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INVESTIGATIONS ON FUNGICIDAL PROPERTIES OF 1,3,4-THIADIAZOLE DERIVATIVES

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Abstract: The results of the research concerning the effect of 1,3,4-thiadiazole derivatives on fungicidal *Candida albicans* are discussed. In all the conducted tests the reference strain of *Candida albicans* ATCC 10231 was used. To obtain the desired results, the following standard and synthesized thiadiazole derivatives were tested: 2-acetylamino-1,3,4-thiadiazolo-5-sulfonamide; 2-acetylamino-5-chloro-1,3,4-thiadiazole; 2-amino--1,3,4-thiadiazole; 2-acetylamino-1,3,4-thiadiazole; 2-acetylamino-1,3,4-thiadiazolo-5-sulfonic acid and bis(2--acetylamino-1,3,4-thiadiazolo)-5,5'-disulfonamide. In all the examined compounds the increase in the *Candida albicans* inhibition zone proportional to the increase of the compound concentration was observed. The only exception is 2-acetylamino-1,3,4-thiadiazole demonstrating the opposite tendency. While carrying out the research, it was found that 2-amino-1,3,4-thiadiazole proved to be the most effective of all the compounds within the tested group. It was also found that the higher the concentration of the compound, the higher the growth control zone (y) of *Candida albicans.* This phenomenon can be described by means of the equation: $y = 0.9167x^3 - 7.4286x^2 + 10.655x + 23.1$ (where: x – concentration of 2-amino-1,3,4-thiadiazole).

Keywords: *Candida albicans*, antifungicidal activity, 1,3,4-thiadiazole derivatives, MIC

It is known that 1,3,4-thiadiazole derivatives have biological activity with their antifungal action [1, 2]. Also, metal complexes with 1,3,4-thiadiazole derivatives as ligands showed *in vitro* antifungal activity against *Candida* spp [3]. The published literature discusses the fact that such substance as β-amino 21 acid (BAY 10--8888/PLD-118) demonstrates a high antifungal activity against *Candida albicans* [4]. Additionally, the strains of the bacteria *Candida albicans* are susceptible to such triazoles as: fluconazole 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazole-1-ilo)-propane--2-ol [5]. Frequent infections, occurring as a result of *Candida albicans*'s opportunistic pathogen (attacking weakened organism), are widely treated by antifungal preparation –

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fluconazole, which is an ergosterol biosynthesis inhibitor, the basic sterole compound existing in the fungi cell membrane [6–8].

It is known that the protection against *Candida albicans* was observed in the glucan-treated groups of patients. These observations suggest that the biological response modifiers (BMRs) such as glucan may be effectively applied in case of the patients in risk of the post-operative infection [9–11].

The characteristic features of a *Candida albicans* bacterial colony are as follows: cream-yellow colour, slightly convex above agar, not growing into the substrate, smooth, glossy surface, which becomes plicate while aging; usually smooth edges, (the fuzzy one exist only for a few bacterial strains); it releases characteristic yeast odor. The maximum growth temperature is 43–46 ^{o}C; the growth is stimulated by biotin and some of the bacterial strains are stimulated by thiamine; cells form bacterial culture tolerate the osmotic pressure of 8–12 % of sodium chloride solution [12].

Pathogenesis. The species is regarded as the most frequent etiological agent of generalized human and organ candidiasis. *Candida albicans* invasions may concern all the tissues, organs and human body systems in various stages of their development. The perfect stage is not known [12].

Yeast and fungi are also solid components of the soil microflora. Microflora complex is governed by the natural ecosystem; fungus population is dynamic which is a result of the sanitary condition connected with the fertilization of the soil polluted with sewage. Liquid animal excrements used as an organic fertilizer highly pollute cultivable soil with yeast fungi and mould. However, the fungi settled only in connection with 15 species from *Cryptococcaceae* family (including *Candida albicans* and others).

Fertilizations by the use of the following: the fermented liquid manure in a form of agricultural waste, sewage from food production and urban wastewater are potentially dangerous for animals and human beings [13].

The amount of the microorganisms in sewage is assessed on the basis of the bacteria detection regarded as the sanitary indicators. In western European countries, *Salmonella* is such an indicator; in Poland – *Escherichia coli,* the presence of which makes it possible to determine the *Coli* titre. In the USA, the fungus *Candida albicans* was accepted a relevant indicator for water and sewage assessment [14–16].

