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Synthesis and in Vitro Antimicrobial and Cytotoxicity Activities of 2-[(2-nitro-1-phenylalkyl)thio]benzoic Acid Derivatives

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In this research 14 compounds were tested for their antimicrobial activity. The synthesis of 7 compounds corresponding to 2-[(2-nitro-1-phenyl-propyl)thio]benzoic acids **4** has not been reported before and 7 compounds corresponding to 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** were reported by our research group. The antibacterial activity of the title compounds was evaluated by 2 Gram (+) (Staphylococcus aureus, Bacillus subtilis) and 2 Gram (-)(Pseudomonas aeruginosa, Escherichia coli) microorganisms. The antifungal activity of the compounds was also determined against yeast-like fungi (Candida albicans, C. krusei). For antibacterial activity, ampicillin and for antifungal activity, fluconazole and ketoconazole have been used as reference compounds.

All of the 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** derivatives were more active than the reference compound ketoconazole in the antifungal activity test. The title compounds were also screened by consecutive dilution to explore their toxicity to a Vero cell line. Except for **3d** and **3e** all of the compounds **3** exhibited lower toxicity than ketoconazole.

Key Words: 2-[(2-nitro-1-phenylalkyl)thio]benzoic acid derivatives, β-Nitrostyrenes, Antibacterial Activity, Antifungal Activity, Cytotoxic Activity.

Introduction

$\beta-$Nitrostyrenes **1** are known for their antimicrobial activities[1,−3], and the addition products of β-nitrostyrenes exhibit antibacterial and antifungal activities[4,−6]. Michael- type addition reactions of β-nitrostyrenes **1** and their β-methyl derivatives **2** with thiol groups of aromatic and aliphatic character were investigated by our research groups[7−11].

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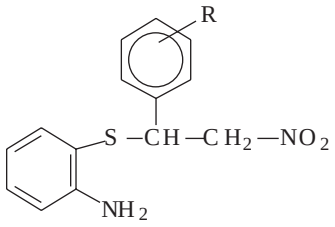
In our previous studies, we synthesized 1-[2-aminophenyl)thio]-1-phenyl-2-nitroethane **5** derivatives, which are the Michael type addition product of $\beta-$ nitrostyrenes with 2-aminothiophenol. Some of the **5** derivatives had significant antifungal activity against *Candida albicans*, *C. stellatoidea*, *C. parapsilosis, C. pseudotropicalis*[7]. We also reported synthesis of 1-[2-aminophenyl)thio]-1-phenyl-2-nitropropane **6** derivatives[8]. It found that **6** derivatives showed significant antibacterial activities against Gram-positive microorganisms (*Streptıcoccus faecalis, S. aureus*).



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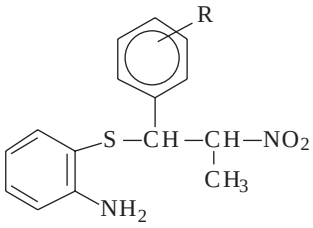

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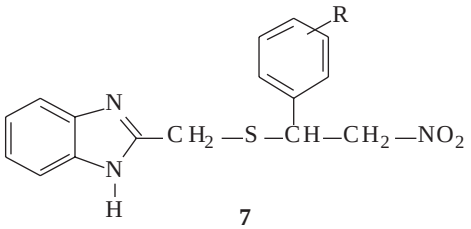
In addition we synthesized 2-[(2-Nitro-1-phenylethyl)thiomethyl]benzimidazole **7** and 2-[(2-nitro-1-phenylpropyll)thiomethyl]benzimidazole **8** derivatives[9,10]. These compounds are the Michael-type addition products of 2-mercaptomethyl-benzimidazole with β-nitrostyrenes and β-methyl-β-nitrostyrenes respectively.

Some of the **7** and **8** derivatives were found to be active as ampicillin against *S. aureus* and *S. faecalis.* Among the derivatives, 2-[(2-nitro-1-phenylethyl)-thiomethyl]benzimidazole **7a** showed antifungal activity close to ketoconazole against *C. albicans and C. parapsilosis.* However, its cytotoxic activity is lower than that of ketoconazole[11].



