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EXTRACTS OF EDIBLE PLANTS STIMULATORS FOR BENEFICIAL MICROORGANISMS

O. V. Pallah[1, 2]
T. V. Meleshko[1, 2]
V. V. Bati[2]
N. V. Boyko[1, 2]

[1]Uzhhorod National University, Department of Clinical and Laboratory Diagnostics and Pharmacology, Faculty of Dentistry, Ukraine
[2]Uzhhorod National University, Research Development and Educational Centre of Molecular Microbiology and Mucosal Immunology, Ukraine

E-mail: ssarvash@gmail.com

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The aim of the work is to determine the content of biologically active compounds in berries and fruits collected in ecologically clean areas, to find out their potential usage for the creation of targeted pharmabiotics, and the ability of such extracts to stimulate the growth of probiotic lactic acid bacterial strains and other representatives of commensal intestinal microbiota.

The content of biologically active compounds was determined by thin-layer chromatography. The plant-specific effect of methanol extracts from berries and fruits on the selected strains *L. acidophilus, L. catenaformis, L. casei, L. fermentum, E. coli* 058, *E. faecalis* (gut commensals), *B. subtilis* 090 (component of biopreparation), which are perspective for the creation of modern pharmabiotics, according to the results of its cultivation in the specified extracts had been shown.

It was also found that tested berry extracts were characterized by a higher content of polyphenols, compared to anthocyanins. Alycha' extract mainly inhibited the growth of most of studied bacterial strains, except *B. subtilis* 090. Extracts of red currant, sweet cherry, and jostaberry stimulated the growth of *L. catenaformis*, while extracts of sweet cherry and jostaberry, in addition to that of the above-mentioned lactobacilli strains, also stimulated the growth of *L. casei* and *L. fermentum*. Blueberry and plum extracts activated the growth of all lactobacilli strains. The ability to stimulate the growth of *B. subtilis* 090 was noted only for the extracts of alycha, jostaberry, and plum.

***Key words*:** berries, fruits, flavanoids, anthocyanins, lactobacteria, pharmabiotics.

It is known that peptides, enzymes, and amino acids are used as separate medications and as prebiotic components in the composition of synbiotics [1]. Today the most frequently used plant extracts are characterized by various preventive and medicinal effects [2]. The objective of our paper was the screening (selection) of berries grown in the mountainous regions of Zakarpattia, which would be characterized by high biologically active compounds (BAC) contents and the ability to stimulate the growth of lactic bacteria strains of different origin, and thus could be perspective components for modern pharmabiotics.

Natural biologically active compounds of antimicrobial action include plant antibiotics, phytoncides, essential oils, balsams, resins, tannins, organic acids, alkaloids, and glycosides [3]. Usually, the BAC content varies depending on the plant species and the region of cultivation [4].

Therefore, our main task was to select berries and fruits, the extracts of which were rich in BAC and had antibacterial properties against opportunistic pathogens, as was shown earlier [5, 6], and at the same time stimulated the growth of beneficial microorganisms, which was the objective of this paper.

Agroclimatic conditions of Zakarpattia Region are favorable for the cultivation of berries rich in BAC. Gardening is well-developed in the region, which, in turn, provides the opportunity to obtain a large number of raw materials for BAC extraction, namely from alycha, cherry, jostaberry, red and black currant. Fruits of these plants are sources of antioxidants, including anthocyanins and polyphenols, which possess antibacterial properties [7].

The quantitative composition of the studied berries is underinvestigated. Determination of the total content of polyphenolic compounds in plant raw material allows pre-evaluating the prospects of their use as a source of BAC for future use in the production of pharmabiotics.

So, the objective of our study was to determine the BAC content in the berries and fruits selected for the study and to find out their potential ability to stimulate the growth of selected microorganisms.

Materials and Methods

Methanol extraction

We determined the quantitative content of polyphenols and anthocyanins in methanol extracts by thin-layer chromatography (TLCH) [8].

We prepared the extract from 50 g of each kind of berries and used 100 ml of 80% methanol for extraction. In our subsequent experiments, we used the methanol liquid extract, not containing precipitate [9].

