ORIGINAL ARTICLE

Veterinary Research Forum. 2021; 12 (2) 241 – 246
doi: 10.30466/vrf.2019.103449.2460
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Synergistic effects of dual antimicrobial combinations of synthesized *N*-heterocycles or MgO nanoparticles with nisin against the growth of *Aspergillus fumigatus: In vitro* study

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Article Info

Article history:

Received: 06 February 2019
Accepted: 30 October 2019
Available online: 15 June 2021

Keywords:

Aspergillus fumigatus
MgO nanoparticle
Nisin
Synergistic antifungal effect
Thiazole

Abstract

Introduction of new inhibitory agents such as peptides, heterocyclic derivatives and nanoparticles (NPs) along with preventive proceedings are effective ways to deal with standard and drug-resistant strains of microorganisms. In this regard, inhibitory activities of some recently synthesized 4-thiazolylpyrazoles, imidazolidine- and tetrahydropyrimidine-2-thiones and magnesium oxide (MgO) NPs alone and in combination with nisin have been assessed against *Aspergillus fumigatus*. Antimicrobial susceptibility tests were done *via* broth microdilution, disk diffusion and streak plate methods according to the modified Clinical and Laboratory Standards Institute (CLSI) guidelines. Synergistic effects were also determined as fractional inhibitory concentration (FIC) and fractional fungicidal concentration (FFC) values. Inhibitory potentials of all heterocycles and NPs against *A. fumigatus* were proved based on inhibition zone diameter (IZD) values in the range of 7.72 - 16.85 mm, minimum inhibitory concentration (MIC) values in the range of 64.00 - 512 μg mL^{-1} and minimum fungicidal concentration (MFC) values in the range of 256 - 2048 μg mL^{-1}. Tetrahydropyrimidine derivative 3f showed the best inhibitory properties. Inhibitory activity was not significant with nisin. While antifungal effects of major derivatives were improved by combination with it. The results indicated that the combined treatment of heterocycles used in the present study with nisin might be efficient for mold prevention and removal in foodstuffs or other products.

Introduction

Aspergillus fumigatus is a saprophytic fungus that inhabits in soil and organic residuals. It plays a vital role in recycling nitrogen and carbon, and releases many conidia in air. Shehu and Bello studied effect of environmental factors on the growth of *Aspergillus* species.[1] It was found that the growth of *A. fumigatus* was increased under continuous light, 100% relative humidity and temperature up to 40.00 ˚C. This pathogenic microorganism is the most important fungal infection risk factor in respiratory system. Infections can also affect organs such as livers, kidneys, eyes, stomach and skin, and increase mortality especially in patients with immunodeficiency disorders.[2] Strains of *A. fumigatus* resistant to traditional antifungal drugs such as itraconazole, isavuconazole, posaconazole, voriconazole, isavuconazole and amphotericin B are rapidly expanding. Researchers recommend identification and preparation of novel and more efficient antifungal agents to treat aspergillosis.[3]

Thiazole skeleton is present in many biologically active compounds. This ring exists in vitamin B1, which is coenzyme for carboxylase enzyme. Some drugs containing thiazole were applied to treat cancer, high blood cholesterol, high blood pressure and AIDS (acquired immune deficiency syndrome).[4] Antioxidant, anti-inflammatory, antimosquito and antitrypanosomal properties were observed with thiazole derivatives.[5-8] Antimicrobial potencies of thiazoles were proven against bacterial pathogens like *Salmonella typhi, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio cholerae* and *Klebsiella pneumoniae* and fungi such as *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus flavus*.[9]

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There has been a growing interest to synthesize imidazole derivatives due to their inhibitory abilities against tumor cells, *Leishmania parasite*, *Enterococcus faecalis*, *Escherichia coli* and *S. aureus*.[10-12] Some imidazolidinyl isoxazole derivatives were prepared, and their fungicidal activities were evaluated on *Aspergillus niger* and *Rhizopus oryzae*.[13]

Tetrahydropyrimidine derivatives are capable of inhibiting growth of *Bacillus subtilis*, *E. coli*, *Mycobacterium tuberculosis*, *K. pneumonia* and *P. aeruginosa*.[14,15] Several derivatives of them were developed as selective muscarinic agonists for the treatment of Alzheimer's disease.[16] *In vitro* antifungal effects of tetrahydropyrimidine derivatives were also evaluated on *A. niger* and *C. albicans*.[17]

