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Novel Perylene-Based Antimicrobial PDI Chromophores

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Abstract: The main goal of the study was to monitor the Antimicrobial activity of two novel perylene diimides which were synthesized and characterized. Antimicrobial activity was investigated against Four *Mycobacterium tuberculosis* strains (MT) (Mt-H_{37}Rv, Mt-H_{37}Ra and two clinical isolates) and two *Staphylococcus aureus* strains [Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus (SA)*]. Minimum inhibitory concentrations (MICs) and minimum bactericidal activity (MBCs) were determined. Both compounds exhibited bactericidal effects and MICs were found to be changing in the range of 48-96 µg/mL for four MT-strains. Compounds were also effective on *Staphylococcus* strains at MIC = 96 µg/mL.

Key words: Perylene diimide**,** antimicrobial activity, *Mycobacterium tuberculosis***,** methicillin resistant, *Staphylococcus aureus*, MIC, MBC.

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1. INTRODUCTION

Perylene diimides are reddish dyes with very high quantum yields. Red chromophores based on 3,4:9,10-perylenebis(dicarboximide)s have shown great promise in a variety of applications owing to their outstanding chemical, thermal and photochemical stability. They have been used widely in such as reprographic processes (1-2), organic light-emitting diodes (3-4), molecular switches and wires (5-6), light-harvesting arrays (7), photoreactive thin films (8-11), solar cells (12-13), and dye lasers (14-15) and photosensitizers (16). By single or double amine substitution on the perylene core, the absorption maxima at these dyes can be shifted up to 750 nm with further appropriate modifications, solubility can be improved.

Perylene diimides also became important field of interest for antimicrobial activities of perylene derivatives in addition to their role in the chemical industry (18-19). *Mycobacterium tuberculosis* (MT), is a bacteria, causes “Tuberculosis (TB)”, affects the lung. TB causes serious health problems such as multi drug resistance (MDR-TB), and extensive drug resistance (XDR-TB) and leads to many more human deaths than any other microbial disease (20). MDR-TB resistance is a problem of acquired drug resistance and known as tuberculosis whose bacteria are resistant to isoniazid (INH) and rifampin (RIF). Extensive drug resistant TB (XDR-TB) is a form of tuberculosis whose bacteria are resistant to INH and RIF and in addition to any fluoroquinolone (21). Therefore, XDR-TB is much severe then TB and MDR-TB (22). One in three people in the world is infected with dormant TB bacteria. Bacteria might be active and causes disease depend on the several factors such as immunity, poverty and age of a person, and HIV (23). Nowadays, XDR-TB patients can be treated by current drugs, unfortunately reaching the success rate is not high. On the other hand, MRSA is resistant to many beta-lactam antibiotics such as penicillins and cephalosporins. Initially, MRSA was viewed as a hospital-acquired infection, but is now seen as an advanced and community-associated MRSA (24).

Therefore, as mentioned above, It is essential to discover new molecules effective on resistance targets of bacteria. For this aims, two perylene diimide derivatives investigated effects against *M. tuberculosis* strains (Mt-H37Rv, Mt-H37Ra and two clinical isolates) and SA strains. Minimum inhibitory concentrations (MICs) and minimum bactericidal activity (MBCs) were investigated.

2.MATERIALS AND METHODS

2.1.Chemicals and Measurements

All chemicals and solvents obtained from Aldrich and Sigma and used without further purification. Column chromatography of all the products were performed using Merck Silica Gel60 (particle size:0.040-0.063 mm, 230-400 mesh ASTM) penetrated with the eluent. Reactions were monitored by thin layer chromatography using precoated silica gel plates (Merck Silica Gel 60 Kiesel gel F254 TLC Aluminum Sheets 20x20 cm).

^{1}H and ^{13}CN MR spectra were recorded on a Bruker Instruments Avance Series-Spectro spin DPX-400 Ultrashield (400 MHz) High Performance digital FTNMR spectrometer (METU, NMR Laboratory). All chemical shifts are referenced to residual solvent signals previously referenced to TMS and splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Electrospray Ionization (ESI) mass spectra were recorded on Agilent 6500 Series LC-MS spectrometer, Agilent instruments, Paolo Alto, CA, USA. UV-Visible spectra were performed with Perkin Elmer Lambda 25 UV/Vis Spectrometer.

