Antibacterial and fungicidal effect of ethanol extracts from *Juniperus sabina*, *Chamaecyparis lawsoniana*, *Pseudotsuga menziesii* and *Cephalotaxus harringtonia*

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We determined a high antibacterial effect of ethanol extracts of four species of gymnosperms (*Juniperus sabina*, *Chamaecyparis lawsoniana*, *Pseudotsuga menziesii* and *Cephalotaxus harringtonia*) against 23 strains of bacteria of families Enterobacteriaceae (Escherichia coli, Enterococcus faecalis, Salmonella typhimurium, S. adobraco, Proteus vulgaris, P. mirabilis, Serratia marcescens, Klebsiella pneumoniae), Staphylococaceae (Staphylococcus aureus, S. epidermidis), Yersiniaeae (Yersinia enterocolitica), Bacillaceae (Bacillus subtilis, B. cereus), Listeriaeae (Listeria innocua, L. monocytogenes), Corynebacteriaceae (Corynebacterium xerosis), Campylobacteraceae (Campylobacter jejuni), Nocardiaceae (Rhodococcus equi), Pseudomonaceae (Pseudomonas aeruginosa) and one strain of fungi of the Saccharomycetaceae family (Candida albicans). The experiment in vitro revealed zone of inhibition of growth of colonies, measuring over 8 mm, produced by ethanol extracts from *J. sabina* against seven species of bacteria (*S. aureus*, *B. subtilis*, *B. cereus*, *L. innocua*, *C. xerosis*, Rh. equi and *P. aeruginosa*), *Ch. lawsoniana* – against five species (*E. coli*, *B. subtilis*, *L. innocua* and Rh. equi), *P. menziesii* – two species (Rh. equi and *P. mirabilis*), *C. harringtonia* – ten species of microorganisms (*E. coli*, *S. aureus*, *S. epidermidis*, *L. innocua*, *L. monocytogenes*, *C. xerosis*, *C. jejuni*, *P. vulgaris*, *S. marcescens* and *C. albicans*). As a result of the research, the most promising plants for further *in vivo* study of antibacterial activity were *C. harringtonia* and *J. sabina*.

Keywords: growth inhibition zone; bacterial colonies; multi-resistant strains; gymnosperms; candidiasis.

Introduction

Over recent years, due to spread of antibiotic-polyresistant strains of bacteria which are poorly susceptible to treatment, more and more often reports emerge, describing the potential of the search for efficient antibacterial substances in ethanol plant extracts (Boyko et al., 2016; Zazharskyy et al., 2019a, b; Palchykov et al., 2019, 2020). Potentially, a subject of significant interest in this aspect is various groups of gymnosperms used for the greening of settlements.

*Cephalotaxus harringtonii* (Forbes) K. Koch known as Japanese plums-yew is an evergreen coniferous shrub or small tree from the Taxaceae family. Sciolchelophyte, eutrophic, mesohyte. Quite winter-hardy (second category), quite drought-tolerant (second category) species. Needs protection against wind over winter. The plants grow in deep rich soils. *C. harringtonii* can be used in group plantations in parks. The species is native to Japan, but sometimes is used in Western countries. Several forms are grown for this purpose. In Japan, *C. harringtonii* grows from Kyusyu in the south to Hokkaido in the north. Particularly, the species is found in Hondo in Chiba Prefecture on Kiyosami Mountain located in Awa district of Awa province. It is also common in Nagasaki and Hiroshima Prefectures. The range variety occurs in the eastern part of Honshu, and also Hokkaido, especially on coastal rocks and highland areas (Tripp, 1995). In Europe, *C. harringtonii* has been cultivated since 1829. Many gardeners are familiar with this species named after Charles Harrington, the fourth duke Harrington, one of the first who grew the plant in a European garden in Elvaston. Orncatexeine, a substance obtained from the leaves of the plant is a new preparation against leukemia.
along streams. The species is very sensitive to infection by Phytophthora lateralis Tucker & Milbrath.

