Antimicrobial activity of phytoextracts on opportunistic oral bacteria, yeast and bacteria from probiotics

N. M. Vorobets*, M. V. Krytsova**, O. Y. Rivis***, M. Y. Spivak ***, H. V. Yavorska****, H. M. Semenova*****

*Danylo Halysky Lviv National Medical University, Lviv, Ukraine
**Uzhhorod National University, Uzhhorod, Ukraine
***Danylo Zabolotny Institute of Microbiology and Virology, National Academy of Science of Ukraine, Kyiv, Ukraine
****Ivan Franko Lviv National University, Lviv, Ukraine
*****Bacteriological Laboratory of Uzhhorod District Hospital, Uzhhorod, Ukraine

Abstract


Developed experimental assays enable us to compare the antimicrobial activity of herbal medicinal drugs on Lactobacillus and Bacillus strains probiotics, which have been claimed to possess the ability of suppressing the growth of various oral pathogens. In the treatment of periodontal disease it is advisable to use a comprehensive approach which would include the application of herbal remedies and probiotics. The combination of such effects may be a new approach in dentistry due to their complementary antimicrobial activity. In this study, we researched antimicrobial effects of herbal medicinal drugs (tinctures of some medical plants, solutions Rotocanum and Chlorophyllipt) against collection strains and clinical strains isolated from the oral cavity of patients with periodontitis, and probiotic strains Bacillus subtilis UKM B-5007 and Bacillus licheniformis UKM B-5514 that are part of the active base of probiotic Biosporin (Ukraine), Bacillus clausii from the probiotic Normaflore (Hungary), as well as the strains Lactobacillus spp. – from probiotic Lactobacterium (Biopharma, Ukraine). For investigation, the standard agar dilution method was used in modification with glass cylinders. The results of the research showed that among the studied herbal medicinal remedies, tinctures of Eucalyptus vulgaris, Mentha piperita and Chlorophyllipt had the strongest antimicrobial activity. Probiotic strains are also sensitive to herbal tinctures (except the tinctura of wormwood), which indicate the possibility of only consecutive usage (with an interval of time) of herbal remedies and probiotics in combination therapy in the treatment of periodontal diseases.

Keywords: antimicrobial activity; herbal medicinal remedies; probiotics; periodontal diseases.

Introduction

Oral health influences the general quality of life, and poor oral health is linked to chronic conditions and systemic diseases. Dental caries, gingivitis and periodontitis are prominent oral disorders. The current understanding is that the etiology of oral diseases is multifactorial but, in many cases, it includes a pathogenic response to bacterial and Candida infection (Rachalovic et al., 2013; Vorobets & Rivis, 2017). Periodontitis is a common and widespread disease, which occurs due to pathogenic microbial infection established within the gingival sulcus. Treatment of patients with periodontal diseases should include both local and general therapy. It must be based on the understanding of the mechanisms of action of the pharmaceutical or prophylactic agents, and be used to be effective and at the same time safe for the patient. Currently, the drugs of plant origin correspond to such criteria, because active compounds in their composition can act bacteriostatically and bactericidally (Rios et al., 2005; Kačániová et al., 2014; Hleba et al., 2016). Another group of medicines – probiotics have relatively recently started to be used for periodontal treatment. Probiotics are commonly bacteria from genera Lactobacillus (including strains L. salivarius and L. plantarum) and Bifidobacterium (including strains B. bifidum, B. longum) or others, which could be beneficial not only to the digestive system, but to oral health too (Mishra et al., 2014; Alok et al., 2017).

The mechanisms of probiotic action are mainly unknown but the inter-microbial species interactions are supposed to play a key role in this together with their immunostimulatory effects. The proposed mechanisms of action of probiotics on oral health correspond to those shown in studies of the gastrointestinal tract. These are aid in synthesis of vitamins B, and K, and also in the breakdown of bile salts, aid in enhancing innate and acquired immunity, and assistance in inhibition of pro-inflammatory mediators (Alok et al., 2017). It is known that oral diseases, including periodontitis, are often accompanied by qualitative and quantitative changes in the microbiota (Rivis et al., 2012; Curtis, 2014). This makes probiotics an alternative means of correction of microflora of the mouth and of reducing the number of opportunistic pathogens. On the other hand, there is evidence that the occurrence of candidiasis of the oral cavity and its chronic form is caused not only by changes in the balance of microorganisms in the mouth, but also by the influence of intestinal microbiota (Zlatkina et al., 2001).

