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In Vitro Studies of Antifungal Activity of a Bifunctionalized Allene Ethanol Extracts

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Abstract: *Antifungal effects of a Bifunctionalized Allene (Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate) (BA-1) on pathogenic yeast and fungi had been established. BA-1exerted different inhibitory effect on different yearst and fungi cells in vitro. The effects of BA-1on eukaryotic cells have not been studied yet. The present study was aimed to assess the antifungal activity of BA-1on pathogenic yeast and fungi. In vitro antifungal test: Aspergillus niger, Penicillium claviforme, Saccharomyces cerevisae, Candida albicans 8673 and Candida glabrata 72 were treated for 24 hours with BA-1 (1 mg/ml, 2 mg/ml, 3 mg/ml and4 mg/ml), Chloronitromycin (250 mg/ml).The antifungal activity was assayed by the well diffusion method.Determination of minimum inhibitory concentrations(MICs):The MIC of BA-1, that shows antifungal activity, were determined by 2-folddilution methods as described by [12] and MICs were read in µg/ml after over nightincubation at 37°C. All experiments were made in replicate. Determination of Minimum fungal concentration (MFC)**:** The MFC was carried out to check whetherthe test microbes were killed or only their growthwas inhibited. Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) was preparedand sterilized at 121°C for 15 minutes, the medium waspoured into sterile petridishes and were allowed tocool and solidify. The contents of the MIC in theserial dilution were then subcultured onto theprepared medium, incubation was made at 37°C for24 h, after which each plate was observed forcolony growth. The lowest concentration of theextracts without a colony growth was recorded asthe MFC.BA-1had higher antifungal activity than tested antibiotic even from this fourth generation – Chloronitromycin.*

The present study indicated significant antibacterial activity of BA-1on tested pathogenic microorganisms. The inhibitory effect of BA-1 against several yeast and fungi indicates broad spectrum antimicrobial potential.This justified the use of BA-1for the treatment of diseases of microbial origin and also makes it a potential candidate to use in drug development fortreatment of infectious diseases caused by these pathogens.

Keywords: BA-1 (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-diene phosphonate),* antifungal activity, antibiotic.

1. INTRODUCTION

Food and feed spoiling moulds and yeasts cause great economic losses worldwide. Furthermore, the presence of moulds with the concomitant production of allergenic spores and possibly mycotoxins makes them serious potential health hazards [3,8]. The reduction of mould and yeast growth in food and feed production and storage is thus of primary importance and there is great interest in developing e⁄cient and safe strategies for this purpose. Filamentous moulds and yeasts are the main spoilage organisms of various products such as fermented dairy foods (cheese, yogurt), bread, stored crops and feed hay and silage [3,6]. The most common spoiling fungi isolated from bakery products belong to the genera *Penicillium, Aspergillus, Monilia, Mucor,Endomyces, Cladosporium, Fusarium,* and *Rhizopus*. The moulds' growth causes alterations in the texture and harms the external aspect of the products [5,6] which leads to

significant economic losses. Five to ten percent of the world's food production is lost due to fungal contamination [13,14], 27% of the foods produced in U.S. are annually destroyed by fungi [9]. In addition, the toxigenic and spoilage fungi are responsible for numerous diseases and health risks [11], due to the potential production ofmycotoxins or allergenic conidia, sporesand mycelia [6]. The problem of increasing antibiotic resistance is worsens and also there are new legislations which has restricted the use of some currently accepted preservatives in different food products [4]. All these important issues raise the need to seek alternative methods of foods/feeds producing and preserving of the human food chain.

In this paper, the antifungal activity of a BA-1 (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate)* has been studied as part of the exploration for new and novel bio-active compounds.

2. MATERIALS AND METHODS

2.1. Test Organisms

Aspergillus niger, Penicillium claviforme, Saccharomyces cerevisae, Candida albicans 8673 and Candida glabrata 72 were obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria. All the isolates werechecked for purity and maintained in slants of Nutrient agar.

2.2. Media Used

They were maintained on Potato Dextrose Agar (PDA, Oxoid, Hampshire, UK) plates at 29^{o}C and subcultured on a monthly basis until sporulation. The spores were harvested after establishing a good growth rate of each of the fungal cultures and were filtered with sterile cotton filter, to avoid the presence of conidia and mycelia. The spore's suspensions in PBS (pH e 7.0) were adjusted to the final concentrations in the range of 10^5-10^6 spores/mL.

2.3. Compound Tested

Bifunctionalized Allene (*Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate)* (BA-1) was synthesised in the Laboratory of Toxicologycal Chemistry, Department of Organic Chemistry & Technology of theKonstantin Preslavsky University of Shumen, Bulgaria (figure 1) [7].

