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ASSESSMENT OF ANTIFUNGAL ACTIVITY OF EXTRACTS FROM NETTLE (*Urtica dioica* L.) AGAINST *Alternaria solani*

OCENA AKTYWNOŚĆ PRZECIWGRZYBOWEJ EKSTRAKTÓW Z POKRZYWY ZWYCZAJNEJ (*Urtica dioica* L.) WOBEC *Alternaria solani*

Abstract: The aim of research was to assess antifungal activity of nettle extracts at 2.5, 5.0, 10.0, 20.0, 40.0% concentrations obtained from a root and leaves against phytopathogenic strain *Alternaria solani*. Their antifungal activity was assessed on the basis of the growth rate index of mycelium and the spore germination index. It has also been assessed how different sterilisation techniques affect the properties of extracts under study. It has been proved, on the basis of obtained results, that extracts sterilised with saturated steam under pressure did not show antifungal activity. For the root extract, which showed the highest antifungal activity, only 7% of the mycelial growth inhibition have been obtained. Whereas, the root extracts sterilised by filtration limited the mycelial growth by 75% and spore germination of *A. solani* by 38%.

Keywords: antifungal activity, *Urtica dioica*, *Alternaria solani*, growth rate index, spores germination index

Introduction

Tomatoes grown in field conditions are very often infested by *Alternaria* fungi, which cause alternaria diseases. The crops may be decreased due to leaves damage and the tomatoes damage. The pathogen may cause the tomato seedling blight and fruit rot [1]. The protection of plants is mainly based on the proper agricultural technology, an application of resistant plant varieties and the use of conventional chemical fungicides. However, the current schemes of plant disease control are being verified more frequently and natural substances *eg* plant extracts, are introduced as a possibility of plant protection [2]. In ecological cultivations of tomatoes, it is recommended to perform copper-sprayings alternately with grapefruit extracts, once the risk of plant disease occurs. The results of recently conducted research have proved that natural sources of compounds with antibacterial, antifungal and antioxidant properties are herbal plants including *Urtica dioica* L. often called common nettle or stinging nettle [3, 4].

Urtica dioica is widespread through Europe and North America, and also occurs in North Africa and parts of Asia. There are naturalised populations in several other parts of the world. The plant has a long history of use as a source of medicine, food and fibre [3, 5, 6].

The commonly known phytochemical compounds from *U. dioica* are lectins, sterols, terpenes, volatile compounds and fatty acids, polysaccharides, protein, vitamins, minerals and flavonoids [3, 6]. The flavonoids are mainly kaempferol, isorhamnetin, quercetin, isoquercitrin and rutin. The shikimic acid derivatives like phenylpropanes, caffeic acid and various esters of this acid such as chlorogenic acid and caffeoyl malic acid have been identified. The leaves are rich in vitamins B, C, K (phylloquinone), carotenoids and

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minerals such as calcium, iron, magnesium, phosphorus, potassium and sodium [6, 7]. Other main constituents present are amino acids, tannins, glucokinnins and chlorophyll. GC-MS analysis of *U. dioica* essential oil by Gul et al [8] identified 43 compounds. The main components of essential oil of *U. dioica* are carvacrol, carvone, naphthalene, (E)-anethol, hexahydrofarnesyl acetone, (E)-geranyl acetone, (E)-β-ionone and phytol [8].

Due to varied phytochemical composition, the common nettle has been universally applied, however, among herbal plants it has been most underrated. Not many research papers describe its potential application when fighting against phytopathogenic moulds, focusing on its medical properties instead. In folk medicine, nettle plants have been used as a diuretic, antibleeding, stimulating blood circulation, nutritional, anticancer, antiatherosclerotic, antiasthmatic, antidandruff, hemostatic and hypoglycemic. For a long time nettle has been used to treat arthritis and rheumatism. It also inhibits inflammation of the urinary tract and the digestive tract, improves digestion and an absorption of nutrients. It stimulates the immune system, increasing the resistance against infections (immunostimulant) [3, 6, 9, 10].

