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EXPERIMENTAL PAPER

In vitro antimicrobial activity of extracts and their fractions from three *Eryngium* L. species

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Summary

Introduction: Due to increasing resistance against antibiotics and antifungal agents, crude plant extracts, fractions, and isolated pure compounds became a new interest as antimicrobial agents. **Objectives:** The antimicrobial activity of methanolic extracts and fractions of *Eryngium planum* L., *E. campestre* L., and *E. maritimum* L. was evaluated against selected bacteria, yeast and mould, and compared in tested *Eryngium* species and in their organs. **Methods:** The antimicrobial activity was studied with use of broth microdilution method. The antibacterial (*Staphylococcus aureus*, *Pseudomonas aeruginosa*) and antifungal (*Candida albicans*, *Aspergillus niger*) activity of selected extracts and fractions compared with the reference substance was expressed by Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal/Fungicidal Concentration (MBC/MFC).The extract and fraction compounds were identified on the basis of TLC examination. **Results:** The saponin-phenolic acid fractions of *E. maritimum* and *E. planum* and a saponin fraction of *E. planum* showed the highest activity against *S. aureus* (MIC = 1–2.5 mg·ml^{-1}). The growth of *C. albicans* was inhibited by methanolic extract of *E. planum* cell suspension culture (MIC = 7.8 mg·ml^{-1}). **Conclusion:** The antimicrobial activity depends on the *Eryngium* species, tested biomass, and microorganism.

Key words: *Eryngium planum, E. campestre, E. maritimum, antibacterial activity, antifungal activity*

INTRODUCTION

Severe infections caused by multidrug-resistant bacteria continue to pose significant treatment challenges. These difficult-to-treat pathogens include e.g.: methicillin-resistant *Staphylococcus aureus,* Gram-negative rods [1]. Biofilms of *Pseudomonas aeruginosa* can cause chronic opportunistic infections. They often cannot be effectively treated with traditional antibiotic therapy [2]. Numerous reports provide the evidence that the prevalence of antibiotic-resistant bacteria is growing. This phenomenon has led to a major research effort to find alternative antimicrobial therapies. *Candida* species with *C. albicans* as the most common are casual agents of opportunistic human infections. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns [3]. *Aspergillus niger* is likely to cause human disease if large amounts of spores are inhalated due to a serious lung disease. Over the years, amphotericin B has become a standard treatment for aspergillosis, although the response remains poor [4]. There is a dire need to search for new classes of antimicrobial substances, especially from natural sources.

The genus *Eryngium* L. belongs to the subfamily *Saniculoideae* of the family *Apiaceae* [5]. Three of 26 species described in "Flora Europaea" grow in restricted regions in Poland (*E. planum*, *E. campestre*, *E. maritimum*) [6, 7]. The pharmacological activity of *Eryngium* species depends mainly on their saponin content, but the presence of phenolic acids, flavonoids, polyacetylenes, and betalains is also important for their usage in traditional medicine [8]. Roots of field-grown Polish *Eryngium* species contain complex of triterpenoid saponins [9, 10]. The main phenolic acids occurring in *Eryngium* genus are caffeic acid derivatives, mostly rosmarinic and chlorogenic acids [9, 11, 12].

Several species of Eryngo are used in various parts of the world in traditional medicine. The statement regarding the use of *E. planum* species mentioned in 1993 concerns it as a remedy for whooping cough in Transylvania. *E. planum* is used as a whole herb infusion of one teaspoonful of the raw material [13]. The aerial part and roots of *E. maritimum* are used in folk medicine as appetizer, antitussive, diuretic, stimulant and aphrodisiac [14]. Roots of *E. maritimum* were used to prevent scurvy in England and to induce menstruation in Algeria [15]. Moreover, the roots were used to promote flatulence, as an urethritis remedy, stone inhibitor, obstructions liver remover [15]. *E. campestre* is used in the treatment of many diseases instead of *E. maritimum*. The medicinal parts are dried roots, leaves and flowers. Eryngo root is administered in tea mixtures, extracts, decoctions, liquids and tinctures for the treatment of bladder and kidney stones, renal colic, kidney and urinary tract inflammation, and edema. It is also used for coughs, bronchitis, skin and respiratory disorders. The herb of *E. campestre* is administered as an extract and in homeopathic dilutions in the treatment of urinary tract infections and as an adjuvant to treat inflammation [16].

