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Antimicrobial Activity of Wild Plant Seed Extracts against Human Bacterial and Plant Fungal Pathogens

**Valentina I. Pushkareva[1]*, Marina P. Slezina[2]*#, Tatyana V. Korostyleva[2],
Larisa A. Shcherbakova[3], Ekaterina A. Istomina[2], Svetlana A. Ermolaeva[1],
Olga A. Ogarkova[2], Tatyana I. Odintsova[2]**

[1]Gamaleya Research Center of Epidemiology and Microbiology, Russian Academy of Medicinal Sciences, Moscow, Russia
[2]Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, Russia
[3]All-Russian Research Institute of Phytopathology, Russian Academy of Sciences, Bolshie Vyazemy, Russia
Email: #omey@list.ru

Abstract

Five wild plant species belonging to different families (*Chenopodium album*, *Plantago major*, *Elytrigia elongata*, *Filipendula ulmaria* and *Nigella sativa*) widely spread in Russian Federation and the former USSR were evaluated for their ability to inhibit growth of two important human food-borne pathogens (*Escherichia coli* O157:H7 and *Listeria monocytogenes* strain EGD-e) and eight plant pathogens (*Alternaria alternata*, *Alternaria tenuissima*, *Bipolaris sorokiniana*, *Stagonospora nodorum*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium culmorum* and *Phytophtora infestans*). To isolate biologically active compounds from seeds, a step-wise procedure including extraction with hexane, ethyl acetate, ethanol, and 10% acetic acid followed by reversed-phase HPLC was developed. Using disc-diffusion assay, the highest activity against *E. coli* O157:H7 was observed with extracts from *F. ulmaria* (hexane and ethyl acetate extracts and the unbound RP-HPLC fraction) and *P. major* (ethyl acetate extract and the unbound RP-HPLC fraction); *E. elongata* (the unbound RP-HPLC fraction) was less active. The extracts from *P. major* and *E. elongata* (the unbound RP-HPLC fractions) were equally highly active against *L. monocytogenes*, while those of *F. ulmaria* (the unbound RP-HPLC fraction) and *N. sativa* (hexane and ethyl acetate extracts) were less active against this pathogen. The dynamics of *L. monocytogenes* EGD-e and *E. coli* O157:H7 growth in the presence of two most potent extracts (RP-HPLC-unbound fractions of *P. major* and *E. elongata* and the hexane extract of *F. ulmaria*) was studied. All plant extracts caused considerable reduction of the bacterial population already after 24 h of incubation. Antifungal assays showed that the

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*The first two authors contributed equally to this work.

ethanol extract and both RP-HPLC fractions of *F. ulmaria* completely inhibited spore germination in all tested fungi. The RP-HPLC-bound fraction from *N. sativa* completely inhibited spore germination in *P. infestans* and *B. sorokiniana*. Both RP-HPLC fractions of the plants examined (except for *C. album*) completely inhibited spore germination in *S. nodorum*. Significant inhibition of *F. culmorum* spore germination was recorded with both RP-HPLC fractions from *E. elongata* and RP-HPLC unbound fraction of *P. major*. *F. oxysporum* spore germination was also inhibited by the RP-HPLC bound fraction of *C. album*. *B. sorokiniana* and *A. alternata* appeared to be less sensitive to plant extracts. The results obtained expand the repertoire of valuable biological activities discovered in plants for future use in medicine, food preservation and crop protection.

Keywords

Plant Extracts, Antibacterial Activity, Human Food-Borne Pathogens, Antifungal Activity, Plant Pathogenic Fungi

1. Introduction

Despite enormous progress in medicine, infectious diseases pose a serious threat to human health. Intestinal infections caused by pathogenic bacteria occupy a prominent place among them, with nearly 1.7 billion cases of diarrheal diseases registered worldwide annually [1].

Listeriosis is a dangerous human disease caused by the pathogenic bacterium *Listeria monocytogenes* [2] [3]. Listeriosis ranks third in the total number of deaths among food-borne pathogens with overall mortality rate of 20% - 30% [4]. Contaminated food is usually the primary source of infection that mainly occurs through dairy products and meat [5]. However, raw vegetables can also serve as the source of infection.

L. monocytogenes is a Gram-positive, non spore-forming, facultatively anaerobic, rod-shaped bacterium which belongs to the most virulent food-borne pathogens [4]. It is widely spread in soil, water, plants, silage, sewage, milk. It was isolated from cattle, sheep, goats, poultry, hydrobionts, pets, and wild animals [4]. Vegetables appear to be often contaminated with *L. monocytogenes*, where the bacterium forms biofilms [6]. The bacterium can grow and multiply at low positive temperatures and is tolerant to freezing, drying and other stressful abiotic factors. The ability to grow at low temperatures permits the bacterium to multiply even in refrigerated foods.

L. monocytogenes possesses a limited repertoire of pathogenicity factors (seven proteins) [4]. The hemolysin designated listeriolysin O encoded by the *hly* gene is considered the major virulence factor. The listeriolysin produces large transmembrane pores which are supposed to be responsible for its cytolytic properties and destruction of erythrocytes. Human listeriosis may be caused by all 13 serovars of *L. monocytogenes*, however, serovars 1/2a, 1/2b and 4b cause

most of the cases.

Escherichioses caused by pathogenic *Escherichia coli* strains represent another threat to human health. The outbreaks of diseases caused by pathogenic *E. coli* strains are being constantly reported in Europe, North America (1 - 2 cases per 100,000), and Argentina (up to 22 cases per 100,000) [1]. After dramatic epidemic of escherichiosis in Japan (more than 10000 cases) caused by consumption of radish seedlings contaminated with the enterohemorrhagic *Escherichia coli* O157:H7 [7], more than 40 outbreaks of the disease were registered worldwide.

E. coli is a Gram-negative, non spore-forming, facultatively anaerobic, rod-shaped bacterium belonging to the family Enterobacteriaceae. It inhabits human gastrointestinal tract, however, some serovars are human pathogens that cause different disorders, such as urinary tract infections and gastrointestinal diseases [8] [9]. It has been established that the main reservoir of different serovars of enteropathogenic *E. coli* are farm animals (ruminants). The bacteria inhabit their gut not producing any harm to adult animals. Escherichiosis caused by enteropathogenic *E. coli*, which produces Shiga toxins / verotoxins (more than 100 serotypes) capable of killing Vero cells, are particularly life-threatening [10]. The genes responsible for toxin production are located in the locus of enterocyte effacement (LEE) pathogenicity island [11]. The toxins encoded by locus LEE can directly damage renal and endothelial cells. Shiga toxins I and II (Stx1 and Stx2) are responsible for the clinical manifestation of infection—hemorrhagic colitis or hemolytic uremic syndrome.

