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The fresh extracts of Allium species as potential in vitro agents against planktonic and adherent cells of Candida spp.

Świeże ekstrakty gatunków czosnku jako potencjalne czynniki przeciwko planktoidalnym i adherentnym komórkom *Candida spp.* w warunkach *in vitro*

Candida albicans is the predominant etiologic agent of candidiasis. Besides, other species of *Candida* non-*albicans*, e.g. *C. famata, C. glabrata* or *C. krusei* have emerged as opportunistic pathogens. *Candida* spp. is the fourth pathogen involved in nosocomial infections, including those associated with indwelling medical devices [8]. Strains of *Candida* spp. are able to colonize various biomaterials, followed by biofilm formation [2, 3, 6, 13]. The biofilm is a universal, complex, interdependent community of surface-linked microbial cells embedded in the matrix of extracellular polymeric substances [10]. Additionally, biofilm-associated infections are difficult to treat, since fungal cells within the biofilm are intrinsically insensitive to available antifungal drugs after standard dosing [10].

The first step of the biofilm formation is the adhesion. The adherence of *Candida* spp. cells to biomaterials is mediated by both nonspecific factors and by specific adhesins on the fungal cell surface. Attachment of individual fungal cells to a surface is closely followed by cell division, proliferation, and biofilm development [10, 13].

Medicinal plants have been a subject of scientific investigations for many years in order to develop several, alternative, natural drugs, e.g. antimicrobial drugs. Since garlic (*Allium sativum*) is known to possess good antifungal properties [7, 14, 15], the aim of this paper was to compare the activity of fresh extract of this plant against planktonic (free-floating) and adherent cells of *Candida* spp. Also, the activity the fresh extract of three other species of *Allium,* i.e. *Allium ursinum* (the wild garlic) *Allium victorialis* (the victory onion), *Allium scorodoprasum* (the rocambole), was assayed.

MATERIAL AND METHODS

M i c r o o r g a n i s m s a n d c u l t u r e c o n d i t i o n s. A total of 7 strains of *Candida* spp., obtained from nasopharynx of patients with lung cancer undergoing pulmonary resection, included:

C. albicans (3 isolates), *C. famata* (2 isolates), *C. glabrata* (2 isolates), *C. krusei* (1 isolate). The isolates were stored at -20° C in 50% glycerol and cultured on Sabouraud dextrose agar at 30º C for 48 h; before each experiment, the isolates were subcultured on Sabouraud glucose broth (further called Sabouraud medium) at 30º C for 48 h.

P l a n t s. Four species of *Allium*: *Allium sativum, Allium ursinum, Allium victorialis, Allium scorodoprasum* from The Botanical Garden of Lublin (Poland) were examined. From each species a fresh water extract at the concentration 40 mg/ml in phosphate-buffered saline (PBS) at room temperature was obtained according to the method described by Shuford et al. [14]. Briefly, fresh *Allium* cloves were weighed and their cloves were crushed in sterile PBS. The fresh extract of each plant was submitted to sterilized filtration through 45µm filters using a vacuum pump (AGA Labor).

D e t e r m i n a t i o n o f m i n i m a l i n h i b i t o r y c o n c e n t r a t i o n (M I C) a n d m i n i m a l f u n g i c i d a l c o n c e n t r a t i o n (M F C) f o r f r e s h e x t r a c t o f *A l l i u m* s p e c i e s a g a i n s t *C a n d i d a* s p p. Determination of MIC for the extract of *Allium* plants was performed by a broth microdilution method, using serial two-fold dilutions of the extracts in Sabouraud medium. Stock inoculum suspensions of yeasts were prepared in Sabouraud medium and adjusted to the optical density corresponding to 0.5 Mc Farland standard, i.e. 150 x 10^6 CFU (Colony Forming Units)/ml diluted 1:100. After incubation at 35° C for 24 h, the MICs were assessed visually as the lowest extract concentration showing complete growth inhibition. In order to determine the MFC for the fresh extract of *Allium* plants, 10 µl from each tube that showed thorough growth inhibition, from the last positive one and from the growth control was streaked onto Sabouraud dextrose agar plates. After incubation at 35° C for 48 h, the MFCs were assessed visually as the lowest extract of plants concentration at which there was no growth. All experiments were done in triplicates. The representative data are presented.