2.2. Synthesis of *N,N'*-O-*t*-butyl-L-serine *t*-butylester-3,4:9,10-perylene diimide (Compound 1)

A mixture of 0.5 g ($1.274x10^{-3}$ mol) perylene-3,4:9,10-tetracarboxylic acid dianhydride and 0.646 g ($2.548x10^{-3}$mol) O-*t*-butyl-L-serine *t*-butyl ester hydrochloride in 10 mL H_2O, 10 mL n-butanol and 1.5 mL triethylamine was stirred for 48 h at 85°C. The reaction solution was removed by rotary evaporator, and purified by column chromatography ($CHCl_3$:CH_3OH - 97:3). After solvent removal by rotary evaporator, the precipitate was dried in vacuo (Yield: 29%).

(1) $C_{47}H_{53}N_2O_{10}$,ESI-MS:m/z805.37(M^+ + 1).
(2) ^{1}H-NMR (400 MHz, $CDCl_3$), δ[ppm], 1.10 (s, 18H, -O-C), 1.48 (s, 18H, -C(=O)O-CH_3), 4.15-4.20 (m, 4H, -C(=O)O-CH_3), 5.88 (t, 2H, N-CH), 7.26 (d, J= 8Hz, 2H, CH-arom), 8.51 (d, J=8.1 Hz,2H, CH-arom), 8.61 (d, J=8Hz, 2H, CH-arom)
(3) ^{13}C-NMR (100 MHz, $CDCl_3$), δ[ppm], 27.43, 27.97, 38.72, 68.14, 76.98, 82.35, 120.83, 121.91, 128.57, 128.77, 130.24, 130.84, 132.43, 138.18, 167.18, 167.73.

2.3. Synthesis of *N,N'*-O-*t*-butyl-L-threonine *t*-butylester-3,4:9,10-perylene diimide (Compound 2)

A mixture of 0.5 g ($1.274x10^{-3}$ mol) perylene-3,4:9,10-tetracarboxylicacid dianhydride and 0.742 g ($2.548x10^{-3}$ mol) O-*tert*-butyl-L-threonine *t*-butyl ester acetate salt in 10 mL H_2O, 10 mL n-butanol and 1.5 mL triethylamine was stirred for 48 h at 85 °C. The reaction solution was removed by rotary evaporator, and purified by column chromatography ($CHCl_3$:CH_3OH - 97:3). After solvent removal by rotary evaporator, the precipitate was dried in vacuo (Yield 31%).

(1) $C_{49}H_{57}N_2O_{10}$, ESI-MS: m/z 833.40 (M^+ + 1).

(2) ^{1}H-NMR (400 MHz, $CDCl_3$), δ[ppm], 1.27 (s, 18H, -O-C), 1.43 (s, 18H, -C(=O)O-CH_3), 1.54 (s, 6H, CH_3), 4.43-4.47 (m, 4H, -C(=O)O-CH_3), 5.44 (t, 2H, N-CH), 7.24 (d, J= 8 Hz, 2H, CH-arom), 8.55 (d, J=8,1 Hz, 2H, CH-arom), 8.66 (d, J=8Hz, 2H, CH-arom)
(3) ^{13}C-NMR (100 MHz, $CDCl_3$), δ[ppm], 23.75, 27.42, 28.448, 29.616, 31.58, 58.78, 59.32, 64.88, 67.38, 73.75, 77.20, 81.68, 122.96, 126.41, 129.07, 131.49, 134.73, 162.84, 167.55.
Relative absorption of Compound 1 ''*N,N'*-O-*t*-butyl-L-serine *t*-butyl ester-3,4:9,10-perylene diimide'' and Compound 2 ''*N,N'*-O-*t*-butyl-L-threonine *t*-butyl ester-3,4:9,10-perylene diimide'' was measured using by UV-Vis spectrometer as these articles (25-27) (Fig 2).

Before the biological evaluation of synthesized derivatives, the protective groups were removed upon treatment with CF_3COOH:$CHCl_3$ (50:50).