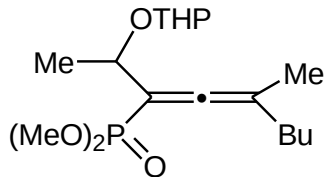

Fig1. *Structural formula of BA-1*

Dimethyl 3-methyl-1-[1-(tetrahydro-2H-pyran-2-yloxy)-ethyl]-hepta-1,2-dienephosphonate

(BA-1). Orangeoil, yield: 72%. R_f 0.43; IR (neat, cm^{-1}): 1120 (C-O-C), 1254 (P=O), 1956 (C=C=C). ^{1}H-NMR($CDCl_3$, 600.1 MHz): δ 0.93 (t, *J* = 7.1 Hz, 3H, Me-$(CH_2)_3$), 1.43 (dd, *J* = 6.3 Hz, *J* = 10.0 Hz, 3H, Me-CHO),1.48, 1.55, 1.64, 3.62, 4.38 (overlapping multiplets, 9H, OTHP), 1.77 (d, *J* = 6.6 Hz, 3H, Me-C=),1.41, 1.74, 2.11 (overlapping multiplets, 6H, Me-$(CH_2)_3$), 3.76 (d, *J* = 11.2 Hz, 3H, MeO), 4.64(m, 1H, CHO). ^{13}C-NMR ($CDCl_3$, 150.9 MHz) δ = 13.8, 18.8 (*J* = 6.5 Hz), 19.5, 22.7, 23.5 (*J* = 7.5 Hz),25.7, 29.6, 30.4, 32.8, 52.3 (*J* = 6.2 Hz), 62.3, 68.6 (*J* = 10.2 Hz), 91.4 (*J* = 191.7 Hz), 95.6, 103.4(*J* = 16.2 Hz), 209.0 (*J* = 5.3 Hz). ^{31}P-NMR ($CDCl_3$, 242.9 MHz): δ 20.5. Anal. Calcd for $C_{17}H_{31}O_5P$(346.40): C 58.94, H 9.02. Found: C 59.01, H 8.96.

2.4. Preparing the Solution of BA-1

The solutions of BA-1(1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml) were freshly preparedin ethanol.

2.5. Assay for Antifungal Activity

Antifungal assay was performed by the well diffusion method using soft 0,8% agar. Agar medium was added to sterile Petri dishes seeded with 100 µl of each test bacterial strains. Wells of equal

distance were dug on the seeded plates. Each well was filled up with 100 µl of the BA-1and antibiotics tested. After adjusting the pH at 6.5 by NaOH, the activity of the plant extracts was checked. The plates were incubated at 37°C for 48 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well [2]. All experiments were performed in triplicate.

2.6. Determination of Minimum Inhibitory Concentrations (MICs)

The minimum inhibitory concentrations of BA-1, that shows antimicrobial activity, were determined by 2-folddilution methods as described by [12] and MICs were read in µg/ml after over nightincubation at 37^{o}C. All experiments were made in replicate.

2.7. Determination of Minimum Fungal Concentration (MFC)

The MFC were carried out to check whetherthe test microbes were killed or only their growthwas inhibited. Potato Dextrose Agar agar was preparedand sterilized at 121^{o}C for 15 minutes, the medium waspoured into sterile petridishes and were allowed tocool and solidify. The contents of the MFC in theserial dilution were then subcultured onto theprepared medium, incubation was made at 37^{o}C for24 h, after which each plate was observed forcolony growth. The lowest concentration of theextracts without a colony growth was recorded asthe MFC.

3. RESULTS

In the present study the effects of BA-1on five pathogenic fungi and were evaluated. The effects were compared with widely used antibiotic Chloronitromycin. According to NCCLS, the antibiotic Chloronitromycin used is known to have broad spectrum antifungal activity [10].

The effects of **BA-1**on the microorganisms were summarized in Table 1.

Table1. *Effect of* ***BA-1****on test organisms.*

Microorganisms	**Zone of inhibition (mm)**
A. niger	**24.91**±0.02
P. claviforme	**18.70**±0.03
S. cerevisae	**29.10**±0.07
C. albicans 8673	**20.61**±0.02
C. glabrata 72	**23.23**±0.03
Ethanol(96%) (Negative control)	**11.12**±0.05
Chlornitromycin 250 µg/ml	**13.06**±0.19

Data are presented as average values ± standard deviation in mm.

The sensitivities of the test organisms to infusions were indicated by clear zone around the wells (Figure 2).

