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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF BENZOYL AND HALOBENZOYL THIOUREA BEARING α- AND β-ALANINE

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ABSTRACT

Antibacterial activity of synthetically synthesized benzoyl thiourea and halobenzoyl thiourea bearing α-alanine and β-alanine compounds were investigated against gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative bacteria (*Esherichia coli*). The study of antibacterial activity of the compounds was conducted by using the disc diffusion method and minimal inhibitory concentration (MIC) as well as minimal bactericidal concentration (MBC). The results indicate that 4-fluorobenzoylthiourea α-alanine, benzoylthiourea α-alanine, 4-fluorobenzoylthiourea β-alanine, 4-chlorobenzoylthiourea β-alanine, 4-bromobenzoylthiourea β-alanine, and benzoylthiourea β-alanine possess weak to strong antibacterial activity on all of tested bacteria. The strongest antibacterial activity was found in the 4-fluorobenzoylthiourea α-alanine, whereas the weakest antibacterial activity was exhibited in 4-fluorobenzoylthiourea β-alanine. *S. aureus* was found the most susceptible toward these thiourea derivatives, indicating bactericidal activity against the bacteria.

KEYWORDS

benzoyl thiourea, halobenzoyl thiourea, antibacterial, disc diffusion test, minimum inhibition concentration.

1. INTRODUCTION

Medicinal chemistry involves chemical identification and thorough synthetic alteration of drugs to produce highly effective therapeutic agents. Scientists chose to produce new drugs by synthesizing complex organic molecules from a simple synthetic or natural precursor. One of the examples is thiourea derivatives, which can be further synthesized into different organic complex molecules with various biological activities. Thiourea can be classified as an organosulfur compound with the general formula of $SC(NH_2)_2$ (Al-hazams et al., 2013). The presence of two free hydrogen atoms, one at each nitrogen atom makes the thiourea as versatile starting materials for the synthesis of many heterocyclic compounds (Vedavathi et al., 2013). A group researcher reported that thiourea derivatives possess a wide range of biological activity including anti-bacterial, anti-fungal, anti-tubercular, anti-helmintic, rodenticidal, insecticidal, and herbicidal (Madabhushi et al., 2014). Other than that, some researcher recorded that thiourea isoxyl has been used clinically to be an effective anti-tuberculosis drug, against a range of multidrug-resistant strains of Mycobacterium tuberculosis (Phetsuksiri et al., 2003). Previous studies also highlighted that N-substituted phenyl thiourea can be used to treat anxiety, mania, depression, panic disorders, migraine and defects associated with AIDS (Jadhav et al., 2010). The rising of bacterial drug resistance has led to the need for an improvement to the existing antibiotics and the development of new ones. In this study, antibacterial activity of newly synthesized benzoyl and halobenzoyl thiourea bearing α-alanine and β-alanine were examined. Six compounds were studied namely, benzoylthiourea α-alanine, 4-fluorobenzoylthiourea α-alanine, benzoylthiourea β-alanine, 4-fluorobenzoylthiourea β-alanine, 4-chlorobenzoylthiourea β-alanine, and 4-bromobenzoylthiourea β-alanine. These compounds were tested for their antibacterial activity using disc diffusion method. The compounds which showed inhibition zone of more than 12 mm were further examined through minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) analyses.

2. METHODS

2.1 Synthesis of benzoyl and halobenzoyl thiourea derivatives

The mol ration for each reactant is 1:1:1. A suspension of ammonium thiocyanate (2.0g, 0.0263mol) was dissolved in acetone (10 mL) and added dropwise into a two-round neck flask containing 4-

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fluorobenzoylchloride (4.2g, 0.0263 mol). The solution mixture was stirred for 15 minutes until the white precipitate observed. Subsequently, the solution of a-alanine in acetone (2.3g, 0.0263 mol) was added to the solution mixture and refluxed for 5 hours. The resulting solution was filtered off into a beaker containing ice cubes and left at room temperature until the formation of precipitate. The precipitate obtained was filtered, dried and kept in dry bottle for analysis and further used. For other derivatives, the reaction will follow the same procedures, only substitute the 4-fluorobenzoylchloride and α-alanine with other aroylchlorides and β -alanine, respectively.