Therefore, a complex treatment should be performed targeting the mouth and lower parts of the gastrointestinal tract. Dysbacteriosis is not only excessive growth of pathogenic microbes in the gut, but also...
involves the overall decline of the immune defence of the body (Alok et al., 2017). At the same time, it can involve such diseases as atopic dermatitis, eczema, thirst, bronchial asthma, food allergy in children. To restore normal intestinal microbiota, probiotics can be used, which are often composed of bifidobacteria and lactobacilli, which are able to show antagonism against pathogenic and opportunistic microorganisms. The results of our previous studies indicate that some industrial strains of spore bacteria that are used as a basis for probiotic products show high antagonistic activity against strains of microorganisms isolated from "periodontal pockets" (Rivis et al., 2013). However, from the clinical practitioner’s point of view, direct recommendations for the use of probiotics cannot yet be given (Curtis, 2014). Beside this, there is a lack of information regarding the contributions of probiotics in oral health and their compatible use with plant origin remedies (Safronova, 2009; Shipradeep, 2012). Our research is focused on the determination of the anti-microbial properties of Tinctura Eucalypti, Tinctura Calendulae, Tinctura Menthae pipertiae, Tinctura Absinthii, and also Tinctures Chlorophyllipt and Rotoceanum against collection and clinical strains isolated from the oral cavity of patients with periodontitis and against probiotic strains that are part of the active base of probiotics Biosporin, Lactobacterin and Normalflor.

Material and methods

The effect of herbal medicinal products such as Rotoceanum, Chlorophyllipt, Tinctura Calendulae, Tinctura Eucalypti, Tinctura Menthae pipertiae, Tinctura Absinthii was investigated on collection and clinical isolated strains of microorganisms. The general chemical profiles of the extracts and their pharmacological effects against oral cavity diseases are summarized in Table 1. To determine the antimicrobial activity of the herbal medicinal remedies, as test cultures we used bacteria from the American Type Culture Collection, USA: Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29223, Enterococcus faecalis ATCC 29212, and yeast Candida albicans ATCC 885-653; clinical strains of bacteria: Staphylococcus aureus, Streptococcus salivarius, Enterobacter sp., Neisseria sp.; yeast Candida albicans, isolated from the oral cavity of periodontitis patients; and bacteria Bacillus subtilis UKM B-5807 and Bacillus licheniformis UKM B-5514, isolated from probiotic Biosporin (Biopharma, Ukraine), and Bacillus clausii from the probiotic Normaflore (Manufacturer: Uniter Laboratory, France; holder of Sanofi-aventis S.p.A., Hungary trade license), as well as the strains Lactobacillus spp. – from probiotic Lactobacterium (Biopharma, France), Positive control were prepared with the same solvents, which were used to prepare the plant Tinctures. The antisepsic drug Decasan (Solution of decamethoxine dihydrochloride 0.02% by weight in water with sodium chloride, Yuria-Pharm Ltd.) also has antimicrobial properties. The agar diffusion method as adapted earlier using glass cylinders (Vorobets & Yavorska, 2016) was used. From the daily culture of microorganisms, a suspension was made in a sterile physiological solution, and every suspension was adjusted to equal 0.5 McFarland standard. Each cup of Muller-Hinton agar was filled with 0.1 ml of a bacterial suspension. The cups were dried at room temperature for an hour. Then on the culture medium with tweezers we carefully arranged sterile glass cylinders, into which 0.1 ml of the substance was contributed. Antimicrobial activity was judged by the presence and size of the growth zone of the studied microorganisms around the cylinder with the extract. To determine the antimicrobial activity of the examined samples, the following scale was used: diameter of the growth retardation zone more than 20 mm – highly sensitive, 10-20 mm – sensitive, up to 10 mm – moderately sensitive. Ethanol of various concentrations was used as solvent control. All tests were performed at least three times.