The aim of this study was to test the standard and synthesized derivatives of 1,3,4-thiadiazoles against the *Candida albicans* mycelium growth inhibition.

Experimental part

The following synthesized and standard compounds (Pharmaceutical Plants Polpharma, Starogard Gdanski, Poland) were used in the biological research for the test fungicidal properties: 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (AcATS); 2-amino--1,3,4-thiadiazole (AT); 2-acetylamino-1,3,4-thiadiazole (AcAT); 2-acetylamino-5-chloro--1,3,4-thiadiazole (AcATCl); 2-acetylamino-1,3,4-thiadiazolo-5-sulfonic acid ($AcATSO_3H$) and bis(2-acetylamino-1,3,4-thiadiazole)-5,5'-disulfonamide (bAcATDS). Reagents of analytically pure class produced by Sigma-Aldrich and POCh Gliwice (Poland) were used for the compounds preparation.

Chemical syntheses of 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (AcATS), of 2-amino-1,3,4-thiadiazole (AT), and of bis(2-acetylamino-1,3,4-thiadiazole)-5,5'-disulfonamide (BAcATDS) were discussed in the other article concerning the chemical characteristics of ^{13}C NMR of above mentioned compounds [17].

Preparing 2-acetylamino-1,3,4-thiadiazole (AcAT)

2-acetylamino-1,3,4-thiadiazole (AcAT) was obtained with the method applied by Kanaoka [18], according to which 1-ethoxymethylenothiosemicarbazide was obtained from the reaction products such as thiosemicarbazide and ethyl orthoformate. 2-acetylamino-1,3,4-thiadiazole (AcAT) was obtained as a result of the ring formation of 1-ethoxymethylenothiosemicarbazide with acetic anhydride. 9.0 g (0.10 mole) of thiosemicarbazide and 16.4 cm^3 (0.10 mole) of ethyl orthoformate (d = 0.8910 g/cm^3) were introduced to the reactor of volume 350.0 cm^3, equipped with a mechanic mixer and a reflux condenser. Next, the mixture was heated in a water bath for 2 hours. After this period of time, 250.0 cm^3 (4.70 mole) of acetonitrile (d = 0.7830 g/cm^3) was introduced to the mixture and the whole mixture was heated to boiling. The mixture was dried thermally. After cooling, the product was dried until the dry mass was attained, the total yield was 3.48 g (24 %) of ethyl orthoformate thiosemicarbazide. 14.0 cm^3 (0.15 mole) of acetic anhydride (d = 1.0840 g/cm^3) was added to 3.0 g (0.02 mole) of ethyl orthoformate thiosemicarbazide. Then, the mixture was being heated at the temperature of 90 ^oC for one hour. The obtained precipitate was filtered, recrystallized from hot water and dried until dry mass was obtained, (yield 90 %) of white crystals of 2-acetylamino-1,3,4-thiadiazole with melting point at the temperature of 265–267 ^oC (literature temperature of 268–269 ^oC [19–21]).

Preparing 2-acetylamino-5-chloro-1,3,4-thiadiazole (AcATCl)

First of all 2.59 mole (80.0 cm^3) of 36 % hydrochloric acid (d = 1.1830 g/cm^3) and 0.33 mole (10.0 cm^3) of 30 % hydrogen peroxide (d = 1.1110 g/cm^3) were introduced to the 350.0 cm^3 volume reactor, equipped with a mechanic stirrer and a thermometer. Reagents were introduced in batches so that the temperature of the mixture should not exceed 10 ^oC. Next, 0.05 mole (8.04 g) of 2-acetylamino-5-mercapto-1,3,4-thiadiazole and 1.32 mole (75.0 cm^3) of 96 % acetic acid (d = 1.0610 g/cm^3) were introduced. The reaction was being carried out for 8 hours at the temperature ranging from 10 to 15 ^oC. The obtained yellow solution was filtered on Buchner's set. White, crystalline substance of 2-acetylamino-5-chloro-1,3,4-thiadiazole was precipitated from the filtrate. Next, it was separated and dried at the ambient temperature until the dry mass was attained. Consequently, 4.32 g of the product (yield 53 %) was obtained with the melting point at the temperature of 245 ^oC (literature temperature 245–246 ^oC) [22].

