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ANTIBIOFILM-FORMING AND ANTIMICROBIAL ACTIVITY OF EXTRACTS OF *Arnica montana* L., *Achillea millefolium* L. ON *Staphylococcus* GENUS BACTERIA

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The purpose of this work was to study the antimicrobial, antibiofilm-forming and antioxidant properties of alcohol extracts of *Arnica montana* L. and *Achillea millefolium* L. The plants for the analysis were gathered in the territory of Velyky Berezny region, Transcarpathia. Ethyl and methyl extracts were produced from inflorescences of *Arnica montana* L. and *Achillea millefolium* L. For the purpose of analysis, we used Staphylococcus genus bacteria that had been isolated from the mouth cavities and pharynx of human patients suffering from inflammatory diseases, by plating into differentially diagnostic nutrient media with subsequent identification. All isolates were characterized to be biofilm-forming.

The subject of the study was extract's antimicrobial activity evaluated by the diffusion-into-agar method in standard 96-well plates using the spectrophotometric method and by measuring of antioxidant activity (DPPH test).

It was established that *Arnica montana* L. extracts exerted more pronounced antimicrobial activity upon the analysed isolates of *Staphylococcus* genus bacteria. It was furthermore shown that *Arnica montana* L. extracts displayed an antimicrobial effect even upon MRSA of *S. aureus*. Extracts of *Arnica montana* L. and *Achillea millefolium* L. were shown to possess anti-biofilm forming properties.

Ethyl and methyl extracts of *Arnica montana* L. and *Achillea millefolium* L. were shown to reveal significant antioxidant activity.

Thus, our results indicated a need in further research of possible application of *Arnica montana* L. and *Achillea millefolium* L. extracts as anti-staphylococcal agents, which could be employed for the treatment of inflammatory processes in mouth cavity and oropharynx.

Key words: antimicrobial effect, antibiofilm formation, plant extracts.

The growing problem of microorganisms' resistance formation to antibiotics determines the importance of searching for alternative antimicrobial agents [1, 2]. Plant-based materials look especially promising in this respect, for they have traditionally been used in folk medicine, they have a number of advantages, including antioxidant activity, and a wide spectrum of biologically active substances, micro- and macronutrient elements. In view of the possibility of the most opportunistic strains to form biofilm, which complicates the course of pathological processes and favours continuous microorganisms' persistence in the host organism, the study of antibiofilm-forming properties of medicinal plants is an extremely topical task. In particular, plants of the *Asteraceae* genus are widely used by folk and conventional medicine and have good prospects from the viewpoint of antimicrobial activity.

Mountain arnica (*Arnica montana* L.), a perennial herbaceous plant of the *Compositae* genus, has been used by folk medicine since ancient times. In natural habitats, it is spread in Central and South Europe and West Ukraine [3]. As medical material, inflorescences, which contain such groups of biologically active substances as carotenoids, terpenoids, essential oils, polyacetylenes, phenolcarbonic acids,

nitrogen-bearing compounds, coumarins, flavonoids, and others, are used. According to literature data, preparations from arnica flowers demonstrate tonic, anti-inflammatory, sedative, and choleretic activity. They have haemostatic effect at external and internal haemorrhages. They are used to treat heart diseases, bronchitis, spasms, neuralgic pains, and gout [4]. Their extracts are known to demonstrate antibacterial, antitumor, antioxidant, anti-inflammatory, antifungal and immune-modulating activity [3].

Common yarrow (*Achillea millefolium* L.) is another well-known medicinal herb of the *Asteraceae* genus, which has long been used in conventional and folk medicine. As medical material, its flower heads are used. Its extracts and infusions are characterized by anti-inflammatory, wound healing, and antiallergic activities. Together with other herbs as part of a set, yarrow is used for gastritis, stomatitis, and stomach ulcer treatment. A wide spectrum of its biological properties is explained by a variety of its chemical composition, in particular, secondary metabolites — isoprenoids, flavonoids, and vitamins [5].