Results

The antimicrobial activity of the herbal medicinal remedies was tested in vitro (Table 2). Tinctura Calendulae showed antimicrobial effect on E. coli, S. aureus, E. faecalis, and probiotic strains. Moreover, such activity was greater than that of the antisepsic Decasan. However, it showed no or low antifungal activity and effects on bacteria of genus Neisseria and Enterobacter compared to controls. Research has shown...
that Tinctura Eucalypti has high antimicrobial activity against Gram-positive, Gram-negative microorganisms, and probiotic strains. The diameters of zones of growth retardation were the highest for streptococci (up to 30 mm), streptococci (up to 30 mm) and B. clausii (up to 40 mm) and significantly exceeded the effect of Decasan on test culture. The tincture did not reveal a pronounced antifungal effect compared to the control. Tinctura Menthae piperitae produces using 90% ethanol and Chlorophyllipt 96% ethanol, but despite the presence of the extractant revealed high antimicrobial activity of these drugs against Gram-positive microorganisms, so that diameters of growth were in the range of 20 to 40 mm. These indicators significantly exceeded the effect of Decasan on test cultures. The Tinctura of peppermint inhibited the growth of Gram-negative microorganisms too. Phytopreparations of mint and Chlorophyllipt also have a high anti-candidal effect.

Table 2
Antimicrobial activity of plant medicinal drugs to test-bacteria

<table>
<thead>
<tr>
<th>Plant medicinal remedies</th>
<th>C. albicans ATCC 858</th>
<th>S. aureus ATCC 29212</th>
<th>E. faecalis ATCC 29212</th>
<th>E. coli ATCC 2922</th>
<th>C. albicans</th>
<th>S. aureus</th>
<th>Enterobacter sp.</th>
<th>Neisseria sp.</th>
<th>S. salivarius</th>
<th>B. subtilis UKM B-5007, B. licheniformis UKM B-5514 (Biosporin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tinctura Absinthii</td>
<td>18.0 ± 1.6</td>
<td>8.8 ± 2.0</td>
<td>16.5 ± 0.6</td>
<td>10.3 ± 0.3</td>
<td>17.6 ± 0.4</td>
<td>12.8 ± 2.9</td>
<td>13.3 ± 1.0</td>
<td>6.7 ± 1.9</td>
<td>19.6 ± 0.9</td>
<td>0 ± 0.1, 13.1 ± 1.0, 37.6 ± 1.0, 28.7 ± 0.7</td>
</tr>
<tr>
<td>Tinctura Calendulæ</td>
<td>19.5 ± 1.8</td>
<td>16.5 ± 0.5</td>
<td>24.5 ± 0.6</td>
<td>14.7 ± 2.7</td>
<td>15.9 ± 3.3</td>
<td>13.1 ± 1.9</td>
<td>12.5 ± 0.5</td>
<td>6.0 ± 0.4</td>
<td>18.2 ± 0.2</td>
<td>23.5 ± 0.6, 28.6 ± 1.3, 25.0 ± 1.7</td>
</tr>
<tr>
<td>Tinctura Eucalypti</td>
<td>18.0 ± 1.8</td>
<td>29.3 ± 2.4</td>
<td>30.0 ± 0.0</td>
<td>18.0 ± 1.7</td>
<td>20.2 ± 0.4</td>
<td>30.0 ± 1.5</td>
<td>17.3 ± 0.3</td>
<td>21.3 ± 0.3</td>
<td>8.4 ± 1.1</td>
<td>26.0 ± 1.9, 37.6 ± 1.0, 28.7 ± 0.7</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>18.3 ± 1.1</td>
<td>100.0 ± 1.9</td>
<td>0.0 ± 0.0</td>
<td>125.1 ± 1.6</td>
<td>18.9 ± 0.7</td>
<td>94.1 ± 1.4</td>
<td>13.3 ± 0.3</td>
<td>10.0 ± 0.7</td>
<td>18.2 ± 0.2</td>
<td>36.3 ± 10.7, 40.5 ± 17.3, 30.3 ± 0.3</td>
</tr>
<tr>
<td>Rotoconicum</td>
<td>10.3 ± 2.4</td>
<td>128.0 ± 0.6</td>
<td>15.5 ± 0.6</td>
<td>100.0 ± 0.0</td>
<td>118.8 ± 8.5</td>
<td>133.1 ± 0.7</td>
<td>100.0 ± 0.7</td>
<td>14.4 ± 1.9</td>
<td>18.2 ± 1.0</td>
<td>13.7 ± 0.3, 16.3 ± 1.6</td>
</tr>
<tr>
<td>40% ethanol</td>
<td>8.1 ± 1.9</td>
<td>5.1 ± 1.9</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>6.1 ± 1.7</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>6.0 ± 0.3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Tinctura Menthae piperitae</td>
<td>35.7 ± 2.7</td>
<td>258.0 ± 13</td>
<td>290.1 ± 3.3</td>
<td>39.3 ± 0.7</td>
<td>39.5 ± 0.7</td>
<td>25.7 ± 1.3</td>
<td>27.0 ± 0.7</td>
<td>21.0 ± 1.0</td>
<td>36.3 ± 0.7</td>
<td>26.4 ± 4.4</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>24.8 ± 1.1</td>
<td>205.0 ± 11</td>
<td>140.2 ± 2.7</td>
<td>215.1 ± 12.2</td>
<td>21.9 ± 0.9</td>
<td>193.1 ± 1.1</td>
<td>193.2 ± 0.4</td>
<td>16.5 ± 0.7</td>
<td>26.3 ± 2.1</td>
<td>25.0 ± 1.6</td>
</tr>
<tr>
<td>Chlorophyllipt</td>
<td>30.8 ± 2.1</td>
<td>401.0 ± 16.7</td>
<td>375.1 ± 17.7</td>
<td>217.1 ± 10.0</td>
<td>233.3 ± 1.0</td>
<td>348.8 ± 2.5</td>
<td>193.2 ± 1.7</td>
<td>32.0 ± 1.2</td>
<td>35.0 ± 2.7</td>
<td>40.4 ± 12.7</td>
</tr>
<tr>
<td>96% ethanol</td>
<td>26.9 ± 0.9</td>
<td>224.0 ± 8.0</td>
<td>120.2 ± 2.5</td>
<td>233.2 ± 2.4</td>
<td>245.5 ± 1.0</td>
<td>192.0 ± 2.7</td>
<td>23.0 ± 1.7</td>
<td>18.4 ± 0.6</td>
<td>30.5 ± 0.5</td>
<td>60.0 ± 3.3</td>
</tr>
</tbody>
</table>

