USAGE OF INNOVATIVE TECHNOLOGIES TO DRY PLANT EXTRACTS WITH ANTIMICROBIAL ACTIVATES

THESIS SUMMARY

Coordinating
Prof. Dr. Carmen Socaciu

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INTRODUCTION

Food and pharmaceutical industries are constantly developing, as new products are put on the market, using more advanced and faster technologies. Along with the increase of production and the increasing complexity of the technological processes, a very important issue is to keep under control the microorganisms that may easily contaminate the products and technological lines. Currently, the products used for the removal of microorganisms are artificial products in the industry, they need to be stored in special places, they are used with much care and attention as they may endanger human health, and after their use, the water deriving from disinfection process contain large amounts of acids, alkaline products and soda, need to be treated before they are discharged into lakes, rivers or ponds.

The products made from plant extracts by means of innovative technologies that are more environmentally friendly than chemical ones, tend to substitute the classical chemical products.

This paper wants to achieve an alternative to classical disinfectants and their replacement with bio-disinfectants that combine advanced technology processing (drying, immobilization, storage) with classical plant extraction, finally achieving a product for the food industry and for domestic consumers. The new product will be much more easily handled and stored, it will not require special transportation conditions, having a small volume, as it is a powder.

The literature demonstrated that many compounds that may be extracted from different plants have harmful effects on the cultures of micro-organisms, some micro-organisms are resistant to antibiotics, but they are very quickly eliminated by the active substances found in plants.

So, as related to the local climate and the great diversity of plants that may be found in Romania, we selected a number plants that are found and used in various domains and applications. The selected plants are ornamental plants Tagetes erecta (Aztec marigold), Tagetes patula (French marigold), plants that are used in the kitchen (Coriandrum sativum (coriander), Rosmarinus officinalis (rosemary), Ocimum basilicum (basil) and Satureja Montana (winter savory), and also wild herbs Urtica dioica (nettle), Chelidonium majus (greater celandine).

The extraction process of the active compounds was carried out by means of an aqueous process, using a mixture of distilled water and hydrochloric acid (1%). The mixture of acetous water (85%) and plants (15%) was allowed to macerate for 24 hours, and then, a great part of the active compounds, including the products of phenolic acids and flavonoids, were extracted.

The studied plants are known to be rich in polyphenolic derivatives with anti-microbial activity (Niamh et al., 2009; Çelik and Wendel, 2005; Pop, 2013). The flavonoids form a large group of chemical compounds of the polyphenolic derivatives family, with antimicrobial potential (including phenolic acids, flavones and isoflavones, dihydroflavones) (Day and Harborne, 1993).
Polyphenols are present in various medicinal plants, including fruits, cereals, tea, coffee, wine (Sarica et al., 2005), as well as in ornamental plants (Areej et al., 2013, Couto et al., 2012), as it is proven that they have antimicrobial activity (Nichenametla, 2006).

The polyphenols that exist in various plants have anti-inflammatory effect, protect against oxidative stress, effect that is related to the prevention of atherosclerosis and other cardiovascular disorders (Qadan et al., 2005; Chizzola et al., 2008; Kale et al. 2012; Amarowicz et al, 2009; Papageorgiou et al., 2008; Werner and Ros, 2010; Deepinderjeet and Sachin, 2013).

At present, advanced technologies are used for the vaporization of the water content of the plant extracts in a controlled environment (at low or high temperatures), to keep the bioactive phytochemicals and proteins intact (Desobry et al. 1997; Hottot et al., 2004; Zhao et al., 2013; Zhang, et al. 2010).

Atomization is the most widely used technology in food and pharmaceutical industries. The technology works in continuous process, the drying and manufacturing temperature of the product should remain constant; it is possible to adjust the parameters during drying. (Masters, 1991; De Souza et al. 2009).

The fluidized-bed drying is a protective method using hot air that comes into upward contact with the product, drying, aggregating or concentrating various products (Grace et al., 2008) at a low temperature (max. 80°C), which may also be used for alcoholic extracts. This process is easily controlled and it is very easy to go from laboratory study to production. The product is in the form of fine powder, which should be handled carefully because it may cause explosions followed by damages or injuries (Araruna et al., 2013). Fluidized-bed dryer is used in food industry to dry food and cover various food products, but is also found in pharmaceutical industry (Desobry et al., 1997; Jaros and Pabisa, 2006).

Freeze-drying (cryodesiccation), also known as lyophilization, removes water by freezing the product at low pressure, a process which occurs in four phases: pre-treatment, freezing, primary drying and secondary drying (Patapoff et al., 2002; Builders et al., 2010). Pre-treatment is used to protect various parts of the product against the formation of ice crystals that may destroy the product. The freezing process is slow; the temperatures are between -50°C and -80°C, at a pressure of a few miliBarr (Hottot et al, 2004). The primary drying removes up to 95% of water in the product. In these conditions, water goes from solid form into the gaseous form and it may take up to 2-3 days. The secondary drying process is used to remove water which is chemically bound to the product (Pathomwichaiwat et al., 2012). Freeze-drying is used for materials sensitive to high
temperatures, such as proteins, enzymes, micro-organisms and blood plasma; it is an expensive procedure, but it protects various active elements.

To confirm that the resulting product has an antimicrobial effect, we used five types of micro-organisms (E. coli, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus and Salmonella).

The purpose of this study was the use of modern drying technologies (atomization, fluidized-bed drying and lyophilization) of some plant extracts of indigenous flora with known antibacterial potential.

**The main objectives of the experimental studies were:**

1. Preparation of aqueous and hydro-alcoholic extracts from seven types of indigenous plants.
2. Characterization of phenolic composition of extracts, spectroscopic, chromatographic footprint and antioxidant activity.
3. Highlighting of antimicrobial action of extracts by qualitative and quantitative tests on growth mediums seeded with various types of bacteria.
4. Use of aqueous extracts of willow, walnut and mistletoe to obtain powders on various matrices (maltodextrin, salt, lactose) by various drying technologies: atomization, fluidized-bed drying and freeze-drying.
5. Physical-chemical characterization of powders obtained from willow, walnut and mistletoe extracts.
6. Testing the antibacterial effect of the willow powder fixed on maltodextrin or salt, with the potential to be used as bio-disinfectants.

**Structure of the thesis.** This paper is structured in two parts, the first represented by the study of the literature and the second focused on original contributions.

**The first part** (literature study) has 3 chapters (1-3)

- **Chapter 1** presents basic information about the types of plants studied Aztec marigold (Tagetes erecta), French marigold (Tagetes patula), nettle (Urtica dioica), greater celandine (Chelidonium majus), coriander (Coriandrum sativum), rosemary (Rosmarinus officinalis), basil (Ocimum basilicum), winter savory (Satureja Montana), mistletoe (Viscum album), common walnut (Juglans regia) and white willow (Salix alba). All these plants were chosen because the literature has shown that a large part of their active elements have many activities, including the antimicrobial activity.
Chapter 2 presents information on modern water evaporation technologies using three different methods, two of which use hot air (atomization and fluidized-bed drying) and one that uses low temperature and pressure (lyophilization). We also show the applicability of these technologies in industry at the moment.

Chapter 3 includes literature data on various micro-organisms that have high frequency of occurrence in food industry (E. coli, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus and Salmonella), their morphological-functional characterization and their pathogenic potential.

The second part (own researches) has 3 chapters (4-6):

Chapter 4 includes experimental data obtained regarding the extracts from the studied plants using different extraction methods, in aqueous medium and alcoholic medium. We studied the most ecological and rapid extraction method, and also which plants had the highest amount of active elements with antibacterial effect.

Chapter 5 contains information about how to obtain the powders using the three methods of water evaporation, and the action of maltodextrin (MD), salt (S) and lactose (L), matrices subjected to various evaporation conditions.

Chapter 6 includes data the obtained on antimicrobial effectiveness of the willow powder fixed on maltodextrin (MD) or salt (S).

