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*Full Length Research Paper*

# Antimicrobial activity of *Melampyrum cristatum*, *Melampyrum bihariense* and *Melampyrum arvense* tinctures

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**The infections with bacteria and fungi resistant to existing chemotherapeutic drug have become a serious problem in the whole world, which leads to the continuous need to develop new antimicrobial agents. The plants extracts may represent future solutions against these pathogens. The purpose of this study was to determine the antibacterial and antifungal activity of *Melampyrum cristatum, Melampyrum arvense* and *Melampyrum bihariense* tinctures by using the diffusion and the dilution methods. The three tinctures inhibited the growth of *Staphylococcus aureus* up to a dilution of 1/8, and *Escherichia coli* up to a dilution of 1/4. The tinctures of *M. cristatum* and *M. arvense* inhibited the development of *Pseudomonas aeruginosa* up to a dilution of 1/4, while the tincture of *M. bihariense* up to a dilution of 1/8. Of the three tinctures, the tincture of *M. cristatum* showed the best antifungal action, inhibiting the development of *Candida albicans* up to a dilution of 1/8.**

**Key words:** Tinctures of *Melampyrum*, antibacterial activity, antifungal activity.

## INTRODUCTION

The species of *Melampyrum cristatum* L., *Melampyrum bihariense* Kern., *Melampyrum arvense* are plants from the spontaneous flora of Romania, being part of the genus *Melampyrum*, family Scrophulariaceae. The name of *Melampyrum* comes from two Greek words: "*melas*"-black and "*pyros*"-wheat, the seeds of this plant having the aspect of wheat kernels, but of black colour (Buia et al., 1960). The bacteria and fungi will continue to develop new mechanisms of resistance to chemotherapeutic drugs, which is why the plants extracts may represent future solutions against these pathogens.

The species of *Melampyrum* have been scarcely used in the traditional Romanian medicine (Butura, 1979). For *M. arvense*, the decoction of flower stalks was used in the Apuseni Mountains, in Ponor against hepatitis; the dried flower stalks were boiled with edible fruit (dried fruit) and the juice was drank. They were also used for baths against rheumatism. In Năsăud region, cattle with feet affections were washed with the plant decoction. *M. bihariense* it used sporadically for baths against rheumatism in combination with other plants in a tea against jaundice. *M. cristatum* decoction is held in the mouth for acute pain and infectious eruptions believed to have been picked up from found objects, were also washed with it.

The traditional use has been confirmed by the present studies regarding the diuretic and anti-inflammatory actions of *M. bihariense* and *M. cristatum* species (Mogoşan et al., 2008).

This research is the first performed in our country, studying the antimicrobial effects of the *Melampyrum* tinctures. In 2010, following a study performed in Turkey on MeOH and $CHCl_3$ extracts from the *M. arvense* species,

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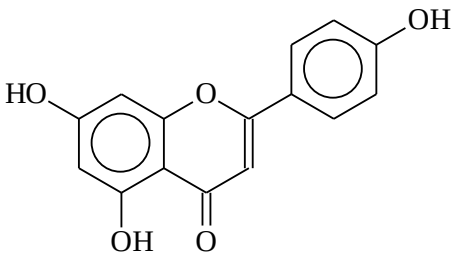



**Figure 1.** Structure of Apigenin(e).

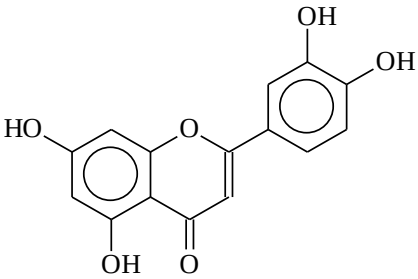



**Figure 2.** Structure of Luteolin.

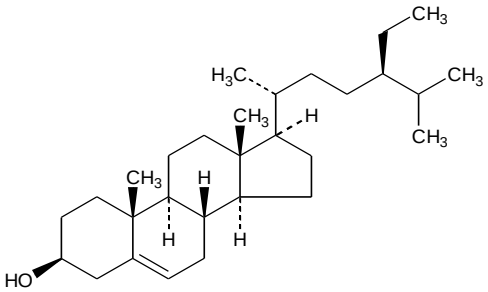



**Figure 3.** Structure of β–Sitosterols.

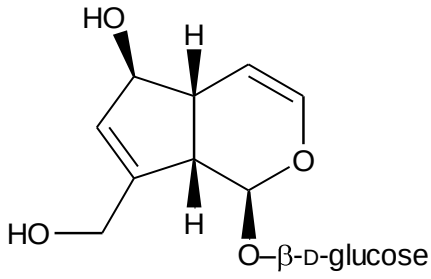



**Figure 4.** Structure of Aucubin.

species, the presence of iridoid glycosides in this species as well as the antiprotozoal effect demonstrated *in vitro* over *Trypanosoma brucei rhodesiense*, *Leishmania donovani* and *Plasmodium falciparum* was highlighted by the gas chromatography-mass spectrometry (GC-MS) analysis (Kirmizibekmez et al., 2011).