Thus, in view of the known anti-inflammatory properties of *Arnica montana* L. and *Achillea millefolium* L. and their traditional application in medicine, it is relevant to study the impact of their inflorescence extracts upon *Staphylococcus* genus bacteria characterized by biofilm-forming ability and multiple resistance to antibiotics.

The purpose of our work was to study antimicrobial, antibiofilm-forming and antioxidant properties of ethyl and methyl extracts of *Arnica montana* L. and *Achillea millefolium* L.

Materials and Methods

The plant materials were collected in the territory of Velyky Berezny region, Transcarpathia, dried at the temperature of 30–35 °C in the shadow, then grounded and placed in tightly closed containers.

Extract manufacturing techniques. Inflorescences of *Arnica montana* L., *Achillea millefolium* L. were used as work material to obtain ethyl and methyl extracts. A 10 g batch of dry plant material was pulverized to powdery mass. In an Erlenmeyer flask, 10 g of plant material were blended with 200 ml of 96° ethyl or methyl alcohol (Sigma, Germany). The flask neck was closed with a food wrap to avoid evaporation. Following a 30 min incubation in the ultrasonic bath (Kraintek) at 35 °C, the blend was filtered through Whatman No. 1 filter paper. The clear solution was placed in an evaporative device (16—17/32″× 34–59/64″ G5B, Coated Dry Ice Condenser Rotary Evaporator) to obtain pure alcoholic extract at 50 °C, 82 rpm. Then, extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove either ethyl or methyl. As a result, the following pure extracts were obtained.

Antimicrobial assay

Antimicrobial activity of plant extracts was determined using agar diffusion test (Rhos and Reico, 2005). The bacterium inocula 100 µl in the physiological solution were adjusted to the equivalent of 0.5 McFarland standard, and evenly spread on the surface of Muller-Hinton agar (incubated at 37 ± 2 °C for 24 hours); yeasts — on SDA agar (incubated at 35 ± 2 °C for 48 hours). The extracts 10 µl were introduced into wells 6 mm in diameter. The diameters of the inhibition zones were measured in millimetres including the diameter of the well. Each antimicrobial assay was performed at least three times.

As test cultures, the following reference bacterial strains were used: *Staphylococcus aureus* ATCC 25923, *S. aureus* CCM 4223 biofilm-forming strain. We also used clinical strains of bacteria (*S. aureus* MRSA, *S. haemolyticus, S. epidermidis, S. hominis*) isolated from the oral cavities of patients suffering from inflammatory periodontiumand pharynx. We chose the clinical strains with multiple resistance at least to two classes of antibiotics. As a positive control gentamicin (10 mg/disk) for Gram-negative bacteria, ampicilin (10 mg/disk) for Gram-positive bacteria, nystatin (100 UI) for *Candida* were used. As a negative control, DMSO was used.

Determination of antibiofilm activity

The antibiofilm activity of the plant extracts were tested in the standard 96-well microtitration plates (Greiner-BioOne, Austria) using a modified staining method according to O' Toole (O'Toole, 2011).

With the purpose to study the antibiofilm-forming activity, a 18-hour culture of the reference *S. aureus* CCM 4223 and clinic biofilm-forming strains grown at 37 °C were used. Into the wells, 180 µl of bacterial suspension, McFarland in broth (TSB, Himedia, India) were introduced. The *Achillea millefolium* L., *Arnica montana* L. extracts were adjusted to the concentrations of 1%, 5% and 10% in dimetylsulfoxide (DMSO; Sigma-Aldrich, USA) and introduced into the wells in a volume

of 20 µl per well. Following the addition of the bacterial suspension, the concentrations of plant extracts in the broth were equal to 0.1%, 0.05% and 0.01%, respectively. The wells with only 180 µl of broth and 20 µl of 10% DMSO served as a control.