Note: mathematical reliability * – P < 0.05, ** – P < 0.01, *** – P < 0.001; the reliability of the difference between the plant medical remedies and the corresponding solution of alcohol P: Tinctura Absinthii, Tinctura Calendulæ, Tinctura Eucalypti – 70% ethanol; Rotoconicum – 40% ethanol; Tinctura Menthae piperitae – 90% ethanol; Chlorophyllipt – 96% ethanol.

Sensitive to the Tinctura Absinthii were S. salivarius (19.6 ± 0.9 mm), E. faecalis (16.5 ± 0.6 mm), and B. clausii (21.3 ± 1.3 mm) from the probiotic Normaflore, as well as C. albicans, S. aureus and Lactobacillus sp., but ethanol also had an effect on these microorganisms. Tinctura Absinthii did not affect the growth of Gram-negative microorganisms, and species of probiotic Biosporin. The drug Rotoconicum, which contains in its composition extracts of chamomile, calendula and yarrow (Calendula officinalis) inhibited bacterial growth and survival, influenced cell morphology and enhanced the autolysis of Gram-positive bacteria, suggesting that bacterial envelopes are the target of its activity. Essential oil from Calendula officinalis was effective against 23 clinical fungi strains tested (Gazim et al., 2008). Other experimental data obtained showed that methanol extract of C. officinalis petals exhibited better antibacterial activity than ethanol extract. However, both extracts showed high antifungal activity in comparison with fluconazole (Elaitriou et al., 2012). In our research Tinctura Calendulæ has shown no or low antifungal activity in comparison with control.

