MODERN METHODS OF EXTRACTION OF BIOACTIVE COMPOUNDS FROM MEDICINAL AND AROMATIC PLANTS, FOR OBTAINING FUNCTIONAL MOUNTAIN FOOD PRODUCTS IN ROMANIA

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Abstract

The paper presents in the first part a bibliographic study on the use in phyto-therapy and aroma-therapy of various medicinal and aromatic plants in mountainous areas in Romania. Various present in Romania are highlighted, the biological significance of primary and secondary metabolites as well as the classical methods of their extraction by steam distillation, with organic or aqueous solvents. In the second part of the paper is presented the modern method of extraction using fluids at subcritical pressure applied to the technological line in positions at the Research Center for Eco-biotechnologies and Equipment for Agriculture and Food at the Research Institute of Transilvania University in Brasov. The paper concludes with the presentation of case studies on the action of bioactive substances in extracts made from sea-buckthorn (Hippophaë rhamnoides L.) and thyme (Thymus serpyllum). The sea buckthorn (oleum hippophaes) oils antioxidant activity was highlighted using the DPPH method, and the antimicrobial activity of volatile thyme oil was demonstrated according to the CLSI standard, M 100 - S 18, 2008, by evaluating the diameter of the Salmonella typh development inhibition zone and Escherichia coli β glucuronidase positive. The conclusions of the paper emphasize the special potential of medicinal and aromatic plants, as well as the superiority of modern methods of extraction of secondary metabolites, an important source of income for farmers in mountain areas in Romania.

Keywords*: medicinal plants, aromatic plants, extraction, functional mountain products*

INTRODUCTION

Plants are well-known sources of pharmaceutical, aromatic and industrial compounds, human society being closely connected to the vegetal world. Veritable biochemical plants powered by solar energy, they produce not only the basic compounds necessary for their own survival, like lipids, proteins and carbohydrates, but also a big number of organic compounds that can be extracted in commensurate volumes to be important for human use with many scientific, technological and commercial applications [2, 3].

Even today, the botanical dowry of the planet stores many still insufficiently known resources, which can be cost-effective alternatives for obtaining deficient raw materials in various economic fields. The plant world still is the most important supplier of phytochemicals used in many industries like pharmaceuticals, food, cosmetics, agro-chemicals, with commercial values expressed in billions of dollars. Plants are a sources that can not be replaced by industrial oils (volatile and fixed), fragrances, flavours, saponins, other surfactants, resins, dyes, hydrocolloid gums, pesticides, natural rubber, medicinal substances and many more [4, 11].

Plant metabolism consists of closely coordinated series of chemical reactions mediated by enzymes that take place in the plant organism, resulting in the synthesis and use of a wide variety of molecules in the category of carbohydrates, amino acids, fatty acids, nucleotides and polymers derived from them (polysaccharides, proteins, lipids, DNA, RNA, etc.). All of these processes are defined as primary metabolism and the respective compounds, which are essential for plant survival, are described as primary metabolites. Recent statistics show that over 1,500 new compounds are identified annually in various plant species and that about a quarter of prescription drugs contain substances of plant origin [1]. At the same time, the rapidity of the process of species extinction and tightening the genetic base of plant resources in the world stimulated both the reconsideration of the vital importance of genetic resources and the interest in obtaining metabolites of interest by unconventional methods [7, 8].

In addition to the "primary" metabolites, with a major role in maintaining the viability of the plant (proteins, carbohydrates and fats), are also synthesized a number of compounds that include terpenes, steroids, anthocyanins, anthraquinones, phenols and polyphenols, which belong to the so-called "secondary-metabolism". Secondary metabolites are present mainly in particular species, often manifesting organ or tissue specificity, can be identified only at a certain stage of growth and development within a species, or can be activated only during periods of stress, caused for example by diseases or the exhaustion of nutrients. Synthesis of secondary metabolites has no direct significance to the normal cell metabolism, but could be very important for the development and functioning of the whole plant [5]. The value of secondary metabolites used in the pharmaceutical industry can reach thousands of dollars per kg. For example, purified opium alkaloids (codeine and morphine) have values of 650 - 1250 USD / kg and volatile essential oils such as rose are valued at over 2000 - 3000 USD / kg. The alkaloids extracted from *Catharanthus roseus* reach values of up to 20,000 USD / g. These high prices are due to both costly isolation methods and extremely low quantities that can be obtained from plant sources.