In this study, the antibacterial activity of newly synthesized benzoyl thiourea and halobenzoylthiourea bearing α-alanine and β-alanine were examined. The structure of benzoyl thiourea and halobenzoylthiourea bearing α-alanine and β-alanine can be described as; the hydrogen atoms at one nitrogen terminals of thiourea are substituted with benzoyl and halobenzoyl carrying different halogen atoms (fluorine, chlorine and bromine). These halogens are substituted at para positions of benzene ring. As for the other hydrogen atom at the nitrogen terminals of thiourea are substituted with either α-alanine or β-alanine. Mainly, there were 6 compounds to be studied namely benzoylthiourea α-alanine, 4-fluorobenzoylthiourea α-alanine, benzoylthiourea β-alanine, 2-fluorobenzoylthiourea β-alanine, 4-chlorobenzoylthiourea β-alanine, and 4-bromobenzoylthiourea β-alanine. Figure 1 depicts the scheme reaction of the synthesis.

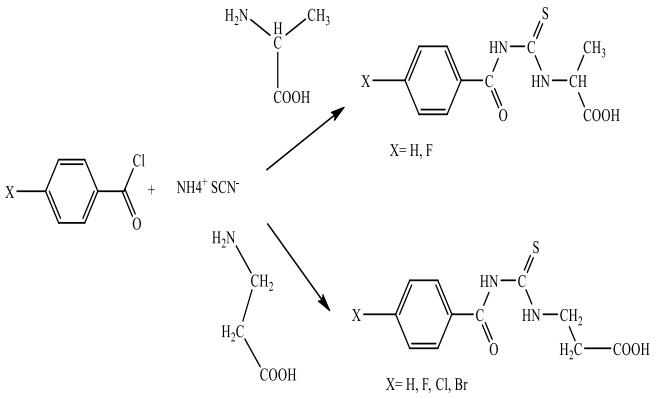


Figure 1: Scheme reaction for the synthesis of benzoyl and halobenzoyl thiourea derivatives

2.2 Preparation of inoculum

The stock solution of gram-positive bacteria: *Bacillus subtilis* ATCC 11778, *Staphylococcus aureus* ATCC 25923, and gram-negative bacteria *Eschericia coli* ATCC 25922 were grown in nutrient agar at 37°C in incubator (Sheldon, USA) for 24 hours. The bacteria were cultured into sterile nutrient broth. Optical density for every broth culture was set from a range of 0.8-1.0. The optical density was checked by using UV/Vis spectrometry (Perkin Elmer, Singapore) at 600 nm.

2.3 Disc diffusion test

The antibacterial qualitative screening was conducted by using disc diffusion method based on Kirby-Bauer (Jorgensen et al., 2007). The thiourea compounds were solubilized in DMSO to a final concentration of 1000 mg/mL. 10 µL of compound solution was impregnated onto Whatman No.1 filter paper (6 mm) disc and left to dry at room temperature for 30 minutes. DMSO was used as negative control. Streptomycin disc (10 µg) was used as positive control for B. subtilis, E. coli and S. aureus. The bacteria were cultured on nutrient agar containing petri dish. After that, the discs were placed on the agar surface pre-inoculated with the suspension of bacteria according to the label respectively. Then, the agar plates which contain the bacteria were incubated invertedly for 24 hours at 37°C. The inhibition zones (in mm) were measured and tabulated. All tests were performed in triplicates and the average was taken as the final reading.

2.4 Minimum inhibitory concentration (MIC)

By following the protocols described the minimum inhibitory concentration (MIC) was determined by two-fold serial dilution (Bădiceanu et al., 2010). 96 wells plate was prepared by dispensing 100 µL of the suspension bacteria and 100 µL of tested compounds (1000 mg/mL) into the first well. Meanwhile, 100 µL of the test suspension bacteria were added into seven consecutive wells. A two-fold serial dilution was carried out until well 8 and the remaining 100 µL of the suspension was discarded. The concentration of tested compound was in the range of 3.9 mg/mL to 500 mg/mL. The concentration in the first well for all compounds was 500 mg/mL. DMSO was used as the negative controls for bacteria, while streptomycin was used as positive control. The plates were incubated overnight at 37°C. Indication of clear solution or decrease in color will indicate inhibition of bacteria. MIC values were recorded by using UV/Vis spectrometry at 600 nm of wavelength.