In this study, the antibacterial and antifungal activities of *Eryngium* methanolic extracts and their fractions were tested and compared in different species, organs and undifferentiated *in vitro* culture – cell suspension. This is a continuation of previous studies on antimicrobial activity of Polish species of *Eryngium* L. genus [17].

MATERIAL AND METHODS

Plant material

Leaves and roots of *Eryngium planum* L. plants were collected in Łukaszewo (Poland) in August 2008. Biomass of *E. planum* cell suspension (passage 9th) cultured in MS [18] with 1.0 mg·l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) was taken for analysis. Plant material of *Eryngium maritimum* L. was collected from Botanical Garden of Adam Mickiewicz University (Poznań, Poland) in September 2009. *Eryngium campestre* L. was collected from steppe reserve Owczary near Kostrzyn nad Odrą (Poland) in August 2009.

The voucher specimens were deposited in the Herbarium of the Medicinal Plants Garden at the Institute of Natural Fibres and Medicinal Plants in Poznań, Poland.

Preparation of extracts and fractions

Extracts

The air-dried and powdered biomass was extracted three times with 30 ml 70% methanol (methanol-water 7:3, v/v) for 1 h at the boiling point temperature under reflux. The combined, cooled and filtered extracts were concentrated under pressure lower than 40°C. The methanolic extracts from *E. planum* leaves (32.4 g), roots (145.45 g), and cell suspension culture (1.0 g) (yields 39.1%, 41.4%, 60%, respectively); *E. campestre* leaves (24.6 g) and roots (33 g) (yields 24.6%, 33%, respectively); *E. maritimum* leaves (31.5 g) and roots (11.6 g) (yields 31.5%, 48.33%, respectively) were assayed for antimicrobial activity.

The extract compounds were identified on the basis of TLC examination (see next section).

Fractions

The portions (0.809 g, 0.400 g, 0.404 g) of the root extracts were separated in Sep-Pak C 18 cartridges (820 mg; 1.6 ml; Waters) using water, methanol-water mixtures (40% and 80% methanol) and methanol for elution. The fraction compounds were identified on the basis of TLC-coumarin fraction (eluted with 40% methanol) and saponin-phenolic acid fraction (eluted with 80% methanol). The respective 40% and 80% methanolic fractions obtained from *E. planum* roots: 0.1429 g and 0.0232 g, *E. campestre* roots: 0.0634 g and 0.0092 g, *E. maritimum* roots: 0.0094 g and 0.0700 g were also tested.

Saponin fraction

The air-dried and powdered roots of *E. planum* (351.0 g) were extracted with boiling 70% ethanol (4 x 3 l). The combined extracts were evaporated to give a dry extract (145.5 g), a portion of which (136.0 g) was separated over a polyamide (SC-6, Macherey-Nagel) column (9 x 32 cm) eluted with water, 100% methanol and 0.01% ammonia in methanol. Column fractions were combined after TLC examination to give sugar, saponin and phenolic acid fractions. For the antimicrobial activity estimation, saponin fraction was used.