Juniperus sabina L. or savin juniper, savin juniper of the Cupressaceae family is a dioecious, humifuse shrub 1.0–1.5 m high. It rapidly grows in width and forms dense thickets. More rarely, small trees 4 m in height with curved trunks are seen. The prostate or shrub-like tree form occurs in many cities of Europe. Evergreen, phytosocial, ground cover, soil protective, winter-hardy (first category) and drought-hardy (first category) species. Heliothyme, mesophyte, xeromosphyte. It is widely used for decorating rocky hills, slopes, in singular or group plantations on lawns or forest edges. Smoke and gas-resistant. Resilient in urban conditions, can be used for greening streets, except in industrial areas. Light-loving,emanding in soil requirements, has soil-protecting abilities. Forests and groves of J. sabina are common in the steppe zone, on rocky mountain slopes and sandy dunes; sometimes the plant extends from the lower to the higher mountain belt (1,000–2,300 m above the sea level), where it forms thickets. The range includes Asia Minor, the Caucasus, Russia (Urals, Siberia and Primorje), South-East Asia, South and Central Europe. J. sabina grows in very rare relic communities, and has a limited distribution, being at the verge of extinction (García-Cervigón et al., 2018; Lambovska-Hristova & Bancheva, 2019). Shoots contain essential oil and are poisonous (San Feliciano et al., 1991; Batatsatsvili et al., 2017). Shoots and juniper berries contain glycosides, saponins, flavonoids, tannins and up to 17% sabinaol, which causes severe poisoning and miscarriages in cattle. Toxic properties of juniper sabina limit its application in therapeutic use. Most often, in folk medicine it is used as an external preparation. Ointment is rubbed into the roots of hair in treatment of alopecia, applied to body areas affected by scabies, tincture is used against warts. Powder is sprinkled over pus ulcer. Attention from fresh branches with leaves is used in homeopathic treatment of diseases of the kidneys and the bladder, strumagia, gout, painful menstruations and impaired monthly cycle (Gabunov et al., 1976). The oils of this plant were dominated by α-pinene, sabinefine, and cedrol.

### Results

As a positive control we used disks with 15.0 µg of azithromycin – macrolid antibiotic of broad spectrum. Disks with 15.0 µg amphotericinin were also used as a second control against Candida albicans. After 24 h growth of the culture was assessed using a multi-angle ruler for measuring growth inhibition zones in microorganisms (Antibiotic Zone Scale-C, model PW297, India) and the program TpsDig2 (2016, F. James Rohlf). The data in tables are presented as x ± SD (standard deviation).

### Materials and methods

Leaves and shoots of four species of coniferous plants were collected in the territory of Botanical Garden of Oles Honchar Dnipro National University (Khirnykh et al., 2018; Boyko & Brygadyrenko, 2019), dried at room temperature, cut, weighed and kept 24 h in 70% ethyl alcohol, filtrated. Antibacterial activity of the plant tinctures were identified using disk-diffusion in agar. Out of the 24 h culture of the ethanol strains of microorganisms of the family, a weighed amount was prepared according to the standard of turbidity of bacterial suspension equaling 0.5 units of density according to McFarland (McF) 1.5 × 10^8 CFU (colony-forming units), which was determined using a densitometer (Densimeter II). The obtained weighed amount was re-inoculated to Muller-Hinton agar (Himedia) followed by cultivation in a thermostat TSO-80/1 over 24 h at the temperature of 37 °C. On top of the re-inoculations, the disks saturated with the tinctures of the extracted ethanol tinctures of the four species of plants were placed (Table 1).

### Table 1

| Family       | Species                     | Used part of plant | The most important literature sources on the effect of the plant on bacteria                                                                 |
|--------------|-----------------------------|--------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Cupressaceae | *Juniperus sabina* L.       | shoots             | Eliseevsky & Brinža (2018), Zivić et al. (2019)                                                                                           |
| Cupressaceae | Chamisa cyarko lawsoniana | leaves             | Smith et al. (2007), Kim et al. (2015), Pača-Paši et al. (2015)                                                                             |
| Prunaceae    | *Pseudotsuga menziesii* (Mirb.) Franco | shoots | Takano et al. (2010), Dwivedi et al. (2015)                                                                                               |
| Taxaceae     | *Cephalotaxus harringtonii* (Forbes) K. Koch | leaves | Watanabe & Fukao (2009)                                                                                                                   |

### Discussion

Research has been conducted on species of gymnosperm plants, which apart from antibacterial and antifungal activity are also charac-

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terized by cytostatic properties against different types of the tumour tissues. Shokrzadeh et al. (2009), Janar et al. (2012) and Huyan et al. (2016) noted moderate inhibiting activity of diterpenoids of C. lawsoniana, toxicity against P. menziesii. Table 2