Following a 24-hour-long incubation in the thermostat at 37 °C, the supernatant was withdrawn and washed 3 to 5 times with distilled water. Following a 30-minute-long incubation, it was dyed with 200 µl of 0.1% solution of crystal violet. Then the dye was withdrawn, and the supernatant washed 3 to 5 times with distilled water. Into every well, 200 µl of 30% acetic acid were added and incubated for 10 minutes. Optical density was measured on the Synergy HT (Biotek, USA) spectrophotometer at 550 nm. Over 50% reduction in absorbance was considered as significant inhibition (Raut et al., 2014). Statistical Analysis Values mentioned are the mean with standard deviations, obtained from three different observations. Values in the control and treatment groups for various molecules were compared using Student's *t*-test. A value of $P < 0.05$ was considered as statistically significant.

Antioxidant activity

Free radical scavenging activity of the samples was measured with 2.2-diphenyl-1-picrylhydrazyl (DPPH) [14]. The antioxidant activity was expressed as percentage (%) of the scavenging activity. Trolox was used as a standard.

Statistical analysis

For the statistical analysis of experimental data, we used statistical software Microsoft Office Excel (2013). The results were calculated and expressed as mean, standard error of mean, or standard deviation.

Results and Discussion

Our studies showed that extract of *Arnica montana* L. had more pronounced antimicrobial effect against typical and clinical bacterial strains of the *Staphylococcus* genus. It is worth noting that arnica extract demonstrated antimicrobial action even against clinical strains of *S. aureus* MRSA characterized by multiple resistance to antibiotics. At the same time, the activities of ethyl and methyl extracts were almost identical.

A significantly lower antimicrobial effect was ascertained for *Achillea millefolium* L., where the inhibition zones never exceeded 10.65 ± 0.15 mm.

Investigation of antibiofilm-forming ability of ethyl extract of *Arnica montana* L. revealed that 0.1% extract lowered the process of biofilm formation by 76%. However the antibiofilm-forming activity of the extract was reducing with lowering of its concentration (Fig. 1).

The study of antibiofilm-forming activity showed that the extracts inhibited the intensity of bacterial biofilm formation (Fig. 2).

Methyl extract of *Arnica montana* L. reduced biofilm formation intensity by 42.3% in the case of an 0.1% concentration, and by 36% — in the case of 0.05% solution. However, introduction of 0.01% had no effects on the process of biofilm-formation.

Thus, we have shown a significant antibiofilm-forming effect of ethyl and methyl extracts of *Arnica montana* L.

High antibiofilm-forming activity of

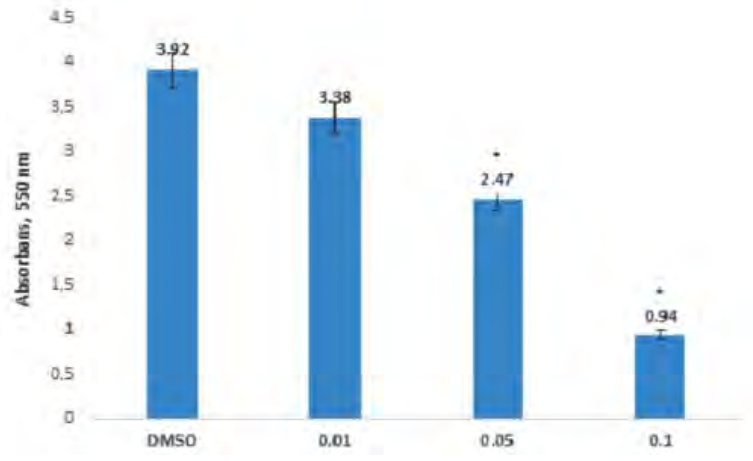

Fig. 1. **Antibiofilm activity of different concentrations of ethyl extract *Arnica montana* L. on *S. aureus* biofilm-forming**

*the data were statistically significant as compared with the control ($P < 0.05$)

Table 1. **Antimicrobial activity of *Arnica montana* L. and *Achillea millefolium* L. extracts, inhibition zones in millimeters, including well diameter, mm ($n = 3$, $x \pm SD$)**