Chromatographic TLC analysis

For detection of phenolic acids and flavonoids, the 5 μl aliquots of each methanolic extract and fraction were applied as 1.0 cm streaks to the cellulose and HPTLC silica gel plates (10 x 20 cm, Merck, Germany). The plates were developed in chambers with ethyl acetate-acetic acid-water (8:1:1 v/v/v) mixture, dried and viewed under UV_{366} nm or day light, before and after spraying with following reagents: (i) NA (Roth) 0.1% solution in ethanol for detection of phenolic acids (blue bands under UV) and flavonoids (yellow bands under UV), (ii) $AlCl_3$ 1% solution in ethanol (followed by heating) for detection of flavonoids. Coumarins were recognized by strong blue fluorescence under UV_{366} nm light. For saponins detection, the 5 μl aliquots of each methanolic extract and fraction were applied as 1.0 cm streaks to the HPTLC silica gel plates (10 x 20 cm, Merck, Germany). The plates were developed with 1-buthanol-acetic acid-water 4:1:5 (v/v/v) mixture and viewed in daylight after spraying with vanillin-sulphuric acid reagent. The spots with violet-pink colour in daylight were considered as those of saponins. TLC analysis for polyacetylenes were carried out on silica gel $60F_{254}$ (Merck), eluted with toluene-ethyl acetate (9:1) mixture. The chromatograms were sprayed with 0.38% $KMnO_4$ solution. The acetylenes were recognized as yellow spots against pink background in daylight.

Antimicrobial activity analysis

Microorganisms and media

The *in vitro* antimicrobial activity of the *Eryngium* extracts and fractions were measured using (i) bacteria strains *Staphylococcus aureus* ATCC 4163, *Pseudomonas aeruginosa* ATCC 6749, (ii) yeast *Candida albicans* ATCC 10231, (iii) mould *Aspergillus niger* ATCC 16404. The test microorganisms were obtained from American Type Culture Collection (ATCC).

Bacterial and yeast strains were stored in Microbank cryogenic vials (ProLab Diagnostics, Canada) at -70±10°C. Moulds were maintained on Sabouraud dextrose agar (SDA; Merck) slants at 10°C.

Bacteria cultures were grown in Brain Heart Infusion broth (BHI, BioMerieux, France) at 34°C for 18 h, yeast cultures were grown in Sabouraud dextrose broth (SDB, Merck) at 34°C for 18 h. After incubation, each culture was diluted in suitable liquid medium (bacteria – Mueller-Hinton broth (MHB; Oxoid, UK); *C. albicans* – Sabouraud dextrose broth (SDB, Merck, Germany) to obtain a final suspension containing about 10^6 CFU/ml.

Moulds were inoculated on Sabouraud dextrose agar (Merck, Germany) and incubated at 34°C for 5 to 8 days for adequate sporulation. After incubation, cultures were covered with sterile 0.9% NaCl solution supplemented with 0.1% Tween 80, carefully rub with a sterile cotton swab and transfer to a sterile flask. Suspensions were homogenized and filtered. Number of spores in the suspension was determined using serial dilution method. Before using, the suspension was diluted in Sabouraud dextrose broth to obtain final suspension containing 2-5x10^5 spores per ml.

Determination of MIC, MBC/MFC

The antimicrobial activity of examined extracts and fractions was studied by employing a broth microdilution method in accordance with EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines with modifications, using: Mueller-Hinton broth (MHB) – bacteria and Sabouraud dextrose broth (SDB) – fungi [19, 20]. The known amount of each extract and fraction was dissolved in methanol (POCH, Poland) to obtain a stock solution. A solution of each extract and fraction was two-fold serially diluted in a culture broth to get the final concentration ranging from 170 mg·ml^{-1} to 1 mg·ml^{-1}. Aliquots of 100 μl of each dilution were distributed in 96-well plates (Kartell, Italy). The sterility control and a growth control (containing culture broth plus solvent, without antimicrobial substance) were performed. Each test and growth control well was inoculated with 100 μl of a microbial suspension. The microdilution trays were incubated at 34°C for: 18 h – bacteria and *C. albicans*; 48 h – *A. niger*. The MIC was defined as the lowest concentration at which visible growth was inhibited.