Applications of nanotechnology are expanding in various fields of science and extensive amount of researches have been allocated to it.[18] The MgO NPs have been applied for bone regeneration, pain relieve and the treatment of cancer and hypertension.[19] The MgO NPs are efficient, cost effective and nontoxic antimicrobial agents with widespread inhibitory effects on Gram-negative and Gram-positive pathogenic bacteria.[20]

Nisin is a bacterial peptide with low molecular weight of 3510 Dalton. It is used as a food preservative without effect on functions of gastrointestinal system and food flavor. Nisin alone or in combination with other anti-microbial agents can inhibit the growth of microorganisms like *Listeria monocytogenes*, *S. aureus*, *Salmonella enterica*, *E. coli* and *Candida lusitaniae*.[21,22]

Biologically importance of N,S-heterocyclic compounds encouraged us to evaluate inhibitory activities of some synthesized thiazole, imidazolidine- and tetrahydro-pyrimidine-2-thione derivatives and MgO NPs alone or in combination with nisin against *A. fumigatus*.[23]

Materials and Methods

General procedure for the synthesis of imidazolidine- and tetrahydropyrimidine-2-thiones 3a-f. 1.00 mmol of both 1,2- or 1,3-diaminoalkanes **1a-f** and carbon disulfide (**2**) and 0.25 mmol of the synthesized MgO NPs (30 - 50 nm, Zabol, Iran) in 2.00 mL of 96.00% ethanol (Merck, Darmstadt, Germany) were stirred at room temperature for 2.50 - 4.00 hr to give imidazolidine- and tetrahydropyrimidine-2-thiones **3a-f**.[24]

Synthesis of imidazolidine- and tetrahydropyrimidine-2-thiones 3a-f:

Imidazolidine-2-thione (**3a**)

4,4-Dimethylimidazolidine-2-thione (**3b**)

Octahydro-2*H*-benzo[*d*]imidazole-2-thione (**3c**)

Tetrahydropyrimidine-2(1*H*)-thione (**3d**)

5,5-Dimethyltetrahydropyrimidine-2(1*H*)-thione (**3e**)

4-Ethyltetrahydropyrimidine-2(1*H*)-thione (**3f**)

General procedure for the synthesis of thiazoles 6a-e. 1.00 mmol of each compounds including: thioamide **4**, *α*-bromocarbonyl compounds **5a-e** and sodium bicarbonate was stirred in 1 mL *N*,*N*-dimethylformamide (DMF) at room temperature for 24.00 - 46.00 hr to afford thiazoles **6a-e** .[25]

Synthesis of thiazoles 6a-e:

3-Methyl-4-(4-methylthiazol-2-yl)-1-phenyl-1*H*-pyrazol-5-amine (**6a**)

1-(2-(5-Amino-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-4-methylthiazol-5-yl)ethan-1-one (**6b**)

Ethyl 2-(5-amino-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-4-methylthiazole-5-carboxylate (**6c**)

2-(5-Amino-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-5-methylthiazol-4(5*H*)-one (**6d**)

2-(5-Amino-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)thiazol-4(5*H*)-one (**6e**)

Preparation of MgO NPs. Sodium hydroxide (Merck) solution (25.00 mL, 0.008 M) was added dropwise to a stirred suspension of starch (0.10 g) and magnesium nitrate (12.83 g, 0.10 mol; Merck) in 100 mL distilled water. The mixture was left at room temperature for 24 hr without stirring. The suspension was centrifuged at 10,000 rpm for 10 min. It was then washed three times using distilled water, then, heated in the furnace at 300 ˚C for 4 hr to yield MgO NPs in the range 30.00 - 50.00 nm based on the results of X-ray diffraction (XRD) and scanning electron microscope (SEM) analysis (Figs. 1 and 2).[25]