The raw materials collected for the research are leaves, stem, root with rhizomes and seeds. Most often, however, leaves and roots are used [3, 6]. The raw material is very often microbiologically infested and may be an additional source of contamination. Therefore, prior to its application, it is recommended to perform a sterilisation process, which in consequence, as it a thermal process, may lead to changes in natural compounds content and their activity. The above mentioned changes depend on the processing conditions of the plant, mainly the length of time and the temperature of heating.

The aim of the research was to assess the antifungal activity of nettle extracts (*U. dioica*) subject different sterilisation processes against phytopathogenic strain *Alternaria solani*.

Materials and methods

In the research, the antifungal activity of commercial extracts from the root and leaf subject to sterilisation process have been assessed. The dry root extract containing 0.8% of phytosterols contained less than 300 CFU/g of microorganisms and the leaf extract of about 100 CFU/g. The analysed extracts were used to prepare aqueous solutions at the following concentrations: 2.5, 5.0, 10.0, 20.0 and 40.0%. The solutions were left for several hours at the room temperature and sterilised afterwards. The sterilisation process was carried out with saturated steam pressure in an autoclave for 15 minutes at 121°C and the pressure of 2 bars. The other sterilisation method employed vacuum filtration with Sartorius membrane filters with pore size of 0.2 µm. Obtained extracts were marked as STA (sterilisation in the autoclave) and STF (filtration sterilisation) respectively.

In the experiment, an indicator strain was *Alternaria solani* - strain isolated from the surface of the tomato fruit infested with alternaria disease.

A fungistatic activity of the extracts under study was assessed with the culture-plate method on PDA medium containing [g/dm^3]: glucose 20.0, potato extract 4.0, agar 15.0. In test trials, certain concentrations of root and leaf extracts were added on the culturing medium and then the plates were inoculated with fungi discs of 10 mm diameter. The control trial contained cultures of *A. solani* with sterile water instead of the supernatant. All

plates were incubated at 25±2ºC for 10 days. Every 1-2 days the measurements of the fungi discs diameter were noted until the mycelium of *A. solani* in the control trial overgrown the plate. All measurements were conducted in 3 replicates, where one replicate was the plate with the culturing medium and one mycelial disc. The activity of nettle extracts on the growth of *A. solani* was assessed against the growth rate index, calculated according to the formula [11]:

$$GRI = \frac{A}{D} + \frac{b_1}{d_1} + \frac{b_2}{d_2} + \ldots + \frac{b_x}{d_x}$$

where GRI represents the growth rate index, A is a mean value of diameter measurements [mm], D is the length of the experiment (number of days), b_1, b_2, b_x denote an increase in a diameter size since the last measurement and d_1, d_2, d_x are the number of days since the last measurement.

The evaluation of fungistatic activity was also carried out on the basis of spores germination of *A. solani* in the presence of tested concentrations of nettle root and leaf extracts. In the control trial the spores were suspended in water. All tests were run in 3 replicates. Microscopic observations were completed after 24 hours of the extracts activity, and the spores germination evaluation was based on the following scale [11]:
0 - non-germinatin conidia,
1 - germ tube shorter than the length of the spore,
2 - the length of germ tube equal to the length of the spore,
3 - germ tube twice as long as the length of the spore,
4 - germ tube branched and many times longer than the spore.

The effect of tested extracts on the spore germination has been evaluated on the basis of germination index, calculated according to the formula [11]:

$$SGI = \frac{\sum (n \cdot a) \cdot 100}{N \cdot 4}$$

where: *SGI* represents the spores germination index, n - number of the spores in the specific grade on the scale, a - grade on the scale, N - general number of the counted spores, 4 - the highest grade of the scale.

The toxicity of the nettle was measured as a percentage of the colony growth inhibition or the germination ability showed in the presence of the analysed extracts against control trials containing water (controls).