To treat infectious diseases caused by food-borne pathogenic bacteria, antibiotics have been extensively used. However, nowadays the resistance of pathogens to antibiotics is rising. The antibiotic-resistance problem stimulates the search for novel highly efficient antimicrobials. To prevent contamination of food products with pathogens causing gastrointestinal disorders, chemical preservatives are commonly used, however, they may affect human health and promote the emergence of resistant strains of pathogens, therefore, efforts are aimed at the development of effective and safe natural food preservatives.

Plant diseases caused by pathogenic fungi result in considerable yield losses in cultivated crops [12]. Furthermore, they decrease the quality of agricultural products. Thus, mycotoxins produced by *Fusarium* species and present in contaminated grain are toxic to humans and livestock; aflatoxins produced by *Aspergillus* species are carcinogenic. *Alternaria alternata* (Fr.) Keissl causes diseases in over 380 host plant species. The symptoms of the disease vary from leaf spots to rots and blights. It can also cause upper respiratory tract infections and asthma in immunocompromised individuals. *Alternaria tenuissima* Samuel Paul Wiltshire is a cosmopolitan species and an opportunistic pathogen of plants. It often colonizes blueberries, strawberries, tomatoes, grapevine, and cereal species. It can cause skin infections in immunocompromised individuals. *Bipolaris sorokiniana* (Sacc.), Shoemaker is the causal agent of a wide variety of cereal diseases. The pathogen can induce disease on roots, leaves, stems and heads. Two most

common diseases are common root rot and spot blotch on wheat and barley. *Stagonospora nodorum* (Berk.) Castellani & E. G. Germane is a devasting pathogen of wheat and related cereals, causing leaf and glume blotch. The genus *Fusarium* comprises a wide and heterogeneous group of fungi. The host range of pathogenic strains of *Fusarium oxysporum* Schitdl. is exceedingly broad, and includes animals as well as plants. However, individual isolates usually cause disease only on a narrow range of plant species. On many plant species *F. oxysporum* causes vascular wilt followed by growth stunting, wilting, defoliation and, finally, death of the plant. Some strains are not primarily vascular pathogens, but cause foot and root rot. *Fusarium culmorum* (W.G.Sm.) Sacc. is the causal agent of seedling blight, foot rot, ear blight, stalk rot, common root rot and other diseases of cereals, and many other monocots and dicots. *Fusarium solani* (Mart.) Sacc. also causes severe crop diseases, such as root and fruit rot of *Cucurbita* spp., root and stem rot of pea, sudden death syndrome of soybean, foot rot of bean and dry rot of potato. *Phytophthora infestans* (Mont.) de Bary is an oomycete that causes the serious disease of potatoes known as late blight or potato blight. The pathogen can also infect other members of the Solanaceae family, such as tomato.

To control fungal diseases, fungicides are currently widely used. Although they reduce yield losses, they contaminate the environment with hazardous substances threatening human health. Ecologically friendly strategies to combat plant diseases are therefore necessary.

Plants extracts serve as a valuable and unique source of various biologically active compounds that can be used for different purposes: (i) to develop novel pharmaceuticals as an alternative or supplement to conventional antibiotics to treat human diseases and preserve food products from infection; (ii) to elaborate novel plant disease control formulations.

Chenopodium album L. (family Amaranthaceae) is a weed widely spread throughout Europe [13]. It is an edible plant. The leaves can be used as a leaf vegetable. Seeds are rich in starch and protein. In India, it is widely grown as a grain crop. In traditional medicine it is used to cure inflammation, cough, bronchitis, digestive tract diseases and relief pain [14]. *Plantago major* L. (family Plantaginaceae) is a perennial common weed native to Europe and northern and central Asia. In Russia, it is found everywhere except the Far North, the Pamirs and deserts. It often grows in lawns and fields and along roadsides. The plant is edible and rich in vitamin B1 and riboflavin. It possesses a wide range of activities, such as anti-inflammatory, antiseptic, antibacterial, cardiac, and diuretic. It quickly stops blood flow and stimulates wound healing. It is used in the treatment of diarrhea, dysentery, gastritis, bronchitis, haemorrhage, cough, and asthma [15]. *Elytrigia elongata* (Host) Nevski (family Poaceae) is a perennial herbaceous forage plant. It grows in Eastern Europe, Crimea, south of Russia, Caucasus, Mediterranean region, and Iran on solonchak steppe, chalk outcrops, and along seashores. Rhizomes are edible. The nutritional value of flour made of *E. elongata* rhizomes is similar to that of wheat. Although the medicinal proper-

ties of this species are poorly studied, it is closely related to the weed *Elytrigia repens* (L.) Gould, which is used in traditional medicine as an antifungal and antibacterial agent to treat arthritis, bronchitis, skin diseases, urinary tract infections, upper respiratory tract, and liver diseases [13]. *Filipendula ulmaria* (L.) Maxim. (family Rosaceae) is a perennial herb growing in damp areas. It is found in most of Europe and Western Asia. It is a medicinal plant widely used for treatment of different disorders. The leaves and flowering stems are anti-inflammatory, antiseptic, diuretic and tonic. The herb is used in traditional medicine as a remedy for acidic stomach, diarrhea, rheumatism, bronchitis, cough, fever and cold. It is also a natural remedy for relieving headaches [16]. *Nigella sativa* L. (family Ranunculaceae) is native to Southern Europe, North Africa and Southwest Asia. Seeds have been used for centuries to treat diseases. The biological activities of *N. sativa* include immunomodulatory, antidiabetic, anticancer, analgesic, antimicrobial, asmolytic, anti-inflammatory, antioxidant, gastroprotective, hepatoprotective and many others. The seeds are used to cure many diseases, such as bronchitis, asthma, diarrhea, rheumatism, and skin disorders [17].

The aim of this study was to examine the antibacterial activity of above-described medicinal plant extracts against human bacterial pathogens *L. monocytogenes* and *E. coli* and their antifungal activity against important plant pathogens in order to evaluate their potential for therapeutic use in healthcare and food preservation and as natural antibiotics for protection of plants against diseases.

2. Materials and Methods

2.1. Plant Materials and Microorganisms

Seeds of *C. album*, *P. major*, *E. elongata* and *F. ulmaria* were collected in 2012 in Moscow region. Seeds of *N. sativa* were collected in Zangiatinskii region of Uzbekistan in 2012.

The microorganisms used in this study were as follows: *L. monocytogenes* strain EGD-e obtained from Prof. J.A. Vazquez-Boland (Department of Molecular Microbiology, University of Bristol, UK), toxin-producing strain of *E. coli* (VT1) (O157:H7) ATCC 43890 (USA). Isolates of *A. alternata*, *A. tenuissima*, *B. sorokiniana*, *S. nodorum*, *F. solani*, *P. infestans* were obtained from the Collection of Phytopathogenic Microorganisms of the All-Russian Research Institute of Phytopathology; *F. oxysporum* strain VKM-137 and *F. culmorum* strain VKM-2303 were received from the All-Russian Collection of Microorganisms of the G.P. Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS.