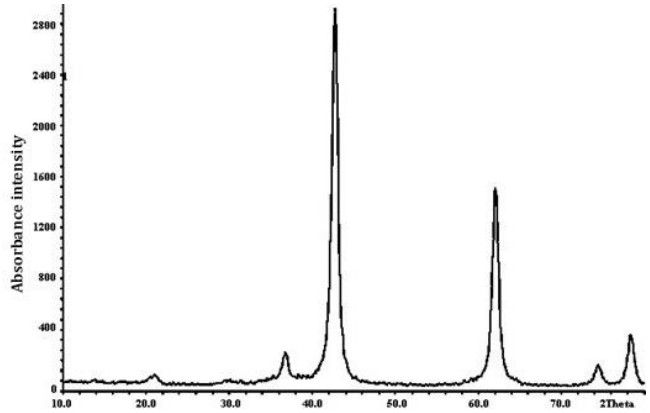

Fig. 1. XRD spectrum of MgO NPs.

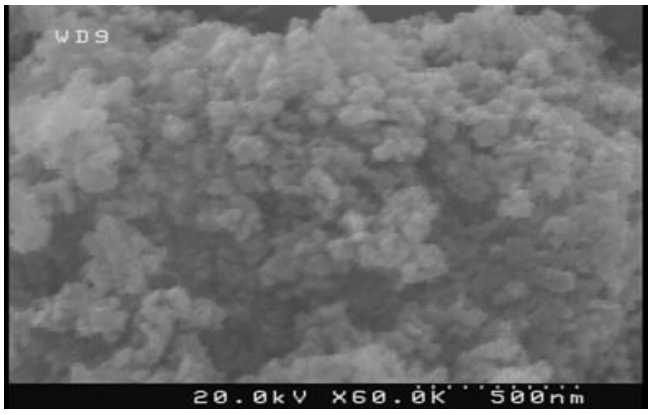

Fig. 2. SEM image of MgO NPs.

Preparation of initial solutions. Nisin was dissolved in sterile 2.00% HCl (Merck) at final concentration 9,011 µg mL^{-1}, incubated in water bath at 80.00 ˚C for 7 min, centrifuged, filtered through a 0.22-µm filter (Millipore, Darmstadt, Germany) and kept at –20.00 ˚C.[13] The solutions of all heterocycles were prepared at initial concentration of 9,011 µg mL^{-1} in 10.00% dimethyl sulfoxide (Merck). Ketoconazole (Sigma-Aldrich, Munich, Germany) as positive control was dissolved in distilled water at concentration of 17.60 µg mL^{-1}.

Preparation of the fungal suspension. *A. fumigatus* (PTCC 5009) was prepared from the Persian Type Culture Collection (PTCC), Karaj, Iran. Fungus was cultured on Sabouraud Dextrose Agar (SDA; HiMedia, Mumbai, India), and incubated for 48 hr at 37.00 ˚C (Fig. 3). Finally, a fungal suspension with concentration 5.00 × 10^6 CFU mL^{-1} in Sabouraud dextrose broth (SDB; HiMedia) was supplied spectrophotometrically which used as a storage source.[25]

Determination of MIC values. 100 µL of SDB was added into all wells of each row of a 96-well plate. Then, 100 µL of initial solutions was added to the first well. After mixing, serial 2-fold dilutions were continued to the final well of each row. Finally, 10.00 µL of fungal suspension was added into all wells. As a result, the final concentrations of compounds and ketoconazole were respectively achieved within range of 4096–32 and 8–0.063 µg mL^{-1}. The plates were incubated under shaking (100 rpm) at 37.00 ˚C for 24 hr. The MICs were detected as the lowest concentration of compounds showing no visible fungal growth.[26]

Determination of MFC values. Samples of all invisible wells in the MIC test were cultured in SDA and then incubated at 37.00 ˚C for another 24 hr. The minimum fungicidal concentration (MFC) values were determined as the lowest concentration without colony.[26]

Measurement of IZD values. 100 µL of fungal suspension was spread on SDA. Sterile blank discs were placed on medium. 10.00 µL of initial solutions were poured onto disks and the plates were then incubated at 37.00 ˚C for 24 hr. Finally, IZDs were measured by caliper.[26]

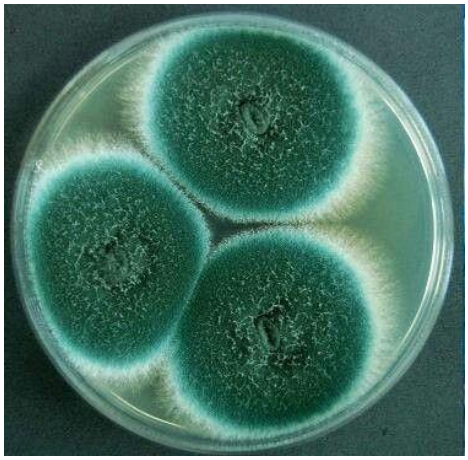

Fig. 3. *Aspergillus fumigatus* colonies on SDA plate.

