

ANTIMICROBIAL ACTIVITY OF *RIBES NIGRUM*, *ROSMARINUS OFFICINALIS*, *BETULA* *PUBESCENS*, *SALIX ALBA*, *VACCINIUM* *MYRTILLUS* GEMODERIVATIVES

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Abstract

In the present paper we report the antimicrobial activity of the aqueous extract of *Ribes nigrum* (black currant) buds, *Vaccinium myrtillus* (cranberry) young shoots, *Betula pubescens* (downy birch) buds, *Rosmarinus officinalis* (rosemary) young shoots, *Salix alba* (willow) aments, products that are part of the S.C. HOFIGAL EXPORT-IMPORT S.A. company portofolio. Antifungal and antimicrobial activity was tested by the diffusimetric method against bacterial strains (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 35218 and *Bacillus subtilis* ATCC 6633) and fungi (*Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 10231).

Rezumat

S-a determinat activitatea antimicrobiană a extractelor apoase ale mugurilor de *Ribes nigrum*, a mlădițelor de *Vaccinium myrtillus*, a mugurilor de *Betula pubescens*, a mlădițelor de *Rosmarinus officinalis*, a amentilor de *Salix alba*. Produsele luate în studiu provin de la compania S.C. HOFIGAL EXPORT-IMPORT S.A. Activitatea antifungică și antimicrobiană a fost testată prin metoda difuzimetrică utilizând următoarele microorganisme: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 35218, *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231.

Keywords: antimicrobial, diffusimetric method, gemoderivatives

Introduction

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in the world. According to the World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. About 80% of the people

from developed countries use traditional medicine that, utilises compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments.

Gemotherapy as a branch of biotherapy, is a treatment method which uses glycerine-hydroalcoholic extracts from fresh embryonary vegetal tissues - meristematic tissues (buds, young shoots, bark of the young shoots, aments, seed, radicles).

In meristematic tissues there are higher quantities of substances and active principles compared to those present in the adult plant. Traditional phytotherapy uses products obtained from the whole plant or certain parts of it (roots, leaves, flowers, fruits, seeds), while gemotherapy starts with embryonary vegetable tissues which are subjected to a glycerine-hydroalcoholic maceration [28, 30].

The complex composition of the gemoderivatives, conditioned with modern, performant techniques, allows the underlining of some growth factors, enzymes, proteins or other essential substances during the embryogenesis process of the plants, these substances having a meaningful biological action over the human body with prophylactic-curative potential in different diseases.

Presently, the gemotherapeutic products are used as well as a remedy on their own, but also accompanying products for allopathic therapy. They are usually extracted from a balanced mix of alcohol, water and glycerine and they are known as glycerine-hydroalcoholic extracts.

Materials and methods

Plant materials and preparation of the glycerine-hydroalcoholic extracts

Plants used in the study, *Ribes nigrum* buds (black currant) [3,28], *Vaccinium myrtillus* young shoots (cranberry), *Betula pubescens* buds (downy birch), *Rosmarinus officinalis* young shoots (rosemary), *Salix alba* aments (willow) are from S.C.Hofigal Export-Import S.A. own crops.

- Harvesting of plant material

Buds, roots and young embryonic tissue are harvest in late winter or early spring period when a maximum concentration of active ingredients exists. In this case we are talking about winter-spring 2008. The used parts of the plant are harvested fresh from our own crops, being kept away from contaminated sources.

For all plants used for therapeutic purposes it is excluded the presence of herbicides and insecticides. We use for these plants a natural

fertilizer. Once harvested, parts of the plants should be transported rapidly in the Quality Control Laboratory, which should start the procedures in the next 48 hours [28].

Freshly harvested parts of the plants are cleaned. Once clean and selected plant material is subjected to trituration for facilitating the solvent extraction operation [28].

- Determination of humidity and dry weight: a specimen plant is used to determine the humidity content. The dry weight of the fresh harvested plant is determined (dried) by heating to 50°C for 24 hours, time required to reach a constant weight.

- Maceration: the material still fresh, clean and triturated is put on maceration for 4-5 days in amber glass bottles and stainless steel lid, with 90°C alcohol. After this first step, a mixture of water and glycerine (1:1) is added to the alcohol which is calculated so that the final product has a weight 20 times greater than the raw material, reported to the dry sample.