Test culture	*Arnica montana* L.		*Achillea millefolium* L.	
	ethyl extract	methyl extract	ethyl extract	methyl extract
S. aureus ATCC 25923	20.20 ± 0.30*	19.30 ± 0.30*	10.33 ± 0.58*	10.67 ± 0.58*
S. aureus CCM 4223 (biofilm formation)	20.50 ± 0.50*	20.20 ± 0.30*	9.33 ± 0.58*	9.83 ± 0.29*
S. aureus MRSA (clinic biofilm formation)	18.60 ± 0.58*	19.50 ± 0.50*	9.50 ± 0.50*	10.33 ± 0.29*
S. haemolyticus (biofilm formation)	20.10 ± 0.15*	20.15 ± 0.50*	10.50 ± 0.30*	10.65 ± 0.15*
S. epidermidis (biofilm formation)	21.50 ± 0.25*	21.60 ± 0.30*	10.70 ± 0.25*	10.55 ± 0.20*
S. hominis (biofilm formation)	21.70 ± 0.50*	20.90 ± 0.15*	11.50 ± 0.15*	11.00 ± 0.10*

Note. An extraction solvent (ethanol or methanol) was used as a control:
control of ethanol — no inhibition; control of methanol — no inhibition; diameter of well 6 mm;
* — the data were statistically significant as compared with the control ($P < 0.05$).

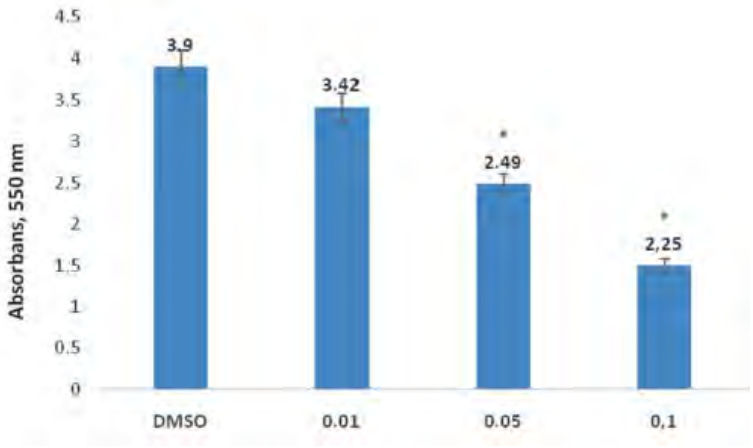

Fig. 2. **Antibiofilm activity of different concentrations of methyl extract *Arnica montana* L. on biofilm-forming *S. aureus***
*the data were statistically significant as compared with the control ($P < 0.05$)

ethyl extract of *Achillea millefolium* was explored (Fig. 3, Fig. 4): e. g., introduction of 0.1% extract solution lowered the process of biofilm formation by 92%. As the extract concentration reduced, the antibiofilm-forming activity was also declined gradually.

A similar trend was observed for methyl extract, though its activity was somewhat lower. For example, 0.1% extract solution reduced the intensity of biofilm formation by 80% (Table 2).

Thus, we have demonstrated high antibiofilm-forming activity of *Achillea millefolium*. It is noteworthy that ethyl extracts showed higher activity than methyl extracts.

The evaluation of antioxidant activity of extracts medicinal herbs may explain their high activity (Table 3).

Other authors also pointed to the antimicrobial properties of arnica against mouth cavity pathogens. For instance, effectiveness of extracts of *Arnica montana* L. against *Streptococcus sobrinus* isolated from the mouth cavity was shown [8, 9]. Essential oil of *Achillea millefolium* L. was established to demonstrate antimycous action against fungi provoking plant diseases [10].