Preparing 2-acetylamino-1,3,4-thiadiazolo-5-sulfonic acid ($AcATSO_3H$)

First, 24.8 cm^3 (0.435 mole) of 99.5 % CH_3COOH (d = 1.0520 g/cm^3), 28.7 cm^3 (0.93 mole) of 36 % HCl (d = 1.1820 g/cm^3), 8.06 g (0.046 mole) of 2-acetyl-

amino-5-mercapto-1,3,4-thiadiazole and 24.1 cm^3 (0. 786 mole) of 30 % H_2O_2 (d = = 1.1110 g/cm^3) were introduced in turn into the 350.0 cm^3 volume reactor, equipped with a thermometer and the stirrer, and placed in the water bath filled with ice. The reaction time was 2 hours from the moment of introducing the oxidizer at the temperature between 5 and 10 ^oC. The reaction precipitate was filtered and washed with a great amount of ice cold distilled water in order to remove the residue of acids and the oxidizer. The product was stored at the temperature from 7 to 10 ^oC. As a result 5.4 g of 2-acetylamino-1,3,4-thiadiazolo-5-sulfonic acid was obtained (yield 53 %), and melting point of substance was determined at the temperature of 282 ^oC.

Characterized by the concentration ranging from 0.5 to 50.0 mg/cm^3, the solutions of the synthesized compounds presented above were prepared in dimethyl sulfoxide (DMSO). A control sample with fungicidal properties was also made. The effect of the tested heterocyclic compounds on the sensitivity of *Candida albicans* fungi was then determined [23]. The ATCC 10231 fungi strains were received from Hohenheim University in Stuttgart, Germany. They were multiplied within 24 hours, diluted in the Standard-I Broth liquid substratum and added to Standard-I Agar (Standard I-Nhragar, Merck No. 7881) solid medium with 0.5 cm^3 of suspended fungi per 250.0 cm^3 of agar. The agar with the inoculated fungi was spilled on the Petri dishes and left to solidify. Then, in the solidified bases, cylindrical wells with the radius of 4 mm were cut and filled with the solutions of the tested compounds. This activity was performed twice each time: 25.0 and 50.0 mg/cm^3; 1.0 and 12.5 mg/cm^3; 0.25 and 0.5 mg/cm^3. The diluent itself was acting as the control. Afterwards, samples were being incubated for 24 hours at the temperature of 37 ^oC [23–25]. After the incubation, the areas of the fungi growth inhibition were measured (in mm) and the minimal inhibition concentrations (MIC) were defined [25].

Results and discussion

The samples with the lowest concentration of the tested chemical compounds (0.25 mg/cm^3) were biologically inactive. Concentrations of 0.5 mg/cm^3 and 1.0 mg/cm^3 were also rather unreliable for most of the chemical compounds, except for 2-amino-1,3,4--thiadiazole (AT). MIC parameter for AT is 0.5 mg/cm^3, and for AcATS, bAcATDS, $AcATSO_3H$, AcATCl and AcAT MIC = 12.5 mg/cm^3.

Figure 1 presents the relationship determined for all the tested chemical compounds in form of concentrations. These compounds demonstrated, respectively: 12.5, 25.0 and 50.0 mg/cm^3 and the size of the *Candida albicans* inhibition growth zone [mm]. It was found that *Candida albicans* was sensitive only to the concentration of 50.0 mg/cm^3 for all the tested 1,3,4-thiadiazole derivatives. Other lower concentrations of 2-acetyl-amino-1,3,4-thiadiazolo-5-sulfonamide (AcATS) did not demonstrate any fungicidal activity.

For most of the heterocyclic compounds, the size of the growth inhibition zone of *Candida albicans* ranged from 5 to 8 mm. Only the 2-amino-1,3,4-thiadiazole the growth inhibition zone exceeded the average limits ranging from about 12 to 27 mm.