In other words, consistently produced solvent ratio should be 1:20 (=5%), with reference to the dry weight of parts of the plant processed. Throughout the process, the amount of glycerol should be maintained in a constant ratio of 1:20, to finally obtain a consistent and reproducible product as pharmacological active principle. Alcohol-glycerol-water solution is able to extract from the buds and stem tissues all contained active ingredients.

Maceration is continued in this alcohol-glycerol solution for three weeks [28].

- Squeezing and filtering: maceration once completed the obtained product is submitted to filtration. The residue is subjected to a constant process of squeezing pressure of approx. 10^{-7} Pa. Afterwards the product is allowed to stay another 48 hours, after which it is filtered again.

- Dilution: maceration glycerol itself is further diluted 1:10 with a mixture of water-alcohol-glycerol prepared separately. This mixture should be composed of 50 parts glycerine, 30 parts alcohol and 20 parts water.

It will finally get a soak in the first decimal homeopathic dilution (1DH).

The French Pharmacopoeia sets a number of ways to control the final product [28].

- Control: at the end of the obtaining process, gemoderivatives are checked before being marketed. Control is based on: organoleptic tests by which we check the smell, taste and color of the glycerine macerate; physical evidence: required to determine the density, degree of alcohol and any residue; chemical tests: allow the characterization of the essential

principles of the gemoderivatives. Through specific chemical reactions it can be checked the content of alkaloids.

A subsequent trial serves to individualise the presence of contaminants such as methanol and 2-propanol by capillary chromatography [28].

- Conservation: gemoderivatives are conserved in glass containers (preferably brown) and are stored in closed places, away from light [28].

The final product in decimal dillution is stored in monodose packing of 1.5-2.0 mL.

- Validity: can be used five years from the date of preparation [28].

Chemical analysis of glycerine -hydroalcoholic extracts

We determined the chemical composition of the glycerine-hydroalcoholic extracts.

The quantitative determination of flavanoids is performed according to FR X by a spectrophotometric method.

The quantitative determination of tannins is achieved by a spectrophotometric method, indirectly, provided by FR X, or by a gravimetric method.

The quantitative determination of polihydroxyphenol compounds is achieved by a spectrophotometric method [32,33].

In quantitative terms, *Ribes nigrum* buds gemoderivatives content is:

-34.17 to 43.18 mg% rutoside/100 mL solution;

-56.89 to 87.13 mg% caffeic acid /100 mL solution.

The chemical analysis performed on *Rosmarinus officinalis* young shoots gemoderivatives showed the presence of the following types of active principles: flavones, tannins, derivatives polyphenolcarboxylic type, polihydroxyphenol compounds, saponosides, proanthocyanins, reducing compounds.

The chemical composition of *Rosmarinus officinalis* young shoots gemoderivatives is:

- 143.8 to 189.14 mg% rutoside/100 mL solution;

- 234.27 to 332.01 mg% caffeic acid/100 mL solution.

Betula pubescens buds gemoderivatives contain:

- 14.23-20.22 mg flavonoids expressed as rutoside mg%/100 mL;

- 85.14-98.29 mg polihydroxyphenol compounds expressed as rutoside mg%/100mL.

The chemical composition of *Salix alba* aments gemoderivatives is:

- 11.23-12.43 mg flavonoids expressed as rutoside mg% /100 mL;

- 15.49-18.23 mg polihydroxyphenol compounds expressed as rutoside mg% / 100 mL.

- 67.5 to 68.4 mg% caffeic acid/100 mL solution;

- 78.0-86.0 mg% rutoside/100 mL solution.

We developed a HPLC method to determine the chemical composition of the gemoderivatives in the following conditions: chromatographic column type RP18, $l = 200$ mm, $\Phi = 4.0$ mm, $5\mu\text{m}$, column temperature 30°C , injection volume $20\mu\text{L}$, flow 2.5 mL / min. , the detection wavelength $\lambda = 280\text{ nm}$, using a mobile phase water: methanol: orthophosphoric acid: chlorogenic acid 98% purity= 1:10:0.01:0.0105.

The method has been applied with good results in order to determine in the gemoderivatives the presence of gallic acid, rosmarinic acid, caffeic acid, p-coumaric acid, chlorogenic acid, anthocyanines, tannins, rutoside, quercetol, salicosides, 3-galactosides, myrcetin.