2.4. Antimicrobial Activities

2.4.1. Antistaphylococcal activity

Microorganisms and culture media: Methicillin resistant *S. aureus* (MRSA, ATCC 33592), *S. aureus* (ATTC 6538P) were used for antistaphylococcal assays. Nutrient agar and Nutrient Broth were used as a culture media.

Preparation of samples: The samples (10 mg) were solubilized in DMSO (1 mL) so as to prepare the stock solution. Final solutions of the samples were prepared as two concentration series, 1.5-768 µg/mL to obtain precisely correct MIC and MBC values.

Preparation of Staphylococcus spp. cultures and inocula: Suspensions of microorganism were prepared from the fresh cultures of bacteria (24 hours) on nutrient agar by suspended in sterile saline solution. The turbidities of bacteria were arranged to a 0.5 McFarland standard (10^8 CFU/mL) then diluted at 100 times to reach of 10^6 CFU/mL. Mueller Hinton Agar and Mueller Hinton Broth were used in vitro antistaphylococcal activity assays.

Antistaphylococcal Activity Assays for S. aureus and MRSA: Mueller Hinton Broth was used for microdilution assays for *S. aureus* susceptible and MRSA. We used a 96-well plate in the experiments. Two-fold dilutions were performed from the first well to the next well (100 µL). The

wells were filled with Mueller Hinton Broth (100 µL) then sample (100 µL) was added to the first well, after mixing several times by pipetting, the procedure was repeated for the dilution series except control wells. Afterwards, inoculum (20 µL) was added all the wells except negative control wells. All plates incubated at 37 °C for 24-48 hours for bacteria (28).

Determination of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Activity (MBCs): To determine of the bacterial growth, Thiazolyl Blue Tetrazolium, a dye for cell growth assays (20 µL, Sigma), was used. To determine the MIC values, dye were added into the wells then incubated at 37 °C for 24-48 h. When the dye turned to pink, bacterial growth was indicated. MIC value is accepted the lowest concentration of the compounds that inhibits visibly the growth of the microorganism after 24-48 h incubation.

To determine MBCs, the wells were filled with fresh Mueller Hinton broth (185 µL) except test compounds, then a bacterial suspension (15 µL) were added to the wells starting from MIC concentration and the higher concentrations in the series. After incubating the microplates at 37 °C for 24-48 h, indicator dye was added to the microplates. MBC was described as the lowest concentration of compounds that there is no growth when subculture in an antibiotic-free growth medium. All tests were performed according to National Committee for Clinical Laboratory Standards for bacteria in triplicate (29). Iecilline (Ulagay, TR)(concentration: 79.36 µg/mL) was used as a standard drug for comparison the activity of the compounds. Standard drug concentrations were given in table 1.

2.4.2. Antimycobacterial activity

Microorganisms and culture media: Four bacteria, *M. tuberculosis* H_{37}Ra (ATCC 25177), *M. tuberculosis* H_{37}Rv (ATCC 25618) and two positive clinical isolates of *M. tuberculosis* MT-strain-1 and MT-strain-2, obtained from hospital, were used for antimycobacterial bioassays. Middlebrook 7H9 Broth (Becton & Dickinson, USA) and Middlebrook 7H10 Agar (Becton & Dickinson, USA) was used as *Mycobacterium* culture media.

Preparation of Mycobacterial inocula: All strains were cultured in MGIT Mycobacteria Growth Indicator Tubes, containing 4 mL of modified Middlebrook 7H9 Broth Base at 37 °C for 5-7 days. OADC supplement (0.5 mL) was added to each tube. Inoculum was prepared from a positive BACTEC *Mycobacteria* Growth Indicator Tube (MGIT) according to the manufacturer's (Becton, Dickinson) instructions (30). To prepare inoculum from a positive BACTEC MGIT tube, the tubes which were day-1 and day-2 positive were used for the susceptibility test. Tubes were checked according to consume the oxygen by actively respiring micro-organisms and allow the fluorescence to be observed using a 365 nm UV transilluminator (31). Positive MGIT 7 mL tubes ranges were changed between 0.8 x 10^5 to 3.2 x 10^5 CFU/mL.