2.2. Preparation of Plant Extracts

Seeds (3 g) were ground into powder in a coffee mill and successively extracted with hexane (Khimmed, Russia), ethyl acetate (Khimmed, Russia), ethanol (Ferein, Russia), and 10% acetic acid (Khimmed, Russia) at a plant/solvent ratio of 1:8 (w/v) under constant stirring at 30 rpm at room temperature for 1h. After

each extraction step the homogenate was centrifuged at 10,000 g at 4°C for 10 min, the pellet was discarded, and the supernatant was filtered through Whatman no.1 filter paper. The extracts obtained with acetic acid were further desalted by reversed-phase HPLC on a NewGuard C18 column (3.2x15 mm) (Applied Biosystems, USA). Elution with 0.1% trifluoroacetic acid (TFA) (Fluka, Switzerland) yielded the unbound fraction, while the bound fraction was recovered with 50% acetonitrile (Panreac, Spain) in 0.1% TFA. All extracts and both RP-HPLC fractions (bound and unbound) were evaporated on a rotary evaporator (Savant, USA), lyophilized, stored at −80°C, and dissolved immediately before use in 1 ml of 5% dimethyl sulfoxide (DMSO) (Amresco, USA).

2.3. Antibacterial Assays

L. monocytogenes and *E. coli* were cultured on liquid and solid BHI (Difco, USA) media at 37°C for 24 h. Inhibition of bacterial growth was assayed by the disc diffusion method developed for commercial antibiotics [18]. Broth cultures adjusted to a concentration of 10^6 colony forming units (CFU) per ml were streaked on Mueller-Hinton Agar in Petri dishes (Himedia, India). Filter paper discs were impregnated with 25, 50 and 75 μl of plant extracts and placed on the inoculated surface of the agar plates. The plates were incubated at 37°C for 24 h, whereupon the diameters (in mm) of zones of microbial inhibition produced by plant extracts were measured. Pure 5% DMSO was used as control.

The survival of the bacteria in the presence of the most active plant extracts was determined by time-kill analysis (survival curve plot), in which the number of viable bacterial cells remaining after the addition of plant extracts was plotted against time. For this assay, agar plates were inoculated with stock solutions of plant extracts mixed with bacterial suspensions at a 1:10 (v/v) ratio. The number of viable cells was calculated after incubation for 2 h, 1, 2, 3, 4 and 5 days and expressed in CFU/ml.

2.4. Antifungal Assays

For antifungal assays spores were washed from the surface of fungal colonies grown on potato-dextrose agar at 25°C for 14 days (*A. alternata*, *A. tennuissima*, *B. sorokiniana*, *S. nodorum*, *F. solani*, *F. oxysporum*, *F. culmorum*) or on oats agar at 21°C - 22°C (*P. infestans*). Spores were separated from mycelium fragments by filtration through the cotton and nylon filters, sedimented by centrifugation at 3000 g for 20 min and resuspended in the minimum volume of distilled water.

Fungitoxicity of plant extracts from *F. ulmaria* and *N. sativa* was assayed by microscopic examination of spore germination in the presence of plant extracts. Plant extracts were diluted 1:10 (v/v) with spore suspensions of *A. tenuissima*, *B. sorokiniana*, *F. solani* and *P. infestans* at a concentration of 2×10^5/ml (the RP-HPLC-bound fraction of *F. ulmaria* was additionally diluted 1:10 and 1:50 (v/v)) on slide plates (four slides per variant). Then the slide plates were placed into humid chamber and incubated at 22°C in the dark for 24 h. Spore suspen-

sions in 5% DMSO were used as controls. The effect of plant extracts on spore germination was evaluated at two time points, after 5 - 6 h and 24 h.

To test antifungal activity of extracts from *C. album, P. major* and *E. elongata,* the assay based on germination of spores in thin agar layers was used. Equal volumes of spore suspensions and plant extracts were mixed to yield the final spore concentration of 10^4/ml for *A. alternata, B. sorokiniana, F. oxysporum,* and *F. culmorum* and 3×10^3/ml for *S. nodorum.* Suspensions were incubated at room temperature for 25 - 30 min at constant gentle stirring, and then 200 µl of the suspension were loaded onto the slide plates covered with 1% agar (3 glass plates for each variant) and placed in a humid chamber. Spores of *S. nodorum* were germinated in the dark at 22°C for 16 - 18, and for 5 - 6 h for *A. alternata, B. sorokiniana, F. oxysporum,* and *F. culmorum.* Suspension of spores in 5% DMSO was used as control. Inhibition of germination induced by plant extracts was monitored under the microscope. Not less than 600 spores were examined in each experimental and control variants. Germination frequency was expressed in per cents from the number of germinated seeds in control.

All antimicrobial assays were conducted in triplicate. Statistical processing of the experimental data was carried out with the STATISTICA 6.0 program ("SoftStat Inc.", USA). The significance of differences at $p \leq 0.05$ was calculated using the *t*-test.

3. Results

3.1. Antibacterial Activity of Plant Extracts against *L. monocytogenes* and *E. coli*

Extracts from seeds of five medicinal plants growing in Moscow region and Uzbekistan were obtained and evaluated for their ability to inhibit growth of important human and plant pathogens. Seeds were successively extracted with different solvents—hexane, ethyl acetate, ethanol, and 10% acetic acid followed by RP-HPLC of the acidic acid extract that produced two fractions—the unbound and bound to the reversed phase. The effect of 24 resulting extracts on growth of Gram+ bacterium *L. monocytogenes* EGD-e and Gram− bacterium *E. coli* O157:H7 was evaluated by disc diffusion assay. The data on *E. coli* growth inhibition are shown in Table 1. Mean values of three independent experiments are presented.

Table 1. Inhibitory activity of plant extracts against *E. coli* O157:H7 (inhibition zone diameter, mm).

Plant Species	10% Acetic Acid		Hexan	Ethyl Acetate	Ethanol
	RP-HPLC-bound	RP-HPLC-unbound			
C. album	0	0	<4	<6	0
P. major	0	20 ± 4	<10	22 ± 2	0
E. elongata	0	18 ± 3	0	0	0
F. ulmaria	0	22 ± 2	22 ± 1	20 ± 2	0
N. sativa	0	0	0	0	0

Taken as control 5% DMSO had no effect on growth of the pathogen (data not shown). The ethanol extracts and the RP-HPLC-bound fractions of all plants studied were inactive against the pathogenic *E. coli* strain O157:H7. The pathogen was not sensitive to the components present in *N. sativa* extracts and was weakly sensitive to the compounds found in *C. album* extracts. On the contrary, the components present in ethyl acetate, hexane, and RP-HPLC-unbound fractions of *F. ulmaria* and *P. major* displayed strong antibacterial activity against *E. coli* O157:H7. The inhibition zone was 20 - 22 mm, except for the hexane extract of *P. major* where it did not exceed 10 mm. In *E. elongata* the active against *E. coli* components were in the unbound RP-HPLC fraction of the acetic acid extract (inhibition zone of 18 ± 3 mm).

