctoranzi și postdoctoranzi (SIMBA)", PhD student entrepreneur (2019-2021)

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