Antimycobacterial activity test for MT strains: The activity of all compounds against *M. tuberculosis* strains was tested using the Microplate Presto Blue Assay (MPBA) by the method described (32). 100 µL of compound was transferred in the first column then 100 µL of 7H9 broth was transferred from the column 1 to column 10. Column 11 and 12 were negative and positive control respectively. 100 µL of compound were transferred from column 1 to column 2 then mixed by pipettes three times and go on the same to provide serial 1:2 dilutions. 100 µL of excess medium was discarded from the wells in column 10. Afterwards, of inoculum (20 µL) was added into the wells from 1 to 10 and positive control wells. Negative control wells were not inoculated with bacteria. Positive and negative control columns were compound-free controls (33-28). Fluorometric susceptibility test procedure carried out according to recommended by the manufacturer, Becton, Dickinson and Company (30).

Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal activity (MBCs): Microplates were incubated at 37 °C for 6 days then presto blue (15 µL, Life Technologies) was added to the bacterial growth control wells (without compound) monitoring the growth of *Mycobacterium.* The microplates were incubated at 37 °C for an additional 24 h. When the dye turned from blue to pink in the positive control tubes (indicating positive bacterial growth); Presto blue solution was added to the other wells to determine the MIC values. All tests were performed in triplicate. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of sample that prevents a color change to pink. The minimum bactericidal concentration (MBC) was corresponded to the minimum compound concentration which is not cause a color change in the subcultures when re-incubated in fresh medium (34). Streptomycin (Concentration: 83 µg/mL; STR, BD), ethambutol (Concentration: 415 µg/mL. EMB, BD), rifampin (Concentration: 83 µg/mLRIF, BD), and izoniazid (Concentration: 8.3 µg/mL.INH, BD) were used as standard drugs for comparison the activity of the compounds. All antibiotics were purchased from BD Company, USA as the BD BACTEC™ MGIT™ 960 SIRE Kit for susceptibility testing of *M. tuberculosis*. Standard drug concentrations were given in Table 1.

3. RESULTS and DISCUSSION

In this work, we designed two different perylene diimides which are expected to show antimicrobial activity (Fig1). Target molecules

were synthesized in a few steps from commercially available materials. Relative absorption of Compound 1 and Compound 2 was shown using by uv-vis spectrometer in Figure 2. Then antimicrobial activities both, antimycobacterial and antistaphylococcal, were given in Table 1. Antimycobacterial activity of the compounds as MIC and MBC (µg/mL) were shown against of two novel perylene diimides in Figure 3.

The synthesis was started with double amine substitution by O-*t*- butyl-L-serine *t*-butyl ester hydrochloride and *O*-*t*-butyl-L-threonine-*t*-butyl ester acetate salt on the perylene core to be improved solubility. We synthesized *N,N'*-O-*t*-butyl-L-serine-*t*-butyl*e*ster-3,4:9,10-perylene diimide and *N,N'-(*O-*t*-butyl-L-threonine-*t*-butyl ester-3,4:9,10-perylene diimide. The chemical structures of two compounds were verified analytically.