The results of antibacterial assays of the plant extracts against the virulent strain of *L. monocytogenes* EGD-e are presented in Table 2. 5% DMSO used as control did not inhibit growth of the bacterium (data not shown). The RP-HPLC-bound fractions of the acetic acid extract and the ethanol extracts of all plants studied were inactive against *L. monocytogenes* EGD-e. Conversely, the unbound RP-HPLC fractions of the acetic acid extracts of *P. major* and *E. elongata* displayed the strongest antibacterial activity, with the inhibition zone of 22 mm. The hexane and ethyl acetate extracts of *N. sativa*, as well as the unbound fraction of the acetic acid extract of *F. ulmaria* were less active (inhibition zone of 16 mm). In *C. album,* only the components extracted with ethyl acetate caused weak inhibition of *L. monocytogenes* (inhibition zone of 8 mm). It should be noted that growth inhibition in both bacterial pathogens was observed only when 75 µl of plant extract solutions were loaded onto the discs. Two-fold dilution considerably reduced the diameter of inhibition zones for both pathogens (data not shown).

Thus, of the plant species studied, only extracts from *C. album* were virtually inactive against two human pathogens. Extracts from *N. sativa* inhibited only *L. monocytogenes*. The highest activity against *E. coli* O157:H7 was observed with extracts from *F. ulmaria* (hexane and ethyl acetate extracts and the unbound RP-HPLC fraction) and *P. major* (ethyl acetate extract and the unbound RP-HPLC fraction); *E. elongata* (the unbound RP-HPLC fraction) was less active. The extracts from *P. major* and *E. elongata* (the unbound RP-HPLC frac-

Table 2. Inhibitory activity of plant extracts against *L. monocytogenes* EGD-e (inhibition zone diameter, mm)

Plant Species	10% Acetic Acid		Hexan	Ethyl Acetate	Ethanol
	RP-HPLC-bound	RP-HPLC-unbound			
C. album	0	0	0	<8	0
P. major	0	22 ± 2	0	0	0
E. elongata	0	22 ± 1	0	0	0
F. ulmaria	0	16 ± 1	0	0	0
N. sativa	0	0	16 ± 2	16 ± 1	0

tions) were equally highly active against *L. monocytogenes*, while those of *F. ulmaria* (the unbound RP-HPLC fraction) and *N. sativa* (hexane and ethyl acetate extracts) were less active against this pathogen.

The dynamics of *L. monocytogenes* EGD-e and *E. coli* O157:H7 growth in the presence of two most potent extracts (RP-HPLC-unbound fractions of *P. major* and *E. elongata* and the hexane extract of *F. ulmaria*) was studied (Figure 1). The abundance of bacterial populations was measured at several time points: 2 h, 1, 2, 3, 4 and 5 days after the addition of plant extracts. Changes in the number of viable *E. coli* cells are shown in Figure 1(a). In control (water), the number of *E. coli* cells gradually decreased by the fourth day of incubation. This tendency is characteristic of the bacteria growing on "poor" media. Both plant extracts caused considerable reduction of the bacterial population already after 24 h of incubation, the hexane extract of *F. ulmaria* being more effective. The effect was much more pronounced following 48 h of incubation with the extracts. Similar population dynamics was observed with *L. monocytogenes* in the presence of RP-HPLC-unbound fractions from *P. major* and *E. elongata* (Figure 1(b)). Cultivation of the bacteria with these extracts caused slower reduction in population abundance in comparison to *E. coli* followed by complete elimination of the bacteria by 96 h. The delayed effect of plant extracts on *L. monocytogenes* is likely to be associated with more complex structure of cell walls in Gram+ bacteria (multilayered structured peptidoglycan) that prevents rapid diffusion of plant components into bacterial cells.

3.2. Antifungal Activity of Plant Extracts

To test the antifungal activity of *F. ulmaria* and *N. sativa* seed extracts, germination of fungal spores in the presence of the extracts was monitored under the microscope at two time points: after 5 - 6 h and 24 h of incubation.

The experiments showed that the RP-HPLC-bound fraction of the acetic acid soluble extract from *F. ulmaria* was highly active against all tested fungi (*F. solani, A. tenuissima, B. sorokiniana*) and the oomycete (*P. infestans*) (Figure 2). Strong inhibitory effect on spore germination persisted after 24 h of incuba-
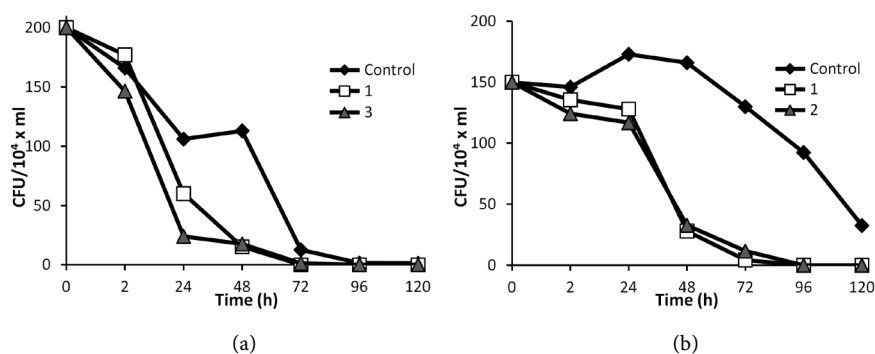

(a)   (b)