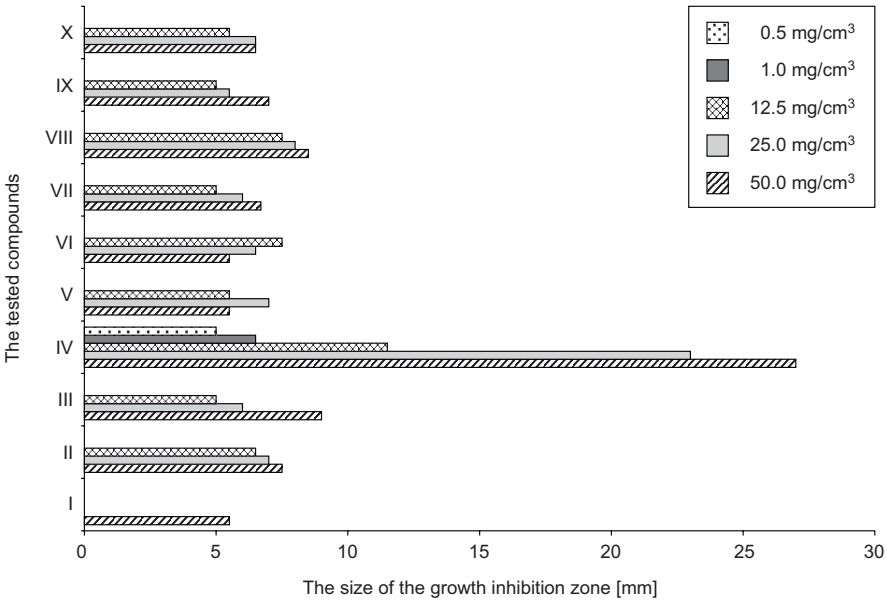

Fig. 1. The size of the growth inhibition zone as a function of 1,3,4-thiadiazole derivative concentration. The tested concentrations were: a) 0.5 mg/cm^3, b) 1.0 mg/cm^3, c) 12.5 mg/cm^3, d) 25.0 mg/cm^3, e) 50.0 mg/cm^3. Markings of the compounds: I – AcATS standard, II – AcATCl standard, III – AcATCl, IV – AT, V – AcAT standard, VI – AcAT, VII – $AcATSO_3H$ standard, VIII – $AcATSO_3H$, IX – bAcATDS standard, X – bAcATDS

For most tested compounds (II, III, IV, VII, VIII, IX), a linear dependence of the growth inhibition zone on *C. albicans* as a function of concentration was observed. Taking into the account the data in Fig. 1, one could notice that two derivatives of thiadiazoles: AcAT and bAcATDS were an exception. For AcAT the lowest concentration 12.5 mg/cm^3 of the standard (V) induced the inhibition equal to 5.5 mm and synthesized (VI) – 7.5 mm (±2.0). The standard (V) of AcAT and the synthesized (VI) of concentration 25.0 mg/cm^3 inhibited the growth of *Candida albicans,* respectively, by 7.0 and 6.5 mm (±0.5). These compounds characterized by the concentration of 50.0 mg/cm^3 inhibited the growth equally by 5.5 mm. For the synthesized AcAT (VI), there was an inverse relationship of the growth inhibition zone from the used concentration of the compound. Standard AcAT did not demonstrate the explicit dependence, which might be a result of the measuring error.

BAcATDS (X) was the other compound which, with the concentration of 12.5 mg/cm^3, inhibited the growth of *C. albicans* by 5.5 mm. But in case of synthesized bis(2-acetylamino-1,3,4-thiadiazole)-5,5'-disulphonamid, the same inhibition was observed for the concentrations of 25.0 and 50.0 mg/cm^3. It was also found out that the standard bAcATDS (IX) has a tendency towards a decrease in the growth inhibition zone along with a decrease in the tested compound concentration. Therefore, the synthesized derivative X probably indicates a similar tendency, as the results of zone measurements were within the limits of permissible error.

Table 1

Activity of 1,3,4-thiadiazole compounds for *Candida albicans*

Compoud	Concentration [mg/cm^3]														
	0.5			1.0			12.5			25.0			50.0		
	R	R/r	Activity	R	R/r	Activity	R	R/r	Activity	R	R/r	Activity	R	R/r	Activity
I	—	—	—	—	—	—	—	—	—	—	—	—	3.0	0.8	+
II	—	—	—	—	—	—	3.5	0.9	+	3.5	0.9	+	4.5	1.1	++
III	—	—	—	—	—	—	2.5	0.6	+	3.0	0.8	+	4.5	1.2	++
IV	2.5	0.6	+	4.0	1.0	++	6.0	1.5	++	14.0	3.5	+++	14.5	3.6	+++
V	—	—	—	—	—	—	3.0	0.8	+	3.5	0.9	+	3.0	0.8	+
VI	—	—	—	—	—	—	3.5	0.9	+	3.5	0.9	+	3.0	0.8	+
VII	—	—	—	—	—	—	2.5	0.6	+	3.0	0.8	+	3.5	0.9	+
VIII	—	—	—	—	—	—	4.0	1.0	++	4.0	1.0	++	4.5	1.1	++
IX	—	—	—	—	—	—	2.5	0.6	+	3.0	0.8	+	3.5	0.9	+
X	—	—	—	—	—	—	3.0	0.8	+	3.5	0.9	+	3.5	0.9	+