B i o m a t e r i a l s. All assays were carried out on two types of catheters – silicone elastomer-coated latex urinary Foley catheter and PCV Thorax catheter. Catheters used were cut aseptically into ca 0.5 cm^2 fragments and placed into Petri dishes.

D e t e r m i n a t i o n o f m i n i m a l c o n c e n t r a t i o n o f f r e s h e x t r a c t o f *A l l i u m* s p e c i e s a g a i n s t a d h e s i o n b y *C a n d i d a* s p p. o n b i o m a t e r i a l s. MTT tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay [12] was used. The standardized yeast suspensions (optical density of 0.5 Mc Farland standard) were prepared in Sabouraud medium. The yeast suspensions (diluted 1:100) containing a given extract of *Allium* plants at various concentrations were incubated in the presence of the catheter fragments for 24 h at 35° C. Non-adherent cells were removed by careful rinsing of the catheter fragments with sterile PBS and then resuspended in Sabouraud medium, followed by overnight incubation (24 h) at 35° C with a drop of 1% MTT solution. After incubation, in the presence of *Candida* spp. viable cells, MTT was reduced to the violet tetrazolium formazan product on the catheter fragments, accompanied by violet colour of the medium. The control assay was carried out without the extracts. All experiments were done in triplicates. The representative data are presented.

C y t o t o x i c i t y a s s a y. The fresh extracts of *Allium* plants cytotoxicity to green monkey kidney (GMK) cells was determined by MTT toxicity assay [16]. Monolayers of GMK cells were grown in Eagle's Minimal Essential Medium (MEM) supplemented with 100 µg/ml of streptomycin

and 100 U/ml of penicillin. After overnight incubation at 37º C, the medium above the cell culture was removed. The fresh extracts of *Allium* plants (*Allium sativum, Allium ursinum, Allium victorialis*) were added to the 2 ml samples of MEM without calf serum to obtain final concentrations ranging from 0.5 to 4.0 mg/ml. Control assays containing only GMK cells in MEM were also carried out. Cell cultures were incubated at 37º C for 24 h. After incubation, the medium above the cell culture was removed and 1 ml of MEM without calf serum and 100 µl of 5 mg/ml MTT solution in PBS were added to the samples and the incubation was continued for another 4 h at 37º C before addition of 1 ml of aqueous solution containing 50% dimethylformamide and 20% solution of SDS to solubilize the insoluble formazan precipitates produced by MTT reduction. The absorbance of converted dye was measured at two wavelengths: 540 nm (A_{540}) and 620 nm (A_{620}), using ELISA plate reader (Organon Technika Microwell system Reader 530). All experiments were done in triplicates.

RESULTS

According to Table 1, the fresh water extracts of *Allium* plants inhibited the growth of planktonic cells all *Candida* spp. strains with MIC ranging from 0.5 to 4.0 mg/ml, depending on the yeast and plants species. The MFC values were also from 0.5 to 4.0 mg/ml, resulting in a low ratio of MFC/MIC values – 1.0 or 2.0. These data suggest the fungicidal effect of fresh extracts of *Allium* plants against *Candida* spp. The most effective against isolates of *Candida* spp. was the extract of *Allium sativum* (MIC = 0.5–2.0 mg/ml, MFC = 0.5–2.0 mg/ml, MFC/MIC = 1.0–2.0). *Allium scorodoprasum* did not inhibit the growth of *Candida* spp. even at the concentration of 4.0 mg/ml. In these experiments requiring the growth of *Candida* spp., the maximal concentration of the extract of *Allium* plantswas 4.0 mg/ml in order to avoid much dilution of the growth medium. In further experiments concerning the effect of the extracts of *Allium* plants on the adhesion of *Candida* spp. isolates on Foley or Thorax catheters, the maximal concentration of the extracts was also 4.0 mg/ml.