Figure 1. *E. coli* O157:H7 (a) and *L. monocytogenes* EGD-e (b) population dynamics in the presence of plant extracts: RP-HPLC unbound fractions of *P. major* (1) and *E. elongata* (2) acetic acid extracts, *F. ulmaria* hexane extract (3).
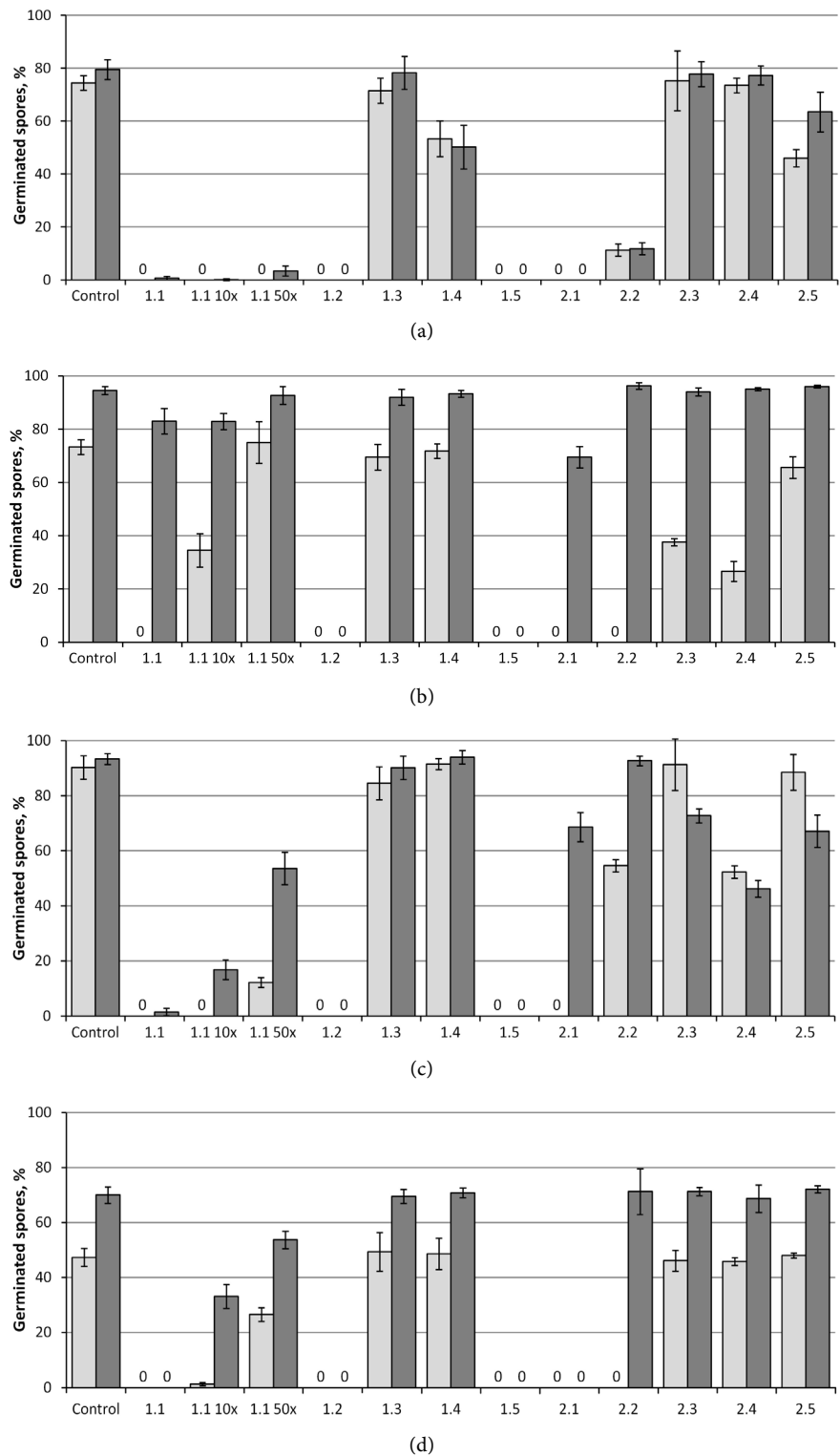
Figure 2. Germination of spores of *P. infestans* (a); *F. solani* (b); *A. tenuissima* (c); and *B. sorokiniana* (d) in the presence of seed extracts from *F. ulmaria* (1) and *N. sativa* (2): RP-HPLC-bound fraction of the acetic acid extract (X.1, diluted 1:10 and 1:50), RP-HPLC-unbound fraction (X.2), hexane extract (X.3), ethyl acetate extract (X.4), and ethanol extract (X.5) (for experimental details, see Materials and Methods). Grey, incubation with extracts for 5 - 6 h, dark grey, for 24 h. Extracts were dissolved in 5% DMSO used as control. Standard deviations are shown.

tion with all fungi, except for *F. solani*. Incubation of the RP-HPLC-bound fraction of the acetic acid soluble extract from *F. ulmaria* with *F. solani* spores caused 100% inhibition of spore germination after 5 - 6 h of observation, however, after 24 h of incubation, inhibition decreased to 11.4%. Despite this fact, we discovered that *F. ulmaria* extract influenced the rate of hyphal elongation, which was considerably reduced: after 24 h, the length of hypha in the presence of the extract was only 91 - 110 μ, while in the control their length reached 650 - 975 μ. Thus, inhibition of *F. solani* was manifested by suppression of hyphal elongation. The RP-HPLC-bound fraction of the acetic acid extract from *F. ulmaria* virtually completely inhibited (98%) spore germination in *A. tenuissima*, and in *B. sorokiniana* it was completely suppressed. Incubation of fungal spores in the presence of RP-HPLC-unbound fraction of the acetic acid extract from *F. ulmaria* and the ethanol extract from this species completely inhibited germination of spores in all tested fungi both after 5 - 6 h and 24 h of incubation. The hexane extract did not inhibit germination of spores of the fungi tested, while the ethyl acetate extract caused 36.7% inhibition of spore germination only in *P. infestans*.

Of *N. sativa* extracts, the acetic-acid-soluble fraction showed the highest inhibitory activity on spore germination (Figure 2). The RP-HPLC-bound fraction completely inhibited spore germination in all fungi tested. However, the effect persisted after 24 h only with *P. infestans* and *B. sorokiniana*. In *F. solani,* incubation with the extract for 24 h resulted in 26% inhibition of spore germination, however, considerable retardation of hyphal growth to 54.3 - 81.5 μ as compared to control, was noted. The inhibitory activity of the RP-HPLC-unbound fraction was weaker on spore germination of *A. tenuissima* and *P. infestans*, and except for *P. infestans* disappeared after prolonged incubation. The hexane and ethyl acetate extracts of *N. sativa* weakly inhibited spore germination in *F. solani* and *A. tenuissima*. It is worth noting that the inhibitory effect disappeared after prolonged incubation (24 h) with *F. solani* spores. In *A. tenuissima*, on the contrary, the hexane extract inhibited growth (22.1%) only after prolonged incubation with the spores. With the ethyl acetate extract, inhibition of spore germination was similar both after 5-6 and 24 h of incubation. The ethanol extract of *N. sativa*, in addition to the above-mentioned fungi, displayed moderate activity against *P. infestans*.