The antimicrobial activity of yarrow has

Table 2. **Biofilm destruction for *S. aureus* clinical strains affected by ethyl / methyl extracts of medicinal herbs**

Plant	Extract concentrations,%		
	0.01	0.05	0.1
ethyl extract			
Achillea millefolium L.	22.00 ± 0.58	52.69 ± 0.72	92.00 ± 0.65
Arnica montana L.	13.70 ± 0.23	36.90 ± 0.55	76.00 ± 0.35
methyl extract			
Achillea millefolium L.	60.00 ± 0.45	76.00 ± 0.50	80.42 ± 0.64
Arnica montana L.	12.30 ± 0.33	36.00 ± 0.55	42.40 ± 0.50

Note: * — the data were statistically significant as compared with the control ($P < 0.05$).

Table 3. **Antioxidant activities of ethyl and methyl extracts of *Arnica montana* L. and *Achillea millefolium* L.**

Plant	%
ethyl extract	
Achillea millefolium L.	85.40 ± 0.50
Arnica montana L.	82.36 ± 0.30
methyl extract	
Achillea millefolium L.	86.62 ± 0.55
Arnica montana L.	79.52 ± 0.60

Note: * — the data were statistically significant as compared with the control ($P < 0.05$).

been earlier described [11], where its antimicrobial effect upon *E. faecalis*, *P. aeruginosa*, *S. aureus*, and *M. luteus* is shown. Besides, the antimicrobial activity of various species of *Achillea* genus versus *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. enteritidis* and two fungi (*Aspergillus niger* and *Candida albicans*) has also been found [12].

The past study [13] has also proved the activity of extracts of medicinal herbs (*Althaea officinalis* L. roots, *Arnica montana* L. flowers, *Calendula officinalis* L. flowers, *Hamamelis virginiana* L. leaves, *Illicium verum* Hook. fruits and *Melissa officinalis* L. leaves) against anaerobic and facultative aerobic periodontal bacteria: *Porphyromonas gingivalis*, *Prevotella* spp., *Fusobacterium nucleatum*, *Capnocytophaga gingivalis*, *Veilonella parvula*, *Eikenella corrodens*, *Peptostreptococcus micros* and *Actinomyces odontolyticus*. In our previous works, we also showed the antimicrobial activity of *Potentilla erecta* L. rhizome and cowberry extract to affect clinical microorganism isolates from oral cavity [14, 15].

Studies of the action mechanisms of plant extracts upon microorganisms are of sporadic character. At the same time, plant extracts are known to contain mixtures of biologic substances having additive effect and varying by their action mechanisms, which factor ensures their bactericidal activity and low probability of development of resistance in microorganisms.

According to the literature data, the antibacterial effect is predominantly provided by polyphenolic substances. At the same time, a correlation dependence has been shown between the antimicrobial and antioxidant activity of plant extracts. The study of kinetics of the death of bacterial cells affected by extracts of medicinal plants proves that the antimicrobial effect is a consequence of the effect upon various physiological processes inside the cell. Currently we are aware of such action mechanism of plant extracts as increase of permeability of the cell membrane, its destruction, and destruction processes in mitochondrial membranes [16].

Experimental works have proved the antimicrobial activity of tannins. A. Scalbert [17] established the antimicrobial activities of tannins upon microorganisms. The

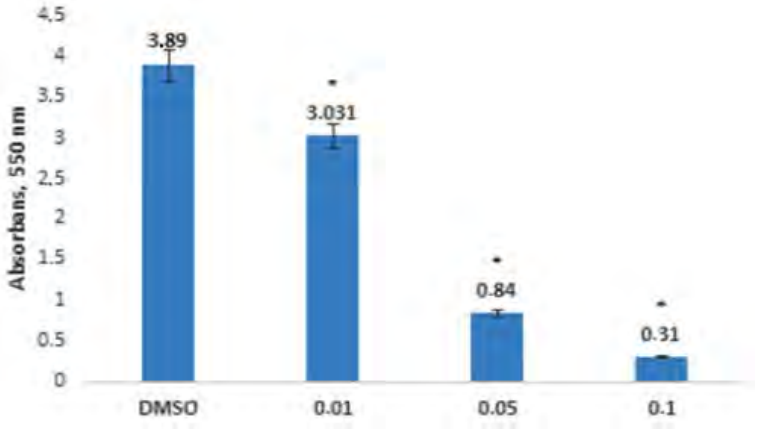

Fig. 3. **Antibiofilm activity of different concentrations of ethyl extract *Achillea millefolium* L. on biofilm-forming *S. aureus***
*the data were statistically significant as compared with the control ($P < 0.05$)