A part of this Ph.D. thesis was performed at VLB Berlin, a research institute for alcoholic and nonalcoholic products, in Germany, in collaboration with the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania, after we obtained a grant made available by Deutsche Bundesstiftung Umwelt (DBU, German Environmental Foundation) between September 2011 and August 2012.
PART I
Chapter 1

MEDICINAL PLANTS WITH ANTIMICROBIAL Potential

In Chapter 1, we present medicinal plants used for the experimental study with reference to morphological-physiological characteristics of the plant Tagetes sp (Tagetes erecta (Aztec marigold) and Tagetes patula (French marigold)), Urtica dioica (nettle), Chelidonium majus (greater celandine), Coriandrum sativum (coriander), Rosmarinus officinalis (rosemary), Ocimum basilicum (basil), Satureja Montana (winter savory), Viscum album (mistletoe), Juglans regia (common walnut) and Salix alba (white willow) and their bioactive compounds.

Chapter 2

MODERN TECHNOLOGIES OF DRYING PLANTS EXTRACTS

In Chapter 2, we presented the technology of drying the plants extracts in a controlled environment (at low or high temperatures) to maintain the bioactive physical-chemical elements and proteins intact (Desobry et al. 1997; Hottot et al., 2004; Zhao et al., 2013; Zhang, et al. 2010).

Atomization is the technology most used in food and pharmaceutical industries. The technology works in continuous process, the drying and product manufacturing temperature remains constant; it is possible to adjust the parameters during drying. (Masters, 1991; De Souza et al. 2009).

Fluidized-bed drying is a protecting method, using hot air that enters into contact with the product upwards, drying, and aggregating or concentrating different products (Grace et al., 2008), at a low temperature (up to 80°C); it may also be used for alcoholic extracts. This process is easy to control and it is very easy to go from laboratory study to production. The product obtained is in the form of fine powder, which should be handled carefully, as it may cause explosions followed by damages or injuries (Araruna et al., 2013). Fluidized-bed drying is used in food industry to dry and cover various foods, but it is also found in pharmaceutical industry (Desobry et al., 1997; Jaros and Pabisa, 2006).

Drying by freeze-drying, also known as lyophilization, removes water by freezing the product at low pressures, a process which occurs in four phases: pre-treatment, freezing, primary drying and secondary drying (Patapoff et al. 2002 Builders et al. 2010). Pre-treatment is used to protect various parts of the product against the formation of ice crystals that may destroy the
product. The freezing process is slow, the temperatures are between -50°C and -80°C, at a pressure of a few millibars (Hottot et al., 2004). The primary drying removes up to 95% of water in the product. In these conditions, water goes from solid form into the gaseous form and it may take up to 2-3 days. The secondary drying process is used to remove water which is chemically bound to the product (Pathomwichaiwat et al., 2012). Lyophilization is used for materials sensitive to high temperatures, such as proteins, enzymes, micro-organisms and blood plasma; it is an expensive procedure, but it protects various active elements.

Plants such as walnut, mistletoe and willow are known to be rich in polyphenolic compounds with antimicrobial activity (Niamh et al., 2009; Çelik and Wendel, 2005; Pop, 2013). Flavonoids form a large group of chemical compounds of the polyphenolic derivatives family, with antimicrobial potential (including phenolic acids, flavones and isoflavones, dihydroflavones) (Day and Harborne, 1993 Harborne and Williams, 2000; Cardona et al., 2013; Neveu et al., 2010). Polyphenols are present in various medicinal plants, including fruits, cereals, tea, coffee, wine (Sarica et al., 2005), as well as in ornamental plants (Areej et al., 2013, Couto et al., 2012), as it is proven that they have anti-microbial activity (Nichenametla, 2006).

The polyphenols that exist in various plants have anti-inflammatory effect, protect against oxidative stress, effect that is related to the prevention of atherosclerosis and other cardiovascular disorders (Deepinderjeet and Sachin, 2013).

Atomization is a process for water evaporation using a flow of hot air. The device sprays a fine vapor screen, the vapors that come into contact with the air flow are turned into a fine powder which is separated gravimetrically in a cyclone.

The lyophilization process uses for water evaporation low temperatures (-60°C) and pressures that almost reach the vacuum value (millibars). The combination of temperature and reduced pressure causes that water in the processed solution to evaporate, resulting a water-free product.

Chapter 3

PATHOGENS IN FOOD

Chapter 3 describes several types of micro-organisms with high incidence in food industry, E. coli, Bacillus subtilis, Staphylococcus aureus, Salmonella and Listeria monocytogenes, which were selected for antimicrobial analyses. We described the morphological particularities and factors determining their proliferation.
PART II
OWN RESEARCHES
Chapter 4
PREPARATION AND CONCENTRATION OF EXTRACTS

4.1. EXPERIMENTAL STUDY 1. PREPARATION AND CHARACTERIZATION OF AQUEOUS AND HYDRO-ALCOHOLIC EXTRACTS OF 7 INDIGENOUS MEDICINAL PLANTS WITH ANTIMICROBIAL POTENTIAL

Taking into consideration the literature data indicating antimicrobial activity of some indigenous medicinal plants, the objectives of this study were:

1. To obtain some aqueous and hydro-alcoholic extracts of 7 indigenous medicinal plants;
2. Spectrophotometric UV-Vis analysis of extracts and determination of phenolic compounds
3. Semi-quantitative evaluation of antimicrobial effect

4.1.1 Materials and methods

<table>
<thead>
<tr>
<th>Plants</th>
<th>Type of extract</th>
<th>Encoding</th>
<th>Representative image of the plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greater celandine (Chelidonium majus)</td>
<td>Aqueous</td>
<td>011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydro-alcoholic</td>
<td>021</td>
<td></td>
</tr>
<tr>
<td>Nettle (Urtica dioica)</td>
<td>Aqueous</td>
<td>012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydro-alcoholic</td>
<td>022</td>
<td></td>
</tr>
<tr>
<td>Plant Name</td>
<td>Extraction Type</td>
<td>Code</td>
<td>Image</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-------------------------</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>Aztec marigold (<em>Tagetes erecta</em>)</td>
<td>Aqueous</td>
<td>013</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>French marigold (<em>Tagetes patula</em>)</td>
<td>Aqueous</td>
<td>014</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Coriander (<em>Coriandrum sativum</em>)</td>
<td>Aqueous</td>
<td>015</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Rosemary (<em>Rosmarinus officinalis</em>)</td>
<td>Aqueous</td>
<td>016</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Basil (<em>Ocimum basilicum</em>)</td>
<td>Aqueous</td>
<td>017</td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>Winter savory (<em>Satureja Montana</em>)</td>
<td>Aqueous</td>
<td>018</td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>
4.1.1. Preparation of raw extracts of the 7 studied medicinal plants

1. Aqueous extract: we weighed 100 g of each dried and finely ground plant (see Table 4.1.) which was mixed with 700 ml of distilled water heated to 80°C. For 7 days, the mixture of plants and water was maintained at 4°C and it was stirred every day.

2. Hydro-alcoholic extract 15%: we weighed 100 g of each dried and finely ground plant (see Table 4.1) which was mixed with 700 ml of a mixture of water and ethanol (v/v 2:5). For 7 days, the plant and solvent mixture was kept at 4°C and it was stirred every day.

Both types of extracts were filtered to remove the vegetal material, and the filtrate obtained in hydro-alcoholic mixture was concentrated in vacuum, in a rotary evaporator at 70°C, ready for concentration.

Table 4.2

The volume of extract (ml) before and after solvent recovery and the concentration factor (%)

<table>
<thead>
<tr>
<th>Plant Code</th>
<th>Initial extract (ml)</th>
<th>Final extract (after the elimination of ethanol) (ml)</th>
<th>Percentage of extract concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>021 Chelidonium majus</td>
<td>452</td>
<td>215</td>
<td>47.57</td>
</tr>
<tr>
<td>022 Urtica dioica</td>
<td>330</td>
<td>127</td>
<td>38.48</td>
</tr>
<tr>
<td>023 Tagetes erecta</td>
<td>355</td>
<td>132</td>
<td>37.18</td>
</tr>
<tr>
<td>024 Tagetes patula</td>
<td>638</td>
<td>293</td>
<td>45.92</td>
</tr>
<tr>
<td>025 Coriandrum sativum</td>
<td>660</td>
<td>347</td>
<td>52.58</td>
</tr>
<tr>
<td>026 Rosmarinus officinalis</td>
<td>570</td>
<td>307</td>
<td>53.86</td>
</tr>
<tr>
<td>027 Ocimum basilicum</td>
<td>632</td>
<td>352</td>
<td>55.70</td>
</tr>
<tr>
<td>028 Satureja Montana</td>
<td>360</td>
<td>160</td>
<td>44.44</td>
</tr>
</tbody>
</table>

4.1.1.2. Analysis methods of extracts

Spectrophotometric UV-Vis analysis, determination of total phenols

Both types of extracts (011-018, 021-028) were analyzed by UV-Vis spectrometry, recording the spectrums in the field 200-400 nm (Perkin Elmer Lambda 25 spectrophotometer). We
determined the content of total polyphenols by Folin-Ciocalteu method according to the procedure below, along with the determination of a calibration curve made with gallic acid (GAE).