After performing MIC test and recording the MIC end point, every well that demonstrated no growth (concentration equal to and higher than MIC) was subcultured onto agar medium: Typcase soy agar (TSA; BioMerieux) – bacteria; Sabouraud dextrose agar (SDA; Merck, Germany) – fungi. The plates were incubated at 34ºC for: 18 h – bacteria and *C. albicans*; 48–72 h – *A. niger*. The MBC/MFC was defined as the lowest concentration at which no growth was observed.

Amikacin for bacteria and fluconazole for fungi were used as a reference positive control. Amikacin showed MIC = 1 μg·ml^{-1} against *S. aureus,* MIC = 2 μg·ml^{-1} against

P. aeruginosa. MIC for fluconazole determined to be 512 μg·ml^{-1} against *A. niger* and >1024 μg·ml^{-1} against *C. albicans*. All tests were performed in duplicate and the antimicrobial activity was expressed as the mean values.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

This is the first report on antimicrobial activity of *E. planum*, *E. campestre*, *E. maritimum* methanol-water fractions from the roots of intact plants, saponin fraction from roots of *E. planum* and additionally methanolic extracts from *in vitro* cell suspension cultures of *E. planum*. Our preliminary studies of antimicrobial activity of ethanolic extracts from leaves and roots of three Polish *Eryngium* species against *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans*, *C. glabra*, *Aspergillus niger, Cryptococcus neoformans* and *Trichophyton mentagrophytes* indicated that those extracts demonstrated a moderate antibacterial and significant antimycotic activity [17].

The antimicrobial activity of *Eryngium* species depends on the qualitative and quantitative content of compounds present in the tested material. TLC analysis of *Eryngium* showed that complex of saponins, phenolic acids and acetylenes are present in all studied extracts. *Eryngium* leaves were characterized, in comparison to roots, by presence of flavonoids, different level of phenolic acids and acetylenes, and various profiles of saponins complex. The separation on a C18 adsorbent of the root extracts yielded to 40% methanolic fractions containing coumarins and 80% methanolic fractions containing saponins and phenolic acids. Column chromatography separation yielded the saponin fraction of *E. planum* roots. Our previous UPLC analysis, indicated that phenolic acid content (rosmarinic and chlorogenic acids) varies between organs of the same species and between different species (*E. planum* – 0.96 mg·g^{-1} for leaves, 0.19 mg·g^{-1} for roots; *E. campestre* – 5.32 mg·g^{-1} for leaves, 3.69 mg·g^{-1} for roots, *E. maritimum* – 0.45 mg·g^{-1} for leaves, 0.72 mg·g^{-1} for roots) [9, 12; for *E. campestre* data unpublished]. Moreover, for saponin complex, there is a difference in composition and content between *Eryngium* species (*E. planum* – 2 saponins in amount of 0.19 mg·g^{-1} for leaves, all 6 saponins 5.63 mg·g^{-1} for roots) and *E. maritimum* – 1 saponin in amount of 0.05 mg·g^{-1} for leaves and 3 saponins 0.67 mg·g^{-1} for roots) [12; for *E. planum* and *E. campestre* data unpublished].

Our results indicated that the methanolic extracts and fractions showed various degrees of antimicrobial activity, depending on the *Eryngium* species, tested biomass and microorganism (tab. 1, 2). The saponin-phenolic acid fractions (80%) of *E. maritimum* and *E. planum* showed the highest antimicrobial activity (MIC = 1–2 mg·ml^{-1}) against *S. aureus*, followed by a saponin fraction of *E. planum* (MIC = 2.5 mg·ml^{-1}). The inhibitory effect of the methanolic extracts from roots of all tested species (MIC = 12.5 mg·ml^{-1}) and *E. planum* cell suspension culture (MIC = 7.8 mg·ml^{-1}) was found against *C. albicans*. In addition, all tested extracts showed a moderate antifungal activity toward *A. niger* (MIC = 21.3–85 mg·ml^{-1}).