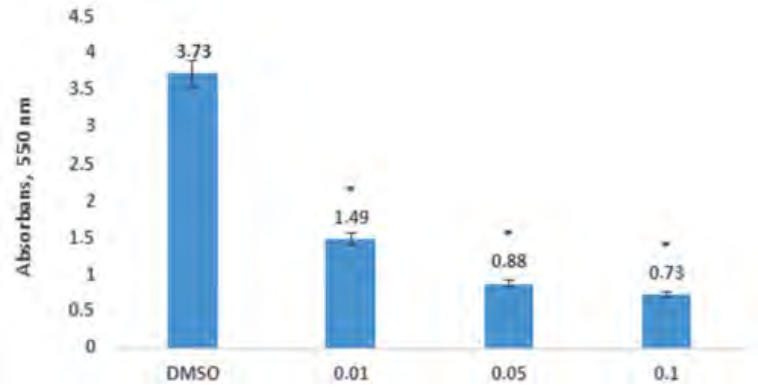

Fig. 4. **Antibiofilm activity of different concentrations of methyl extract *Achillea millefolium* L. on biofilm-forming *S. aureus***
*the data were statistically significant as compared with the control ($P < 0.05$)

antimicrobial activity and toxicity were shown to depend upon the tannin structure. Different mechanisms of explanation of tannin's antimicrobial activity, including inhibition of extracellular microbial enzymes, direct action upon the microbial metabolism via inhibition of oxide phosphorylation, and the mechanism connected with iron fixation have been suggested. Many microorganisms can overcome the plants' defence mechanisms by synthesising the tannin-fixing polymers, oxidation, and tannin biodegradation.

Plant extracts were shown to be able to affect the bacterial quorum sensing QS in biofilm, which was one of its destruction mechanisms. In that case, the destructibility of biofilm does not always correlate with the antimicrobial activity [17].

Another important factor is the choice of optimum extractive agent to ensure the extraction of maximum quantity of biologically active substances. So the works connected with the comparative characteristics of extracts based on various extractive agents are important from the viewpoint of choosing the right ones which will ensure the optimum antimicrobial effect.

The revealed impact of extracts upon biofilm-forming microorganism strains isolated from the mouth cavities of human patients suffering from inflammatory parodentium diseases points to the prospects of their use for oral hygiene products. The antibiofilm-forming activities of extracts seem to be especially promising for prevention of inflammatory parodentium diseases, while the most of the mouth cavity microorganisms are biofilm-forming bacteria. High antioxidant properties of extracts, combined with their antibiofilm-forming and antimicrobial activities, are extremely valuable for the treatment of inflammatory processes of mucous membrane.

Thus, we have established remarkable antimicrobial effect of *Arnica montana* L. against biofilm-forming strains of *Staphylococcus* genus bacteria. We have further shown a high antibiofilm-forming ability of extracts of *Arnica montana* L. and *Achillea millefolium* L. It is noteworthy that extract of *Achillea millefolium* L. showed somewhat higher antibiofilm-forming activity. The revealed trends explain the prospects for the use of herbal extracts to lower the persistence level of *Staphylococcus* genus bacteria, which fact is especially important for inflammatory processes of the mouth cavity.