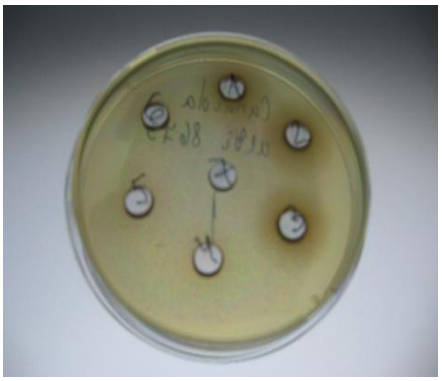

Fig2. Showing Zone of inhibition with BA-1along with tested antibioticChloronitromycinof 24 hours *C. albicans 8673*

Position 1,2 and 3)BA-1; 4,5 and 6) Chloronitromycin; 7) Ethanol

BA-1at concentration 4 mg/ml for 24 hours notably inhibited growth of *S. cerevisae* (29.10 mm mean zone of inhibition), *A. niger* (24.91 mm mean zone of inhibition) and *C. glabrata* 72 (23.23 mm mean zone of inhibition). On the contrary, BA-1 had no activity against *P. claviforme*(18.70 mm mean zone of inhibition).

Our assay for antifungal activity of BA-1was conducted by testing different concentrations of the compound on various pathogens to determine the MICs. We used four concentrations – 1mg/ml; 2mg/ml; 3mg/ml and 4mg/ml. The results are shown in Table 2.

Table2. *The MIC of BA-1*

Microorganisms	**MIC (mg/ml)**			
	BA1mg/ml	**BA2mg/ml**	**BA3mg/ml**	**BA4mg/ml**
A. niger			+	
P. claviforme			+	
S. cerevisae			+	
C. albicans 8673			+	
C. glabrata 72				+

Results are mean ± SEM of three separate trails.

The results revealed variability in the inhibitory concentrations of BA-1for given fungi. MIC of BA-1at concentration 4 mg/ml for 24 hours notably inhibited growth only of *C. glabrata 72*. In contrast, MIC of bifunctionalized allene at concentration 3 mg/ml for 24 hours notably inhibited growth of all other fungi.The probable reason for the higher MIC reported for eukaryotic microorganisms is the complex structure of their cell.

Our next task was to determine the Minimum fungal concentration (MFC) in regards with determining the bactericidal or bacteriostatic activity of the examined BA-1. We used four concentrations – 1mg/ml; 2mg/ml; 3mg/ml and 4mg/ml. The results are shown in Table 3.

Table3. *The MFC of BA-1*

Microorganisms	MBC (mg/ml)			
	BA1mg/ml	**BA2mg/ml**	**BA3mg/ml**	**BA4mg/ml**
A. niger		+		
P. claviforme		+		
S. cerevisae			+	
C. albicans 8673			+	
C. glabrata 72		+		

Results are mean ± SEM of three separate trails.

MFC of BA-1at concentration 3 mg/ml for 24 hours notably inhibited growth of *S. cerevisae* and *C. albicans 8673*. For Fungi Imperfecta from *A. niger* and *P. claviforme* MFC is 2 mg/ml.

Based on the results obtained we can conclude that the examined BA-1has bactericidal activity towards both pathogenic yeast and Fungi Imperfecta, but in different concentrations.

The BA-1possesses biological activity, which is not well studied. We know only from literary data that they are used for inhibiting the biosynthesis of sterol from the pathogen responsible for *Pneumocystis-carinii*pneumonia (PCP) -a disease similar to AIDS[1].

The results obtained show for the first time the existence of antifungal activity of BA-1towards various pathogenic yeast and fungi.

The antifungal agents currently available for clinical use do not necessarily possess adequate antifungal activities against serious systemic fungal infections, or their clinical usefulness is hampered by untoward side effects. Therefore, there is an urgent need for the development of novel antifungal agents with superior therapeutic effect and high safety.

The antifungal agents currently available for clinical use do not necessarily possess adequate antifungal activities against serious systemic fungal infections, or their clinical usefulness is hampered by untoward side effects. Therefore, there is an urgent need for the development of novel antifungal agents with superior therapeutic effect and high safety.

The occurrence of drug resistant strains with less susceptibility to antibiotics due to mutation challenges the researchers to invent newer drugs. At this scenario, evaluation of antimicrobial substances from various sources is considered to be a pivotal role.

However, in the present study results also exhibited the confirmation of the antimicrobial property that showed bactericidal action on the pathogens commonly encountered in hospitalized patients. Nevertheless, further studies are required to explore the mechanism of biochemical active principle in the Bifunctionalized Allenes for the inhibitory action on various pathogens selected in the study.

4. CONCLUSION

The BA-1 at 1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml concentrations showed significant antifungial activity on selected pathogens inclinical isolates.

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