Determination of antibacterial properties of fruit extracts

Using vacuum evaporation, we obtained methanol-free extracts of the following berries: *Ribes rubrum* (red currant), *Prunus avium* (sweet cherry), *Prunus × domestica* (plum), *Ribes × nidigrolaria* (jostaberry), *Vaccinium myrtillus* (blueberry), *Ribes nigrum* (black currant), and *Prunus cerasifera* (alycha).

We studied the ability of the selected berries and fruits to stimulate *L. acidophilus, L. catenaformis, L. casei, L. fermentum* (LAB), *E. coli 058, E. faecalis B. subtilis 090* by culturing them in extracts obtained from these berries and fruits [10]. The initial concentration of the selected LAB strains, *E. coli 058, E. faecalis B. subtilis 090* was 3×10^8 CFU/ml (N0). The obtained data was expressed as the logarithm of the number of surviving bacteria (Nt) to the initial number of bacteria (N0) – lg (Nt/N0) for a certain culturing time (14 h and 24 h).

LAB strains used in the study were isolated by us from various sources, in particular, *L. acidophilus* — from the intestine of a healthy person, *L. catenaformis, L. casei, L. fermentum* — from fermented food [11]; they were identified (MALDI with subsequent sequence analysis) and registered with the Depositary of High-Value Cultures of the Microorganism Collection of IMVNASU as promising strains for the development of modern pharmabiotics. Additionally, the effects of berry and fruit extracts on the representatives of the commensal intestinal microbiota were studied: *E. coli 058*, *E. faecalis*, and *B. subtilis 090*– an integral component of the biological preparation [12].

Statistical processing of the results of experiments was carried out using a software OriginLab 2017 version 94E.

Results and Discussion

Alycha is a relatively unpretentious, frost- and drought-resistant plant [13]. Polyphenols play an important role among the BAC that the alycha fruits are rich in. Plant phenols represent a large group of substances classified as “secondary metabolites”, being of particular interest due to their antioxidant properties. Phenolic compounds are also characterized as antimicrobial, antitumor, and antiviral agents [14, 15]. Jostaberry is also a source of biologically active substances, but to date, the content of BAC in this fruit is almost unstudied. This is also applicable to the unknown content of BAC in the fruits of sweet cherry and currant, but the availability of such data is extremely relevant.

When choosing berries and fruits, we also considered their availability on the market, use in the food industry, and their well-known ability to suppress opportunistic pathogens.

The second stage of the study was obtaining methanolic extracts of alycha, plum, red and black currants, blueberry, sweet cherry, and jostaberry, followed by distillation of the solvent, and a quantitative determination of BAC content by means of thin-layer chromatography. In liquid extracts of berries, the content of biologically active substances was determined, that belonged to the class of anthocyanins and polyphenols, and their quantitative content in each investigated extract was measured.

Black currant and jostaberry fruits were characterized by the same qualitative composition of BAC (delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-rutinoside), but different quantitative content of anthocyanidins and polyphenols. The total content of polyphenols in the fruit of black currant was half as much as the total content of polyphenols in jostaberry fruit. These extracts stimulated the growth of *L. casei*, and jostaberry extract additionally stimulated the growth of *L. fermentum* and *L. catenaformis* strains (Table 2).

The black currant extract (*Ribes nigrum*) contained the following biologically active substances: delphinidin-3-glucoside (20.4%), delphinidin-3-rutinoside (45%), cyanidin-3-