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АНТИБІОПЛІВКОУТВОРЮВАЛЬНІ ТА АНТИМІКРОБНІ ВЛАСТИВОСТІ ЕКСТРАКТІВ *Arnica montana* L., *Achillea millefolium* L. ЗА ЇХНЬОЇ ДІЇ НА БАКТЕРІЇ РОДУ *Staphylococcus*

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Метою роботи було дослідити антимікробні, антибіоплівкоутворювальні та антиоксидантні властивості спиртових екстрактів суцвіть *Arnica montana* L. та *Achillea millefolium* L. Рослини для досліджень було зібрано у Великоберезнянському районі Закарпатської області. Із суцвіть *Arnica montana* L. та *Achillea millefolium* L. було виготовлено етанольні та метанольні екстракти. У досліді використано бактерії роду *Staphylococcus*, ізольовані з ротової порожнини та зіву людей із запальними захворюваннями, шляхом висіву їх на диференційно діагностичні живильні середовища з подальшою ідентифікацією. Встановлено, що всі ізоляти були біоплівкоутворювальними.

Згідно з метою дослідження вивчали антимікробну активність екстрактів (методом дифузії в агар), антибіоплівкоутворювальний ефект (у стандартних 96-лункових планшетах спектрофотометричним методом), антиоксидантну активність (DPPH методом).

Встановлено більш виражену антимікробну активність екстрактів *Arnica montana* L. на досліджувані ізоляти бактерій роду *Staphylococcus*. Показано, що екстракти *Arnica montana* L. мали антимікробний ефект навіть на MRSA *S. aureus*. Доведено антибіоплівкоутворювальні властивості екстрактів *Arnica montana* L. і *Achillea millefolium* L.

Етанольні та метанольні екстракти *Arnica montana* L. і *Achillea millefolium* L. показали високу антиоксидантну активність.

Таким чином, отримані нами результати вказують на необхідність подальших досліджень використання екстрактів *Arnica montana* L. і *Achillea millefolium* L. як протистафілококових засобів при запальних процесах ротової порожнини та ротоглотки.

Ключові слова: антимікробний ефект, антибіоплівкоутворювальна активність, рослинні екстракти.

АНТИБИОПЛЕНКООБРАЗУЮЩИЕ И АНТИМИКРОБНІЕ СВОЙСТВА ЭКСТРАКТОВ *Arnica montana* L., *Achillea millefolium* L. ПРИ ИХ ДЕЙСТВИИ НА БАКТЕРИИ РОДА *Staphylococcus*

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Целью работы было исследовать антимикробные, антибиопленкообразующие и антиоксидантные свойства спиртовых экстрактов соцветий *Arnica montana* L. и *Achillea millefolium* L. Растения для исследований были собраны в Великоберезнянском районе Закарпатской области. Из соцветий *Arnica montana* L. и *Achillea millefolium* L. были изготовлены этиловые и метиловые экстракты. В опыте использованы бактерии рода *Staphylococcus*, изолированные из ротовой полости и зева людей с воспалительными заболеваниями, путем посева на дифференциально диагностические питательные среды с последующей идентификацией. Установлено, что все изоляты были биопленкообразующими.

Согласно цели исследования изучали антимикробную активность экстрактов (методом диффузии в агар), антибиопленкообразующий эффект (в стандартных 96-луночных планшетах спектрофотометрическим методом), антиоксидантную активность (DPPH методом).

Установлена более выраженная антимикробная активность экстрактов *Arnica montana* L. на исследуемые изоляты бактерий рода *Staphylococcus*. Показано, что экстракты *Arnica montana* L. проявляли антимикробный эффект даже на MRSA *S. aureus*. Установлены антибиопленкообразующие свойства экстрактов *Arnica montana* L. и *Achillea millefolium* L. Этиловые и метиловые экстракты *Arnica montana* L. и *Achillea millefolium* L. показали высокую антиоксидантную активность.

Таким образом, установленные результаты указывают на возможность дальнейших исследований использования экстрактов *Arnica montana* L. и *Achillea millefolium* L. как противостафилококковых средств при воспалительных процессах полости рта и ротоглотки.

Ключевые слова: антимикробный эффект, антибиопленкообразующая активность, растительные экстракты.