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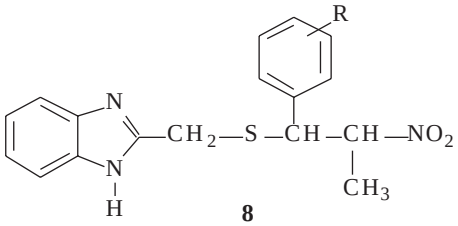

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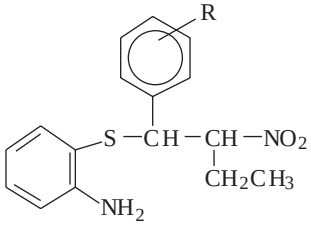
Moreover, 1-[(2-aminophenyl)thio]-1-phenyl-2-nitrobutane **9** derivatives were synthesized[12]. The antimicrobial activities of compounds **9** were investigated against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis*, *Bacillus subtilis*, *S. aureus* and *C. albicans* by the microdilution method. All of the 1-[(2-aminophenyl)thio]-1-phenyl-2-nitrobutane **9** derivatives showed varying degrees of inhibition against the tested bacteria and fungi. The antiviral activity of the synthesized compounds was also determined. All of them were found to be almost 100-fold more active than standard compound acyclovir[12].



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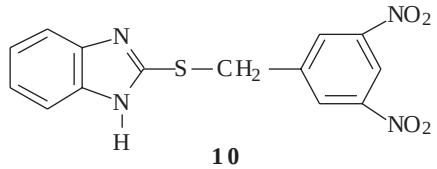
Since it is well known that the aromatic or heteroaromatic thioether groups play an important role in antimicrobial activity, a number of compounds were synthesized and evaluated as antibacterial and antifungal agents[13–16]. Among such compounds the highest antimicrobial activity was observed for the structures bearing a thioethereal function position 2 aromatic or heteroaromatic ring.

For example, a series of 2-alkylsulfanylbenzimidazoles was synthesized and the compounds were evaluated for their in vitro antimycobacterial activity[13]. The substances exhibited appreciable antimycobacterial activity, in particular, against non-tuberculous mycobacteria. The activity of the most active compound in the set, 3,5-dinitro derivative **10**, exceeded that of standard isoniazide against *M. kansasii* and *M. avium*.

Recently 2-methylthio-1*H*-benzimidazoles were synthesized and evaluated for antiparasitic activities[14]. These compounds were more active against *G. lambia* than metronidazole. Especially compound **11**, with a methylthio group at the 2-position and a chlorine at the 5-position, was 244 times more active than Metronidazole and 7.4 times more active than albendazole. Furthermore, selective anti-*helicobacter pylory* acting 2-[[(2-piridyl)metyl]thio]-1H-benzimidazoles **12** were synthesized[15]. These compounds were also tested against *E. coli* and *S. aureus*. Some of them displayed a minor growth inhibition of both *E. coli* and *S. aureus*.

In another study 2-(4-nitrophenyl)ethyl-mercapto-4,6-dibromo-1H-benzimidazole **13a** and 2-(4-nitrophenyl)ethyl-mercapto-4,6-dichloro-1H-benzimidazole **13b** derivatives were synthesized and evaluated for antimicrobial activity. The minimum inhibitory concentration values of the two compounds for all the Gram-positive bacteria were lower than those of nitrofurantoin (the reference agent)[16].



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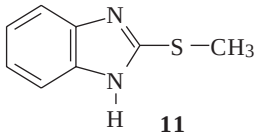

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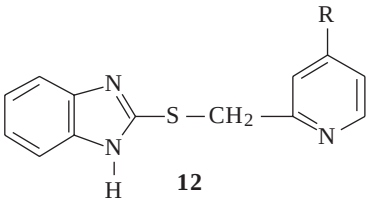

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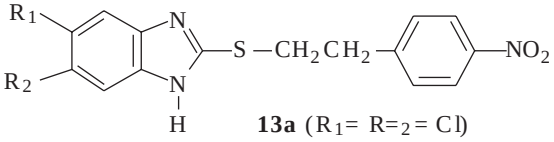


13a (R_1= R=$_2$= Cl)
13b (R_1= R=$_2$= Br)

In order to investigate the contribution of the side chain and the substitution pattern of the sulfur bearing aromatic ring to antimicrobial activity we synthesized a number of new 2-[(2-nitro-1-phenyl-propyl)thio]benzoic acid **4** derivatives. The synthesis of 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** derivatives was reported in our previous study[17]. All of the **3** and **4** derivatives were screened for antimicrobial activity, and cytotoxic evaluation of the title compounds was performed.