Table 1. **The results of anthocyanins content**

Serial Number	Isolated BAC	Tested Extracts					
		Ribes rubrum	*Prunus avium*	*Prunus domestica*	*Ribes × nidigrolaria*	*Vaccinium myrtillus*	*Ribes nigrum*
1	Cyanidin-3-monoboside, μg/ml	26.8	–	–	–	–	–
2	Cyanidin-3-xylosylrutinoside, μg/ml	118.5	–	–	–	–	–
3	Cyanidin-3-rutinoside, μg/ml	33.6	78.0	9.4	310.1	–	846.2
4	Cyanidin-3-glucoside, μg/ml	–	0.58	1.63	106.5	529.9	165.0
5	Cyanidin-3-arabinoside, μg/ml	–	–	–	–	298.5	–
6	Cyaniding-3-galactoside, μg/ml	–	–	–	–	429.5	–
7	Delphinidin-3-glucoside, μg/ml	–	–	–	56.9	697.8	590.1
8	Delphinidin-3-rutinoside, μg/ml	–	–	–	86.5	–	1285.1
9	Delphinidin-3-galactoside, μg/ml	–	–	–	–	651.8	–
10	Delphinidin-3-arabinoside, μg/ml	–	–	–	–	446.9	–
11	Petunidin-3-glucoside, μg/ml	–	–	–	–	472.5	–
12	Peonidin-3-rutinoside, μg/ml	–	–	7.8	–	–	–
13	Petunidin-3-galactoside, μg/ml	–	–	–	–	186.5	–
14	Peonidin-3-galactoside, μg/ml	–	–	–	–	40.7	–
15	Petunidin-3-arabinoside, μg/ml	–	–	–	–	113.9	–
16	Peonidin-3-glucoside, μg/ml	–	–	–	–	210.1	–
17	Malvidin-3-galactoside, μg/ml	–	–	–	–	149.6	–
18	Malvidin-3-glucoside, μg/ml	–	–	–	–	461.5	–
19	Malvidin-3-arabinoside, μg/ml	–	–	–	–	89.7	–

glucoside (5.6%), and cyanidin-3-rutinoside (29%). The total content of polyphenols was 5042 μg/ml. The black currant extract stimulated the growth of *L. casei* (Table 2).

The red currant fruit (*Ribes rubrum*) contained the following anthocyanidins: cyanidin-3-sambubioside (15%), cyanidin-3-xylosylrutinoside (66%), and cyanidin-3-rutinoside (19%). The total content of polyphenols was 3962 μg/ml. This extract was characterized by pro-bacterial properties in relation to *L. catenaformis* (Table 2).

The fruit of sweet cherry had the ability to stimulate the growth of *L. casei*, *L. catenaformis* and *L. fermentum*. However, in contrast to the extracts of black currant and jostaberry, only two types of anthocyanidins were identified in the fruit of sweet cherry:

Table 2. **Biological influence of fruit and berry extracts on the growth of lacto acid bacteria, in dynamics**

	L. acidophilus		*L. catenaformis*		*L. casei*		*L. fermentum*	
N0 (CFU/ml)	$3{\times}10^8$		$3{\times}10^8$		$3{\times}10^8$		$3{\times}10^8$	
Time of cultivation, hours	14	24	14	24	14	24	14	24
Tested Extracts	lgNt/N0	lgNt/N0	lgNt/N0	lgNt/N0	lgNt/N0	lgNt/N0	lgNt/N0	lgNt/N0
Prunus cerasifera	−5.5 ±0.5*	−5.5 ±0.5*	−4.5±1.4*	−5.5 ±0.79*	−5.5 ±1.7*	−5.5 ±0.19*	0.67± 0.25*	−5.5 ±0.17*
Ribes × nidigrolaria	−3.7 ±1.1	−3.2 ±1.1	−3.5 ±0.5	−0.1 ±0.5	−2.8 ±0.35	0±0.3	−2.8 ±0.2	0.52 ±0.3
Prunus avium	−0.78 ±0.79	−1.7 ±0.5	−3.4±1.1	−0.78 ±0.26	−3.7 ±0.3	0±0.29	−2.5 ±0.42	−0.78 ±0.4
Ribes nigrum	−3.5 ±0.5	−4.1 ±0.76	−4.1 ±0.5	−0.1 ±0.5	−0.1 ±0.35	0.52±0.28	−2.8 ±0.38	−2.5 ±0.57
Ribes rubrum	−3.9 ±0.5	−4.1 ±1.2	−0.78 ±0.26	0.2±0.5	−4.8 ±0.5	−4.8 ±0.17	−0.78 ±0.38	−5.5 ±0.45
Vaccinium myrtillus	−2.8 ±0.83	0.34±0.5	−3.5 ±0.29	−0.78 ±0.29	−2.8 ±0.35	0.52±0.5	−2.8 ±1.0	0.52± 0.92
Prunus domestica	−1.5 ±0.2	0.34±0.5	−0.48 ±0.26	−0.78 ±0.29	−0.78 ±0.5	−0.48 ±0.5	−0.48 ±0.78	−0.78± 0.38