Some varieties of imported plants present in Romania, in the mountain area can be mentioned: *Angelica (Angelica Archangelica); Artichoke (Cynara Scolymus); Arnica (Arnica Montana); Catina (Hippophaë Rhamnoides L.); Thyme (Thymus Serpyllum); Chicory (Cichorium Intybus); Feriga (Dryopteris Filix); Gentiana (Gentiana Luted); Juniper (Juniperus Communis); Cranberry (Vaccinum Vitis Idaea); Mountain Cormorant (Sorbus Aucuparid); Yarrow (Millefolii Herba, Millefolii Flos), St. John's wort (Hyperici Herba); Hawthorn (Crataegi Folium, Crataegi Flos, Crataegi Fructus); Lemon balm (Melissa Officinalis); Rosehip (Rosa Canina)* [6].

The paper has the following objectives: presentation of a modern method of extraction of secondary metabolites, under vacuum, with high energy efficiency; results on the antimicrobial activity of thyme essential oil *(Thymus Serpyllum):* results on the antioxidant action of sea buckthorn extract *(Hippophaë Rhamnoides L.)* [10].

MATERIALS AND RESEARCH METHOD

Bioactive substances are obtained through two complementary extraction methods, in conditions of room temperature and in the absence of oxygen, thus keeping all the biologically active properties of plant material used unaltered.

a. Extraction with liquefied gas under subcritical pressure

This method allows the extraction of liposoluble substances and essential oils, with certain antioxidant and antimicrobial properties, from lavender, sea buckthorn, walnut core, pine

sprouts, St. John's-wort, etc. [9] The most important advantage that liquefied gas extraction method with the subcritical pressure offers, is that the oils can be extracted in pure state at room temperature and in the absence of air, which allows obtaining in extract of new categories of bioactive substances.

In table 1 it is presented the flow chart of this method, use by Eco-Biotechnologies And Equipment In Food And Agriculture research center, from Transilvania University of Brasov, Romania. In figure 1 can be observed the main extraction facility, FC 100 Timatic extractor, TECNOLAB Italy.

Table 1

Technological Flow In Research Activity, Ecobiotefa Research Center

1. Drying system - by computer-assisted dryer
2. Sterilization system - by bactericidal lamps
3. Crushing system - through laboratory mill / with interchangeable screens
4. Fat-soluble extraction system - FC100 subcritical pressure fluid extractor
5. System for obtaining acidic or basic hydroalcoholic solutions
6. Water-soluble extraction system - by TIMATIC Extractor
7. Extract filtration system
8. Primary analysis system - laboratory equipment physical-chemical analysis
9. Extract packaging system
10. Storage system - standard and refrigerated cabinets

Figure 1. Extraction facility, FC 100 Timatic, TECNOLAB Italy

b. Extraction into liquid solvents under pressure

Exhausted results after extraction into subcritical (delipolyzed) fluids are processed further by extraction into liquid solvents under pressure (using MicroTimatic extractor) for the extraction of bioactive soluble substances. The two categories of extracts, liposoluble + water-soluble sum up a complex of biocomponents that act synergistically in the human body, thus being superior to dietary supplements existing on the market [12].

Tests performed on extracts obtained from plant resources aim to quantify the **antiviral, antibacterial or antifungal** effect for each newly identified compound [13].

As a method of analysis of antimicrobial activity it was used the method of evaluating the diameter of the area of inhibition of microorganism growth CLSI standard, M100 - S18, 2008. In agar plates previously inoculated with the studied microorganism, paper disks impregnated with solutions of different concentrations of the studied antimicrobial compound are applied, using both a negative control (DMSO) and a positive one (amphotericin B).

After incubation at 35℃ for 24 or 48 hours, depending on the present microorganism, the size of the inhibition zone is evaluated (the portion around the disc in which the microorganism did not proliferate), and if its diameter exceeds 6.5 mm more than the diameter of the disc), the result is interpreted as positive. Thus, the minimum inhibitory concentration of any compound is given by the lowest concentration of that compound at which a positive result is found.

For the test of the antibacterial action, the extract of Thyme (*Thymus Serpyllum*) was used. The antibacterial action was tested on various bacterial strains isolated from food products, following standardized laboratory expertise.

The microorganisms whose susceptibility was tested were the following: *Salmonella typh and Escherichia coli β glucuronidase positive.*

The method dwells in using decimal dilutions, afterword inoculating 2 Petri dishes with TBX medium from each consecutive decimal dilution, incubating it from 18 to 24 hours at 44°C ± 1°C, then looking for the presence of colonies which, could be regarding their characteristics, E.coli positive for β - glucuronidase. The representative β-glucuronidase-positive *E.coli* colony forming units (cfu) of each plate consisting of more than 150 representative cfu and less than 300 total cfu (typical and atypical) are counted from two successive serial dilutions. Next, using mathematical formula the microbial load will be calculated, the obtained values being confirmed using Vitek 2 Compact apparatus. The action of essential oils on the isolated microorganisms was evaluated according to the provisions of the CLSI standard, M 100 - S 18, 2008, depending on the inhibitions zone diameter. This method it is named also the diffusion method, and the work principle is based on inhibition of the bacterial development, in the presence of essential oils at different decimal concentrations. The Müeller-Hinton special medium was used, because has no influence against micro-organism or essential oils activity. For seeding the Müeller-Hinton medium, the flooding method with 1 - 1.2 ml dilution was used (dilution of 10 - 3).