In *Rosmarinus officinalis* young shoots gemoderivatives we determined: rosmarinic acid, gallic acid and tannins (Fig.1).

In *Vaccinium myrtillus* young shoots gemoderivatives we determined the presence of anthocyanines, chlorogenic acid, caffeic acid, p-coumaric acid, routine, kaempferol coumarin and tannins (Fig.2).

In *Salix alba* aments gemoderivatives we determined the salicosides, p-coumaric acid, ferulic acid, kaempferol [28] and tannins (Fig. 3).

In *Betula pubescens* buds gemoderivatives we determined the presence of galactosides myrcetin, chlorogenic acid, p-coumaric acid, coumaric acid, quercelats, rutoside (Fig. 4).

The presence of rosmarinic acid in *Rosmarinus officinalis* young shoots gemoderivatives explains the antioxidant properties, the suppression of oxide and peroxide radicals involved in aging processes and infections, the antimicrobial action [21] and activates the cerebral circulation.

Antocianosides, flavanoids, tannins, p-coumaric acid explain the antioxidant activity of *Vaccinium myrtillus* young shoots gemoderivatives [28].

The presence of salicosides in *Salix alba* aments gemoderivatives explains the antiinflammatory and analgesic action and its use in rheumatic diseases.

The diuretic action of *Betula pubescens* buds gemoderivatives [28] is explained by the presence of flavonosides, saponosides and antocianosides. The diuretic action is accompanied by the elimination of uric acid [28].

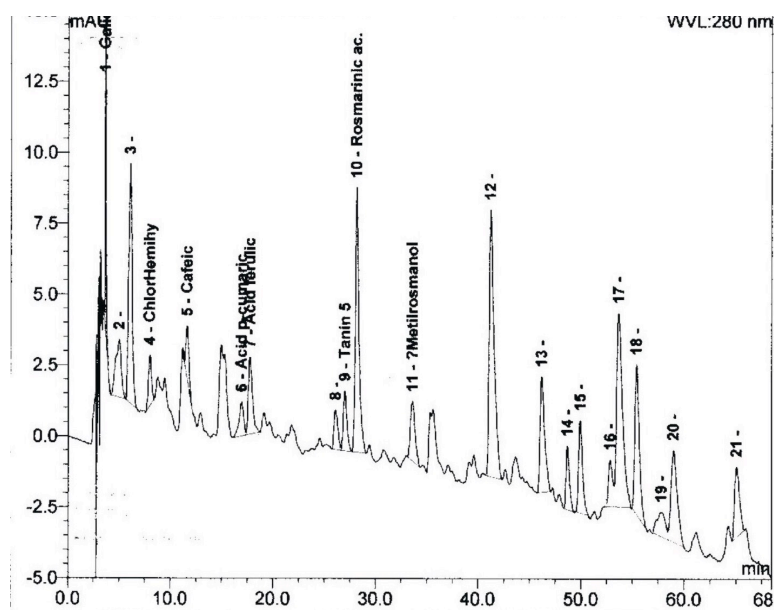


Figure 1
HPLC determination of the amount of active principles of
Rosmarinus officinalis young shoots gemoderivatives