Note. Significant differences with the *Prunus cerasifera* extracts on growth of lacto bacteria by *(P*<* 0.05).

cyanidin-3-glucoside (0.7%), and cyanidin-3-rutinoside (99.3%), while the total content of polyphenols was only 525 μg/ml.

Plum fruits (*Prunus domestica*) contained three types of anthocyanins in their composition: cyanidin glycoside (8.6%), cyanidin-3-rutinoside (50%), and peonidin-3-rutinoside (41.4%). The total content of polyphenols was 668 μg/ml. As to the effect of the plum extract on strains of lactobacilli, we observed the stimulation of growth of all tested LAB and *B. subtilis 090* strains (Table 2).

Analyzing the obtained data of the quantitative study of extracts composition (Table 1), we could note that the number of compounds of anthocyanins and polyphenols is significantly lower in the extracts of the *Pr nus* genus than in the extracts of the *Ribes* genus, but according to the experimental data obtained, plum and sweet cherry extracts were better in stimulating the growth of probiotic strains of microorganisms. Analyzing the obtained chromatogram of the plum extract and comparing it with literature sources, based on chromatographic analysis of extracts of different varieties of plums, we observed that the substances of the first priority here were cyanidin-3-xyloside and cyanidin-3-glycoside in various amounts [16, 17]. However, while examining our extract of *Prunus domestica*, we see that the peak on the chromatogram corresponds to the cyanidin-3-glycoside substance. This can be explained by the fact that, depending on the geographical location, variety, and the year of life of the plant, the quantitative composition of compounds of the anthocyanins may change [3].

Blueberries (*Vaccinium myrtillus*) contained the largest gross anthocyanin content in their composition. The extract contained 15 compounds of anthocyanins: delphinidin-3-galactoside (13.7%), delphinidin-3-glucoside (15%), cyanidin-3-galactoside (8.9%), delphinidin-3-arabinoside (9%), cyanidin-3-glucoside (11%), petunidin-3-galactoside (4%), cyanidin-3-arabinoside (6.4%), petunidin-3-glucoside (9.8%), peonidin-3-galactoside (1.0%), petunidin-3-arabinoside (2.4%), peonidin-3-glucoside (4.3%), malvidin-3-galactoside (3.1%), malvidin-3-glucoside (9.6%), and malvidin-3-arabinoside (1.8%) (Table 1). The total content of these compounds exceeded 5000 μg/ml, and the amount of polyphenols was 8945 μg/ml. We noticed the ability of the blueberries to stimulate the growth of all LAB strains we selected.

Table 3. **Biological influence of fruit and berry extracts on growth of *E. coli* 058, *E. faecalis*, *B. subtilis* 090, in dynamics**

	E. coli		***E. faecalis***		***B. subtilis* 090**	
N0 (CFU/ml)	3×10^8		3×10^8		3×10^8	
Time of cultivation, hours	14	24	14	24	14	24
Tested Extracts	lg (Nt/N0)	lg (Nt/N0)	lg (Nt/N0)	lg (Nt/N0)	lg (Nt/N0)	lg (Nt/N0)
Prunus cerasifera	−6.7 ±0.29	–	−6.7±0.5	–	−3.7 ±0.78	−0.48 ±0.83
Ribes × nidigrolaria	−6.7 ±0.29	−6.7* ±0.79	−6.7±0.5	−6.7±0.5	−0.78±0.5	−0.78±0.2
Prunus avium	−6.2±1.1	−6.7* ±0.29	−6.5±0.5	–	0.91±0.85	−5.5±0.74
Ribes nigrum	−4.8±0.5	–	−6.7±0.5	–	−3.7±0.3	−3.7±0.57
Ribes rubrum	−6.7±0.5	−6.7 ±0.5*	−4.8±0.29	−4.8±0.5	−0.78 ±0.87	−5.5±1.14
Prunus domestica	−4.8±0.79	−6.7 ±0.79*	−4.8±0.29	−4.8 ±0.79	−0.78±1.4	0.93±0.44
Vaccinium myrtillus	0.59±0.2	−3.8 ±0.5*	−4.8±0.29	−6.7 ±0.29	−5.5±0.93	−6.7±0.83