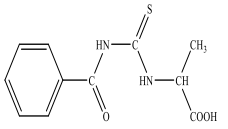
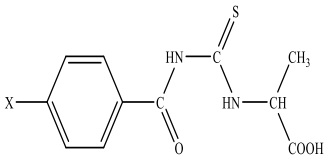
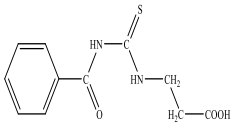
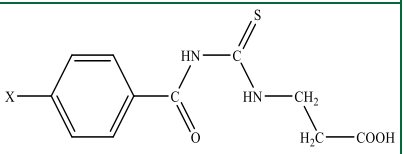
2.5 Minimum bactericidal concentration (MBC)

The values for compound that acted as bactericidal were determined by selecting the well that showed clear solution for MIC test. Then, inoculums were sub-cultured onto sterile nutrient agar plates. The plates were incubated overnight at 37°C. The lowest concentration from which the microorganisms did not grow when transferred to sterile nutrient broth medium will be identified as MBC.

3. Result and Discussion

The chemical structures of the compounds can be referred to Table 1.

Table 1: The structures of used benzoyl thiourea and halobenzoylthiourea bearing α-alanine and β-alanine

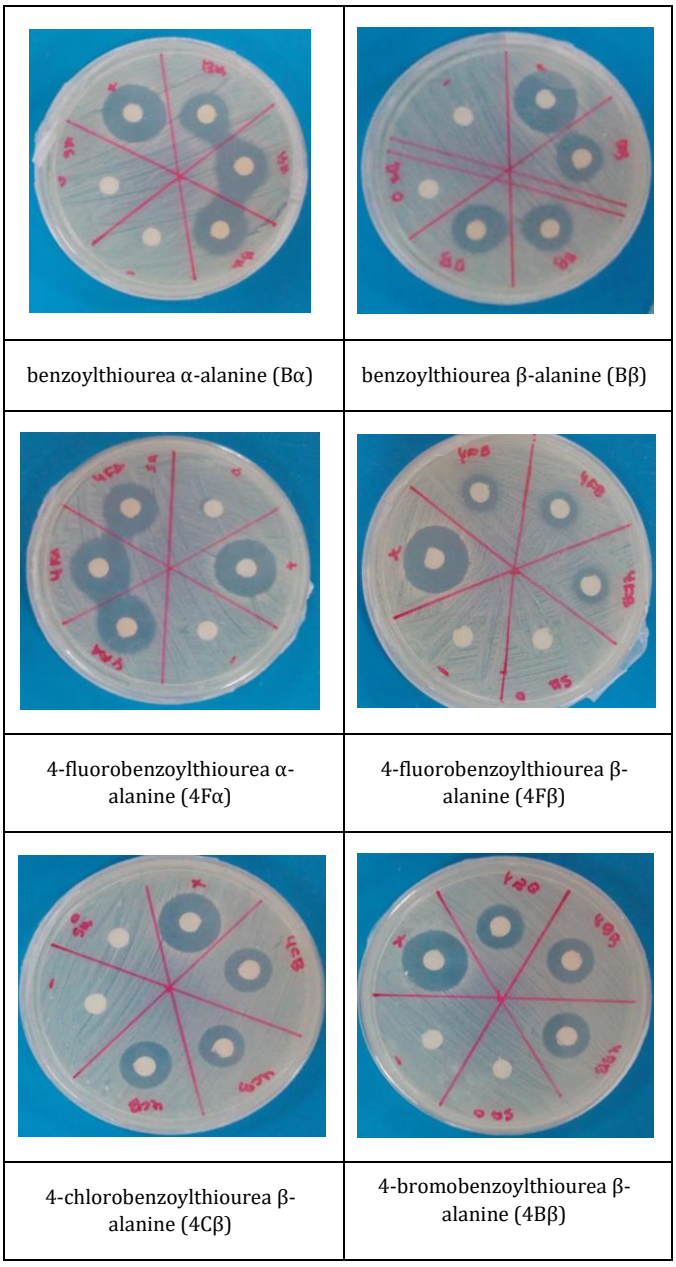
 benzoyl thiourea α-alanine	 halobenzoyl thiourea α-alanine X=fluorine
 benzoyl thiourea β-alanine	 halobenzoyl thiourea β-alanine X=fluorine, chlorine, bromine