Table 2
Width of the growth inhibition zone (mm) determined by the extracts of Juniperus sabina, Chamaecyparis lawsoniana, Pseudotsuga menziesii and Cephalotaxus harringtonia against 24 strains of microorganisms (n = 12)

| Strains of microorganisms | Juniperus sabina | Chamaecyparis lawsoniana | Pseudotsuga menziesii | Cephalotaxus harringtonia |
|---------------------------|-----------------|-------------------------|-----------------------|---------------------------|
| Enterococcus faecalis ATCC 19433 | 4.3 ± 0.65 | 0 ± 0 | 1.1 ± 0.23 | 0 ± 0 | 25.9 ± 2.45 |
| Enterobacter aerogenes ATCC 10006 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 15.9 ± 1.67 |
| Escherichia coli F50 | 4.8 ± 0.35 | 8.8 ± 0.67 | 4.3 ± 0.34 | 2.7 ± 0.21 | 17.8 ± 1.87 |
| E. coli 055 | 4.5 ± 0.34 | 9.3 ± 0.56 | 3.7 ± 0.42 | 10.7 ± 1.45 | 15.6 ± 1.62 |
| Staphylococcus aureus ATCC 25923 | 11.5 ± 0.64 | 4.4 ± 0.32 | 4.1 ± 0.33 | 21.2 ± 2.41 | 21.6 ± 2.45 |
| S. epidermidis ATCC 14990 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 13.4 ± 1.45 | 103 ± 1.34 |
| Bacillus subtilis ATCC 6633 | 8.4 ± 1.12 | 10.6 ± 1.55 | 0 ± 0 | 0 ± 0 | 30.3 ± 0.05 |
| B. cereus ATCC 10702 | 1.4 ± 0.87 | 1.6 ± 0.62 | 0 ± 0 | 5.7 ± 0.76 | 18.3 ± 0.86 |
| Listeria ivanovii | 2.3 ± 0.43 | 0 ± 0 | 9.3 ± 0.76 | 14.7 ± 1.21 |
| L. innocua ATCC 33090 | 12.4 ± 1.55 | 10.6 ± 1.18 | 0 ± 0 | 25.1 ± 1.98 |
| L. monocytogenes ATCC 19112 | 0 ± 0 | 0 ± 0 | 8.1 ± 0.87 | 0 ± 0 |
| Corynebacterium xerosis 1911 | 10.6 ± 0.56 | 6.4 ± 0.43 | 6.8 ± 0.57 | 9.7 ± 0.89 | 93.3 ± 1.34 |
| Campylobacter jejuni ATCC 11322 | 0 ± 0 | 11.4 ± 1.12 | 0 ± 0 | 14.0 ± 1.12 |
| Rhodococcus equi ATCC 6093 | 27.5 ± 2.89 | 14.3 ± 1.54 | 8.7 ± 0.83 | 4.4 ± 0.42 | 19.1 ± 1.98 |
| Proteus vulgaris ATCC 13315 | 2.8 ± 0.54 | 5.1 ± 0.43 | 0 ± 0 | 8.3 ± 0.87 | 0 ± 0 |
| P. mirabilis ATCC 14153 | 2.1 ± 0.23 | 0 ± 0 | 10.5 ± 0.93 | 0 ± 0 | 0 ± 0 |
| Salmonella typhimurium ATCC 14028 | 3.4 ± 0.21 | 2.2 ± 0.13 | 1.9 ± 0.13 | 0 ± 0 | 20.3 ± 1.54 |
| S. adelaide 1 | 2.7 ± 0.19 | 2.7 ± 0.39 | 1.4 ± 0.12 | 0 ± 0 | 26.3 ± 2.76 |
| Pseudomonas aeruginosa ATCC 2353 | 10.5 ± 0.98 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| P. aeruginosus ATCC 2799 | 0 ± 0 | 6.4 ± 0.78 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Klebsiella pneumoniae ATCC 13883 | 2.5 ± 0.21 | 1.7 ± 0.12 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Yersinia enterocolitica ATCC 9610 | 2.5 ± 0.22 | 0 ± 0 | 0 ± 0 | 12.8 ± 1.27 |
| Serratia marcescens ATCC 8100 | 1.2 ± 0.23 | 0 ± 0 | 2.8 ± 0.23 | 10.4 ± 0.94 | 0 ± 0 |
| Candida albicans ATCC 2091 | 2.2 ± 0.41 | 3.7 ± 0.34 | 3.4 ± 0.31 | 9.8 ± 0.92 | 2.4 ± 0.21* |

Note: * – discs with 15.0 µg of azithromycin were used for all bacteria as positive control; ** – discs with 15.0 µg amphotericin were used as positive control for Candida albicans (Valle et al., 2015).