The research has not included tests on the control trials which were not sterile, as preliminary analysis showed that plant material was heavily contaminated microbiologically, which prevented the measurement of the mycelium and conidia observation.

In order to determine the significant statistical differences ($p < 0.05$) between *U. dioica* extracts sterilised with different methods, ANOVA analysis has been applied with Tukey's HSD test.

Results and discussion

Presented papers show a pilot research, in which fungistatic activity of *U. dioica* root and leaf extracts against pathogenic strain *A. solani* have been evaluated. Conducted tests allowed to determine how sterilisation methods of the extracts affect their activity and the growth pace of tested strain.

Table 1

Influence of extracts of *U. dioica* sterilised in the autoclave (STA) and by filtration (STF) on the mycelial growth rate index of *A. solani*

	STA				STF			
	Root extract		Leaf extract		Root extract		Leaf extract	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	57.5 [a]	±2.2	58.2 [a]	±2.1	58.8 [a]	±1.2	58.8 [a]	±1.2
2.5%	55.54 [a]	±0.64	55.4 [a]	±1.6	15.14 [b]	±0.81	15.80 [b]	±0.69
5.0%	54.7 [a]	±1.3	54.9 [a]	±1.4	19.5 [c]	±2.8	16.47 [b]	±0.82
10.0%	54.6 [a]	±3.8	55.9 [a]	±3.2	15.60 [bc]	±0.45	16.80 [b]	±0.86
20.0%	54.9 [a]	±1.9	53.9 [b]	±1.6	17.97 [bc]	±4.3	15.5 [b]	±2.0
40.0%	53.3 [a]	±2.4	55.6 [a]	±3.2	14.7 [bd]	±1.4	17.09 [b]	±0.86

Different letters indicate significant differences (ANOVA, $p < 0.05$, Tukey's HSD test)

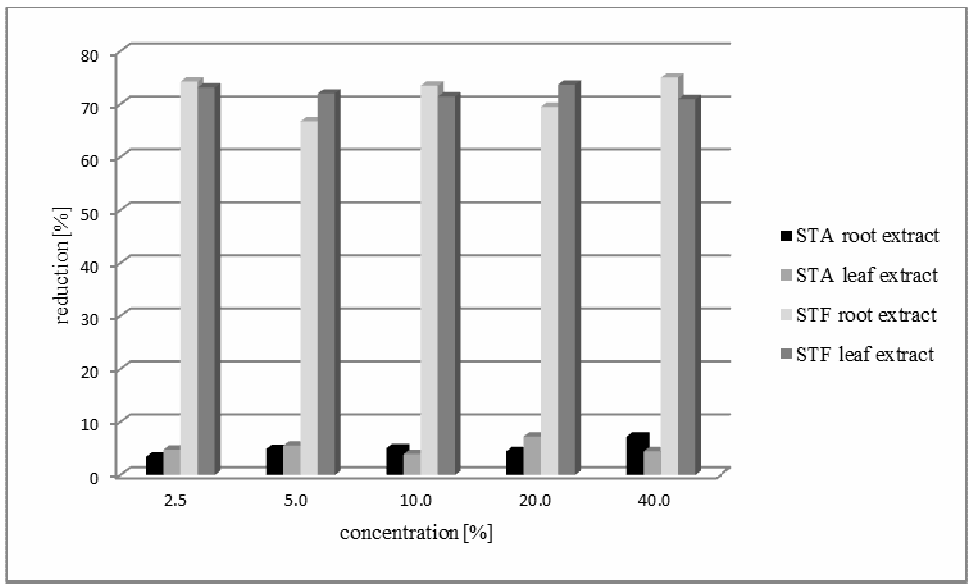

Fig. 1. The reduction of the mycelial growth rate index of *A. solani* induced by *U. dioica* extracts sterilised in the autoclave (STA) and by filtration (STF)

The values of GRI characterising the mycelial growth pace of *A. solani*, for the consecutive concentrations of the roof and leaf extracts sterilized in autoclave (STA) and by filtration (STF) have been presented in Table 1. The table presents arithmetic mean values of GRI and standard deviation for the trial. The highest noted values of GRI, not exceeding