Using the qualitative MTT method, we found that all *Candida* spp. isolates were able to adhere to the catheters used – silicone elastomer-coated latex urinary Foley catheter and PCV Thorax catheter, followed by biofilm formation. This was monitored by the appearance of the violet tetrazolium formazan product and violet-colored medium due to cutting off the adherent or biofilm-embeded cells.

The inhibition of cell adhesion of *Candida* spp. isolates to both catheters was monitored by lack of the violet tetrazolium formazan product after incubation with MTT. Data presented in Table 2 showed that adhesion of *Candida* spp. cells was prevented by extract of *Allium sativum* at the concentration 1.0 or 2.0 mg/ml, i.e. 1.0–4.0 x MIC or MFC, depending on yeast species, except for the adhesion of two isolates – *C. famata* (CF1) and *C. glabrata* not prevented by this extract even at the maximal concentration of 4.0 mg/ml. The adhesion of all *Candida* spp. isolates to both catheters was not prevented by the extract of *Allium ursinum* or *Allium victorialis* even at the maximal concentration of 4.0 mg/ml.

It was found that extracts of *Allium* plants exerted only a slight inhibitory effect on GMK cells even at the concentration of 4.0 mg/ml (cell growth inhibition < 20%).

Table 1. *In vitro* activity of the fresh extract of *Allium* species against planktonic cells of *Candida* spp.

Plant species	Parameter	Isolates						
		CA1	CA2	CA3	CF1	CF2	CG	CK
Allium sativum	MIC (mg/ml)	0.5	1.0	0.5	1.0	0.5	2.0	2.0
	MFC (mg/ml)	1.0	1.0	0.5	2.0	0.5	2.0	2.0
	MFC/MIC	2.0	1.0	1.0	2.0	1.0	1.0	1.0
Allium ursinum	MIC (mg/ml)	2.0	1.0	2.0	4.0	1.0	4.0	4.0
	MFC (mg/ml)	2.0	2.0	2.0	4.0	1.0	4.0	4.0
	MFC/MIC	1.0	2.0	1.0	1.0	1.0	1.0	1.0
Allium victorialis	MIC (mg/ml)	2.0	2.0	2.0	4.0	2.0	4.0	4.0
	MFC (mg/ml)	2.0	2.0	2.0	4.0	2.0	4.0	4.0
	MFC/MIC	1.0	1.0	1.0	1.0	1.0	1.0	1.0

CA1, CA2, CA3 *Candida albicans* isolates; CF1, CF2 – *Candida famata* isolates; CG – *Candida glabrata* isolate; CK – *Candida krusei* isolate

Table 2. The *in vitro* effect of the fresh extract of *Allium* plants on the adherent cells of *Candida* spp.

Plant species	Catheter	Isolates						
		CA1	CA2	CA3	CF1	CF2	CG	CK
Allium sativum	Fc	1.0	1.0	2.0	>4.0	1.0	>4.0	2.0
	Tc	1.0	1.0	2.0	>4.0	1.0	>4.0	2.0
Allium ursinum	Fc	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
	Tc	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
Allium victorialis	Fc	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0
	Tc	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0	>4.0

CA1, CA2, CA3 *Candida albicans* isolates; CF1, CF2 – *Candida famata* isolates; CG – *Candida glabrata* isolate; CK – *Candida krusei* isolate; Fc – Foley catheter, Tc – Thorax catheter

DISCUSSION

Allium plants can be regarded as a source of potential medicinal agents. Garlic (*Allium sativum*) is reported to be a wonderful medicinal plant owing to multiple biological properties, including antifungal activity [7,15]. Our data indicate that the extractof *Allium sativum* was the most effective against planktonic cells of *Candida* spp. isolates compared to other *Allium* species studies – *Allium ursinum* and *Allium victorialis*.*Allium scorodoprasum* appears to be ineffective against yeasts. According to the literature [1, 9], antifungal activity of *Allium sativum* is attributed to the action of sulfur-containing compound – allicin.