3.1. The Synthesis of Two Novel Compounds

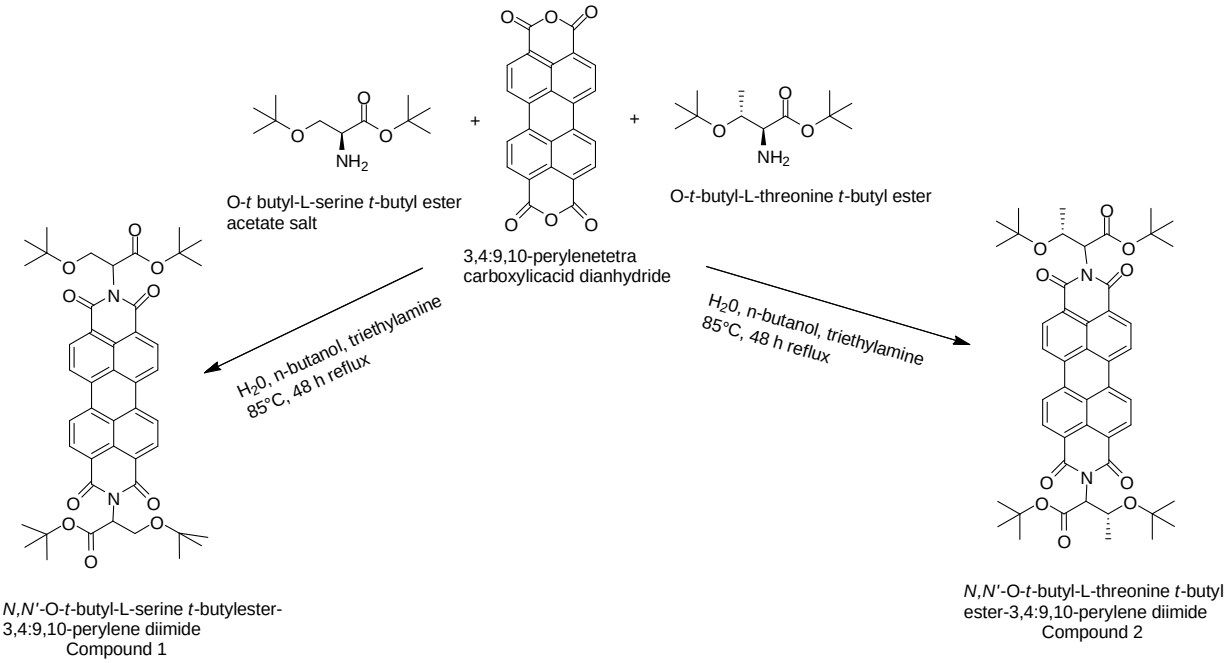

Figure 1: Synthesis of perylene diimide derivatives (*N,N'*-O-*t*-butyl-L-serine *t*-butyl ester-3,4:9,10-perylene diimide and *N,N'*-O-*t*-butyl-L-threonine *t*-butyl ester-3,4:9,10-perylene diimide).