58 units, were obtained in control trials. Plant extracts sterilized by saturated steam under pressure did not inhibit the mycelial growth of *A. solani* and no statistically significant differences were noted for the root and leaf extracts in comparison with the control trials. The opposite has been observed when sterilisation applied filtration method. In this case statistically significant differences were obtained between tested and control trials. However, no clear correlation has been found between the type of the extract, its concentration and GRI. The highest value of GRI has been noted for the root extract at 0.5% concentration and the lowest at 40.0% concentration obtaining the growth reduction of 75% (Table 1, Fig. 1). For the remaining extracts under study and their concentrations, measured values of GRI fluctuated at a similar level and obtained differences were not statistically significant. Antifungal activity of the nettle extracts have also been proved by other researchers [12, 13]. Hadizadeh et al [12] obtained full inhibition of the mycelial growth of *A. alternata* after the application of 0.9% nettle extract. It should be noted, however, that applied extract was the ethanol extract not the aqueous extract used in the presented paper. The same results were obtained by Hadizadeh et al [13] with the application of 1500 and 2000 ppm essential nettle oil. Similar results were noted by Tapwal et al [14], who observed the highest mycelial growth inhibition of *A. solani* and *A. zinniae* (41.67 and 29.87% respectively) when 20.0% aqueous nettle extract was applied.

In conducted studies, it has also been assessed how *U. dioica* extracts affect spore germination abilities of *A. solani* depending on the concentration of the extracts. As shown in Table 2 the extracts sterilised in the autoclave did not show antifungal activity. Although no statistically significant differences have been noted between the control trial and tested trial, it should be noted that SGI values in tested trials were higher than the values obtained in the control trials. On the basis of microscopic observations, it has been noted that in the control trials dominated germinating spores of the 4th type on the scale and single non-germinating spores were present. In the test trials only spores constructing very long and multi-branched tubes were visible. It has been assumed that all spores germinated as type 4 and the value of SGI amounted 100. The above results show, that extracts sterilised in the autoclave simulated germination of *A. solani* only slightly. Therefore Figure 2 does not include any data concerning values of SGI obtained when sterilisation in the autoclave was applied. However, extracts sterilized with filtration inhibited spores germination. Siginificant statistical differences in values of SGI have been noted for all concentrations of the root extracts and for 5.0% concentration of the extract from the nettle leaf. For the root extracts, the lowest value of SGI amounting 60.21 units have been obtained for 40.0% extract, which caused reduction higher that 37% (Table 2, Fig. 2). However, according to other research papers, the spores transported with the rain drops or by wind are responsible for spreading the pathogen and their growth reduction would allow to inhibit the alternaria diseases [1, 15]. For this reason, 37% reduction obtained in own research might not be sufficient in practice, as proved by research of Pati and Kolte [16], who obtained 86.6% reduction of the germinating spores of *A. brassicae* after the application of *U. dioica* extract and described it as a poor result. The essential nettle oil used by Hadizadeh et al [13] did not allow to completely reduce germinating spores either. The authors obtained the dicrease in germinating spores of 93.9% and the reduction in the length of germinating hyphae. The above show that spores of *Alternaria* spp. are less sensitive to the nettle extracts than the mycelium.

Table 2

The effect of *U. dioica* extracts sterilised in the autoclave (STA) and by filtration (STF) on the spore germination index of *A. solani*

	STA				SF			
	Root extract		Leaf extract		Root extract		Leaf extract	
	mean	SD	mean	SD	mean	SD	mean	SD
Control	96.2 [a]	±3.9	96.2 [a]	±3.9	97.09 [a]	±0.61	97.09 [a]	±0.61
2.5%	100 [a]	±0	100 [a]	±0	82.03 [bc]	±0.78	94.8 [a]	±3.2
5.0%	100 [a]	±0	100 [a]	±0	89.72 [b]	±0.14	74.0 [b]	±2.5
10.0%	100 [a]	±0	100 [a]	±0	92.2 [b]	±1.2	96.4 [a]	±1.7
20.0%	100 [a]	±0	100 [a]	±0	91.3 [b]	±1.3	95.7 [a]	±1.7
40.0%	100 [a]	±0	100 [a]	±0	60.2 [bd]	±1.3	94.3 [a]	±1.6