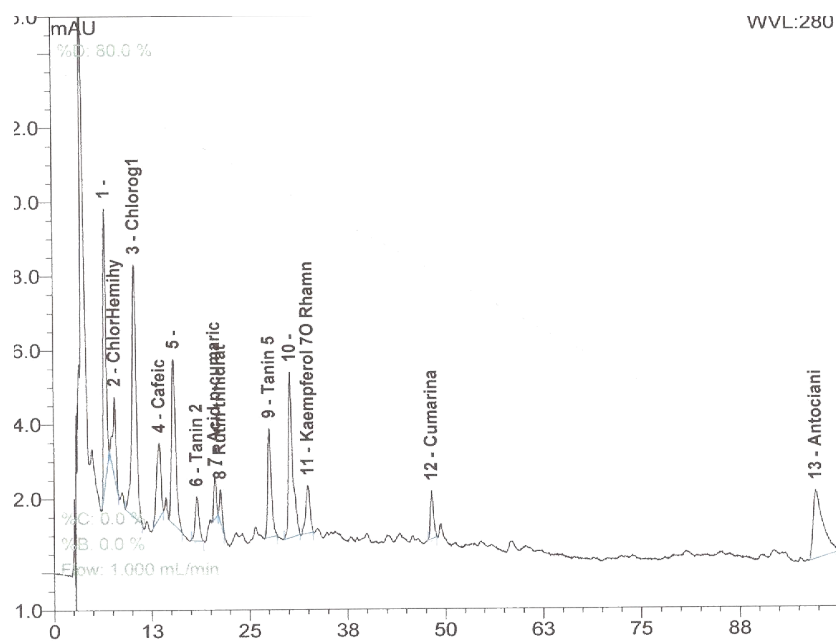


Figure 2
HPLC determination of the amount of active principles of
Vaccinium myrtillus young shoots gemoderivatives

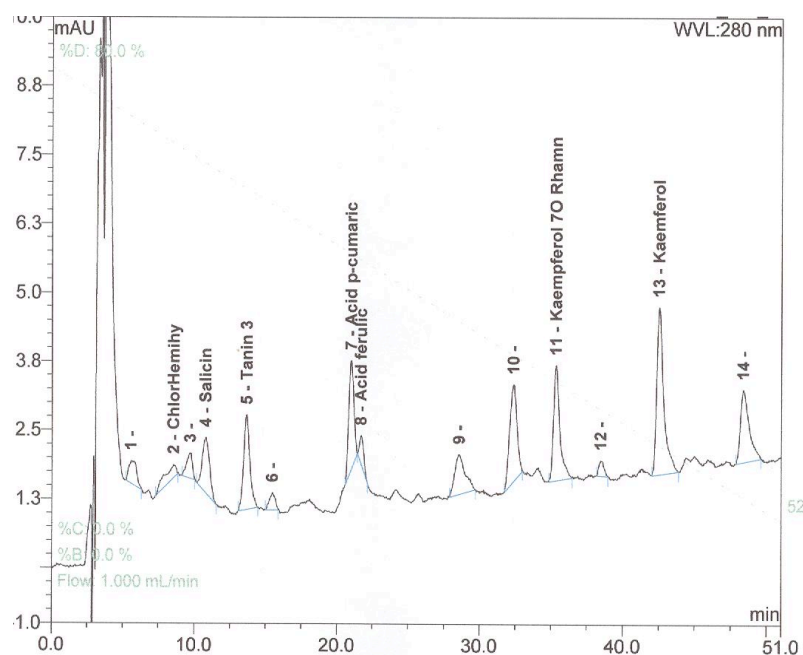


Figure 3
HPLC determination of the amount of active principles of
Salix alba amens gemoderivatives

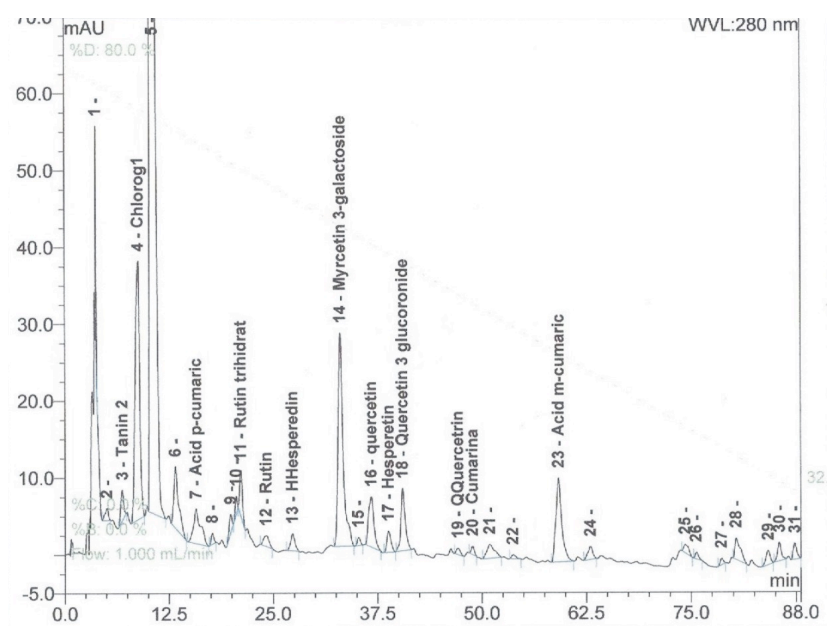


Figure 4
HPLC determination of the amount of active principles of
Betula pubescens buds gemoderivatives

Bacterials cultures

Four bacterial strains (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 35218 and *Bacillus subtilis* ATCC 6633) and two fungal strains (*Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231) have been used for testing the antimicrobial activity [2,4,5,6,7,8].