As for the chemical composition of the *Melampyrum* genus species, the bibliographical data have shown the presence of several classes of compounds such as iridoid glycosides expressed in aucubin (Figure 4); flavonoids and carboxylic and polyphenolic acids (Figures 1 and 2) (Munteanu et al., 2008) expressed in luteolin and apigenin; sterols expressed in β-sitosterols (Figure 3), carotenoids and mucilages (Munteanu and Vlase, 2011). The analyses carried out to determine these classes of compounds were performed through the HPLC-MS technique.

The purpose of this study was to determine the antibacterial and antifungal activity of *M. cristatum, M. arvense* and *M. bihariense* tinctures.

## MATERIALS AND METHODS

The species (*M. bihariense and M. cristatum*, were harvested in 2004 during the month of June; *M. arvense* was harvested during the month of May 2009) have been harvested from Mureş county, Romania, close to Lake Fărăgău, and analyses were carried out on the first species in 2004 and on the latter in 2009.

From the aerial parts of the species: *M. cristatum, M. arvense* and *M. bihariense* tinctures were prepared 1:10 (mg:ml) in ethylic alcohol of 70°C, according to FR X (Romanian Pharmacopeia, 1993). In order to test the antibacterial and antifungal activity, the following microorganisms have been used *Staphylococcus aureus* ATCC (American Type Culture Collection) 25933, *Enterococcus faecalis* ATCC 51299, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Proteus mirabilis* ATCC 43071, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 11778, *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16404. Testing the antimicrobial activity was performed through the difusimetric method and the dilution method (in Mueller Hinton broth). The following substances were tested through the difusimetric method: tincture of *M. cristatum* (TMC), tincture of *M. arvense* (TMA), tincture of *M. bihariense* (TMB), and the ethylic alcohol.

Dilutions of 1/2, 1/4 and 1/8 were done using as diluent the alcohol in each tincture and 20 µl were applied in each well. The strains tested by the diffusion method were Gram positive bacteria: *S. aureus* ATCC 25933, Gram negative Bacteria: *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, and yeast: *C. albicans* ATCC 10231. The results were obtained in duplicate.

The three tinctures used were prepared as follows: from the aerial part of the three species, *M. cristatum*, *M. arvense* and *M. bihariense* tinctures were prepared 1:10 in 70° ethyl alcohol, according to FR X (Romanian Pharmacopeia, 1993).

### Diffusion method

Diffusion method is also called the cup-plate agar diffusion method (Reeves and Reeves, 1983). The medium used was the simple Mueller-Hinton growth medium for screening *S. aureus, E. coli*, *P. aeruginosa*, and for *C. albicans* the medium used was Mueller-Hinton supplemented with 2% glucose (which provides a proper growth of yeasts) and 0.5 µg/ml methylene blue (provides a better delimitation of the diameter of the zone of inhibition). Six millimeter diameter wells were cut from the agar using a sterile cork-borer, and a predetermined (20 µl) volume of each compound solution were delivered into the wells.

For each of the tested microorganisms, a few colonies (maximum 5) were suspended from the pure culture in 5 ml of 0.85% sodium chloride (NaCl) sterile solution in order to obtain a suspension with standard turbidity of 0.5 McFarland at 530 nm (inoculum that corresponds to a population of $1\times10^6$ to $5\times10^6$ CFU).

After obtaining a suspension with standard turbidity, the medium was inoculated with a sterile nontoxic swab well impregnated in the prepared inoculum. The surface of the medium was inoculated in three directions in order to obtain a uniform distribution on the whole surface of the medium. After drying by maintaining at room temperature for 5 to 15 min, 20 µl from each tested substance was deposited in the well.

The plates incubated at 35°C for 20 to 24 h (Mareş, 2007), and the results were obtained in duplicate.