In summary, the hexane and ethyl acetate extracts from *F. ulmaria* showed no inhibition of spore germination in the fungi examined, however the ethyl acetate extract was moderately active against *P. infestans.* Conversely, the ethanol extract and both RP-HPLC-bound and unbound acetic acid soluble fractions completely inhibited spore germination in all tested fungi and the oomycete (Figure 2). The most convincing results were obtained with the RP-HPLC-bound fraction of the acetic acid extract of *F. ulmaria* when tested against *P. infestans*. Even 50-fold dilution of the extract caused 95.7% inhibition of spore germination in the oomycete after 24 h of incubation (Figure 2(a)). The extracts from *N. sativa* seeds, except for the RP-HPLC-bound fraction of the acetic acid extract, were

less potent against the fungi studied in comparison to *F. ulmaria*. The RP-HPLC-bound acetic-acid-soluble fraction was the most active: it completely inhibited spore germination in *P. infestans* and *B. sorokiniana* after 24 h of incubation (Figure 2(a) and Figure 2(d)). Other *N. sativa* extracts showed low inhibitory activity, in some cases, the effect decreased with time depending on the fungus. It should be specifically noted that although acetic-acid-soluble fractions of *F. ulmaria* and *N. sativa* caused weak inhibition of spore germination in *F. solani*, the rate of hyphal elongation was significantly affected.

The results of antifungal assays with RP-HPLC-bound and -unbound fractions of the acetic-acid extracts from *C. album, P. major*, and *E. elongata* against fungal pathogens are shown in Figure 3. As seen from this Figure, the acidsoluble plant extracts differ considerably in their ability to inhibit spore germination in pathogenic fungi.
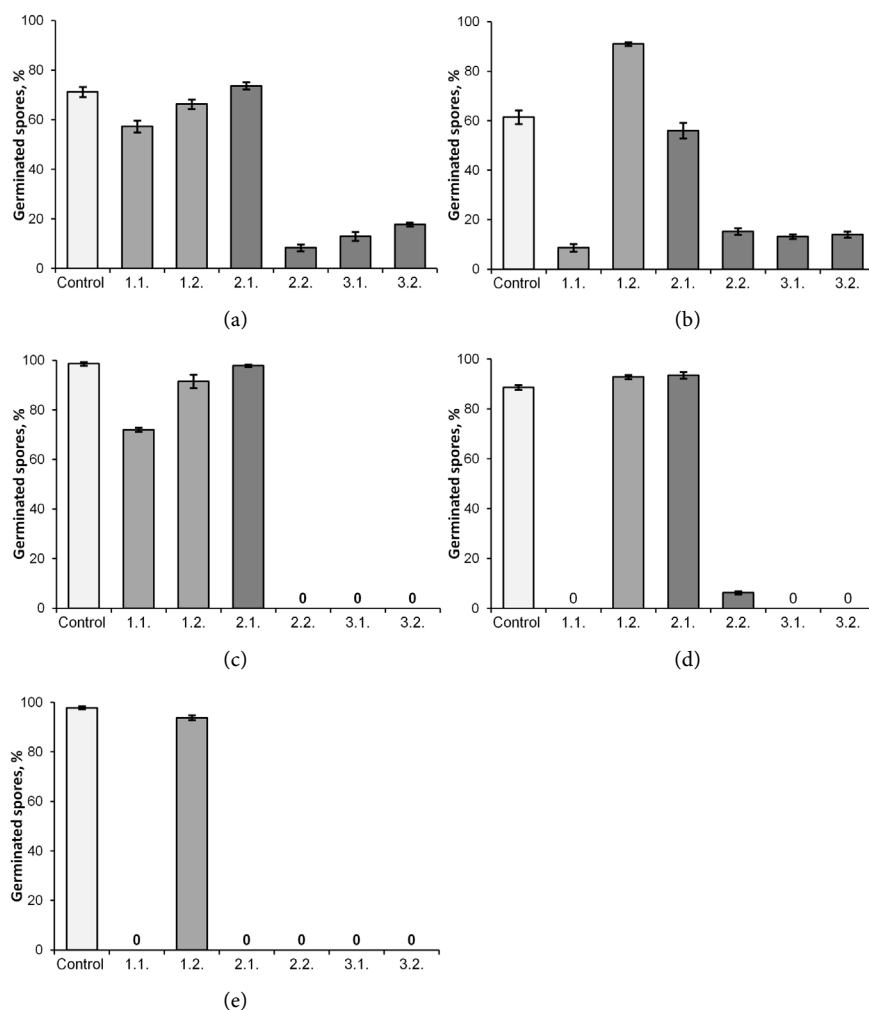

Figure 3. Germination of spores of *A. alternata* (a); *B. sorokiniana* (b); *F. culmorum* (c); *F. oxysporum* (d); and *S. nodorum* (e) in the presence of seed extracts from *C. album* (1), *P. major* (2) and *E. elongata* (3): RP-HPLC-bound fraction (X.1), RP-HPLC-unbound fraction (X.2) (for experimental details, see Materials and Methods). Incubation with extracts for 5 - 6 h. Extracts were dissolved in 5% DMSO used as control. Standard deviations are shown.

The RP-HPLC-bound fraction of *C. album* was active against all five fungi, while the unbound fraction was inactive. *A. alternata* was the least sensitive fungus to this fraction (**Figure 3(a)**). Conversely, it was highly active against *B. sorokiniana* (**Figure 3(b)**), while the germination of spores in *F. oxysporum* and *S. nodorum* was completely inhibited (**Figure 3(d)** and **Figure 3(e)**). Inhibition of *F. culmorum* spore germination was moderate - 27% (**Figure 3(c)**). However, similarly to the effect of the RP-HPLC-bound fractions from *F. ulmaria* and *N. sativa* seeds on *F. solani*, the extract from *C. album* considerably decreased the rate of mycelium growth (data not shown).

The RP-HPLC-bound fraction of *P. major* completely inhibited germination of *S. nodorum* spores, but had no effect on other pathogens (**Figures 3(a)-(e)**). The antifungal activity in this plant was mainly associated with the RP-HPLC-unbound fraction (**Figures 3(a)-(e)**). It completely inhibited spore germination in *F. culmorum* and *S. nodorum* and strongly suppressed this process in other tested fungi (**Figures 3(a)-(e)**).

It is interesting that incubation of *S. nodorum* spores with the RP-HPLC-bound fractions from *C. album* and *P. major* led to disturbance of spore morphology (data not shown). In all probability, the inhibitory effect of these plant fractions on germination is due to loss of spore viability.

Both RP-HPLC fractions obtained from *E. elongata* strongly inhibited spore germination in *A. alternata* and *B. sorokiniana* (**Figure 3(a)** and **Figure 3(b)**) and caused 100% inhibition of spore germination in *S. nodorum* and two *Fusarium* species (**Figures 3(c)-(e)**).

Plant fractions obtained by RP-HPLC of acid-soluble plant extracts displayed antifungal activity against *A. alternata*, *B. sorokiniana*, *F. culmorum*, *F. oxysporum* and *S. nodorum*. The latter was the most sensitive fungus to the extracts tested. Both bound and unbound RP-HPLC fractions of plants examined (except for *C. album*) completely inhibited germination of spores in this fungus. In the case of the RP-HPLC-bound fractions from *C. album* and *P. major*, this effect was associated with changes in spore morphology. Significant inhibition of *F. culmorum* spore germination was recorded with both RP-HPLC fractions from *E. elongata* and RP-HPLC-unbound fraction of *P. major*. *F. oxysporum* spore germination was also inhibited by the components of *C. album* present in the RP-HPLC-bound fraction. *B. sorokiniana* and *A. alternata* appeared to be less sensitive to plant extracts, however more than 75% inhibition of spore germination was achieved with both RP-HPLC fractions of *E. elongata*, the RP-HPLC-unbound fraction of *P. major* and the bound fraction of *C. album* (against *B. sorokiniana*).