The results of antibacterial activity against *S. aureus* of saponin-phenolic acid fraction (80% methanol fraction) and saponin fraction from *E. planum* clearly

Table 1.

Determination of MIC (mg·ml^{-1}) of *Eryngium* species methanolic extracts, methanol-water fractions (40%, 80%) and saponin fraction against different microorganisms

Microorganism	*E. planum*						*E. campestre*				*E. maritimum*			
	Methanolic extracts			Fractions			Methanolic extracts		Fractions		Methanolic extracts		Fractions	
	Cell suspension	Leaves	Roots	Roots 40%*	Roots 80%*	Saponin fraction·	Leaves	Roots	Roots 40%*	Roots 80%*	Leaves	Roots	Roots 40%*	Roots 80%*
S. aureus	7.8	10.6	-	>25	2	2.5	12.5	12.5	>25	4	10.6	50	12.5	1
P. aeruginosa	15.6	15.6	-	>25	>8	20	25	25	>25	>4	21.3	50	12.5	>4
C. albicans	7.8	85	12.5	>25	>8	20	25	12.5	>25	>4	>170	12.5	12.5	>4
A. niger	>15.6	85	25	>25	>8	>40	50	50	-	-	21.3	50	-	-

MIC – Minimal Inhibitory Concentration; * – fractions eluted from Sep-Pak C18 cartridge with 40% methanol (coumarin fraction) and 80% methanol (saponin-phenolic acid fraction); – fractions separated over a polyamide column

Table 2.

Determination of MBC/MFC (mg·ml^{-1}) of *Eryngium* species methanolic extracts, methanol-water fractions (40%, 80%) and saponin fraction against different microorganisms

Microorganism	*E. planum*						*E. campestre*				*E. maritimum*			
	Methanolic extracts			Fractions			Methanolic extracts		Fractions		Methanolic extracts		Fractions	
	Cell suspension	Leaves	Roots	Roots 40%*	Roots 80%*	Saponin fraction·	Leaves	Roots	Roots 40%*	Roots 80%*	Leaves	Roots	Roots 40%*	Roots 80%*
S. aureus	31.3	>170	-	>25	8	10	25	>50	>25	>4	>170	>100	12.5	1
P. aeruginosa	31.3	85	-	>25	>8	20	>25	>50	>25	>4	>170	>100	12.5	>4
C. albicans	>15.6	>170	12.5	>25	>8	20	>50	>50	>25	>4	>170	>100	12.5	>4
A. niger	>15.6	>170	>100	>25	>8	>40	>50	>50	-	-	>170	>100	-	-

MBC/MFC – Minimal Bactericidal/Fungicidal Concentration; * – fractions eluted from Sep-Pak C18 cartridge with 40% methanol (coumarin fraction) and 80% methanol (saponin-phenolic acid fraction); · – fractions separated over a polyamide column

indicated that the antimicrobial activity did not depend only on saponin content. Moreover, the content of saponins in *E. planum* roots is 8.5-fold higher than in the roots of *E. maritimum* as it is clear from our previous quantitative research [see above] but the fraction from roots of *E. maritimum* showed higher antibacterial and antifungal activities. However, as is apparent from the same UPLC analysis *E. maritimum* roots have 3.5 times more phenolic acids. *E. campestre* roots have a strong antimicrobial activity although UPLC analysis did not detect the presence of tested saponins in the material, in contrast to the two other species. However these results cannot be explained in this simple way, since this species have saponins containing D-rhamnose instead of L-rhamnose as a terminal carbohydrate in contrast to *E. planum* and *E. maritimum*, which was published in the article of Kowalczyk *et al.* [10]. The activity is probably based on a synergistic action of saponins and phenolic acids. In all cases the saponin-phenolic acid fractions (80% methanol fraction) characterized considerably higher inhibition of tested microbe growth than coumarin fraction (40% methanol fraction). The treatment of bacteria and fungi with the fractions gave better results than with extracts.