**Determination of the calibration curve.** We weighed the analytical balance, 25 mg of gallic acid and introduced it into a 25 ml volumetric flask; we added 15 ml of 40% ethanol, sonicated and made up to volume with ethanol, and we obtained a solution at 1 mg / ml GAE. This represents the standard parent solution, of which we prepared 5 dilutions: 1mg/100 ml; 0.5 mg/100 ml; 0.25 mg/100 ml; 0.125 mg / ml 0.0625 mg / ml.

To 1 ml of standard solution, we added GAE 60-70 ml distilled water, mixed it, then we added 5 ml of Folin-Ciocalteu reagent and homogenized it. After 1 minute and 8 minutes before, we added 15 ml of 7.5% sodium carbonate. We recorded this as time “0” and we homogenized it again. We made it up to the volume of 100 ml with distilled water and after 30 min, we read the absorbance at \( \lambda = 750 \) nm compared to the blank. The blank was prepared in the same manner, using 1 ml 40% ethanol. For the other 3 dilutions, we used standard solutions of 0.5 ml, 0.25 ml, 0.125 ml and 0.0625 ml.

The calibration curve was drawn by graph representation of the absorbance read depending on the concentration of the gallic acid (mg / ml).

To determine the concentration of phenolic compounds in the concentrated extracts (011-018 and 021-028), we proceeded similarly, on the determination, using 1 ml of extract.

**4.1.1.3. Semi-quantitative microbiological analysis, on solid medium**

The reagents used were: Murashige-Skoog growth medium (MS medium) and sterile distilled water. The cultures of micro-organisms *E. coli* and *Bacillus subtilis* came from the Microbiology Laboratory of the Research Center “Biochemistry and Agri-food Biotechnology”.

Materials used: laminar flow hood, Petri dishes, incubation thermostat, autoclave, Nanodrop spectrometer.

The method used is based on the properties of antimicrobial substances to diffuse in a solid growth medium seeded with a bacterial culture.

The growth medium used was Mueller-Hinton (30% meat extract, 1.75% casein, 0.15% starch and 1.7% agar), without glucose and not supplemented. We used micro-organisms from pure culture, having a concentration of about 0.5 on the MacFarland scale.

Work method: the medium melt and cooled to 50°C was poured into Petri dishes to a thickness of 4 mm; after solidification it was seeded with bacterial suspension on the entire the surface of the plate. With a clamp, we placed micro-tablets with concentrated extracts (011-018 and
021-028). They were incubated at 35-37°C for 18-24 hours, the diameter of zone of inhibition was measured with a ruler.

The readings were compared with the interpretation tables (in mm), appreciating the bacterial strain as sensitive or resistant to the extract tested. (Zarnea G., 1984)

4.1.2. Results and discussions

4.1.2.1. Description of aqueous and hydroalcoholic extracts

Fig. 4.1. Representation of aqueous extracts (011 - 018) from left to right

Fig. 4.2. Image of alcoholic extracts (021 - 028) from left to right, after concentration
4.1.2.2. UV-Vis spectrometric analysis and concentration of phenolic compounds

<table>
<thead>
<tr>
<th>Bioactive compound</th>
<th>Characteristic wave length (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gallic</td>
<td>217, 272</td>
</tr>
<tr>
<td>protocatechuic</td>
<td>218, 260, 295</td>
</tr>
<tr>
<td>gentisic</td>
<td>213, 239, 332, 370</td>
</tr>
<tr>
<td>caffeic</td>
<td>220, 240, 294, 326</td>
</tr>
<tr>
<td>vanillic</td>
<td>219, 261, 294, 320</td>
</tr>
<tr>
<td>syringic</td>
<td>218, 276, 328</td>
</tr>
<tr>
<td>p-coumaric</td>
<td>226, 312, 361</td>
</tr>
<tr>
<td>sinapic</td>
<td>238, 326</td>
</tr>
<tr>
<td>ferulic</td>
<td>218, 236, 295</td>
</tr>
</tbody>
</table>

*** Rebecca J. Robbins, 2003

UV – Vis spectrum of plants (Figures 4.1 - 4.2) are characterized by the presence of phenolic acids, showing intense absorption at 200-400 nm, absorptions corresponding to phenolic acids. Similar results are found in the literature (Table 4.3) (Rebecca J. Robbins, 2003).

Also, we may notice that the absorption forces of hydro-alcoholic extracts were higher than aqueous ones.

Table 4.3

<table>
<thead>
<tr>
<th>Extract code</th>
<th>mg Polyphenol/1ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>011 Chelidonium majus</td>
<td>0.078</td>
</tr>
<tr>
<td>012 Urtica dioica</td>
<td>0.106</td>
</tr>
<tr>
<td>013 Tagetes erecta</td>
<td>0.065</td>
</tr>
<tr>
<td>014 Tagetes patula</td>
<td>0.085</td>
</tr>
<tr>
<td>015 Coriandrum sativum</td>
<td>0.042</td>
</tr>
<tr>
<td>016 Rosmarinus officinalis</td>
<td>1,543</td>
</tr>
<tr>
<td>017 Ocimum basilicum</td>
<td>2,236</td>
</tr>
</tbody>
</table>
4.1.2.3. Comparative evaluation of the antimicrobial activity of the extracts on solid medium with agar

The comparative effect of aqueous and hydro-alcoholic extracts on the growth of bacteria, tested on *E. coli* and *Bacillus subtilis*

The antimicrobial effects of aqueous extracts (011-018) and hydro-alcoholic extracts (021-028) were evaluated on agar plates seeded with *E. coli* and *Bacillus subtilis*. The extracts were added as such, but we also made different dilutions of 1:5, 1:10, 1:50 and 1:100 in each extract. Unfortunately, the dilutions of 1:10, 1:50 and 1:100 did not have any results. In each plate we was introduced 3μl of each sample.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>018 <em>Satureja Montana</em></td>
<td>2,484</td>
</tr>
<tr>
<td>021 <em>Chelidonium majus</em></td>
<td>0,591</td>
</tr>
<tr>
<td>022 <em>Urtica dioica</em></td>
<td>0,032</td>
</tr>
<tr>
<td>023 <em>Tagetes erecta</em></td>
<td>0,873</td>
</tr>
<tr>
<td>024 <em>Tagetes patula</em></td>
<td>1,039</td>
</tr>
<tr>
<td>025 <em>Coriandrum sativum</em></td>
<td>0,044</td>
</tr>
<tr>
<td>026 <em>Rosmarinus officinalis</em></td>
<td>8,216</td>
</tr>
<tr>
<td>027 <em>Ocimum basilicum</em></td>
<td>7,522</td>
</tr>
<tr>
<td>028 <em>Satureja Montana</em></td>
<td>13,939</td>
</tr>
</tbody>
</table>

Table 4.5

<table>
<thead>
<tr>
<th>E. coli</th>
<th>Bacillus subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not filtered</td>
<td>filtered</td>
</tr>
<tr>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>013 <em>Tagetes erecta</em></td>
<td>0</td>
</tr>
<tr>
<td>017 <em>Ocimum basilicum</em></td>
<td>0,7</td>
</tr>
</tbody>
</table>

Table 4.5 shows that aqueous extracts (011, 012, 014, 015, 016, 018) did not inhibit the growth of micro-organisms. The extracts (013 and 017) showed selective antibacterial inhibition, thus, 013 presented an action on *Bacillus subtilis* (0.7 cm), but it did not have an action on *E. coli*,
while 017 inhibited the growth of *E. coli* (0.7 cm), but it did not inhibit the growth of *Bacillus subtilis*.