**Dilution method**

The dilution method had two parts: the first part was the simple dilution, which uses small glass beads, on the surface of which the microorganism to be tested was dried for 15 min. The beads were then exposed to the substance whose antimicrobial action was tested, at a temperature of 20°C, time of action at 1, 5, 10 and 30 min, and were rinsed with water, and then transferred into tubes with growth medium. After incubating at 37°C for 48 h, the tubes were examined for growth.

**Reference method**

***Germ carrier test***

The strains tested through the simple dilution method with glass beads were *S. aureus* ATCC 25933, *E. faecalis* ATCC 51299, *E. coli* ATCC 25922, *S. enteritidis* ATCC 13076, *P. mirabilis* ATCC 43071, *P. aeruginosa* ATCC 27853, *B. cereus* ATCC 11778, *C. albicans* ATCC 10231, and *A. niger* ATCC 16404. The tested substances were undiluted TMC, TMA, and TMB.

A suspension was prepared in 0.85% NaCl with a measured density of $10^7$ CFU/ml for bacteria and of $10^7$ to $10^8$ CFU/ml for fungi for inoculum from 18 to 24 h culture. The microorganism's bearer is the glass beads.

The glass beads (bearer) were immersed into the bacterial suspension for 15 min, after which they were allowed to dry. The beads were introduced into the testing substance (or dilutions of this substance) and allowed to contact for different time periods. A culture witness was prepared with glass beads removed from the microbial suspension and which were not put in contact with the testing substance. Elimination of the antimicrobial substance was done by introducing the beads into sterile distilled water for a minute. For subcultures, the beads were each introduced into a tube with liquid medium: broth for bacteria and Sabouraud liquid medium for fungi. The tubes with liquid medium were incubated at 37°C for 18 to 72 h for bacteria and 7 days for fungi. The same procedure was used with culture control beads. The cultures have been microscopically examined and subcultures have been created on adequate mediums. In "high" means the development of the microorganism in the corresponding tube and in "absent" means lack of development of the microorganism in the corresponding tube and in subcultures. The second part of this method was the broth dilution method.

The inoculum was prepared from the pure culture of 18 to 24 h of the testing strain, adapted to the density of the standard of 0.5 McFarland. The inoculum thus standardized was diluted (1:10) in a sterile isotonic saline solution. The standardized culture was diluted as follows: 1/200 for *Enterobacteriaceae* and *Staphylococci* and 1/1000 for *Pseudomonades*, which ensured an inoculum of approximately $5\times10^5$ CFU/ml. Each tube received 0.1 inoculum from the binary dilutions from the antimicrobial substance in Mueller Hinton broth of 1 ml; the last tube was used as culture witness, and it contains 1 ml Mueller Hinton broth and 0.1 inoculum (it does not contain antimicrobial substance). The tubes were incubated at 37°C for 20 h and the smallest concentration was incubated as antimicrobial agent that inhibits development of the germ (lack of any macroscopically noticeable growth represents the minimum inhibitory concentration (MIC)) (Buiuc and Neguț, 1999). By using broth dilution method, the tested strains were *S. aureus* ATCC 25933, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 10231. The tested substances were TMC, TMA, and TMB in various dilutions (Reeves and Reeves, 1983).

## RESULTS AND DISCUSSION

### Diffusion method

After the incubation period, the area of complete inhibition (mm) was measured with the measuring rule in the light reflected against matte black background. The complete inhibition of the growth was followed. It was checked if the diameters of the zones of inhibition of each antimicrobial substance were within the limits of permissible variation (in accordance with the CLSI standard). The results obtained for *S. aureus* ATCC 25933 are shown in Table 1 (ethylic alcohol = 10 mm). The results obtained for *E. coli* ATCC 25922 are shown in Table 2 (ethylic alcohol = 10 mm). The results obtained for *P. aeruginosa* ATCC 27853 are shown in Table 3 (ethylic alcohol = 10 mm). The results obtained for *C. albicans* ATCC 10231 are shown in Table 4 (ethylic alcohol = 10 mm).

The results obtained following the simple dilution, with glass beads, the three analysed tinctures in undiluted form, after the contact time of 20 min, showed an antibacterial action (over Gram positive cocci: *S. aureus*, *E. faecalis*, Gram negative bacilli: *E. coli*, *S. enteritidis*, *P. mirabilis*, *P. aeruginosa*, and Gram positive bacilli: *B. cereus*) and an antifungal action (over the yeasts, *C. albicans* and filamentous fungi, *A. niger*).