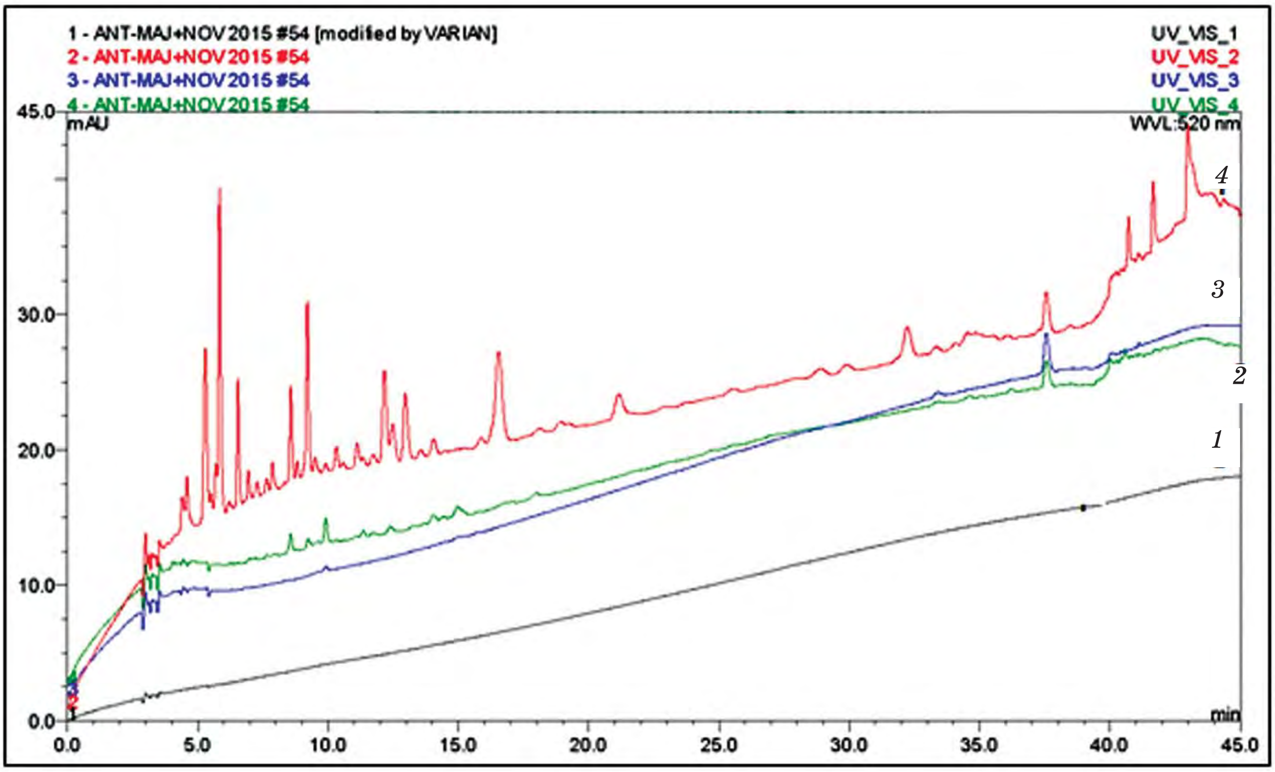
Note. Significant differences with the of fruit and berry extracts on growth (24 hours of cultivation) of *E. coli 058* by (*$P < 0.05$).