### Table 4.6

<table>
<thead>
<tr>
<th>Plant</th>
<th><em>E. coli</em></th>
<th></th>
<th><em>Bacillus subtilis</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not filtered</td>
<td>Filtered</td>
<td>Not filtered</td>
<td>Filtered</td>
</tr>
<tr>
<td>1:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0,7</td>
</tr>
<tr>
<td>1:5</td>
<td>0,6</td>
<td>0,7</td>
<td>1,2</td>
<td>1,4</td>
</tr>
<tr>
<td>1:1</td>
<td>0</td>
<td>0</td>
<td>0,9</td>
<td>0,7</td>
</tr>
<tr>
<td>022 <em>Urtica dioica</em></td>
<td>0</td>
<td>0</td>
<td>0,7</td>
<td>0</td>
</tr>
<tr>
<td>026 <em>Rosmarinus officinalis</em></td>
<td>0,6</td>
<td>0,7</td>
<td>1,2</td>
<td>1,4</td>
</tr>
<tr>
<td>027 <em>Ocimum basilicum</em></td>
<td>0</td>
<td>0</td>
<td>0,9</td>
<td>0,7</td>
</tr>
<tr>
<td>028 <em>Satureja Montana</em></td>
<td>0,8</td>
<td>0</td>
<td>0,9</td>
<td>0</td>
</tr>
</tbody>
</table>

According to table 4.6, the filtered extracts 022 (0.7 cm), 026 (1.4 cm) and 027 (1.3 cm) of 1:1 showed bacterial inhibition on *Bacillus subtilis*; unfiltered extracts 026 (1.2 cm) and 028 (0.9 cm) presented significantly lower bacterial inhibition.

In the case of *E. coli*, the bacterial inhibition was noticed for extracts 026 (0.6 cm) unfiltered ratio 1:1, 028 (0.8 cm) unfiltered ratio 1:1. The filtered extract 026 (0.7 cm) ratio 1:1 showed antimicrobial action for *E. coli*.

Analyzing the results obtained, we estimate that the hydro-alcoholic extract of rosemary had the best antimicrobial effect against *Bacillus subtilis* and basil showed similar antibacterial action.

We may also notice that the filtered extracts had higher antibacterial efficiency for *Bacillus subtilis*.

The various degrees of bacterial inhibition of the bio-elements on the two bacteria are influenced by the structure of the bacteria. Thus, as *E. coli* has lipopolysaccharide bacterial membrane prevented the passage of the compounds into the interior of the cell, being less affected by bio-elements.
4.1.3 Conclusions

1. UV-Vis analysis of extracts from the plants studied confirms stronger absorptions in the case of hydro-alcoholic extracts than in the case of aqueous extracts, which are characterized by the presence of phenolic acids.

2. The difference in the extraction of the bioactive elements (UV-Vis spectrum) is due to the different polarity of the solvents used for the extraction.

3. The content of total polyphenols was higher for hydro-alcoholic extracts of rosemary, basil and marigold.

4. Upon the completion of the study, we may say that hydro-alcoholic extracts had more intense antibacterial effects than aqueous extracts.

5. Hydro-alcoholic extracts of Rosmarinus officinalis, Ocimum basilicum and Satureja Montana had good results, inhibiting the bacterial development of E. coli or Bacillus subtilis. Generally, the effect was more intense on bacteria Bacillus subtilis.

6. The variants of filtered extracts were more active, the most intense antibacterial effect against Bacillus subtilis was noticed in the filtered extracts 026 and 027, Rosmarinus officinalis and Ocimum basilicum.

7. The best results with antibacterial effect on both strains of E. coli and Bacillus subtilis were noticed with hydro-alcoholic extracts of winter savory Satureja Montana - (028) and Rosmarinus officinalis (026).
4.2. EXPERIMENTAL STUDY 2. PREPARATION AND CHARACTERIZATION OF WILLOW, MISTLETOE AND COMMON WALNUT LEAVES EXTRACTS

**Table 4.7**

*Studied plants morphological aspect and codification of samples*

<table>
<thead>
<tr>
<th>Studied plants</th>
<th>Encoding</th>
<th>Representative image of the plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mistletoe <em>(Viscum album)</em></td>
<td>031</td>
<td><img src="image" alt="Mistletoe Image" /></td>
</tr>
<tr>
<td>Leaves of common nut <em>(Juglans regia)</em></td>
<td>032</td>
<td><img src="image" alt="Leaves Image" /></td>
</tr>
<tr>
<td>Willow <em>(Salix alba)</em></td>
<td>033</td>
<td><img src="image" alt="Willow Image" /></td>
</tr>
</tbody>
</table>

4.2.1.2. UV-Vis spectrum and determination of concentrations of total phenolic compounds

**Table 4.8**

*Total phenolic content of aqueous extract*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mg Polyphenols/ 1 ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salcie</td>
<td>2,6</td>
</tr>
<tr>
<td>Vâsc</td>
<td>1</td>
</tr>
<tr>
<td>Nuc</td>
<td>1,21</td>
</tr>
</tbody>
</table>
4.2.1.3. HPLC chromatography analysis and photodiode detection (HPLC_DAD)

Chromatographic analyzes were performed on an Agilent 1200 HPLC chromatograph connected with a detection diode (DAD) and two eluents A and B: methanol / acetic acid / water (10:2:88) (solvent A) respectively methanol / acetic acid / water (90:3:7) (solvent B). The flow rate was 1 ml / min at 280 nm. The gradient used was as follows: 100% to 85% (0-10 min) at 85% - 50% (min 10-30) from 50% to 15% (min 30-45) and the 15% - 100% (minutes 45-55).

4.2.1.4. Microbiological analysis on solid medium (semi-quantitative) and liquid (quantitative)

To achieve microbiological tests we used 5 types of micro-organisms (E. coli, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus and Salmonella enteritis); for each investigation we used revitalized micro-organisms (grown on fresh growth medium at 37°C, 24 hours before the study). The studies were made on solid and liquid medium, and before adding the bacteria, pH value was adjusted to 7.

We used micro-organisms of Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, E. coli and Salmonella enteritis provided by the department of microbiology of the University of Agricultural Sciences and Veterinary Medicine of Cluj. The extracts were tested to see their inhibition on the selected microorganisms over a 24-hour period.

4.2.1.5. Analysis of metals from aqueous extracts of willow, mistletoe and common walnut

The dried and ground sample of vegetal material (leaves of common walnut, willow and mistletoe) is extracted with a solution of HCl 1% by keeping it for 16 hours at room temperature and stirring it periodically. The extract was filtered and subject to the determination of metals by atomic absorption. The resulting solution is suitable for the determination of metals using adequate techniques of atomic spectrometry. General instrumental analytical conditions are shown in Table 4.9.

The selection of this method for any of these elements depends on the amount of the said element that might be in the sample and the need to include all elements in a single sample.
Table 4.9

General condition for spectroscopy of atoms in flame

<table>
<thead>
<tr>
<th>Element</th>
<th>Wave length (nm)</th>
<th>Flame type</th>
<th>Lanthanum chloride</th>
<th>Main interfaces</th>
<th>Background correction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>228.8</td>
<td>Air/oxidant acetylene</td>
<td>No</td>
<td>Fe</td>
<td>Deuterium</td>
</tr>
<tr>
<td>Cobalt</td>
<td>240.7</td>
<td>Air/oxidant acetylene</td>
<td>No</td>
<td></td>
<td>Deuterium</td>
</tr>
<tr>
<td>Copper</td>
<td>324.8</td>
<td>Air/oxidant acetylene</td>
<td>No</td>
<td></td>
<td>Deuterium</td>
</tr>
<tr>
<td>Lead</td>
<td>217.0</td>
<td>Air/oxidant acetylene</td>
<td>No</td>
<td></td>
<td>Deuterium</td>
</tr>
<tr>
<td>Manganese</td>
<td>279.5</td>
<td>Air/oxidant acetylene or reducing acetylene /N₂O</td>
<td>Yes</td>
<td>Fe, Si</td>
<td>Deuterium</td>
</tr>
<tr>
<td>Iron</td>
<td>248.3</td>
<td>Air/oxidant acetylene</td>
<td>No</td>
<td>Al, Mn</td>
<td>Deuterium</td>
</tr>
<tr>
<td>Nickel</td>
<td>232.0</td>
<td>Air/oxidant acetylene</td>
<td>No</td>
<td>Fe</td>
<td>Deuterium</td>
</tr>
<tr>
<td>Zinc</td>
<td>213.9</td>
<td>Air/oxidant acetylene</td>
<td>No</td>
<td></td>
<td>Deuterium</td>
</tr>
</tbody>
</table>

Preparation of standard solutions for each element

The stock solution for each metal has a concentration of 1000 mg/l. Of this solution, we prepared a standard solution of the 40 mg/l by diluting 20 ml of the stock solution to 500 ml. These solutions are stable 6 months as of the date of preparation.