Biofilm-associated yeast cells are intrinsically insensitive to traditional antifungal agents [4, 6, 13] or different chemical agents [5]. Shuford et al. [14] studied the *in vitro* activity of the fresh garlic extract against planktonic and adherent cells of *C. albicans*, and found antifugal activity of this extract against *C. albicans* biofilm formation at the adherence phase at higher concentrations (0.5–1.0 mg/l) compared to those active against planktonic cells (0.0625–0.125 mg/l), using semiquantitative tetrazolium reduction assay. Our data, based on the qualitative tetrazolium reduction assay, suggest that among the fresh extracts of *Allium* plants, that of *Allium sativum* appears to be a potential, effective agent preventing *in vitro* adhesion to the biomaterials of *C. albicans* isolates and to a lesser extent by *C.* non-*albicans* isolates, at concentrations ranging from 1.0 to >4.0 mg/ml, that is higher than those effective against planktonic cells, ranging from 0.5 to 2.0 mg/ml, depending on the yeast species. The obtained differences between our data and those of Shuford et al. [14], regarding the effective concentrations of garlic extract, may be connected with the type of medium used; in studies by Shuford et al. [14], yeast nitrogen base was used but in our studies – Sabouraud medium.

CONCLUSIONS

Data by Shuford et al. [14] and data presented in this paper suggest that the fresh water extract of *Allium sativum* seems to be an effective agent preventing *in vitro* adhesionof *Candida* spp., mainly *C. albicans*. Further studies are needed to assess if garlic extract may be use in the prophylaxis of yeast infections associated with some medical devices *in vivo*, also taking into account the half-life of this extract at 37° C [11].

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SUMMARY

The aim of this paper was to compare the activity of fresh water extract of *Allium sativum, Allium ursinum, Allium victorialis, Allium scorodoprasum* against planktonic cells of *Candida spp*. (*C. albicans, C. famata, C. glabrata, C. krusei*) and those attached to the catheters in relation to cytotoxicity of the plant extracts to green monkey kidney (GMK) cells. The extracts of *Allium* plants, besides *Allium scorodoprasum*, inhibited growth of planktonic cells of *Candida* spp. at the concentrations ranging from 0.5 to 4.0 mg/ml, while that of adherent cells – at the concentrations ranging from 1.0 to > 4.0 mg/ml, depending on the yeast and plant species. The most effective was the extract of *Allium sativum*. Extracts of *Allium* plants exerted only a slight inhibitory effect on GMK cells even at the concentration of 4.0 mg/ml.

STRESZCZENIE

Celem pracy było porównanie aktywności świeżych ekstraktów wodnych *Allium sativum, Allium ursinum, Allium victorialis, Allium scorodoprasum* wobec planktoidalnych komórek *Candida spp.* (*C. albicans, C. famata, C. glabrata, C. krusei*) oraz komórek, które uległy adhezji do cewników. Badano również cytotoksyczność ekstraktów roślinnych w stosunku do linii komórkowej GMK. Badane ekstrakty, oprócz ekstraktu z *Allium scorodoprasum*, hamowały wzrost planktoidalnych komórek *Candida* w zakresie stężeń od 0,5 to 4,0 mg/ml, natomiast komórek, które uległy adhezji – w zakresie stężeń od 1,0 do >4,0 mg/ml, w zależności od gatunku drożdżaka i czosnku. Najbardziej aktywny był ekstrakt z *Allium sativum*. Badane ekstrakty wywierały tylko nieznaczny efekt toksyczny na komórki GMK, nawet w stężeniu 4,0 mg/ml.