Culture media

In order to determine the antimicrobial activity, the following culture media were necessary:

- Casein soya bean digest agar medium;
- Sabouraud dextrose agar;
- Casein soya bean digest broth;
- Sabouraud- dextrose broth;
- Agar medium O (Baird Parker agar);
- Agar medium H (MacConkey agar);
- Agar medium N (Cetrimide agar).

Working method

The antimicrobial activity was determined by the diffusimetric method.

Diffusimetric method

The principle of this method consists in the contact of a standard medium from the microorganism-test with decreasing concentrations of the test sample.

Microorganism-suspension test: in the isotonic sodium chloride solution, suspensions have been prepared with a concentration of 10^7 colony forming units /mL micro-test, corresponding to the tube's turbidity of 0.5 Mac Farland.

The samples for analysis: have been dilutions of 1/2, 1/4 and 1/8.

In the Petri dishes, either bacteria were inoculated onto 20 mL of agar medium, or fungi were inoculated onto 20 mL of Sabouraud medium. On the surface of the media, discs of sterile filter paper were placed, soaked with various concentrations of the test products. Simultaneously, control Petri dishes were used, which were inoculated with the same microorganisms [12], without having test samples placed upon them.

The Petri dishes with the agar medium for bacteria were incubated at 30-35°C, while those with Sabouraud medium for fungi were incubated at 20-25°C in aero-biotic conditions. The Petri dishes were examined after 24-48 hours, by recording the diameter of the inhibition zones for each micro-organism and for each dilution of the test sample [1, 19, 20, 34, 35].

We studied the antimicrobial action [7,13,16] of the aqueous extracts (5%) of *Ribes nigrum* buds (black currant) [3,28], *Vaccinium myrtillus* young shoots (cranberry), *Betula pubescens* buds (downy birch), *Rosmarinus officinalis* young shoots (rosemary), *Salix alba* aments (willow). The aqueous extract 5% was obtained by eliminated the alcohol-glycerin from the system by rotary evaporation under reduced pressure.

We took 10 g of the aqueous extract 5% in a sterile tube and suspended it in 90 mL sodium chloride peptone solution, pH = 7.00 to obtain the working dilution 1: 10 [16]. From this, we prepared serial dilutions 1: 100 and 1: 1000.

In Petri dishes 9 cm in diameter, we transferred 1 mL of each diluted solution. As reference substance was used the volatile oil of thyme. Sterile filter paper disks were treated with the above aqueous extracts and then placed in a culture incubated for 48 hours at 37°C [26].

Results and discussion

Inhibition zone diameter was calculated for each dilution and was determined in millimeters. *Oleum Thymi* was used as a reference substance. The results obtained are presented in table I and fig.5-15. Statistical analysis: results were assessed using Student t-test.

Table I

Antimicrobial activity of the plant extracts on different bacterial and fungal strains

Studied germs	Buds of <i>Ribes nigrum</i> inhibition zone diameter (mm) A	Young shoots of <i>Vaccinium myrtillus</i> inhibition zone diameter (mm)	Buds of <i>Betula pubescens</i> inhibition zone diameter (mm)	Young shoots of <i>Rosmarinus officinalis</i> inhibition zone diameter (mm) B	Aments of <i>Salix alba</i> inhibition zone diameter (mm)	Volatile oil of thyme <i>Thymus vulgaris</i> inhibition zone diameter (mm) Reference substance
<i>Staphylococcus aureus</i> ATCC 6538	30	0	0	31	0	32
<i>Pseudomonas aeruginosa</i> ATCC 9027	21	0	0	22	0	20
<i>Escherichia coli</i> ATCC 35218	18	0	0	19	0	10
<i>Aspergillus niger</i> ATCC 16404	33	0	0	29	0	40
<i>Candida albicans</i> ATCC 10231	29	0	0	27	0	33

Where: A=The column that contains the values diameter inhibition of *Ribes nigrum* buds against microorganisms using *Oleum Thymi* as a reference substance

B= The column that contains the values diameter inhibition of *Rosmarinus officinalis* young shoots against microorganisms using *Oleum Thymi* as a reference substance

Through Student t-test we determined that the results are statistically significant, $p < 0.05$.