4. Discussion

Food-borne diseases caused by pathogenic microorganisms contaminating food products induce most gastrointestinal infections causing diarrhea, fever and other clinical manifestations. Pathogenic bacteria, such as *Salmonella enteritidis, Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Campylobacter*

spp. are responsible for most of them. Over the last decades antibiotics traditionally used to cope with infections have become ineffective due to the emergence of pathogen resistance. *Salmonella* spp. became resistant to chloramphenicol and ampicillin. Methicillin-resistant *S. aureus* strains appeared. *E. coli* and related bacteria have developed resistance to third-generation cephalosporins. Enterobacteriaceae acquired resistance even to carbapenems. Resistance results from the global increase in antibiotic consumption and inappropriate use of antibiotics both in medicine to treat diseases and in agriculture to intensify food animal production. In addition to antibiotics, other chemical preservatives, such as chlorine dioxide and sodium nitrate in combination with physical preservation methods, such as freezing, thermal treatment, high hydrostatic pressure, pulsed electric fields are used in food industry to eliminate infection. Taking into consideration antibiotic resistance problem and consumer's demand for food safety the need for novel natural antimicrobials to eliminate food-borne pathogens is increasing. Plant diseases are another global problem. Pathogenic fungi dramatically decrease yields of cultivated crops and deteriorate quality of agricultural products. Fungicides, although effective, contaminate the environment and threaten human health. The search for novel ecologically friendly measures of crop protection is therefore necessary.

Plants represent a valuable but still insufficiently explored source of biologically active compounds that can serve, on the one side, as the basis for the development of novel pharmaceuticals for treatment of diseases and food preservation, on the other side, as novel plant disease control agents. Plant extracts represent enormous natural libraries of biologically active compounds that can be used for these purposes. Plants have been used for centuries in traditional medicine. Antimicrobial effect of plant essential oils and extracts has been successfully used in preservation of food products, pharmaceuticals and alternative medicine [19] [20] [21]. However, only 5% - 15% of existing plant species have been assayed for biologically active compounds. Substances that plants use to protect themselves from pathogens are structurally diverse. Secondary metabolites (phytoalexins and phytoanticipines) play an important role in plant defense strategy. They do not participate in key metabolic processes but are important for adaptation to stressful environmental factors and include compounds that protect plants from microorganisms, pests and other predators. These natural compounds can be used to control growth of pathogenic microorganisms in food products, either by inhibiting growth or killing them. Research in this field is in progress (see, for example [22] [23] [24]). Plant secondary metabolites fall into three main chemical classes: (1) phenolic compounds, the most abundant group of metabolites which protect plants from microbes, (2) terpenes and terpenoids, mono- and sesquiterpenes are important constituents of essential oils, (3) alkaloids. Not less important but much less studied group of plant components that can be used in medicine and agriculture are represented by polypeptide-based molecules. A special role in the development of novel drugs and crop protectants is occupied by endogenous antimicrobial peptides AMPs—effector

molecules of innate immunity both in plants and animals that provide defense against a wide range of pathogens—bacteria, fungi and viruses. One of their most important advantages is that they do not promote resistance development in microorganisms.

In our work, we tested antimicrobial activity of several medicinal plants belonging to different families and widely spread in Russian Federation and former USSR: *C. album, P. major, E. elongata, F. ulmaria,* and *N. sativa*. The first three species are weeds, and all of them are edible plants, and thus safe for human health. To the best of our knowledge, before our study these plant species have not been examined simultaneously against important human bacterial pathogens (*L. monocytogenes* and *E. coli*) and plant fungal pathogens. To extract most completely biologically active compounds from these species, we have developed a stepwise extraction procedure including different types of solvents that allowed us to isolate both hydrophobic and hydrophilic seed components. For the first time, the RP-HPLC step was additionally included in the procedure to recover polypeptide-based molecules (RP-HPLC-bound fraction). We discovered that the antimicrobial activity of these seed extracts against *E. coli* O157:H7 and *L. monocytogenes* EGD-e was highly variable: from complete absence or low antibacterial activity (*C. album*) to strong inhibition by extracts from *P. major*, *E. elongata* and *F. ulmaria* (Table 1 and Table 2) with the highest activity observed in unbound fractions of acetic acid extracts. This fraction mainly contains hydrophilic non-protein components. The compounds present in the ethanol extract and in the RP-HPLC-bound fractions of the acetic acid extracts of all plant species did not display antibacterial activity. This indicates that ethanol-soluble components of seeds do not exhibit antibacterial activity, and that this activity is not associated with proteins. Seed extracts also differed in their ability to inhibit Gram-positive and Gram-negative bacteria. Both Gram-positive and Gram-negative bacteria were inhibited by the components present in RP-HPLC-unbound fractions of the extracts from *P. major*, *E. elongata* and *F. ulmaria*. *N. sativa* extracts suppressed growth of only Gram-positive *L. monocytogenes*, while hexane and ethyl acetate extracts of *F. ulmaria* and *P. major* were active only against *E. coli*.

Extracts from *F. ulmaria* exhibited the highest of all tested plants inhibitory activity against *E. coli* O157:H7. The hexane and ethyl acetate extracts as well as the RP-HPLC-unbound fraction of the acetic-acid-soluble extract suppressed growth of the bacterium. In the case of *L. monocytogenes*, only the RP-HPLC-unbound fraction was active. It should be noted that the identified chemical constituents in *F. ulmaria* are salicylic acid, methyl salicylate, essential oils, flavone glycosides, flavonoids avicularin, quercetin and luteolin, and tannins [14]. Most of these components exhibit antibacterial/antifungal activity and may contribute to the antibacterial activity of the active extracts. We may suggest that essential oil components soluble in hexane are responsible for the antibacterial activity against *E. coli*. Essential oil constituents of a number of plants have been shown to display antibacterial activity. For example, carvacrol present in essen-

tial oils of *Origanum vulgare* L. inhibits *L. monocytogenes in vitro* due to permealization of membranes [25]. We may suggest a similar mechanism for the hexane extract of *F. ulmaria*. Tannins also show antimicrobial activity, antilisterial, in particular, and they are present in *F. ulmaria* [5]. The antifungal activity of *F. ulmaria* resided mainly in the ethanol extract and RP-HPLC fractions. They caused complete inhibition of spore germination in all fungi and the oomycete *P. infestans*. The antifungal activity of RP-HPLC-unbound fraction is associated with hydrophilic seed components soluble in 0.1% TFA, while the activity of RP-HPLC-bound fraction, with polypeptide-based molecules. Ethanol-soluble biologically active compounds, presumably salicylic acid readily soluble in ethanol, are responsible for the potent antifungal activity of the ethanol extract of *F. ulmaria* seeds. These data are in accordance with literature data on inhibition of soil-borne bacterial and fungal pathogens with salicylic acid [26] [27].