Next, the transfer was done on the surface of selective culture media, used to isolate bacteria of the genus *Salmonella* and *E. coli*, respectively, Rambach medium and TBX medium.

The inoculated medium was placed into the thermostat with the lid of the semi-open plate, for drying up to 20-30 minutes, after which the disks impregnated with essential oils were distributed. The disks taking should be placed at 15 mm distance from the periphery of the environment and approximately 30 mm each other. The plates were covered with a lid, after which they were left on the work table for 15-20 minutes for pre-diffusion so that the essential oils diffused into the medium. The plates were then placed at constant temperature for a period of 18-24 hours, after which the experimental values were evaluated.

RESULTS AND DISCUSSIONS

The results of the presented experimental methodology were evaluated by measuring the diameter of the inhibition zone induced by the used essential oil. If the diameter of the inhibition zone was greater than 6 mm, it was considered that the essential oil, at the used

concentration, had a sensitive effect on the respective microorganism and was denoted by S in table 2.

In the situation where the diameter of the area of inhibition was between 2 and 5 mm it was considered that the essential oil had a moderate sensitive effect (MS), and if the diameter was less than or equal to 2, the effect was considered negligible, being denoted by R.

Table 2

Experimental results about inhibition effect of essential oils upon different pathogenic micro-organism

Name of the microorganism	**Essential oils**								
	Thyme (Thymus Serpyllum)			***Basil (Ocimum Basilicum)***			***Narrow-leaved paperbark (Malaleuca Alternifolia)***		
	S	**MS**	**R**	**S**	**MS**	**R**	**S**	**MS**	**R**
Salmonella typhymurium		x			x				x
Escherichia coli ß glucuronidase positive		x				x		x	

It is observed that *Thymus Serpyllum* essential oil has the most important inhibition activity, with moderate sensitivity on *Salmonella typhymurium* and *Escherichia coli ß positive glucuronidase*. *Occimum basalicum* essential oil, had moderate sensitivity to *Salmonella typhymurium* and resistance to *Escherichia coli ß glucuronid*ase positive was found, and in Maleluca, moderate susceptibility to *Escherichia coli ß positive glucuronidase* and resistance to *Salumella typhymurium* were found. Regarding *Hippophae rhamnoides*, the determined vitamin C content was: 114 - 1550 mg per 100 grams with an average of 695 mg / 100 grams.

The antioxidant activity determined by the DPPH method was: 92.7 ± 3.1 for fat-soluble extract and 71.7 ± 2.1 water extract PH 9.5.

Other research directions of EBIOTEFA research center are:

- Study of food, designed on the basis of eco-biotechnologies, more specific from the group of sanogenic foods (functional foods, nutraceuticals, supplements, etc.) or from the group of composite foods (prepared from classical gastronomy or the one of excellence);
- Biodiversity, especially of plant origin, through biological and biotechnical methods, but also through traditional non-polluting and non-destructive technological processes from agri-food products;
- Research in the field of efficiency and maintenance of equipments specific to agri-food facilities;
- Study of foods designed in the basis of eco-biotechnologies, like the group of composite foods (culinary products) and innovative foods (personal gastronomic products) functional foods, etc).

CONCLUSIONS

Raw material extracts obtained by the mentioned methods can be used as pure active ingredients in future food supplements, sanogenic and functional foods, natural sanogenic refreshments, medicines and cosmetics, etc.

Bioactive substances are obtained through two complementary extraction methods, in conditions of room temperature and in the absence of oxygen, thus keeping all the biologically active properties of plant material used unaltered.

This methods allows the extraction of liposoluble substances and essential oils, with certain antioxidant and antimicrobial properties, from lavender,sea buckthorn, walnut core, pine sprouts, St. John's-wort, etc. The most important advantage that liquefied gas extraction method with the subcritical pressure offers, is that the oils can be extracted in pure state at room temperature and in the absence of air, which allows obtaining in extract of new categories of bioactive substances.

The interest for obtaining secondary metabolites by unconventional methods is stimulated by the pronounced decrease of the consecrated vegetal resources, as a result of the disturbance of the balance of the natural environment, of the unlimited exploitation of these resources, of the increase of labor cost and of technical and economic difficulties in the cultivation of plants from the spontaneous mountain flora.

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