Experimental

Chemistry

The fine chemicals and all the solvents used in this study were purchased locally from Merck A.G. and Aldrich. β-nitrostyrene, β-methyl-β-nitrostyrene derivatives were synthesized in our laboratory according

to the reported data[18,19].

The melting points of the compounds were determined on an electrothermal 9200 melting points apparatus and the values were given uncorrected.

The IR spectra of the compounds were recorded on a Bruker Vector 22 IR (Opus Spectroscopic Software Version 2.0) spectrophotometer. The ^{1}H-NMR spectra of the compounds were recorded in DMSO-d_6 on a Jeol 500 MHz NMR spectrometer using tetramethylsilane as internal standard. All the chemical shifts were recorded as δ (ppm). Microanalyses for C, H and N were performed by the TÜBİTAK Analytical Laboratory, Turkey, and were within $\pm$ 0.4% of the theoretical values.

Synthesis of 2-[(2nitro-1-phenylpropyl)thio]benzoic acid 4 derivatives

0.01 mole thiosalicylic acid and 0.01 mole appropriate β-methyl-β-nitrostyrene derivative were dissolved in 20 mL ethanol at room temperature stirring vigorously and a clear solution was obtained. The stirring continued for 3 h at room temperature, and was followed by refrigeration overnight. Precipitated crystals were filtered the next day and washed with cold ethanol.

Microbiology

The following bacteria were used for antibacterial study: *Staphylococcus aureus* ATCC 25923, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 27833 and *E. coli* 25882.

The following yeast-fungi were used for antifungal study: *C. albicans* ATCC 64550 and *C. krusei* ATCC14243.

Inoculation suspensions

The microorganism suspensions used for inoculation were prepared at 10^6 cfu/mL concentrations by diluting fresh cultures at MacFarland 0.5 density (10^8 cfu/mL). It was known that there were 5 x 10^4 cfu/mL microorganisms in each well after inoculation.

Medium

Mueller-Hinton Broth (Oxoid) was used for the dilution of microorganism suspensions and 2-fold dilutions of the compounds. Sabouraud liquid medium (Oxoid) was used for yeast-like fungi for the same purpose.

Equipment

Falcon^R microplates, which have 96 wells, were used for the microdilution method.

A Brinkmann transfer pipette was used for 2-fold dilution of compounds in the wells.

Method

Microdilution was employed for antibacterial and antifungal activity tests[20a,b]. The synthesized compounds and the stock solution of the standards were dissolved in dimethylsulfoxide (ketoconazole), in water (fluconazole) and in phosphate buffer saline (ampicillin) at 1000 μg/mL final concentration. The solutions of each compound at 500,....., 3.9 μg/mL concentrations were prepared in the wells by diluting with the media. Suspensions of the microorganisms at 10^6 cfu/ml concentrations were inoculated to the two

fold–diluted solutions of the compounds; consequently the microorganism suspensions in each well was approximately 5x10^5 cfu/mL. The solutions of DMSO-microorganism mixture, the pure microorganisms, and pure media were used as control wells.

The microplates were then covered and incubated at 36 $^\circ$ C for 24-48 h. Wet cotton-wool was placed in the incubation chamber in order to keep it sufficiently humid to avoid evaporation. After a certain period, the wells were evaluated. The concentrations of the wells where no growth was observed were evaluated as the MIC of the respective compounds.

Cytotoxicity assay

The compounds were screened by serial dilution to assess toxicity to a Vero cell line.

Test material

All of the 14 compounds were tested to detect the cytotoxicity. Concentrations were arranged to provide 1000 μg/mL of each compounds. Each of the synthesized compounds was sterilized by filtering through a 0.2 μm membrane (Sartorius, Germany).

Cell culture

Vero cell culture (African green monkey kidney) was used to detect the cytotoxicity of the test compounds. Eagle's Minimal Essential Medium (EMEM) and 5% fetal bovine serum were used to test the growth and continuity of the cells.

Method

The cytotoxicity of the synthesized compounds was determined by the method described by Walker et al.[21] with small modifications[22]. The test was performed in 96-well microplates (Greiner, Germany). The 14 compounds were fed into the wells, 100 μL in each. In another set of 11 rows, 50 μL of EMEM was dispersed. Then 100 μL of the test compounds was transferred to the next row with a multichannel pipette and mixed. From this 50 μL of compounds was transferred to the next row and this was repeated until the last row. After mixing the material of the last row, 50 μL of the compounds was removed and thus compound dilutions of 50 μL were determined in each well. With this application a 12-step dilution of each compound was obtained according to Log_2 on the microplates.