P. major ethyl acetate extract and the RP-HPLC-unbound fraction were highly active against *E. coli*, while the hexane extract was moderately active. Only the RP-HPLC-unbound fraction was active against *L. monocytogenes*. This RP-HPLC fraction was also highly active against the fungal pathogens. It completely inhibited spore germination in *F. culmorum* and *S. nodorum*. The RP-HPLC-bound fraction was active only against *S. nodorum*. The biologically active compounds responsible for the antimicrobial activity of the extracts have not been identified. We may suggest that the biological activity of the RP-HPLC-unbound fraction is mainly associated with water-soluble iridoid glycoside aucubin, although this plant species has many other valuable components with bactericidic and fungicidic activities, such as flavonoids, organic acids (benzoic, salicylic, citric), oxy-cinnamic acids (chlorogenic, ferulic, caffeic acid), saponins, and even alkaloids in small amounts [28] [29]. The antimicrobial activity of *P. major* extracts have been analyzed in a number of studies, and the antibacterial activity against some human pathogens was shown that is in accord with our results [30] [31] [32] [33] [34].

To the best of our knowledge, the antimicrobial properties of *E. elongata* have not been studied so far, and its chemical composition is unknown, however, its close relative *E. repens* contains carbohydrate triticin, flavonoid tricin, organic acids, volatile oil, consisting of agropyrene and monoterpenes carvacrol, carvone, thymol, menthol, menthone, and sesquiterpenes. The active substances of the aqueous extract are polysaccharides, flavonoids and tannins [35]. We discovered strong antibacterial activity of the RP-HPLC-unbound fraction of *E. elongata* against *E. coli* and *L. monocytogenes* and suppose that it may be associated with the antibiotic effect of agropyrene and some other mentioned-above compounds. Furthermore, both RP-HPLC fractions of the acetic acid extract of seeds displayed potent antifungal activity against tested plant pathogens: they completely inhibited spore germination in both *Fusarium* species and *S. nodorum*, and strongly suppressed this process in *A. alternata* and *B. sorokiniana*. We assume that the antifungal activity of the RP-HPLC-unbound fraction is due to agropyrene and other bioactive secondary metabolites, while that of RP-HPLC-

bound fraction, with antimicrobial peptides. However, isolation and comprehensive structural and functional analysis of all active components is necessary to prove this suggestion.

N. sativa is called a miracle herb [17] because the seeds of this plant are widely used in treatment of various diseases. The therapeutic properties of *N. sativa* are associated with the presence of thymoquinone, which is the major component of essential oil [17]. Other active components of *N. sativa* seeds are thymohydroquinone, dithymoquinone, carvacrol, 4-terpineol, sesquiterpene longifolene, thymol and others. Seeds also contain two types of alkaloids—nigellicimine and nigellicimine-N-oxide, as well as nigellidine and nigellicine. In addition to secondary metabolites, we demonstrated that *N. sativa* seeds contain two highly active antifungal peptides—defensins Ns-D1 and Ns-D2 [36]. In this work, we showed that seed extracts from *N. sativa* were inactive against Gram-negative *E. coli* O157:H7, while potent inhibitory activity was shown for hexane and ethyl acetate extracts against Gram-positive *L. monocytogenes*. We suppose that this activity is associated with thymoquinone present in essential oil and extracted with hexane. Our results agree with those of other researchers. The antibacterial activity of *N. sativa* seeds against other Gram-positive bacteria, such as *S. aureus*, was reported [37]. The involvement of thymoquinone in bactericidal activity of *N. sativa* seeds against Gram-positive cocci *S. aureus* and *S. epidermitis* was shown in a separate study with purified thymoquinone [38]. In our work, we also showed that the antifungal activity of *N. sativa* seeds against plant pathogens was mainly associated with the RP-HPLC-bound acetic-acid-soluble fraction, which contains polypeptide-based molecules. This fraction completely inhibited spore germination in *P. infestans* and *B. sorokiniana* after 24 h of incubation (**Figure 2(a)** and **Figure 2(d)**). We suppose that the antifungal activity of this fraction is due to highly potent defensins Ns-D1 and Ns-D2 [36]. Antifungal activity of *N. sativa* extracts was analyzed in a number of studies. The antifungal activity of aqueous extracts of *N. sativa* against *Candida* sp. was reported by Bita *et al.* [39]. The main components of *N. sativa* essential oil—thymoquinone and thymohydroquinone—were shown to possess strong antiyeast activity [40].

C. album was reported to possess a number of valuable compounds [41] [42], among them a novel phenolic glycoside named chenoalbuside isolated from the methanol extract of seeds [43], saponins [44], cinnamic acid amides [45], alkaloid chenoalbicin [46], phenols and lignans [47], free phenolic acids, gallic acid, caffeic acid, syringic acid and vanillin [48]. However, in our antibacterial tests against *E. coli* O157:H7 and *L. monocytogenes* EGD-e the extracts of *C. album* seeds showed no activity, except for weak activity (diameter of inhibition zone was less than 8 mm) of the ethyl acetate extract against *L. monocytogenes*. Singh et al (2011) reported antibacterial activity of aqueous and methanol extracts of *C. album* leaves against several human pathogens including *E. coli* [49]. However in another study, methanolic and ethanolic extracts from leaves and flowers were inactive against selected bacterial strains [50]. Although inactive against human bacterial pathogens, the extracts from this species displayed potent anti-

fungal activity. The RP-HPLC-bound fraction of the acetic acid extract completely inhibited spore germination in *F. oxysporum* and *S. nodorum*, and in *B. sorokiniana* germination was strongly inhibited by this fraction. The results on high antifungal activity of *C. album* extracts agree with those of Javaid A and Amin M (2009) who showed strong antifungal activity of the methanol inflorescence extract of *C. album* against *Macrophomina phaseolina* [51].

5. Conclusion

Different extracts from five medicinal edible plants widely spread in Russian Federation and former USSR were evaluated for antibacterial activity against two important bacterial food-borne pathogens (*E. coli* and *L. monocytogenes*) and potent antimicrobial activity for four of them (*P. major, F. ulmaria, E. elongata, N. sativa*) was shown. Furthermore, strong antifungal activity against six fungal and one oomycete pathogen of extracts from all plants was demonstrated. Further detailed analysis of biologically active fractions will form the basis for the development of environmentally safe natural products for medicine, food industry and agriculture.

Acknowledgements

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