Different letters indicate significant differences (ANOVA, $p < 0.05$, Tukey's HSD test)

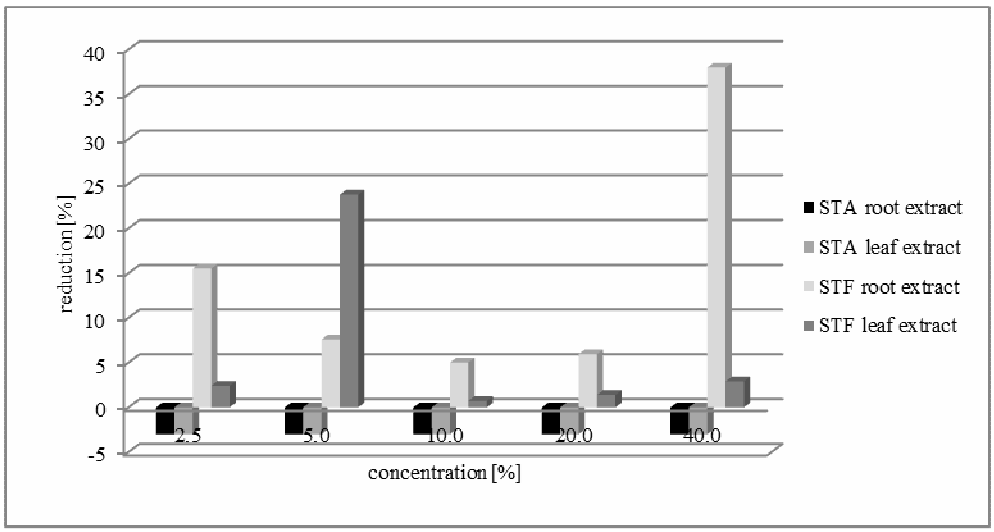

Fig. 2. Reduction of the spore germination index of *A. solani* induced by *U. dioica* extracts sterilized by filtration (STF)

In Poland, the tomato field plantations are protected against alternaria disease with numerous conventional fungicides. In order to reduce the risks associated with the application of pesticides and their effects on people's health it is advised to develop and introduce an integrated plant protection or techniques, which aim at reducing the amount of applied pesticides. A good solution might be extracts prepared on the basis of common herbal plants. However, natural extracts used in plant protection, may be microbiologically contaminated and become a new source of contamination. The contamination may be primary and may depend on the natural environment or secondary, caused for example by an improper storage of plants, their packaging or transportation. In order to avoid the contamination of herbal material, the plants are sterilised. It should be noted, that it is a thermal process and may lead to change in the content of natural ingredients, which will directly affect their properties. These changes depend largely on the conditions of the process, that is the time and the temperature of warming the plant material, which has been