Where: +++ – high activity; ++ – medium activity; + – low activity; — – deficiency in activity; indications of the compounds as in Fig. 1.

Table 1 presents the determined activities of the tested chemical compounds for different concentrations reacting on the fungi and the values necessary to determine biological activity. The values presented in the table describe, respectively: R – half of the zone of the fungi growth inhibition, r – radius of the well. Basing on R/r, the activity of a given compound was determined for tested fungi strains within the range of 1,3,4-thiadiazole concentration. Interpreting the results: the low activity is found when the ratio of the studied chemical substance $R/r < 1$. The medium activity is for $1 \leq R/r < 2$, and the ratio $R/r \geq 2$ indicates high activity [23]. As it results from the data, after 24 hours of incubation at 37 ^{o}C, the zone of fungi growth inhibition, being a minimum inhibitory concentration (MIC), depended on a tested compound type and the fungi strain.

Among all the tested heterocyclic compounds AcATS showed the lowest activity against *Candida albicans*. It demonstrated the low activity only for the concentration of 50.0 mg/cm^3. The next chemical compound demonstrating low activity against *Candida albicans* was bis(2-acetylamino-1,3,4-thiadiazole)-5,5'-disulfonamide (bAcATDS), both as a standard and a synthesized substance. However, the compound differed from AcATS because it demonstrated low activity not only for the concentration of 50.0 mg/cm^3 but also for 25.0 and 12.5 mg/cm^3. This situation might be perceived as a result of a similar composition of these substances. Another heterocyclic compound, showing the low activity against *Candida albicans* in the same range of the tested concentrations as for bAcATDS was the standard and the synthesized 2-acetyl-amino-1,3,4-thiadiazole. All of the presented chemical compounds have in their structure: acetylamine (CH_3CONH-), sulfonamide (NH_2SO_2-) or just hydrogen (H-) as the final group at the heterocyclic ring. Such chemical composition may result in the low activity of these compounds. Both the standard and the synthesized compound of 2-acetylamino-5-chloro-1,3,4-thiadiazole showed medium and low activity against *Candida albicans*. For concentrations 12.5 and 25.0 mg/cm^3 it showed the low fungicidal activity, but for the concentration of 50.0 mg/cm^3 – the medium activity. The concentration growth of 2-acetylamino-5-chloro-1,3,4-thiadiazole resulted in the activity growth of this compound. Such behavior with respect to *Candida albicans* may be interpreted by the presence of chlorine as the final function group in position 5 of 1,3,4-thiadiazole ring. Interesting results were obtained for 2-acetylamino-1,3,4-thia-diazolo-5-sulfonic acid. The sulfonic acid standard showed a low activity against *Candida albicans* for the concentrations ranging from 12.5 to 50.0 mg/cm^3. However, the same chemically obtained substance showed the medium activity. This difference can be attributed to non-perfect purity of the obtained substance the separated 2-acetylamino-1,3,4-thiadiazolo-5-sulfonic acid ($AcATSO_3H$) and the presence of the compounds occurring in equilibrium:

2-acetylamino-1,3,4-thiadiazolo-5-sulfonic acid $\rightleftharpoons$
$\rightleftharpoons$ 2-acetylamino-1,3,4-thiadiazolo-5-sulfochloride,

$$R\text{-}SO_3H \rightleftharpoons R\text{-}SO_2Cl,$$

which may consequently result in the fungicidal activity growth. The highest and the most diversified activity with *Candida albicans* was found for 2-amino-1,3,4-thiadiazole (AT). This compound has the simplest chemical structure and is the most toxic compound among the tested heterocyclic substances [25]. It was characterized by the low activity for the concentrations of 0.5 mg/cm^3, the medium activity for concentrations of 1.0 and 12.5 mg/cm^3, and the high activity for the concentrations of 25.0 and 50.0 mg/cm^3. This state of being permits the selective elimination or has an influence on *Candida albicans* for 2-amino-1,3,4-thiadiazole. This fact is better illustrated by Figure 2, presenting the *Candida albicans* inhibition growth zone [mm] against the used 2-amino-1,3,4-thiadiazole concentration. The growth tendency was determined by the following cubic polynomial equation: $y = 0.9167x^3 - 7.4286x^2 + 10.655x + 23.1$ (where: y – size of the inhibition growth zone, x – AT concentration) with the high correlation coefficient $R^2 = 0.9895$.

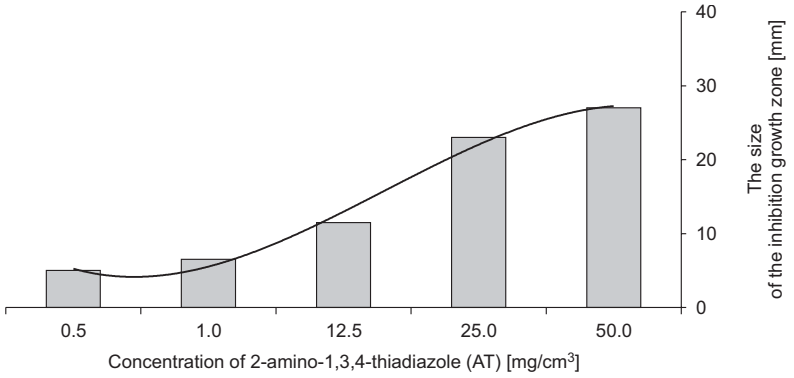

Fig. 2. Fungicidal activity of 2-amino-1,3,4-thiadiazole against *Candida albicans*

Conclusion

The fungicidal activity of 1,3,4-thiadiazole derivatives was tested within the concentration ranging from 0.25 to 50.0 mg/cm^3. The tested compounds causes the increase in the growth inhibition zone of fungus *Candida albicans* along with an increase in their concentrations. Additionally, 2-acetylamino-1,3,4-thiadiazole (AcAT) is an exception and shows an opposite tendency. 2-Amino-1,3,4-thiadiazole (AT) of MIC = 0.5 mg/cm^3 was characterized by the highest and the most diversified activity against *Candida albicans*. This compound may be potentially applied to treat *Candida albicans* mycosis and to reduce the sanitary environment pollution. The highest MIC value (50.0 mg/cm^3) was obtained for 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (AcATS), which showed the low activity against *Candida albicans*.

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Abstrakt: W prezentowanej pracy określano działanie grzybobójcze pochodnych 1,3,4-tiadiazoli. We wszystkich testach używano referencyjnego szczepu *Candida albicans* ATCC 10231. W tym celu przetestowano następujące syntetyzowane i wzorcowe związki heterocykliczne: 2-acetyloamino-1,3,4-tiadiazolo-5-sulfonamid; 2-acetyloamino-5-chloro-1,3,4-tiadiazol; 2-amino-1,3,4-tiadiazol; 2-acetyloamino-1,3,4-tiadiazol; kwas 2-acetyloamino-1,3,4-tiadiazolo-5-sulfonowy oraz bis(2-acetyloamino-1,3,4-tiadiazolo)-5,5'-disulfonamid. Badane związki proporcjonalnie ze zwiększeniem stężenia zwiększają strefę zahamowania wzrostu grzyba *Candida albicans*. Wyjątkiem jest 2-acetyloamino-1,3,4-tiadiazol, który wykazuje odwrotną tendencję. Najskuteczniejszym z badanej grupy związków okazał się 2-amino-1,3,4-tiadiazol. Wykazano, że ze zwiększeniem stężenia tego związku rośnie rozmiar strefy zahamowania wzrostu (y) *Candida albicans* zgodnie z równaniem $y = 0{,}9167x^3 - 7{,}4286x^2 + 10{,}655x + 23{,}1$ (gdzie: x – stężenie 2-amino-1,3,4-tiadiazolu).

Słowa kluczowe: *Candida albicans*, aktywność fungistatyczna, pochodne 1,3,4-tiadiazoli