Calculation of FIC and FFC values. The synergistic effect of dual antimicrobial combinations was determined using the microdilution checkerboard method. Initially, 40.00 µL of SDB was added to all wells of a 64-well plate. Then, 25.00 µL of each compound at various concentrations (MIC × 8, MIC × 4, MIC × 2, MIC, MIC / 2, MIC / 4, MIC / 8, MIC / 16) was added horizontally into all wells of each row. Similarly, 25.00 µL of nisin was added vertically into all wells. Finally, 10.00 µL of fungal suspension was added into them. The plates were incubated under shaking (100 rpm) at 37.00 ˚C for 24 hr. The FIC and FFC values were determined and calculated according to MIC and MFC tests with the following formula:

$$FIC = \frac{MIC\ compound\ in\ combination}{MIC\ compound\ alone} + \frac{MIC\ nisin\ in\ combination}{MIC\ nisin\ alone}$$

$$FFC = \frac{MFC\ compound\ in\ combination}{MFC\ compound\ alone} + \frac{MFC\ nisin\ in\ combination}{MFC\ nisin\ alone}$$

In this experiment, FIC or FFC ≤ 0.50, 0.50 < FIC or FFC ≤ 0.75, 0.75 < FIC or FFC ≤1.00, 1.00 < FIC or FFC ≤ 4.00 and FIC or FFC ≥ 4.00 indicated synergistic, relative synergistic, incremental, ineffective and antagonist effects, respectively.[27]

Results

As shown in Table 1, acceptable to good inhibitory effects on *A. fumigatus* were observed with nisin, MgO NPs and heterocyclic derivatives. The IZD, MIC and MFC values were found in the range of 7.72 to 16.85 mm, 64.00 to 512 µg mL^{-1} and 256 to 2048 µg mL^{-1}, respectively. Heterocycles **3a-d**, **6a**, **6c**, **6d** and MgO NPs displayed similar results to block *A. fumigatus*. The most and the least antifungal potentials were belonged to tetrahydropyrimidine **3f** and thiazole **6b**. Relative synergistic effects (FIC and FFC values = 0.75 µg mL^{-1}) were observed by MgO NPs. A variety of interactions was observed between heterocycles and nisin according to their FIC and FFC values. It was determined that antifungal effects of thiazoles (except **6d**) were significantly improved in combination with nisin.

Discussion

The discovery of new inhibitors against *A. fumigatus* is essential. In this study, inhibitory activities of some synthesized heterocycles and NPs were evaluated against this pathogen and their interactions were also studied in combination with nisin.

A variety of interactions was observed between chemicals and nisin. As expected, nisin alone showed moderate inhibitory activity against *A. fumigatus*. Nisin is often known as an antibacterial agent. It has been found that it can block the growth of a variety of Gram-positive

Table 1. Antifungal effects of compounds combined with nisin.

Compounds	IZD (mm)	MIC (μg mL^{-1})	MFC (μg mL^{-1})	FIC	FFC
3a	10.22	256	1024	-	-
3b	11.94	256	1024	-	-
3c	10.12	256	1024	-	-
3d	11.01	256	1024	-	-
3e	12.10	128	512	-	-
3f	16.85	64	256	-	-
6a	10.35	256	1024	-	-
6b	7.72	512	2048	-	-
6c	10.53	256	1024	-	-
6d	10.61	256	1024	-	-
6e	14.28	128	512	-	-
MgO NP	10.18	256	1024	-	-
Nisin	8.11	512	2048	-	-
Ketoconazole	19.68	4.00	8.00	-	-
3a+Nisin	-	64.00	256	0.62, R	0.62, R
3b+Nisin	-	64.00	512	0.62, R	0.75, R
3c+Nisin	-	64.00	512	0.50, S	0.75, R
3d+Nisin	-	64.00	256	0.62, R	0.62, R
3e+Nisin	-	32.00	256	0.31, S	0.62, R
3f+Nisin	-	16.00	64.00	0.28, S	0.28, S
6a+Nisin	-	64.00	256	0.37, S	0.37, S
6b+Nisin	-	128	1024	0.50, S	1.00, I
6c+Nisin	-	64.00	256	0.37, S	0.37, S
6d+Nisin	-	64.00	256	0.62, R	0.62, R
6e+Nisin	-	32.00	256	0.31, S	0.62, R
MgO NP+Nisin	-	128	512	0.75, R	0.75, R