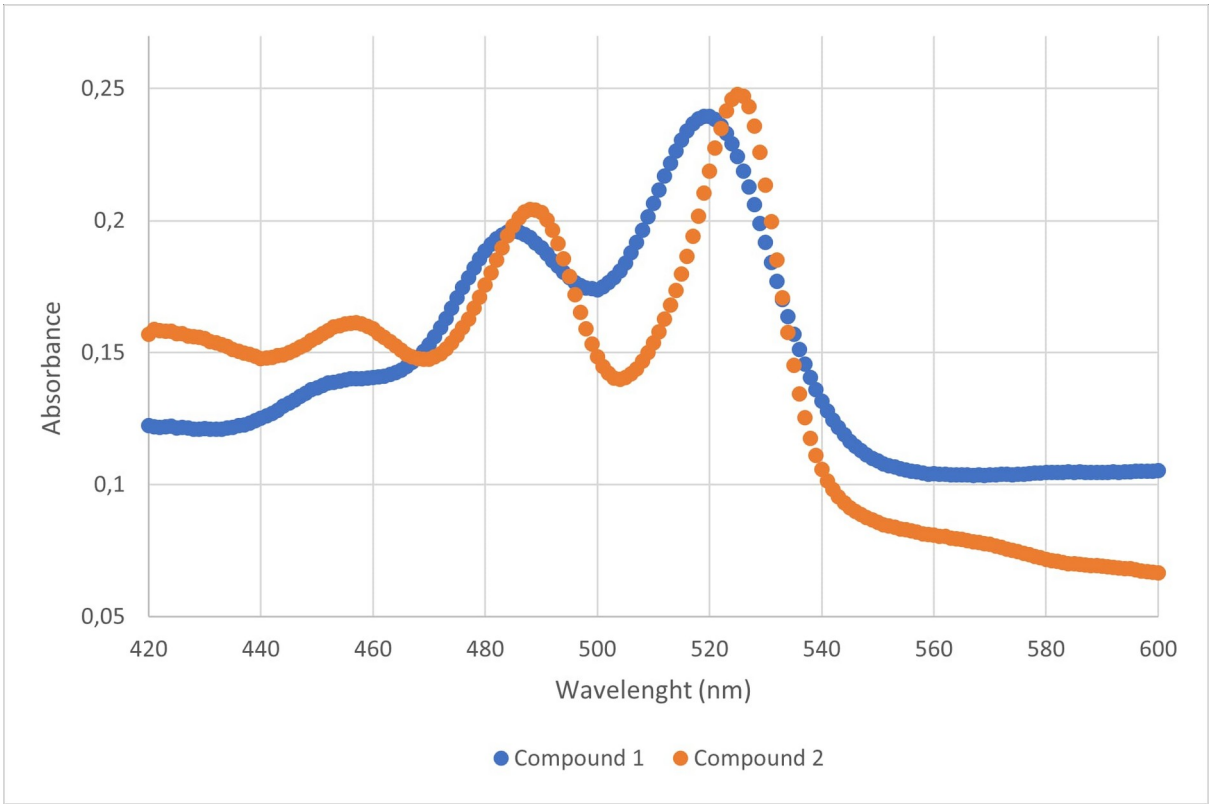

Figure 2: Relative Absorbance Graph of Compound 1 ''*N,N'*-O-*t*-butyl-L-serine *t*-butyl ester-3,4:9,10-perylene diimide'' and Compound 2 ''*N,N'*-O-*t*-butyl-L-threonine *t*-butyl ester-3,4:9,10-perylene diimide''

3.2. Antimycobacterial Activity Assays

All biological activity assays performed for two compounds. Compounds were more effective on all the microorganisms.

Efficacy of the compounds against drug resistant (Mt-H_{37}Rv), drug susceptible (Mt-H_{37}Ra*)* and clinical isolates showed that the MICs were changing between 48-192 µg/mL. MBC values were a little bit higher than MIC values and changed between 96-384 µg/mL (Table 1). For compound **1**, the lowest MIC values were determined against Mt-H_{37}Ra and MT-strain-1 (MIC 48 µg/mL)**,** MBC was 192 and 96 µg/mL respectively. Compound 2 showed the lowest MIC values (MIC 48 µg/mL) against Mt-H_{37}Ra. MBCs were varies between 48-384 µg/mL). The highest MBCs determined in MT- Strain-1 and MT- Strain-2.

3.3. Antistaphylococcal Activity Assays

While, compound **1** and compound **2** exhibited the same MIC and MBC values 96, 192 µg/mL respectively against MRSA. The efficacy of the compound **1** against *S. aureus* was 96 µg/mL (MIC) and 384 µg/mL (MBC) and the compound **2** against *S. aureus* was 96 µg/ mL (MIC) and 192 µg/mL (MBC) (Table 1). As a results, both of the compounds showed bactericidal effects and kill the bacteria depended the concentrations.

Table 1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the samples (µg/mL),

Compounds	Antimicrobial activity (µg/mL)											
	Mt-H_{37}Rv		Mt-H_{37}Ra		MT-Strain 1		MT-Strain 2		S. aureus		MRSA	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Compound **1**	96	384	48	192	48	96	96	192	96	384	96	192
Compound **2**	96	192	48	96	96	384	96	384	96	192	96	192
	Standard drugs											
Streptomycin	0.65	0.65	0.65	1.29	2.59	5.18	0.65	**nt**	nt	nt	nt	nt
Izoniazid	0.13	1.03	0.51	1.03	0.10	1.03	0.51	0.51	nt	nt	nt	nt
Rifampin	0.65	5.18	0.32	2.59	0.65	0.65	0.65	5.18	nt	nt	nt	nt
Ethambutol	3.74	7.48	1.87	1.87	3.74	3.74	1.87	nt	nt	nt	nt	nt
Iecilline	nt	nt	nt	nt	nt	nt	nt	**nt**	0.31	0.62	10	20

nt: Not tested;