Palá-Paúl et al. (2015) researched Ch. lawsoniana. They showed that the essential oils from young shoots and leaves of Ch. lawsoniana exert high antibacterial and antifungal activity towards Candida albicans, Bacillus subtilis, Staphylococcus aureus and Micrococcus luteus: essential oil was more effective against gram-positive rather than gram-negative bacteria. Recently, interest has developed to the use of essential oils from shoots of Ch. lawsoniana in commercial products, such as preparations against pests and cosmetics (Duringer et al., 2015). To assess the relative toxicological risk from these oils for fresh-water algae equaled 1.25 (72 h) and 0.63 mg/L (96 h). The emergence of antibiotic-resistant bacteria has caused difficulties in the treatment of infectious diseases (Kim et al., 2015). Methicillin-resistant Staphylococcus aureus is one of the commonest recognized antibiotic-resistant bacteria. New antibiotics for the treatment of MRSA in humans are urgently needed. Raw materials obtained from natural sources can be used for the development of new antibiotics, for example Chamaecyparis obtusa (Siebold & Zucc.) Endl., which is traditionally used for treating asthma. Kim et al. (2015) studied antibacterial activity of essential oil from leaves of Ch. obtusa against MRSA. Growth of MRSA and production of acid during metabolism of glucose was inhibited in Staphylococcus by the concentration of 0.1 mg/mL of Ch. obtusa. Smith et al. (2007) showed that within the framework of a project aimed at characterizing the antibacterial or immune-modulating activity of compounds of immature conifer cones against multi-drug resistance (MDR) strains of S. aureus, eight compounds were extracted from Ch. lawsoniana. Active compounds are mostly diterpenes with minimum inhibitory concentrations equaling 4–128 µg/mL against strains of S. aureus which are resistant to mexiticillin (EMRSA). Out of Ch. lawsoniana, diterpene ferrugiol, pisiferol and its epimer 5-epipisiferol, formazan oxide, trans-
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Ferruginol and 6,12-dihydroxiabieta-5,8,11,13-tetraene-7-one and abieta-8,11,13-trien-7β-ol. They conducted in vitro studies on these compounds against clinically isolated bacteria and strains of Candida. Ferruginol and 6,12-dihydroxiabieta-5,8,11,13-tetraene-7-one exerted antimicrobial activity against several gram-positive bacteria. None of the six diterpenes was active against gram-negative organisms and tested species of yeasts.

Evanova et al. (2008) observed significant antifungal activity of harringtonolide—a complex poly cyclic condensed norsterpenes from C. harringtonia var. drupacea. Ram and Kumar (2001) also confirmed anti- turnour activity of C. harringtonia. Smith et al. (2008) extracted a new abietane diterpen, from the bark of the stem of Prumnopitys andina (Poeppl. ex Endl.) de Laub. This new compound presented antibacterial activity in 8 µg/mL against two strains of S. aureus, but it is interesting that it was not active in 128 µg/mL against a strain of wild-type and Methicillin-resistant S. aureus. Smith et al. (2008) note that ferruginol was active against these four strains of S. aureus. Presence of acetoxy group caused harmful effect on the antibacterial activity towards certain strains.

The antibacterial in vitro effect which we observed from J. sabina exceeded a 8 mm wide zone of inhibition of growth of 7 species of bacteria (S. aureus, B subtilis, B cereus, L. innocua, C. xerosis, Rh. equi and P. aeruginosa). Ch. lawsoniana had the same effect against 5 species (E. coli, B. subtilis, L. innocua and Rh. equi), P. menziesii—only against 2 species (Rh. equi and P. mirabilis) and C. harringtonia—against 10 species of microorganisms (E. coli, S. aureus, S. epidermidis, L. ivanovii, L. monocytogenes, C. xerosis, C. jejuni, P. vulgaris, S. marcescens and C. albicans). Further detailed study of individual chemical substances present in those plants should be undertaken in both in vitro and in vivo experiments.

**Conclusion**

None of the four species of gymnosperms produced notable (growth inhibition zone of over 8 mm) effect towards E. faecalis, E. aerogenes, S. typhimurium, S. adolbraco, K. pneumoniae and Y. enterocolitica. Negative effect (growth inhibition zone of over 8 mm) on E. coli was exerted by alcohol extracts of Ch. lawsoniana and C. harringtonia, extracts of J. sabina and C. harringtonia took effect on S. aureus, only C. harringtonia affected S. epidermidis, against B. subtilis—J. sabina and Ch. lawsoniana were effective, against B. cereus—only J. sabina, against L. ivanovii—only C. harringtonia, against L. innocua—J. sabina and Ch. lawsoniana, against L. monocytogenes—only C. harringtonia, against C. xerosis—J. sabina and C. harringtonia, against C. jejuni—only C. harringtonia, against Rh. equi—J. sabina, Ch. lawsoniana and P. menziesii, against P. vulgaris—only C. harringtonia, against P. mirabilis—only P. menziesii, against P. aeruginosa—only J. sabina, against S. marcescens—only C. harringtonia, and against C. albicans only alcohol extract of C. harringtonia had negative effect. We consider that it is practical to recommend ethanol extracts from J. sabina, Ch. lawsoniana, P. menziesii and C. harringtonia, or individual compounds present in them, to be used in further studies on treatment against polyresistant strains of the abovementioned microorganisms.
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