Determining the sample for analysis

Separately, we aspirated the solution for the blank sample and analysis sample in the flame, and then, we measured the absorbance for the element. We read the solutions at least twice, and if the values were within an acceptable range, we made their average. After each measurement, the water was aspirated and, if necessary, the zero was reset. If the concentration of the sample to be analyzed exceeded the calibration range, solution to be analyzed was diluted with the blank for calibration.

4.2.3. Results and discussions

4.2.3.1. Characterization of the UV-Vis spectrums and HPLC-DAD chromatograms of the three extracts

The concentration of polyphenols is between 1 and 2.6 mg / ml (Pop et al, 2013), the high concentration is for the willow extract. This extract has the highest quantity of eridiocites,
quercetin, catechins, salicyl and isohamantine demonstrated following the investigation by means of HPLC-DAD, all these bioactive molecules are responsible for antimicrobial activities.

The common walnut extract was the next extract in terms of polyphenol quantity, represented by derivatives of hydrojuglones (P₃ and ₄), derivatives of quercetines (P₈ and ₉) and theaflavines (P₁₀). The data are consistent with the Duke international basis.

The mistletoe extract has been characterized as being rich in quercetine and theaflavine.

**Identification peak:**
1- cafeo quinic acid; 2- cumaroil quinic acid; 3- glucosidic hydrojuglone; 4- quercetin 3-rhamnozid; 5- kaemferol 3-glucosid; 6- kaemferol 7-glucozid; 7- juglone; 8- quercetin 3-glucozid; 9- quercetin 3-arabinozid; 10- theaflavin 3-galate

**Identification peak:**
1- acido dihidroascorbic; 2- glucosid p-coumaroi; 3- eriodictiol 7-glucozid; 4,5- naringenin 7- si 5-glucozid; 6- catechin; 7- catechingalate; 8- naringin; 9- quercetin 3-glucozid; 10- naringenin dimer; 11- salicilat; 12- taxifolin; 13- isorhamnetin

*Fig. 4.2. HPLC – DAD/MS chromatograms at 280 nm for Salix alba, Juglans regia and viscum album*
4.2.3.2. Semi-quantitative evaluation of the antimicrobial activity on solid medium

Fig. 4.4. Inhibition area of *de Salix alba* against *Listeria monocytogenes*, dil. 1:1 și 1:5

Fig. 4.5. Inhibition area of *de Salix alba* against *Bacillus cereus*, dil. 1:1 și 1:5

The inhibition area of the bacteria in agar depended on the concentration and type of extract. The extract of *Salix alba* had the largest growth inhibition areas for *Bacillus cereus* (2.72 cm), *Staphylococcus aureus* (1.10 cm), *Listeria monocytogenes* (3.08 mm) and *E. coli* (0.85 cm). Figures 4.4 and 4.5 show the inhibition of *Salix alba* extract against *Listeria monocytogenes* and *B. cereus*.

**Table 4.10**

<table>
<thead>
<tr>
<th>Tested micro-organism</th>
<th>Common walnut extract</th>
<th>Willow extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
<td>1:5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Salmonella enteritis</em></td>
<td><strong>0.3</strong></td>
<td>0</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td><strong>0.8</strong></td>
<td>0</td>
</tr>
</tbody>
</table>

The data presented in table 4.10 suggest that in the case of walnut extract (1:1) the bacterial inhibition area was 0.8 cm in listeria; the dilution 1:1 showed weak bacterial inhibition against *E.coli* and *Salmonella enteritis* (0.1 and 0.3 cm), while the inhibition was not noticed in *Bacillus cereus* and *Staphylococcus aureus*. The differences occurred in the inhibition zones are due to the differences in membrane structure of the two bacteria.
The willow extract had negative influence on bacterial growth where *Listeria monocytogenes* (dilution 1:1 - 3.08 cm) and *Bacillus cereus* (dilution 1:1 - 2.72 cm). We may notice that the willow extract had a weak inhibition on salmonella, staphylococcus and E. coli. (0, 1.1 and 0.8 cm). In case of dilution 1:5 and 1:10, we noticed areas of inhibition only in the case of *Bacillus cereus* and *Listeria monocytogenes*. The weak anti-bacterial effect, shown by the common walnut extract, may be attributed to low concentrations of total polyphenols, compared with the extract of willow (three times).

4.2.3.3. **Quantitative evaluation of antimicrobial activity on the liquid medium**

**Growth of micro-organisms on liquid medium**

Figures 4.6 - 4.7 represent the dynamics of bacterial growth for the five types of bacteria studied.

Following the evaluations in liquid medium, the walnut extract showed an inhibiting effect on all plants studied. The effect of walnut extract against *Staphylococcus aureus* showed a strong inhibiting effect for a 24-hour period for all 3 concentrations; in the left part of the figure, we may notice that mistletoe extract does not have an inhibiting effect on *Staphylococcus aureus*, all three concentrations have values comparative with the control values. (Fig.4.6)

![Growing rate of Staphylococcus aureus in vitro using tree concentration of Juglans regia and Viscum album](image)

**Fig. 4.6. Growing rate of Staphylococcus aureus in vitro using tree concentration of Juglans regia and Viscum album**

Braga et al., (2005) used *Punica granatum* to inhibit the growth of *S. aureus* just as Zuo et al. (2008) showed a decrease in the growth of *Staphylococcus* using extracts of medicinal plants.

Mistletoe has a rather low content of phenolic acids; it does not have any effect against *Staphylococcus aureus*.
Figure 4.7 shows the increase curve of *Listeria monocytogenes* grown in *vitro* by adding *Juglans regia* and *Viscum album* extracts at various concentrations (1:1, 1:5 and 1:10); it shows a low inhibition thereof. Only common walnut extract (1:1) shows a more pronounced inhibition of *Listeria monocytogenes*; similar results were reported (Alvarez *et al.* 2006 Zuo *et al.*, 2008). Mistletoe had a low inhibiting effect on Listeria.

![Graph showing increase curve of Listeria monocytogenes in vitro using tree concentration of Juglans regia and Viscum album](image1)

Fig. 4.7. *Growing rate of Listeria monocytogenes in vitro using tree concentration of Juglans regia and Viscum album*

The walnut extract could to inhibit the development of *Bacillus cereus* for the entire period of the study, the best results were given by dilution 1:1 (Fig. 4.7); similar results were reported by Hyejung *et al.*, 2013.

Mistletoe extract had a weaker inhibiting effect, but a positive result was noticed for dilution 1:1 that could finally stop the growth of the micro-organism; similar results were also reported by Gutiérrez-Larraínzar *et al.*, 2013.

![Graph showing growing rate of Bacillus cereus in vitro using tree concentration of Juglans regia and Viscum album](image2)

Fig. 4.8. *Growing rate of Bacillus cereus in vitro using tree concentration of Juglans regia and Viscum album*
Walnut extract inhibited the growth of *E. coli* (Figure 4.9) in a controlled manner; similar results were also noticed by (Wong and Kitts 2006), compared with mistletoe extracts which had a weaker inhibition effect (Fig 4.9).

![Fig. 4.9. Growing rate of *E coli* in vitro using tree concentration of *Juglans regia* and *Viscum album*](image)

After 24 hours of the study, in figure 4.10 we may notice as walnut extract inhibited the growth of *Salmonella enteritidis*. Recent studies demonstrated the inhibition of *Salmonella* with an extract of *Vaccinium corymbosum* (Shen et al., 2014).