3.1 Antibacterial activity

Benzoyl thiourea and halobenzoyl thiourea bearing α-alanine and β-alanine compounds were screened for antibacterial activity against B. subtilis and *S. aureus* for Gram-positve bacteria and *E. coli* for Gram-negative bacteria. As depicted in Table 2, all halobenzoyl thiourea bearing α-alanine and β-alanine compounds showed antibacterial activity against all tested bacteria by exhibiting inhibition zone in the range of 8.8 to 19.0 mm. It was also observed that *B. subtilis* and *E. coli* were inhibited by all tested compounds with 4-bromobenzoylthiourea β-alanine compound producing the highest inhibition zone against *E. coli* (19.0 mm). This compound also showed the lowest inhibition zone against S. aureus, which was 8.8 mm. However, all tested compounds except for 4-fluorobenzoylthiourea α-alanine showed weak to moderate inhibition against S. aureus (8.8-11.7 mm). According to another study, in antibacterial screening test, it can be classified as weak; 7-8 mm, moderate; 9-11 mm and strong; >12 mm of inhibition zones (Sinam et al., 2014).

Table 2: Inhibition zone of benzoyl and halobenzoyl thiourea bearing α-alanine and β-alanine

Compounds	Gram positive bacteria Zone of Inhibition (mm)		Gram negative bacteria Zone of Inhibition (mm)
	S. aureus	***B. subtilis***	***E. coli***
benzoylthiourea α-alanine	9.3	13.7	16.7
benzoylthiourea β-alanine	9.0	11.7	14.8
4-fluorobenzoylthiourea α-alanine	15.0	14.0	18.2
4-fluorobenzoylthiourea β-alanine	11.7	11.7	11.7
4-bromobenzoylthiourea β-alanine	8.8	15.0	19.0
4-chlorobenzoylthiourea β-alanine	9.7	13.7	14.0

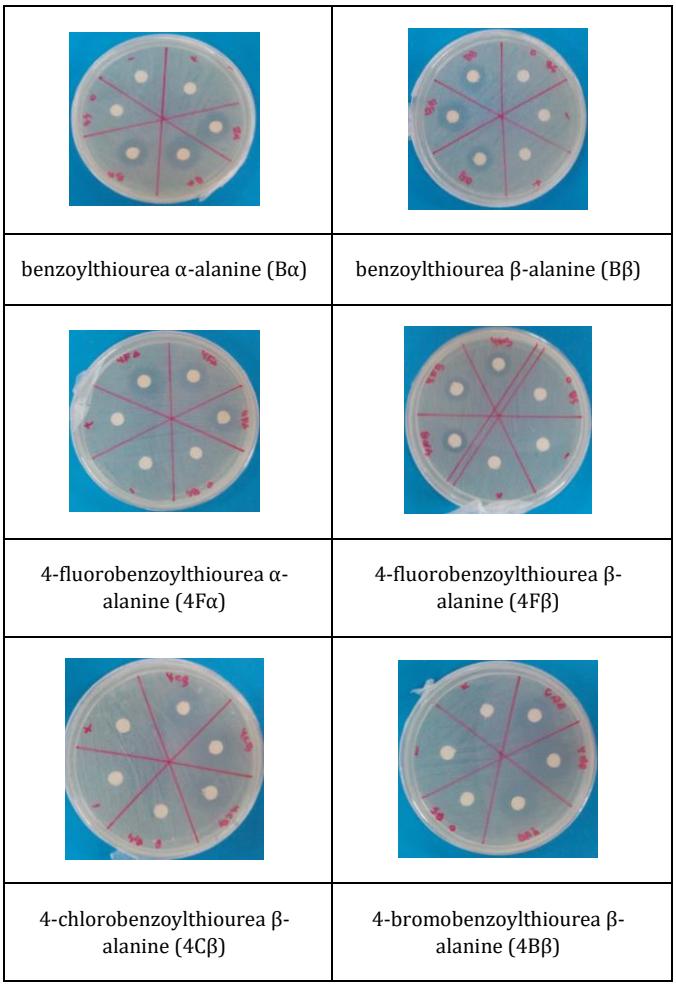


benzoylthiourea α-alanine (Bα) | benzoylthiourea β-alanine (Bβ)