NP: Nanoparticles, IZD: Inhibition zone diameter, MIC: Minimum inhibitory concentration, MFC: Minimum fungicidal concentration, FIC: Fractional inhibitory concentration, FFC: Fractional fungicidal concentration, S: Synergistic effect, R: Relative synergistic effect, I: Incremental effect.

bacteria, however, it is less effective for Gram-negative bacteria, viruses and fungi due to the presence of outer membrane permeability barrier.[28] Antimicrobial effects of nisin on *A. fumigatus* have not been studied well so far. Nisin Z is able to resist oral gingival cells against *C. albicans*.[29] Inhibitory properties of nisin and propionic acid were evaluated on aflatoxin produced by *Aspergillus parasiticus*, *Aspergillus ochraceus* and *Fusarium moniliforme,* and fungistatic activities were improved in a special combination of both agents.[30] Antifungal effects of nisin alone and in combination with red ginger essential oil (*Zingiber officinale var. rubrum*) were proved against *A. niger.*[31] Nisin can reduce or change ATP production and the concentration of vital ions through the perforation of the cell membrane of microorganisms.[32] Specific cell wall proteins of yeast forms a barrier to small peptides such as nisin.[33]

Relative synergistic effects were recorded by MgO NPs. Synergistic effect of nisin on *S. auras* and *E. coli* have also been reported in combination with MgO NPs.[34] Antimicrobial activity of MgO NPs is related to its ability to damage cell membrane, increase pH value and produce active oxygen species.[31] Factors including size, concentration and pH affect antimicrobial activities of NPs.[35] Inhibitory properties of MgO, CaO and ZnO powders have been studied against *C. albicans*, *Saccharomyces cerevisiae*, *A. niger* and *Rhizopus stolonifer*.[36]

Effective interactions were observed by some thiazole derivatives combined with nisin according to their synergistic effects. These heterocycles as enzyme or protein inhibitors can block the growth of microorganisms.[37] Substituents such as phenyl, chloro, fluoro, bromo and nitro on thiazole ring improved antimicrobial effects.[38] Good to excellent results were reported with thiazoles against *A. fumigatus*.[39,40]

In the present study, synergistic effects on *A. fumigatus* were recorded with tetrahydropyrimidine derivatives **3d-f**. They act as channel and surface inhibitors. Some synthesized tetrahydropyrimidine derivatives have shown antifungal effects on *A. niger* and *A. flavus* with MICs in the range of 12.50 to 100 μg mL^{-1}.[41]

In our study, imidazolidine derivatives **3a-c** also showed synergistic effects on *A. fumigatus*. It was proposed that they could inhibit the synthesis of lipid or dihydrofolate reductase (DHFR) enzyme.[42] Antifungal effects of some synthetic imidazolidine derivatives on *A. fumigatus* were increased because of the binding of phenyl groups to their ring.[43]

To conclude, inhibitory potentials of all tested chemicals were proved against standard strains of *A. fumigatus*; while their antifungal effects were reinforced in combination with nisin. These combinations could be used as antimicrobial agents to treat fungal infections. In addition, nontoxic MgO NPs might be applied as preservatives to prevent microbial decomposition of food, beverages, biological samples, pharmaceutical drugs, cosmetics, paints and wood. The potential of nisin has also been proven to block one of the most important pathogenic fungi.

Acknowledgments

This study was supported by the University of Zabol under Research Project No: IR-UOZ96-10 and Grant No: UOZ-GR-9718-9.

Conflict of interest

The authors declare there are no conflicts of interest.

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