The antimicrobial activity of extracts has been described for some *Eryngium* species [21-28]. The results of Meot-Duros studies on *E. maritimum* antimicrobial activity of leaves extracts showed that apolar fractions are generally more active, as compared to polar fraction. *Pseudomonas aeruginosa* and *P. fluorescens* were the most sensitive bacteria to root extracts [21]. Kholkhal *et al.* showed the high antibacterial activity of methanolic extract from *E. maritimum* root against *P. aeruginosa* and moderate activity against *S. aureus* [22]. The ethanolic extracts from *E. maritimum* roots exhibited activity against *S. aureus* (MIC 2.5 $mg \cdot ml^{-1}$) [23]. According to other authors, the ethanolic extracts of *E. caucaseum* and *E. bungei* have shown moderate antibacterial activity against *S. aureus* and *Streptococcus pyogenes*, which cause the most bacterial infections of the skin [24]. Furthermore, the methanolic extracts from aerial parts and roots of *E. palmatum* showed activity toward *S. aureus*, *P. aeruginosa* and *C. albicans* with MIC values in the range of 7.8–15.6 $\mu g \cdot ml^{-1}$ [25]. The antifungal activity (MIC) of the volatile extract of *E. duriaei* was $>20\ \mu g \cdot ml^{-1}$ for *C. albicans* [26]. The essential oil of *E. thorifolium* showed considerably higher activity compared to *E. campestre* and *E. creticum*. The anti-MRSA (*S. aureus*) activity of this species was comparable with those of reference antibiotic vancomycin [27].

The chemical structure of antimicrobial compounds found in plants belongs to most common classes of secondary metabolites such as phenolic acids, flavonoids, coumarins and triterpenoid saponins. Phenolic compounds inhibit microbial adhesions and inactive cell envelope transport protein [29].

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WŁAŚCIWOŚCI PRZECIWBAKTERYJNE EKSTRAKTÓW I ICH FRAKCJI TRZECH GATUNKÓW *ERYNGIUM* L.

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Streszczenie

Wstęp: Z powodu rosnącej oporności na antybiotyki i środki przeciwgrzybicze rośnie zainteresowanie ekstraktami roślinnymi, frakcjami i wyizolowanymi czystymi związkami jako środkami przeciwdrobnoustrojowymi. **Cel:** Badano aktywność przeciwdrobnoustrojową ekstraktów metanolowych i frakcji *Eryngium planum* L., *E. campestre* L. i *E. maritimum* L. w stosunku do wybranych bakterii, drożdżaka i grzyba pleśniowego oraz porównywano badane gatunki *Eryngium* i ich organy. **Metody:** Aktywność przeciwdrobnoustrojową badano z zastosowaniem metody seryjnych rozcieńczeń w bulionie. Aktywność przeciwbakteryjna (*Staphylococcus aureus, Pseudomonas aeruginosa*) i przeciwgrzybicza (*Candida albicans*, *Aspergillus niger*) wybranych ekstraktów i frakcji, w porównaniu z substancją referencyjną, wyrażona została za pomocą minimalnego stężenia hamującego (MIC) i minimalnego stężenia bakterio- lub grzybobójczego (MBC/MFC). **Wyniki:** Frakcje saponinowo-fenolokwasowe *E. maritimum* i *E. planum* oraz frakcja saponinowa *E. planum* wykazały najwyższą aktywność wobec *S. aureus* (MIC = 1–2,5 mg·ml $^{-1}$). Wzrost *C. albicans* był hamowany przez metanolowy ekstrakt zawiesiny komórkowej *E. planum* (MIC = 7,8 mg·ml $^{-1}$). **Wnioski:** Aktywność przeciwdrobnoustrojowa zależy od gatunku *Eryngium*, testowanej biomasy i mikroorganizmu.

Słowa kluczowe: *Eryngium planum, E. campestre, E. maritimum, aktywność przeciwbakteryjna, aktywność przeciwgrzybicza*