After dilution 50 μL of the cell suspension of 300,000 cells/mL, which was prepared in EMEM + 5% fetal bovine serum, was put in each well and the plates were incubated in 5% CO_2 atmosphere at 37 $^\circ$ C for 5 days. At the end of this time the cells were examined using an inverted optical microscope, comparing treated and control untreated cultures based on cellular morphologic alterations.

Results and Discussion

Chemistry

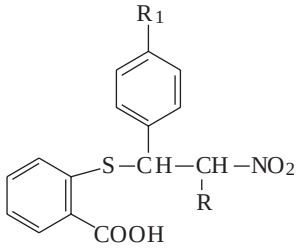
The target compounds were synthesized according to Scheme.

The $\beta-$ methyl-β-nitrostyrene derivatives, the starting material, were synthesized according to literature methods[18,19]using corresponding benzaldehyde derivatives and nitromethane in the presence of a base.

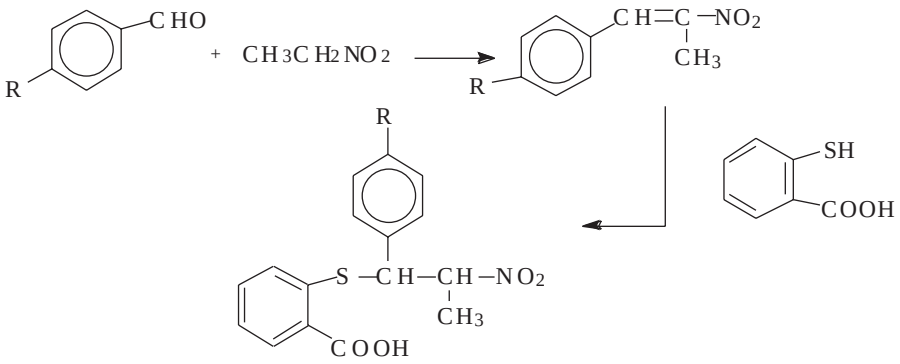
The addition products of $\beta-$ nitrostyrene and β-methyl-β-nitrostyrene derivatives with thiosalicyclic acid were obtained by Michael-type addition reaction.

The addition products, of which the antimicrobial activity was investigated in this study, are listed in Table 1. Spectral data and elemental analysis of newly synthesized 2-[(2-nitro-1-phenylpropyl)thio]benzoic acid derivatives **4** are given in Table 2.

Table 1. 2-[(2-Nitro-1-phenyl-alkyl)thio]benzoic acid derivatives and their Mps and yields.



Comp.	R	R_1	Mp. (°)	Yields %	Molecular Formula Formula	Analysis
3a	H	H	136-138	75	$C_{15}H_{13}NO_4S$ (303.05)	C, H, N
3b	H	4-Cl	135	73	$C_{15}H_{12}NclO_4S$ (337.02)	C, H, N
3c	H	4-CH_3	138-140	90	$C_{16}H_{15}NO_4S$ (317.07)	C, H, N
3d	H	4-C_2H_5	108-110	87	$C_{17}H_{17}NO_4S$ (331.09)	C, H, N
3e	H	4-OCH_3	115	83	$C_{16}H_{15}NO_5S$ (333.07)	C, H, N
3f	H	4-OC_2H_5	161	81	$C_{17}H_{17}NO_5S$ (347.08)	C, H, N
3g	H	4-NO_2	128	70	$C_{15}H_{12}N_2O_6S$ (348.04)	C, H, N
4a	CH_3	H	130	75	$C_{16}H_{15}NO_4S$ (317.06)	C, H, N
4b	CH_3	4-CH_3	135	73	$C_{17}H_{17}NO_4S$ (331.38)	C, H, N
4c	CH_3	4-OCH_3	138	60	$C_{17}H_{17}NO_5S$ (347.38)	C, H, N
4d	CH_3	4-OC_2H_5	110	77	$C_{18}H_{19}NO_5S$ (361.41)	C, H, N
4e	CH_3	4-$N(CH_3)_2$	115	73	$C_{18}H_{20}N_2O_4S$ (360.48)	C, H, N
4f	CH_3	4-OH, 3-OCH_3	161	71	$C_{17}H_{17}NO_6S$ (363.38)	C, H, N
4g	CH_3	4-NO_2	128	65	$C_{16}H_{14}N_2O_6S$ (362.35)	C, H, N



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Scheme. Synthesis of 2-[(2-nitro-1-phenylpropyl)thio]benzoic acid **4** derivatives.