Among the studied herbal medicinal products, the strongest antimicrobial activity was found in Tinctura Eucalypti, Tinctura Menthae piperitae and Chlorophyllipt. Eucalyptus oil, which is known for its antibacterial, antiviral (Cermelli et al., 2007; Astani et al., 2009) and antifungal (Ashour, 2008) properties, and has a long history of use for the treatment of colds, flu, rhinitis, sinusitis and other respiratory tract diseases. The obtained results have shown that essential oils of the leaves of E. globulus have antimicrobial activity against Gram-negative bacteria (E. colii) as well as Gram-positive bacteria (S. aureus) (Bachir & Benali, 2012). The effectiveness of eucalyptus oils on caries and periodontitis pathogens was also investigated. In particular, essential oils of Eucalyptus calandulae showed antibacterial activity against Streptococcus mutans and significantly retard its biofilm formation (Rasooli et al., 2009). Takarada et al. (2004) earlier showed that eucalyptus oil inhibited the growth of the following oral bacteria: Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Streptococcus mutans, Streptococcus sanguinis and also inhibited the adhesion of S. mutans (Takarada et al., 2004). The antifungal effect of eucalyptus essential oils has been investigated. Ashour et al. (2008) showed that essential oils of E. sideroxylon and E. torquata generally exhibited moderate to high antifungal activities against Candida albicans, A. flavus and A. niger (Ashour et al., 2008). Agarwal et al. (2008) inves-

tigated the ability of eucalyptus essential oil to suppress the formation of *C. albicans* biofilm. Takashahi et al. (2004) reported that extracts of *Eucalyptus globulus, E. maculata* and *E. viminalis* significantly inhibited the growth of six Gram-positive bacteria (*Staphylococcus aureus, MRSA, Bacillus cereus, Enterococcus faecalis, Alcylcocillus acidaederres*, *Propionibacterium acnes*), and of a fungus (*Trichophyton mentagrophytes*), but they did not show strong antibacterial activity against Gram-negative bacteria (*Escherichia coli, Pseudomonas putida*) (Takahashi et al., 2004). In our findings, Gram-negative bacteria showed lower sensitivity to *Tinctura Eucalypti* than Gram-positive bacteria.

Our results showed that the Tinctura of peppermint inhibited the growth of Gram-positive, Gram-negative microorganisms and had a high anti-candidal effect. Caretto et al. (2010) also showed antimicrobial activity of hydroalcoholic extract of *Mentha piperita L.* against *Candida* spp. (*C. albicans, C. tropicalis* and *C. glabrata*). Shahayal et al. (2017) showed the potential antibacterial activity for *Mentha piperita* extracts against *MDR S. pyogenes, E. faecalis, MRSA, MRSE* and carbepe	

nem-resistant *E. coli*, and *Klebsiella pneumonia* clinical isolates. Sujana et al. (2013) earlier demonstrated that the organic extracts of the leaves of *Mentha piperita L.* possessed strong antibacterial activity against a range of pathogenic bacteria, such as *Bacillus subtilis*, *Strep
tococcus pneumonia*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Klebsiella pneumonia* (Sujana et al., 2013). The mint leaf methanolic extract showed considerable antimicrobial activity against human oral pathogens, such as: *Escherichia coli, Actinetobacter sp., Staphylococcus aureus* and two fungi such as *Candida albicans, C. glabrata* (Pratmila et al., 2012). Miloš Nikolić et al. reported antimicrobial activity of essential oil of peppermint *Mentha piperita* against pathogenic microorganisms isolated from the oral cavity (8 bacteria and 58 Candida spp.) and referent strains (Nikolić et al., 2013).

According to usage instruction, Chlorophyllipt inhibits staphylococci infections that are resistant to antibiotics and is used in washing, rinsing, lotion, wet tampons and douching, and in dental stomatitis to treat gangrenous pulps, abscesses, boils, inflammation of the oral mucosa. Our results suggested its antibiotic activity not only against collection and clinical strains of Gram-positive and Gram-negative bacteria, but also against investigated probiotic strains. By investigation of the antagonistic activity of probiotics it was established that bacteria, being the background of the biopreparations, have demonstrated different levels of suppression effect on various strains of test-cultures.

Conclusions

It was confirmed that each of the proposed health benefits should be studied separately for each probiotic bacterial strain, especially if the treatment protocol stipulates the use of drugs with herbal remedies. At the same time, all probiotic strains proved to be sensitive to the action of plant extracts, indicating the possibility of consecutive use of medicinal products according to their activity on the microbiome and antibacterial potential of methanolic leaf extract of peppermint (*Mentha piperita L.*). The mint leaf methanolic extract showed considerable antimicrobial activity against human oral pathogens, such as: *Escherichia coli, Actinetobacter sp., Staphylococcus aureus* and two fungi such as *Candida albicans, C. glabrata* (Pratmila et al., 2012).