According to the data obtained, the fruits of alycha did not contain anthocyanins in their composition. This is confirmed by the fact that due to the presence of anthocyanins in the fruits, the fruits were of red to violet color, and alycha fruit was yellow. Analyzing the obtained chromatogram (Figure), we see that it has a very small amount of flavonoids and a small amount of coumarin. We also observed here the content of polar phenols and, according to the analysis of literature data, they could be phenolic acids, for example, catechin [18]. The total content of polyphenols was 219 µg/ml. The alycha extract inhibited the growth of all beneficial microorganisms we selected, and therefore when using it as a prebiotic component of pharmabiotics, such undesirable effect on the LAB tested by us should be taken into account (Table 2, 3).

Analyzing the quantitative and qualitative composition of BAC, included in the extracts tested by us, it can be concluded that the content of polyphenols significantly exceeded the content of anthocyanins, with no anthocyanins in the alycha extract detected whatsoever.

We determined the contents of anthocyanins and polyphenols in extracts of fruits and berries, and compared them with a database of BAC content in plant extracts ePlant LIBRA [19]. According to the analysis of the content of BAC in the following extracts: *Ribes rubrum, Ribes nigrum, Ribes × nidigrolaria, Vaccium myrtillus, Prunus avium*, which were included in the above mentioned database, the cyanidin-3-rutinoside compound was not identified [20, 21], but it was found in the extracts of the fruits and berries that we studied; there was also a difference in a quantitative content of anthocyanins and polyphenols. Comparing the BAC content of the *Prunus domestica*, and *Prunus cerasifera* extracts studied by us, with the ePlant LIBRA database, we noticed a difference, however, not in the qualitative composition of extracts, but in their quantitative indices [22].

While investigating the probacterial properties of extracts in relation to beneficial strains of microorganisms, we found that extracts of red and black currants, jostaberry, plum, and blueberry possessed such properties. In other words, these are the extracts, that contain anthocyanins and polyphenols in their composition, and therefore, we can conclude that the prebiotic properties are the properties of anthocyanins. This hypothesis is confirmed by the experimental data we obtained: since the extract of alycha had no anthocyanins in its composition, it had an inhibitory action on the strains of lactic bacteria.



The Chromatogram of the alycha extract:
UV-VIS *1* — 520 nm (anthocyanins); UV-VIS *2* — 280 nm (phenols); UV-VIS *3* — 360 nm (flavanoids);
UV-VIS *4* — 320 nm (coumarin)

So, comparing the results of our research with literature data, we can argue that namely the compounds of anthocyanins in the extracts of the berries we have studied have probacterial properties. The previously-published papers show that anthocyanidins, namely cyanidin-3-glucoside, inhibit the growth of only *E. coli* and do not inhibit the growth of probiotic strains of microorganisms and other representatives of human commensal microbiota [23, 24]. But, analyzing the experimental data we obtained, we can conclude that it is the compound of cyanidin-3-rutinoside that not only does not inhibit the growth of lactic bacteria, but rather contributes to their growth. This is confirmed by the fact, that the fruits of sweet cherry and plum stimulated the growth of the beneficial microorganisms we selected.

Thus, comparing the experimental data obtained with our previous studies, we can suggest that there are compounds of anthocyanins in the extracts of the studied berries that have probacterial properties.

The data obtained testify to possible further testing of berry extracts for their use as new generation pharmabiotics. After all, biologically active compounds that are part of the extracts, not only do not inhibit the growth of beneficial (probiotic and commensal) microorganisms, but rather stimulate it.

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ЕКСТРАКТИ ЇСТІВНИХ РОСЛИН ЯК СТИМУЛЯТОРИ РОСТУ КОРИСНИХ МІКРООРГАНІЗМІВ

О. В. Паллаг[1, 2], *Т. В. Мелешко*[1, 2], *В. В. Баті*[2], *Н. В. Бойко*[1, 2]

[1]Ужгородський національний університет, кафедра клініко-лабораторної діагностики та фармакології, стоматологічний факультет, Україна
[2]Ужгородський національний університет, науково-дослідний і навчальний центр молекулярної мікробіології та імунології слизових оболонок, Україна

E-mail: ssarvash@gmail.com

Метою роботи було визначення вмісту біологічно активних речовин у ягодах і плодах, зібраних в екологічно чистих зонах, для з'ясування перспективності їх використання для створення фармабіотиків спрямованої дії та здатності одержаних з них екстрактів стимулювати ріст пробіотичних штамів лактобактерій і представників коменсальної кишкової мікробіоти.