Table 2. Spectral data of 2-[(2-nitro-1-phenyl-alkyl)thio]benzoic acid derivatives.

Comp.	IR(KBr)cm^{-1}		^{1}H-NMR *(DMSO-d_6)* ppm
	C=O	NO_2	
4a	1680	1540-1375	13.06 (s, 1H, COOH), 7.69-7.10 (m, 9H, arom. protons), 5.25-5.13 (m, 1H, -CH-NO_2), 5.02-4.99 (d, 1H, S-CH), 1.22-1.12 (d, 3H, CH_3).
4b	1680	1545-1360	13.30 (s, 1H, COOH), 7.91-7.09 (m, 8H, arom. protons), 5.22-5.18 (m, 1H, -CH-NO_2), 5.06-5.02 (d, 1H, S-CH), 2.25 (s, 3H, aromatic methyl), 1.34-1.22 (d, 3H, CH_3).
4c	1670	1550-1370	13.53 (s, 1H, COOH), 8.03-7.05 (m, 8H, arom. protons), 5.15-5.10 (m, 1H, -CH-NO_2), 4.95-4.75 (d, 1H, S-CH), 4.70 (s, 3H, OCH_3), 1.32-1.20(d, 3H, CH_3).
4d	1690	1540-1370	13.12 (s, 1H, COOH), 7.52-6.85(m, 8H, arom. protons), 5.20-5.13 (m, 1H, -CH-NO_2), 5.10-5.03(d, 1H, S-CH), 4.00-3.95(q, 2H, O$\underline{CH_2}CH_3$), 1.35-1.32(d, 3H, CH_3), 1.30-1.27(t, 3H, $OCH_2\underline{CH_3}$),
4e	1670	1550-1370	13.31 (s, 1H, COOH), 7.96-6.68 (m, 8H, arom. protons), 5.11-5.00 (m, 1H, -CH-NO_2), 4.87-4.68 (d, 1H, S-CH), 2.91 (s, 6H, $N(CH_3)_2$, 1.34-1.23 (d, 3H, CH_3).
4f	1680	1540-1370	13.40 (s, 1H, COOH), 8.23-6.94 (m, 7H, arom. protons) 5.40-5.15 (m, 1H, -CH-NO_2), 4.63-4.40 (d, 1H, S-CH), 3.99-3.81(s, 3H, OCH_3), 1.49-1.43 (d, 3H, CH_3).
4g	1670	1550-1370	13.33 (s, 1H, COOH), 8.19-7.21(m, 8H, arom. protons), 5.41-5.38 (m, 1H, -CH-NO_2), 5.33-5.26 (d, 1H, S-CH), 1.39-1.37(d, 3H, CH_3).

Microbiology

Almost all the major classes of antibiotics have encountered resistance in clinical application[23−28]. The emergence of bacterial resistance to β-lactam antibiotics, macrolides, quinolones and vancomycin is becoming a major worldwide health problem[28−31]. In particular, antibiotic resistance among Gram-positive bacteria is becoming increasingly serious[32−36]. In order to overcome these emerging resistance problems, there is an urgent need to discover novel antibacterial agents in structural classes distinct from existing antibiotics. These finding have inspired us to synthesize new class potential antibiotics such as 2-[(2-Nitro-1-phenylalkyl)thio]benzoic acid derivatives (**3** and **4)**.

Compounds **3a-g** and **4a-g** were tested for antibacterial and antifungal activities using various strains

with the microdilution method[20,21]. For the determination of antibacterial activity *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 27833 and *E. coli* ATCC 25882 strains were utilized. All the compounds were also tested for in vitro antifungal activity against *C. albiccans* ATCC 64550 and *C. krusei* ATCC 14243 strains. Ampicillin, ketoconazole and fluconazole were used as reference compounds. There was no inhibitory activity in the wells containing only DMSO. Microbial growth occurred in the respective wells, and the medium was not contaminated during the tests. The MIC values of 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** and 2-[(2-nitro-1-phenyl-propyl)thio]-benzoic acid **4** derivatives are given as μg/mL in Tables 3 and 4.

Table 3. The MIC (μg/mL) values of 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** derivatives.