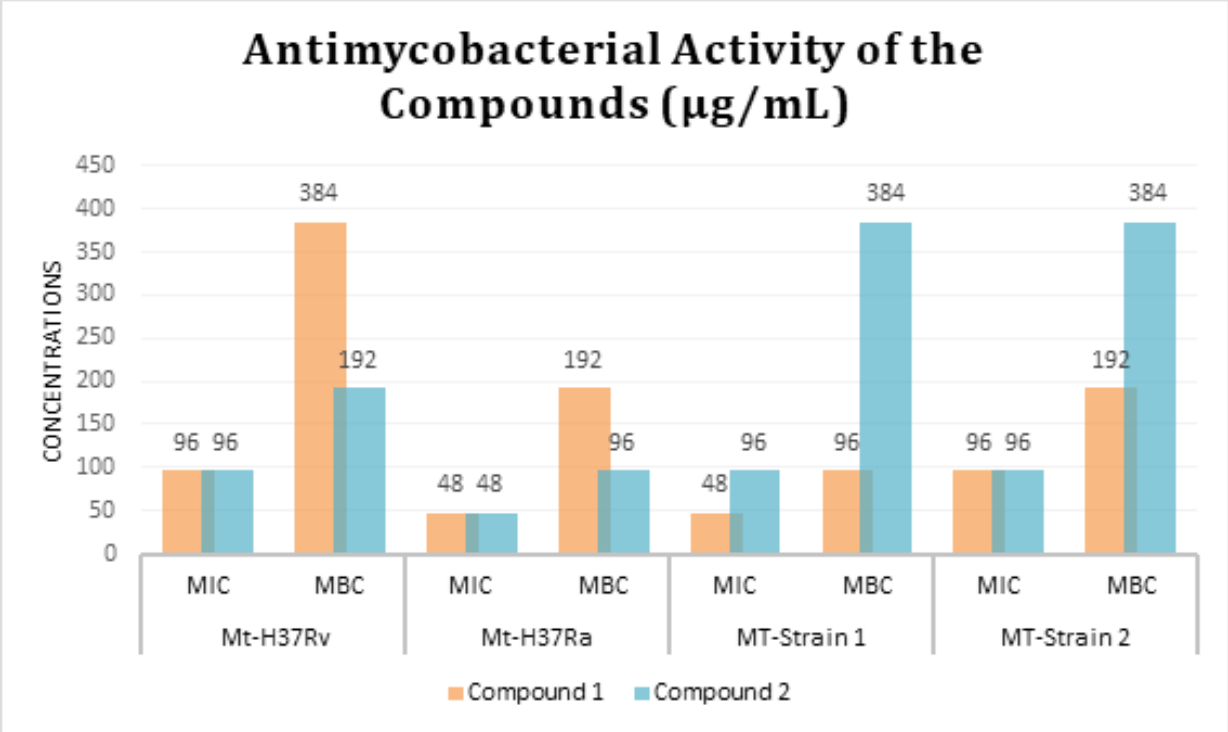

Figure 3: Antimycobacterial activity of the compounds as MIC and MBC (µg/mL).

Recently, perylene diimide derivatives have been attracted great interest and find lots of area to use Yukruk et al, that Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of perylene diimides for Staphylococcus aureus were determined respectively (35). Keskin et al. studied potential therapeutic advantages of perylene diimide derivatives on cancer therapy as a drug (36). Liu et al. reported that the potential usege of PDI derivatives as a recyclable specific Hg^{2+} ion

sensor and an efficient DNA delivery transporter (37).

We researched possible antimicrobial activity of two novel perylene diimides. This activity might be occurred from the moiety of serine or threonine part of PDI. A serine/threonine protein kinase enzyme, exist in procaryotes. It works by phosphorylating the OH group of serine or threonine (38). Ohlsen&Donat showed that serine/threonine phosphorylation and dephosphorylation are of great importance for *S. aureus* strains on the cell wall metabolism, cell proliferation, citrate cycle, apoptosis, and translation (38).

The results from this study will be beneficial for further development of new complexes.

4. CONCLUSIONS

In this study, two novel perylene diimides were synthesized and characterized. Accumulating evidence has demonstrated the potential antimicrobial activities of two novel PDI. They displayed a clear, concentrations-depended bactericidal activity against the bacteria resistant to the medicine. More consideration should be given to the research on PDI that could produce more valuable data to expand on their usage area.

5. ACKNOWLEDGEMENTS

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6. CONFLICT of INTEREST

The authors have declared no conflict of interest.

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