Вміст біологічно активних речовин визначали методом тонкошарової хроматографії. Вплив метанольних екстрактів ягід та плодів на відібрані нами штами: *L. acidophilus, L. catenaformis, L. casei, L. fermentum, E. coli 058, E. faecalis* (коменсали кишечнику), *B. subtilis 090* (складова біопрепарату), які є перспективними для створення сучасних фармабіотиків, визначали за результатами їх культивування в зазначених екстрактах.

Виявлено, що досліджувані ягідні екстракти характеризувалися більш значним вмістом поліфенолів порівняно з антоціанами. Екстракт аличі пригнічував ріст усіх тестованих нами штамів, окрім *B. subtilis 090*. Екстракти червоної смородини, черешні та йошти сприяли росту *L. catenaformis*, а екстракти черешні і йошти на додаток до вищезазначених штамів лактобактерій додатково стимулювали ріст *L. casei* та *L. fermentum*. Екстракти чорниці та сливи активували ріст усіх штамів лактобактерій, а чорниці, чорної та червоної смородини пригнічували ріст відібраних нами коменсальних мікроорганізмів. Здатність стимулювати ріст *B. subtilis 090* було відзначено лише в екстрактах аличі, йошти та сливи.

Ключові слова: ягоди, плоди, флаваноїди, антоціани, лактобактерії, фармабіотики.

ЭКСТРАКТЫ СЪЕДОБНЫХ РАСТЕНИЙ КАК СТИМУЛЯТОРЫ РОСТА ПОЛЕЗНЫХ МИКРООРГАНИЗМОВ

А. В. Паллаг[1, 2], *Т. В. Мелешко*[1, 2], *В. В. Бати*[2], *Н. В. Бойко*[1, 2]

[1] Ужгородский национальный университет, кафедра клинико-лабораторной диагностики и фармакологии, стоматологический факультет, Украина
[2] Ужгородский национальный университет, научно-исследовательский и учебный центр молекулярной микробиологии и иммунологии слизистых оболочек, Украина

E-mail: ssarvash@gmail.com

Целью работы было определение содержания биологически активных веществ в ягодах и плодах, собранных в экологически чистых зонах, для выяснения перспективности их использования для создании фармабиотиков направленного действия и способности полученных из них экстрактов стимулировать рост пробиотических штаммов лактобактерий и представителей комменсальной кишечной микробиоты.

Содержание биологически активных веществ определяли методом тонкослойной хроматографии. Влияние метанольных экстрактов ягод и плодов на отобранные нами штаммы: *L. acidophilus, L. catenaformis, L. casei, L. fermentum, E. coli 058, E. faecalis* (комменсалы кишечника), *B. subtilis 090* (составляющая биопрепарата), которые являются перспективными для создания современных фармабиотиков, устанавливали по результатам их культивирования в указанных экстрактах.

Выявлено, что исследуемые ягодные экстракты характеризовались более значительным содержанием полифенолов по сравнению с антоцианами. Экстракт алычи подавлял рост всех тестируемых нами штаммов, кроме *B. subtilis 090*. Экстракты красной смородины, черешни и йошты способствовали росту *L. catenaformis*, а экстракты черешни и йошты в дополнение к вышеупомянутым штаммам лактобактерий дополнительно стимулировали рост *L. casei* и *L. fermentum*. Экстракты черники и сливы активировали рост всех штаммов лактобактерий, а черники, черной и красной смородины подавляли рост отобранных нами комменсальных микроорганизмов. Способность стимулировать рост *B. subtilis 090* была отмечена только в экстрактах алычи, йошты и сливы.

Ключевые слова: ягоды, плоды, флаваноиды, антоцианы, лактобактерии, фармабиотики.

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