Comp.	A	B	C	D	E	F
3a	15.6	15.6	15.6	15.6	3.9	3.9
3b	3.9	15.6	15.6	15.6	3.9	3.9
3c	15.6	7.8	15.6	15.6	3.9	3.9
3d	3.9	7.8	15.6	15.6	3.9	3.9
3e	3.9	15.6	15.6	15.6	3.9	3.9
3f	3.9	15.6	31.2	31.2	3.9	3.9
3g	7.8	7.8	15.6	15.6	3.9	3.9
AMP	3.9	3.9	3.9	3.9	-	-
KET	-	-	-	-	7.8	7.8

A: *Staphylococcus aureus* ATCC 25923
B: *Bacillus subtilis* ATCC 6633
C: *Pseudomonas aeruginosa* ATCC 27833
D: *Escherichia coli* ATCC 25882
E: *Candida albicans* ATCC 64550
F: *Candida krusei* ATCC 14243

Table 4. The MIC (μg/mL) values of 2-[(2-nitro-1-phenylpropyl)thio]benzoic acid **4** derivatives.

Comp.	A	B	C	D	E	F
4a	15.6	15.6	31.2	31.2	15.6	15.6
4b	15.6	15.6	62.5	62.5	31.2	31.2
4c	15.6	15.6	31.2	31.2	15.6	15.6
4d	7.8	7.8	62.5	62.5	15.6	15.6
4e	7.8	7.8	62.5	62.5	15.6	15.6
4f	7.8	7.8	62.5	62.5	15.6	15.6
4g	7.8	7.8	62.5	62.5	15.6	15.6
AMP	1.9	1.9	7.8	7.8	-	-
FLUC	-	-	-	-	1.9	1.9

A: *Staphylococcus aureus* ATCC 25923
B: *Bacillus subtilis* ATCC 6633
C: *Pseudomonas aeruginosa* ATCC 27833
D: *Escherichia coli* ATCC 25882
E: *Candida albicans* ATCC 64550
F: *Candida krusei* ATCC 14243

The results of this study showed that 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** derivatives had

significant antibacterial activity. The activity of compounds **3b**, **3d**, **3e** and **3f** is equal to that of ampicillin in terms of antibacterial activity against *S. aureus.*

Entire derivatives of 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** had pronounced antifungal activity and exceeded that of ketoconazole, which was used as the reference compound. It is well known that the antifungal drug ketoconazole is used clinically to treat or suppress various fungal infections[37−42]. These results suggest that compound **3** derivatives may be worth studying further in terms of their antifungal activity.

As seen in Table 4 2-[(2-nitro-1-phenyl-propyl)thio]benzoic acid derivatives **4** exhibited no important antibacterial activity. It appears a methyl substitutent on the side chain of compound **4** derivatives reduced antibacterial activity. The derivatives of 2-[(2-nitro-1-phenyl-propyl)thio]benzoic acid **4** were assayed for antifungal activity against 2 different fungi, using fluconazole as the reference compound. All the derivatives **4** are less active than fluconazole.

Cytotoxicity

The MIC (μM) values of compounds **3** and **4** in the cytotoxicity test are reported in Table 5. Ketoconazole and fluconazole, which were used as standard compounds in the antifungal activity test, were tested for cytotoxicity. The cytotoxicity effects of the synthesized compounds were determined in a VERO cell line.

Table 5. Minimum toxic concentrations of 2-[(2-nitro-1-phenyl-ethyl)thio]benzoic acid **3** and 2-[(2-nitro-1- phenyl-propyl)thio]benzoic acid **4** derivatives.

Comp.	Cytotoxicity (μM)
3a	$6.39X10^{-2}$
3b	$2.31X10^{-2}$
3c	$8.36X10^{-2}$
3d	$1.17X10^{-2}$
3e	$1.17X10^{-2}$
3f	$2.24X10^{-2}$
3g	$5.50X10^{-2}$
4a	$2.46X10^{-2}$
4b	$1.17X10^{-2}$
4c	$1.12X10^{-2}$
4d	$1.07X10^{-2}$
4e	$1.08X10^{-2}$
4f	$1.07X10^{-2}$
4g	$5.38X10^{-2}$
KET	$1.46X10^{-2}$
FLU	$5.44X10^{-2}$

The MIC (μM) is defined as the lowest concentration of cytotoxic effect. The cytotoxicity data of the tested compounds indicate that all of the compounds exhibited various degrees of toxicity. Except for **3d** and **3e**, the all of the **3** derivatives exhibited lower toxicity and higher antifungal activity than standard comparison compound, ketoconazole. Our investigations continue to determine the in vivo antifungal activity of compounds **3**.

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