proved by the authors' own research. It has been proved that extracts of *U. dioica* under study, sterilised with saturated steam under pressure do not show antifungal properties against phytopatogenic strain *A. solani* contrary to extracts sterilized with the use of filters. Furthermore, in the research, the root extract of *U. dioica* was more active than the extract from the leaf. This is probably associated with the differences in chemical content of the extracts and further changes in this content which may occur under the high temperature. The influence of the temperature on antimicrobial activity of plant extracts is not clear though. It may be stated, referring to other research papers, that the activity is closely connected with chemical composition of the extract. In most cases, extracts sterilised with the filtration method show higher inhibitory activity against fungi than extracts sterilized in the autoclave [17]. For example, Venturoso et al [18] proved high antifungal activity of garlic extract sterilised with filtration method, whereas sterilisation method applied to cinnamon and clove extracts had no effect on their activity. In the research of both Abimbola [19] and Hashemi et al [20] plant extracts sterilised in the autoclave showed lower antimicrobial activity in comparison with the filtered ones. The authors explained that the differences in the antimicrobial activity induced by high temperature may be the result of many factors involved. The heating process and higher pressure may cause damage to the cell wall and thus increase the bioavailability of antimicrobial substances. Moreover, during the thermal process new compounds with antifungal properties may be formed. Furthermore, filtration is a mechanical or physical operation. Mechanical filtration is typically achieved by passing water or solvent through materials which act as a sieve, physically trapping the particulate matter. Contaminants or bacteria are removed by filter through a membrane having microscopic holes that allow water or solvent molecules, but not larger compounds, to pass through. It is also possible that some phytochemical compounds cannot pass through the filter [20]. The results obtained in Kim et al [21] study seem to suggest that any loss in the antimicrobial activity of a compound which had undergone hydrolysis is being compensated by the increase in activity of some other compounds as a result of the high temperature.

Chemical analyses of *U. dioica* revealed the presence of many valuable chemical compounds like phytosterols, saponins, flavonoids and tannins [6]. Some of these compounds have been reported to be hydrolysable at high temperatures either into more active compounds or less active compounds. In conducted research the extracts sterilised in the autoclave have not shown antifungal properties, therefore there is high probability that inhibitors in the nettle are thermolabile. High temperature caused damage in their structure, which led to either complete or partial loss of antifungal activity.

Conclusions

The paper presents obtained results in conducted research on the assessment of antifungal activity of extracts from *U. dioica,* which were subject to the sterilisation process in the autoclave and with the use of filters. The following has been concluded:

- root extract from *U. dioica* shows higher antifungal activity in comparison with leaf extract. An application of 40.0% aqueous root extract caused 75% mycelial growth inhibition and 38% inhibition of spores germination of *A. solani*;

- antifungal activity of nettle extracts is not inhibited when extracts are sterilized with filtration; it may be an indication that filtration is a suitable choice for the sterilisation of *U. dioica* root extract;
- sterilisation with the saturated steam under pressure caused a complete loss of inhibitory properties of extracts under study;
- extracts from nettle have the potential application in the protection of tomato plant against *A. solani*. The future for using plant extracts and plant products is promising, because they are less expensive and less hazardous to the environments.

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OCENA AKTYWNOŚĆ PRZECIWGRZYBOWEJ EKSTRAKTÓW Z POKRZYWY ZWYCZAJNEJ (*Urtica dioica* L.) WOBEC *Alternaria solani*

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Abstrakt: Celem pracy była ocena aktywności przeciwgrzybowej ekstraktów z korzenia oraz z liścia pokrzywy zwyczajnej (*Urtica dioica* L.) w stężeniach 2,5, 5,0, 10,0, 20,0, 40,0% wobec fitopatogennego szczepu *Alternaria solani*. Aktywność przeciwgrzybową ekstraktów z pokrzywy określono w oparciu o indeks tempa wzrostu grzybni oraz indeks kiełkowania zarodników. W badaniach określono również wpływ metod sterylizacji testowanych wyciągów na ich aktywność. Na podstawie uzyskanych wyników wykazano, iż ekstrakty sterylizowane parą wodną pod ciśnieniem nie wykazują aktywności przeciwgrzybowej. W przypadku ekstraktu z korzenia, który charakteryzował się większą aktywnością inhibicyjną, uzyskano zaledwie 7% redukcję tempa wzrostu grzybni. Natomiast ekstrakt z korzenia poddany sterylizacji przez sączenie ograniczał o 75% wzrost grzybni i o 38% kiełkowanie zarodników *A. solani*.

Słowa kluczowe: aktywność przeciwgrzybowa, *Urtica dioica*, *Alternaria solani*, indeks tempa wzrostu, indeks kiełkowania zarodników