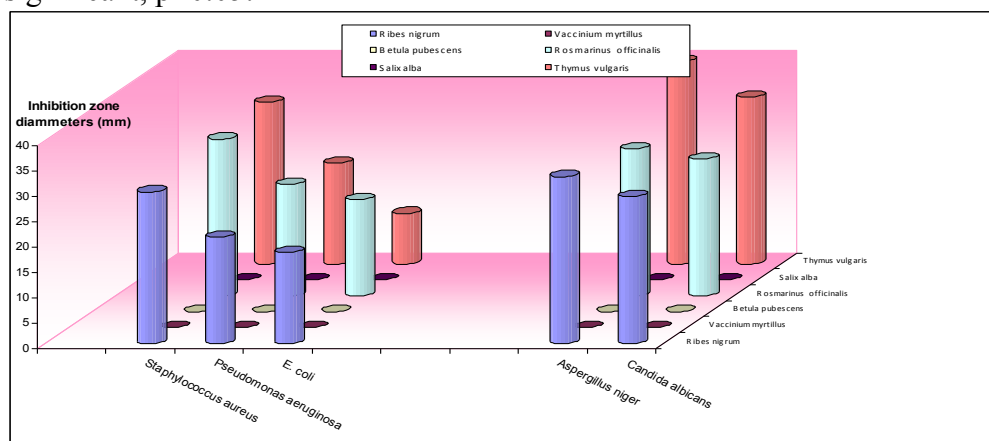


Figure 5

Antimicrobial activity of the plant extracts on different bacterial and fungal strains

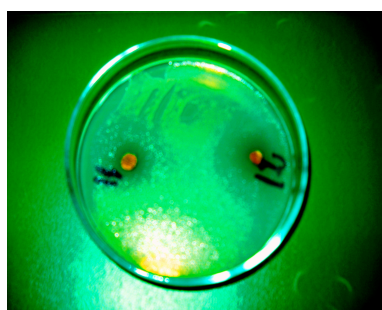


Figure 6

The action of aqueous extract (5%) of *Ribes nigrum* buds against *Staphylococcus aureus*

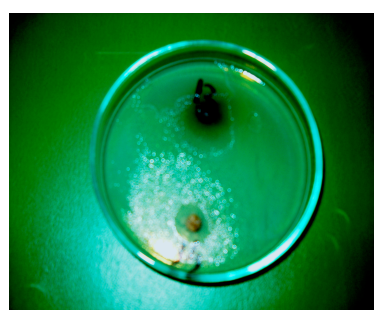


Figure 7

The action of aqueous extract (5%) of *Ribes nigrum* buds against *Pseudomonas aeruginosa*

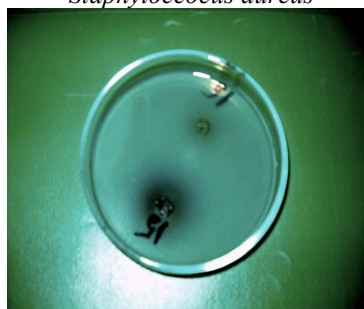


Figure 8

The action of aqueous extract (5%) of *Ribes nigrum* buds against *Escherichia coli*

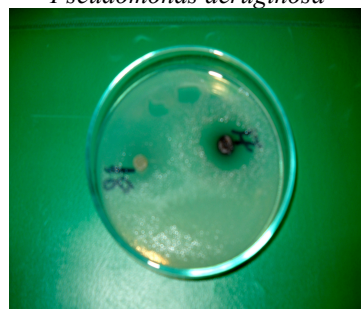
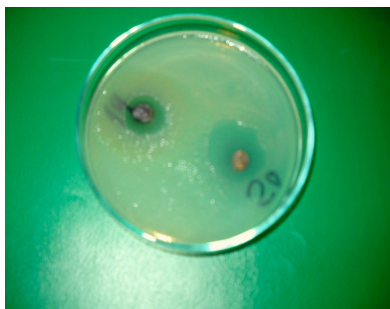
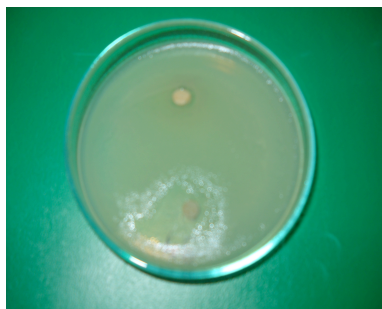