The results obtained following Mueller-Hinton broth dilution method. The three tinctures analysed cause a growth inhibition up to the dilution of 1/8, as far as *S. aureus* is concerned. Regarding the behaviour of *Melampirum* species tinctures over *E. coli*, the data obtained were slightly different. Thus, for a dilution of ¼, the tinctures obtained from *M. cristatum* and *M. bihariense* showed a higher inhibition than in the case of *M. arvense* tincture. In the case of *P. Aeruginosa*, the best inhibition of the bacterial growth was obtained with *M. cristatum* tincture, for a dilution of 1/8. In the case of *C. albicans*, the *M. arvense* and *M. bihariense* tinctures inhibited the fungal growth in a dilution of 1/2, the *M. cristatum* tincture determined the growth inhibition to a dilution of up to 1/8.

**Table 1.** Diffusion method results obtained for *S. aureus*.

| Tincture | Dilution (mm) | | | |
|---|---|---|---|---|
| | Undiluted | 1/2 dilution | 1/4 dilution | 1/8 dilution |
| *M. cristatum* tincture (TMC) | 16 | 18 | 19 | 19 |
| *M. arvense* tincture (TMA) | 16 | 19 | 19 | 19 |
| *M. bihariense* tincture (TMB) | 16 | 18 | 18 | 19 |

*S. aureus* ATCC 25933 (10 µg Gentamicin 19 to 27 mm, 30 µg Cefoxitin 23 to 29 mm, 10 µg Fusidic acid 24 to 32 mm).

**Table 2.** Diffusion method results obtained for *E. coli*.

| Tincture | Dilution (mm) | | | |
|---|---|---|---|---|
| | Undiluted | 1/2 dilution | 1/4 dilution | 1/8 dilution |
| *M. cristatum* tincture (TMC) | 12 | 12 | 14 | 15 |
| *M. arvense* tincture (TMA) | 12 | 12 | 12 | 14 |
| *M. bihariense* tincture (TMB) | 12 | 12 | 14 | 14 |

*E. coli* ATCC 25922, 10 µg Gentamicin 19 to 26 mm, 30 µg Cefoxitin 23 to 29 mm, 10 µg Colistin 11 to 17 mm.

**Table 3.** Diffusion method results obtained for *P. aeruginosa*.

| Tincture | Dilution (mm) | | | |
|---|---|---|---|---|
| | Undiluted | 1/2 dilution | 1/4 dilution | 1/8 dilution |
| *M. cristatum* tincture (TMC) | 12 | 14 | 14 | 15 |
| *M. arvense* tincture (TMA) | 12 | 12 | 14 | 14 |
| *M. bihariense* tincture (TMB) | 12 | 12 | 14 | 14 |

*P. aeruginosa* ATCC 27853: 10 µg Gentamicin 16 to 21 mm, 10 µg Colistin 11 to 17 mm.

**Table 4.** Diffusion method results obtained for *C. albicans*.

| Tincture | Dilution (mm) | | | |
|---|---|---|---|---|
| | Undiluted | 1/2 dilution | 1/4 dilution | 1/8 dilution |
| *M. cristatum* tincture (TMC) | 12 | 15 | 18 | 18 |
| *M. arvense* tincture (TMA) | 12 | 15 | 18 | 18 |
| *M. bihariense* tincture (TMB) | 12 | 16 | 18 | 18 |

*C. albicans* ATCC 10231: 25 µg Fluconazole 25 to 30 mm.

## Conclusions

According to our knowledge, this is the first research performed in our country, studying the antimicrobial effects of the *Melampyrum* tinctures. Following the tests, it was noticed that *M. cristatum*, *M. bihariense* and *M. arvense* tinctures have shown an antibacterial and antifungal action. All the three tinctures inhibited the development of *S. aureus* up to a dilution of 1/8, the development of *E. coli* up to a dilution of 1/4. The *M. cristatum* and *M. arvense* tinctures inhibited the development of *P. aeruginosa* up to a dilution of 1/4, and the *M. bihariense* tincture up to a dilution of 1/8. Of the three tinctures, the *M. cristatum* tinctured showed the best antifungal action, inhibiting the development of *C. albicans* up to a dilution of 1/8. Thus, our study has shown that *Melampyrum* tinctures presented antibacterial and antifungal activity, which justifies further studies in this direction.

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