Mistletoe has a weak inhibiting effect of *Salmonella*. Similar studies conducted with extract of *Aloe secundiflora* showed an inhibiting effect on *Salmonella* (Waihenya et al. 2002).

![Fig. 4.10. Growing rate of *Salmonella* in vitro using tree concentration of *Juglans regia* and *Viscum album*](image)

4.2.3.4. Analysis of metals from the raw willow, common nut and mistletoe extracts

The values of the metal concentrations, expressed in mg/L (ppm) and µg/L (ppb) are shown in Table 4.11.

<table>
<thead>
<tr>
<th>Table 4.11</th>
<th>The metal quantise detected in the three extracts expressed in ppm (mg/l)</th>
</tr>
</thead>
</table>

XXIII
<table>
<thead>
<tr>
<th>Element / Plant</th>
<th>Ppm (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Willow</td>
</tr>
<tr>
<td>Li</td>
<td>0.006</td>
</tr>
<tr>
<td>Na</td>
<td>5.511</td>
</tr>
<tr>
<td>Mg</td>
<td>119.091</td>
</tr>
<tr>
<td>Ca</td>
<td>640.561</td>
</tr>
<tr>
<td>Mn</td>
<td>23.841</td>
</tr>
<tr>
<td>Fe</td>
<td>4.456</td>
</tr>
<tr>
<td>Co</td>
<td>0.039</td>
</tr>
<tr>
<td>Ni</td>
<td>0.064</td>
</tr>
<tr>
<td>Cu</td>
<td>0.215</td>
</tr>
<tr>
<td>Zn</td>
<td>23.085</td>
</tr>
<tr>
<td>Pb</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cd</td>
<td>0.216</td>
</tr>
</tbody>
</table>

### 4.2.5. Conclusions

The study confirms that aqueous extracts of common walnut (*Juglans regia*), mistletoe (*Viscum album*) and willow (*Salix alba*) have a good antimicrobial effect. All three extracts have a high content of polyphenolic derivatives presented in HPLC analyzes. After studying the effects on agar, the willow extract showed the highest antimicrobial effect especially against *Listeria monocytogenes* and *Bacillus cereus*.

The quantitative antimicrobial assessment in liquid medium, measuring the absorbance of the micro-organisms mixture in the extract, showed that depending on the concentration of polyphenols and phenolic derivatives, the activity is different for the extract of common walnut, mistletoe and willow, as they could inhibit the growth of micro-organisms.

In conclusion, the aqueous extract of willow, common walnut and mistletoe may be used as natural preservative or bio-disinfectant against a wide range of micro-organisms, which may be found in food. These extracts may be used as ingredients in the manufacturing of products used to obtain biod-disinfectants,
Chapter 5
PREPARATION AND PHYSICAL - CHEMICAL CHARACTERIZATION OF THE POWDERS OBTAINED, COMPARATIVELY, THROUGH THE THREE DRYING TECHNIQUES

The purpose of the research was to obtain and characterize the powders obtained from extracts of common walnut (Juglans regia), mistletoe (Viscum album) and willow (Salix alba), using various methods of drying (atomization, fluidized-bed drying and lyophilization).

Objectives of the study:
1. Use of three different drying methods (atomization, fluidized-bed drying and lyophilization) to obtain powders, selecting the most effective method of drying, fixation and conservation of antioxidant and antimicrobial activity of the polyphenols present in powders, derived from the three plant extracts.
2. The determination of the antioxidant powders obtained as compared with raw extracts of walnut (Juglans regia), mistletoe (Viscum album) and willow (Salix alba).
3. The screening analysis by infrared spectrometry (FTIR) of powders, as compared with the raw extracts of walnut (Juglans regia), mistletoe (Viscum album) and willow (Salix alba).

5.1. MATERIALS AND METHODS
5.1.1. Preparation of extracts and spectrometric analysis of phenolic elements
Described in chapter 4.1.1.2.

5.1.2. Determination of antioxidant activity (DPPH)

5.1.3. FT-IR spectrometric analysis

All raw extracts and the resulting powders were characterized using IR spectrophotometry, in the MIR field (600-4000cm\(^{-1}\)). The recording of the FTIR absorption spectrums was made directly by using Shimadzu IR Prestige -21 spectrophotometer with diamond horizontal ATR accessory (Horizontal Attenuated Total Reflectance) with a single reflection. We used volumes 10 l evaporated sample in the HATR system. The spectrums were recorded in the wavelength range of 600-4000 cm\(^{-1}\), and the absorption bands characteristic for the type of links and the functional groups (expressed in cm\(^{-1}\)) were identified. The fingerprint area was identified in the 600-1800 cm\(^{-1}\).
5.1.4. Equipment and techniques used for the drying of extracts and preparation of powders

Fig. 5.1. *Spray drying Mobile Monor from GEA* 1 - Main chamber, 2 - separation cyclone, 3 - suction fan

Fig. 5.2 *Fluid bed dryers* 1 - drying chamber, 2 – control panel
5.2. RESULTS AND DISCUSSIONS

5.2.1. Characterization of the powders obtained

Figure 5.4. presents the powders obtained following the dehydration of the extracts (walnut, willow, mistletoe) fixed on maltodextrin (MD), lactose (LA) and salt (S) through specific processes of atomization (SD), fluidized-bed drying (FB), and freeze-drying (DF).

Salt was good in all three dehydration processes SD, FB and FD. Lactose gave good results for FB and FD, unlike maltodextrin that had good results only on SD. The highest percentage of polyphenols recovered after processing 94-96% was after atomization (Fang and Bhandari, 2011).
Atomization (SD) | Lyophilization (FD) | Fluidized-bed drying (FB)
---|---|---
**Drying process**
Entry temperature: 180°C  
Drying temperature: 45°C  
Pump debit: 380 ml/min
Main drying – 55°C,  
vacuum pressure: 0.12 mbar  
Final drying – 5°C,  
vacuum pressure: 0.12 mbar
Entry temperature: 80°C  
Drying temperature: 40°C  
Pump debit: 28 ml/min

<table>
<thead>
<tr>
<th>Matrix</th>
<th>MD</th>
<th>S</th>
<th>LA</th>
<th>MD</th>
<th>S</th>
<th>LA</th>
</tr>
</thead>
</table>
| Willow  
(*Salix alba*) |  |  |  |  |  |  |
| (Viscum album) |  |  |  |  |  |  |
| Nut  
(*Juglans regia*) |  |  |  |  |  |  |

Fig. 5.4. *Powder made from extract (Salix alba, Viscum album and Juglas regia) on different matrix (salt (S), lactose (LA), maltodextrin (MD)) using Spray drying (SD), Freeze drying (FD) and fluid bed drying (FB)*

### 5.2.2. Total polyphenol content of powders, compared to the raw extracts

Table 5.1

Polyphenolic concentration (mg GAE/ml) from the initial extract \(c_i\), compared with theoretical value \(c_D\) and value obtained in powder \(c_R\) using SD, FD and FB

<table>
<thead>
<tr>
<th>Extract</th>
<th>(c_i)</th>
<th>SD</th>
<th>FB</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mg GAE / ml extract)</td>
<td>(mg GAE / ml SD powder extract 10%)</td>
<td>(mg GAE / ml FB powder extract 10%)</td>
<td>(mg GAE / ml FD powder extract 10%)</td>
<td></td>
</tr>
</tbody>
</table>
| Willow  
(*Salix alba*) | 2,60 |  |  |  |
| \(c_D = 2,44\)  
\(c_R = 1,53\) | 2,44  
1,50 | -  
- | 2,44  
1,32 | 2,44  
1,61 | -  
- | 2,44  
1,51 | 2,44  
1,88 |
Thus, in the case atomization method is used (SD):

- For the willow, the capacity to recover the phenols was of 62.7% on MD, respectively 61.47% on S.
- For the mistletoe, the capacity to recover the phenols was of 82% on MD, respectively 52% on S.
- For the nut, capacity to recover the phenols was of 50.4% on MD, respectively 58.8% on S.