Figure 9

The action of aqueous extract (5%) of *Ribes nigrum* buds against *Aspergillus niger*

**Figure 10**

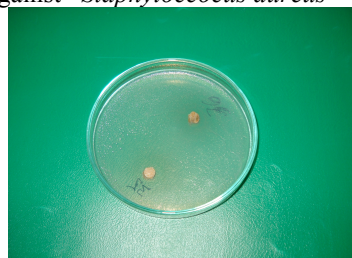
The action of aqueous extract (5%) of *Ribes nigrum* buds against *Candida albicans*

**Figure 11**

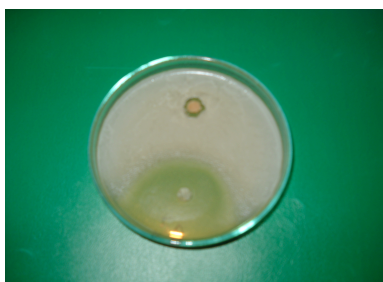
The action of aqueous extract (5%) of *Rosmarinus officinalis* young shoots against *Staphylococcus aureus*

**Figure 12**

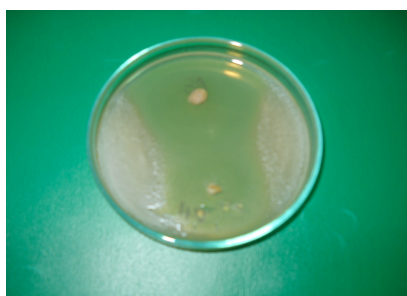
The action of aqueous extract (5%) of *Rosmarinus officinalis* young shoots against *Pseudomonas aeruginosa*

**Figure 13**

The action of aqueous extract (5%) of *Rosmarinus officinalis* young shoots against *Escherichia coli*

**Figure 14**

The action of aqueous extract (5%) of *Rosmarinus officinalis* young shoots against *Aspergillus niger*

**Figure 15**

The action of aqueous extract (5%) of *Rosmarinus officinalis* young shoots against *Candida albicans*

After testing using the diffusimetric method, the antimicrobial and antifungal action were observed on aqueous extracts (5%) of *Ribes nigrum* buds (black currant), *Rosmarinus officinalis* young shoots (rosemary) [15,21], against *Staphylococcus aureus*, *Pseudomonas aeruginosa* [18], *Escherichia coli* [25,29], *Aspergillus niger*, *Candida albicans*. The

antimicrobial activity of the studied extracts is explained by high levels of essential oils, polyphenols, flavonoids, campherol, myricetin, quercetin, isoquercetin, isohesperidin, ascorbic acid [10, 11, 14, 22, 23, 24, 27, 31, 34].

The volatile oil from *Ribes nigrum* (black currant) gemoderivatives buds is rich in sabinen, limonene, alpha and beta pinene, fenandren giving the antimicrobial activity [9, 17, 31].

Conclusions

Buds and young shoots of *Ribes nigrum* (black currant) and *Rosmarinus officinalis* (rosemary) at the same concentrations, showed an antimicrobial activity on the bacterial strains of : *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, as well as on the fungal strains of : *Aspergillus niger* and *Candida albicans*.

Ribes nigrum (black currant) contains flavonoids, including quercetin, myricetin, and kaempferol; and at least 15 different phenolic acids, including anthocyanins and proanthocyanidins; p-coumaric acid has been identified as one of the predominant phenolic acids. The main components of *Thymus vulgaris* are thymol, carvacrol and borneol and those of *Rosmarinus officinalis* are pinene, limonene and piperitone, all of which are phenolic compounds that confer antimicrobial properties to the natural extracts or essential oils obtained from these plants. *Vaccinium myrtillus* young shoots (cranberry), *Betula pubescens* buds (downy birch), *Salix alba* aments (willow) have no antimicrobial and antifungic activity.

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