In the case of using the fluidized-bed drying method:

- For the willow, capacity to recover the phenols was of 54% on S, respectively 66% on LA.
- For the mistletoe, capacity to recover the phenols was of 51% on S, respectively 55% on LA.
- For the nut capacity to recover the phenols was of 58.8% on S, respectively 68.9% on LA.

### 5.2.3. Characterization of the FT-IR spectroscopic footprint of raw extracts and powders obtained

**Table 5.2**

*FT – IR absorbance pro powder and extract Juglans regia, Viscum album and Salix alba*

<table>
<thead>
<tr>
<th>Extract</th>
<th>Area 1 600-970 cm(^{-1})</th>
<th>Area 2 1000-1250 cm(^{-1})</th>
<th>Area 3-4 1300-1700 cm(^{-1})</th>
<th>Area 5 2900-3300 cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willow</td>
<td>634, <strong>763</strong>, 819, 865</td>
<td>1041, <strong>1232</strong></td>
<td><strong>1446</strong>, 1608, 1718</td>
<td>2937, 3238</td>
</tr>
<tr>
<td>Nut</td>
<td>632, <strong>667</strong>, 775, <strong>812</strong>, 871</td>
<td>1045, <strong>1224</strong>, <strong>1319</strong></td>
<td>1647, 1732</td>
<td>2935, 3278</td>
</tr>
<tr>
<td>Mistletooe</td>
<td><strong>590</strong>, <strong>605</strong>, 632, <strong>786</strong>, 866</td>
<td>1047, 1215</td>
<td><strong>1508</strong>, <strong>1543</strong>, 1645, 1716</td>
<td>2933, 3238</td>
</tr>
</tbody>
</table>
5.2.4. Antioxidant activity of the powders obtained, as compared to the content in polyphenols

**Table 5.3**

*Average concentration of polyphenols from powder extract using SD, FB, FD*

<table>
<thead>
<tr>
<th>Powder</th>
<th>mg GAE/1 ml dissolved powder</th>
<th>μM Trolox/1ml dissolved powder</th>
<th>Ratio Polyphenols / antioxidant activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willow (SD) on MD matrix</td>
<td>1,501</td>
<td>13,55</td>
<td>9,02</td>
</tr>
<tr>
<td>Nut (SD) on MD matrix</td>
<td>0,698</td>
<td>6,02</td>
<td>8,62</td>
</tr>
<tr>
<td>Mistletoe (SD) on MD matrix</td>
<td>0,520</td>
<td>5,24</td>
<td>10,07</td>
</tr>
<tr>
<td>Willow (FD) on S matrix</td>
<td>1,317</td>
<td>11,90</td>
<td>9,03</td>
</tr>
<tr>
<td>Nut (FD) on S matrix</td>
<td>0,701</td>
<td>5,87</td>
<td>8,37</td>
</tr>
<tr>
<td>Mistletoe (FD) on S matrix</td>
<td>0,509</td>
<td>5,41</td>
<td>10,62</td>
</tr>
<tr>
<td>Willow (FB) on S matrix</td>
<td>1,512</td>
<td>13,26</td>
<td>8,75</td>
</tr>
<tr>
<td>Nut (FB) on S matrix</td>
<td>0,700</td>
<td>5,98</td>
<td>8,54</td>
</tr>
<tr>
<td>Mistletoe (FB) on S matrix</td>
<td>0,512</td>
<td>5,60</td>
<td>10,93</td>
</tr>
</tbody>
</table>

We found that willow powders have the most intense antioxidant activity (11.9 to 13.55 μM Trolox/1ml dissolved powder) and this is correlated with a maximum content of polyphenols (1.317 to 1.512 mg GAE / 1 ml dissolved powder). Using FB and SD techniques, we obtained better results using MD, respectively S matrixes.

The minimum antioxidant activity (reduced by approx. 50%) was found in mistletoe powders, irrespective of the drying technique or matrix.

The ratios obtained between the concentration of polyphenols and the antioxidant activity were relatively constant for a given drying technique, but they were different depending on the type of plant from which the powder was obtained. Thus, the largest ratio was obtained for mistletoe (10.07 to 10.93), although the absolute value of the phenolic compounds was the lowest, while willow and walnut extracts had similar values, within the interval of 8.5-9. These differences suggest that the antioxidant activity of mistletoe is higher per unit of phenolic compound, i.e., the antioxidant potential of polyphenolic units of mistletoe (flavonoids) is more intense. Instead, the antioxidant potential of polyphenolic units of willow (phenolic acids) is lower, although their concentration is higher.
5.3. CONCLUSIONS

1. We obtained powders of aqueous extracts 15% of walnut (*Juglans regia*), mistletoe (*Viscum album*) and willow (*Salix alba*), using three drying technologies: atomization (SD), fluidized-bed drying (FB) and freeze-drying (FD). A total of 21 powders were obtained using such techniques on three different matrixes: maltodextrin, salt or lactose.

The matrixes used had different behavior in the drying process, the most suitable for atomization were the salt and maltodextrin; the salt and lactose behaved well in the fluidized-bed drying. The good results were obtained on salt and lactose and in freeze-drying process, but the technology is expensive. We believe that the atomization is the most appropriate method to fix polyphenol-rich extracts on salt or maltodextrin support, because it is a quick and continuous drying process, finally, being the most convenient.

The quickest evaporation method was SD in continuous systems. By this procedure, we obtained powders with the maltodextrin (MD) and salt matrixes.

Fluidized-bed drying gave good results when we used lactose and salt matrixes, but it did not have a satisfactory result for MD. The disadvantage of FB is that we work in small charges 300-450 g / charge.

Freeze-drying is a slow process and a major energy consumer. Salt and lactose were the matrixes suitable for this process.

2. The content of phenolic compounds was different depending on the plant extracts and stability of the powder and matrixes used.

Thus, the willow powder had maximum polyphenols content of 1.53 mg GAE / ml powder obtained from maltodextrin, dissolved and 1.88 mg GAE / ml powder obtained on lactose, dissolved. A better stability of the polyphenols was noticed in the SD process, with the highest recovery of polyphenols, up to 82% on a MD substrate.

3. FT-IR spectrometry was used to obtain the specific fingerprint of plant extracts before and after drying. According to the spectroscopic data, the best drying technology proved to be atomization due to high processing speed and an ongoing action as compared to lyophilization, which is a slow process, because of evaporation at low temperatures.

4. Willow powders had the most intense antioxidant activity (11.9 to 13.55 μM Trolox/1ml dissolved powder) and this is correlated with a maximum content of polyphenols (1.317 to 1.512 mg GAE / 1 ml powder dissolved). The SD and FB techniques had good results, using MD, respectively S matrixes.
The minimal antioxidant activity (reduced by approx. 50%) was found in mistletoe powders, irrespective of the drying technique or matrix.

The ratios obtained between the concentration of polyphenols and antioxidant activity were relatively constant for a given drying technique, but they were differ depending on the type of plant from which the powder was obtained. Thus, the highest ratio was obtained for mistletoe (10.07 to 10.93), although the absolute amount of phenolic compounds was the lowest, while willow and walnut extracts had similar values within the interval 8.5-9. These differences suggest that the antioxidant activity of mistletoe is higher per unit of phenolic compound, i.e. the antioxidant potential of the polyphenolic units of mistletoe (flavonoids) is more intense. Instead, the antioxidant potential of polyphenolic units of willow (phenolic acids) is lower although their concentration is higher.

The product obtained from willow extract is the richest in polyphenols and it is recommended to be used as bio-disinfectant.

Chapter 6
ANTIMICROBIAL EFFICIENCY OF THE WILLOW POWDER FIXED ON MALTODEXTRINE (MD) OR SALT (S) TESTED ON FIVE TYPES OF MICRO-ORGANISMS

6.1 MATERIALS AND METHODS

To correlate the data previously obtained (content of total phenolic compounds in the three kinds of powders, dependent on the drying technique (SD, FB and FD), of the matrix used (MD, S or LA) and antioxidant capacity, with their antimicrobial potential, we selected willow powder because it has the highest concentration of phenolic compounds, it was obtained by SD technique, on matrixes of maltodextrin (MD) and salt (S).

After 5 and 10 min, we collected samples using sterile buffers, then suspended in saline solution, of which we made 5 dilutions and of each dilution we incubated 1 ml suspension on solid growth medium (agar) for 24 hours. At the same time, we considered the correlation between the number of colony forming units (CFU / ml), and the absorbance at 600 nm according to the literature,1 unit of absorbance corresponding to $10^8$ cfu / ml per OD600 (Chia-Liang Cheng, et al., 2009). Based on the absorbance criteria of these solutions at 600 nm, we measured the number of CFU / mL, also referred to as “colonies”.

The testing of the antimicrobial effect was done as follows:
On the surface of a clean and disinfected piece of tile (10x10cm), we applied 1 ml growth medium with micro-organisms. The medium was homogenized on the entire plate surface. After homogenization, the medium and micro-organism were left 5 and 10 minutes, and then, we sprayed solution of water + dissolved powder (10% dissolved powder) on the tile surface. After the solution drains, we collected samples with cotton swabs, we made 6 dilutions, the solution was placed on the agar growth medium and they were incubated.

6.2. RESULTS AND DISCUSSIONS

6.2.1. The effect of the willow powder extract fixed on MD or S on the development of micro-organisms: study on solid medium – qualitative evaluation

Fig. 6.1. $a$ – $b$ E. coli colonies after incubation and collection of samples after 5 min ($a$) and 10 min ($b$) exposure

Fig. 6.2. $a$ – $b$ S. aureus colonies after incubation and collection of samples after 5 min ($a$) and 10 min ($b$) exposure
5 min after the application of the willow powder / MD rehydrated on the tile, on solid medium, 62 colonies of Salmonella, respectively 321 colonies of Listeria developed. After 10 minutes, 12 colonies of Salmonella, respectively 9 colonies of Listeria developed. In the case of willow powder / S rehydrated, on solid medium, 8 colonies of Salmonella, respectively 9 colonies developed after 5 minutes and after 10 minutes, 2 colonies of Salmonella, and respectively 5 Listeria colonies developed.
6.2.2 Semi-quantitative evaluation of the effect of the willow powder extract fixed on MD or S, on the development of micro-organisms on agar plates

Fig. 6.5. *Number of colonies (m – sample and 1 – 4 dilutions) after applying Salix alba powder solution (using as matrix MD and salt) after 5 and 10 min exposure*
Fig. 6.6. *Number of colonies (m – sample and 1 – 4 dilutions) after applying Salix alba powder solution (using as matrix MD and salt) after 5 and 10 min exposure*  
![Bar graph showing B. cereus](image)

Fig. 6.7. *Number of colonies (m – sample and 1 – 4 dilutions) after applying Salix alba powder solution (using as matrix MD and salt) after 5 and 10 min exposure*  
![Bar graph showing Salmonella](image)

Fig. 6.8. *Number of colonies (m – sample and 1 – 4 dilutions) after applying Salix alba powder solution (using as matrix MD and salt) after 5 and 10 min exposure*  

6.4. CONCLUSIONS

The antibacterial effect was tested with willow powders on maltodextrin and salt matrixes, on five types of bacterial cultures which were initially dispersed on tiles. The evaluation of the effect was done by taking swabs after spraying dissolved willow powder, on intervals of 5 and 10...
minutes, cotton swabs that were dissolved in distilled water and incubated on agar plates. From the data obtained, correlated with absorptions at 600 nm, we found the different efficiency of powders, ie the powders with the salt matrix were more efficient, time action of 10 minutes was better. The most sensitive micro-organisms were *S. aureus and Salmonella*.

In conclusion, we recommended the use of willow powder on salt matrix as potentially biodisinfectant, with applications in the food industry and the biomedical field.

**GENERAL CONCLUSIONS**

In relation to the purpose and objectives of the work, we may mention the following conclusions

1. We obtained aqueous and hydro-alcoholic extracts of native plants, namely willow, mistletoe, walnut. The plants were mixed with acidified water, having a concentration of 15% dry plant and allowed to macerate for 24 hours.

2. The extracts were characterized by calculating the efficiency of extraction, composition of the phenolic compounds of the extracts, the spectroscopic, chromatographic footprint, and antioxidant activity. The physical-chemical analyses, followed by microbiological tests showed that hydroalcoholic extracts are most effective than the aqueous ones. The best results were obtained for the extracts of *Rosmarinus officinalis, Urtica dioica, Ocimum basilicum* and *Satureja Montana*.

3. We marked out antimicrobial effects of extracts through qualitative and quantitative tests on growth medium seeded with different types of bacteria.

The hydro-alcoholic extract of rosemary (026) was the only extract that had good results against micro-organisms used for *E. coli* and *Bacillus subtilis*. As noted in the literature, the essential oils obtained from *Rosmarinus officinalis* have good activity against other micro-organisms such as *Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus fecalis, Escherichia coli, Staphylococcus epidermidis, Bacillus subtilis and Candida albicans*. A good result was also obtained in the case hydro-alcoholic extract of *Satureja Montana* (028), as it is the only extract that had good results against micro-organisms used for test *E. coli* and *Bacillus subtilis*.

In conclusion, hydroalcoholic extracts of *Rosmarinus officinalis, Ocimum basilicum* and *Satureja Montana* had good results against micro-organisms inhibiting their development.

The study on the aqueous extract of walnut, mistletoe and willow showed that all three extracts have antibacterial effect. The extracts studied had high content of polyphenols, particularly flavonoids and triterpenoids, a result obtained following the HPLC analysis. The studies made on
agar medium showed that willow extract had a good effect against *Listeria monocytogenes* and *Bacillus cereus*.

The quantitative evaluation of the antimicrobial effect in liquid medium, using UV-Vis spectrometry method, demonstrated that the willow extract is most rich in polyphenolic compounds (salicylic acid) and had the best antimicrobial effect. The nut and mistletoe had specific microbial activities, depending on the concentration, respectively the type of micro-organism used.

4. The preparation of powders from aqueous willow, nut and mistletoe extracts using different matrixes (maltodextrin, salt, lactose) through drying, atomization, fluidized-bed drying and freeze-drying technologies

The powders were obtained from the extract with acidified water (1% HCl) with 15% plant and mixed with the matrix (MD, L and S). Following dehydration processes, 21 powders were obtained. The matrixes had different behavior during processing, salt reacted well to all three processes of evaporation (SD, FB and FD), MD to SD and FD and L to FD and FB.

After the studies made on powders to establish the polyphenol content remaining after processing, it resulted that the willow extract had the highest percentage of active compounds.

As related to the performance, the best results were obtained on SD, with S and MD matrixes. Atomization has several advantages compared to similar procedures as continuous flow processing (charges process for the two processes), permanent control of the liquid flow and drying temperatures, low energy consumption (lyophilization consumes a large quantity of energy to cool the product at -50 - 60°C), during the process the concentration of the extract may be easily changed, the product may be replaced or the temperature may be modified.

5. The physical-chemical characterization of powders obtained from extracts of willow, walnut and mistletoe.

Willow powders had the best results as antioxidant activity (11.9 to 13.55 μM Trolox/1ml dissolved powder) and this is correlated with a maximum content of polyphenols (1.317 to 1.512 mg GAE / 1 ml dissolved powder).

The ratios obtained between the concentration of polyphenols and the antioxidant activity were relatively constant for a given drying technique, but it was different according to the type of the plants from which the powder was obtained.

6. The testing of the antibacterial effect of the willow powder on maltodextrin or salt, with the possibility of being used as bio-disinfectants.
After the rehydration of powders, we conducted a controlled test to establish the efficiency of the willow extract on salt or maltodextrin matrix.

On ceramic surface cleaned of any dirt, we placed micro-organisms with a known concentration, we homogenized their distribution, then we applied the solution obtained after rehydration. The willow extract on salt matrix had a superior result than that on maltodextrin matrix, as it has a very good action for four of the five micro-organisms used (Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus and Salmonella enteritis). The rehydrated powder of willow extract on salt matrix may be easily used to eliminate micro-organisms.

In conclusion, we may say that the aqueous extract and especially willow powder in concentrated form has antibacterial potential and it may be used as natural “instant” bio-preservative or bio-disinfectant, efficient against pathogenic micro-organisms in food production areas, biomedical areas, etc.

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