4-fluorobenzoylthiourea α-alanine (4Fα) | 4-fluorobenzoylthiourea β-alanine (4Fβ)

4-chlorobenzoylthiourea β-alanine (4Cβ) | 4-bromobenzoylthiourea β-alanine (4Bβ)

Figure 2: Inhibition zone produced by compounds against *S. aureus*

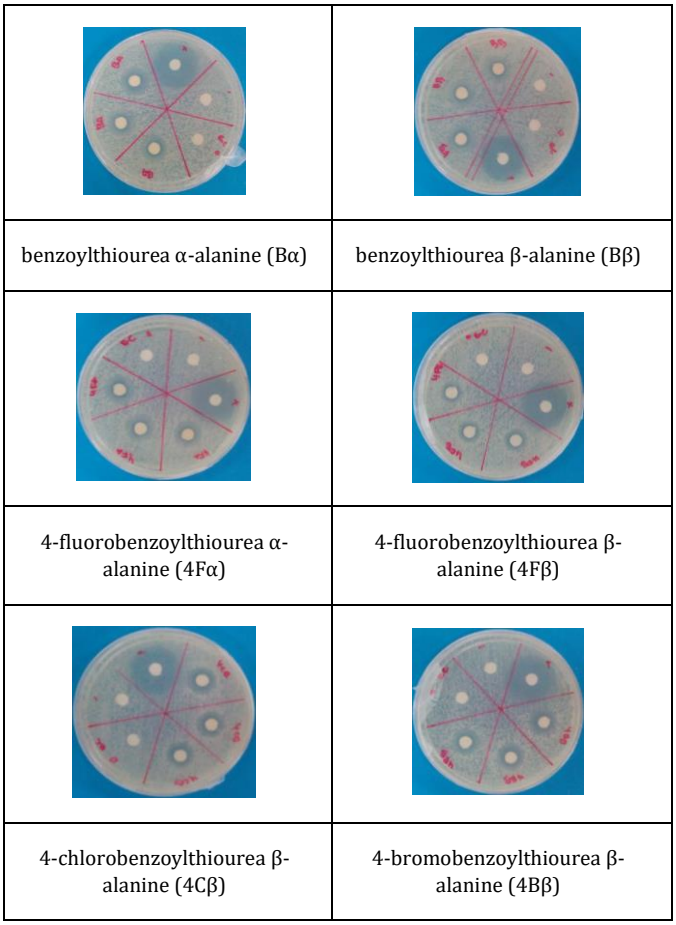


benzoylthiourea α-alanine (Bα) | benzoylthiourea β-alanine (Bβ)

4-fluorobenzoylthiourea α-alanine (4Fα) | 4-fluorobenzoylthiourea β-alanine (4Fβ)

4-chlorobenzoylthiourea β-alanine (4Cβ) | 4-bromobenzoylthiourea β-alanine (4Bβ)

Figure 3: Inhibition zone produced by compounds against *B. subtilis*



benzoylthiourea α-alanine (Bα) | benzoylthiourea β-alanine (Bβ)

4-fluorobenzoylthiourea α-alanine (4Fα) | 4-fluorobenzoylthiourea β-alanine (4Fβ)

4-chlorobenzoylthiourea β-alanine (4Cβ) | 4-bromobenzoylthiourea β-alanine (4Bβ)

Figure 4: Inhibition zone produced by compounds against *E. coli*

In the Figures 2-4, the results show that 4Fα, 4Fβ, 4Chβ and 4Bβ compounds exhibited good inhibition activities against E. coli and B. subtilis due to the inclusion of halogen as the substituent possesses good inhibitory activity. The substitution of halogen at para position of benzoyl thiourea derivative plays significant role in increasing the antibacterial activity (Wan Zullkiplee et al., 2021). Based on the inhibition zone, the inclusion of bromine shows the highest antibacterial activity against gram-positive bacteria compared to chlorine and fluorine. As for the gram-negative bacteria, apparently the substitution of fluorine to the benzoyl thiourea derivative showed greater inhibition zone compared to chlorine and bromine. As highlighted by the incorporation of fluorine into organic molecule can enhance the lipophilicity and eventually the rate of cell penetration and transportation of a drug to an active site increase (Li et al., 2013).

Lipophilicity is particularly important for the penetration of gram-negative bacteria as it has membrane with an efficient permeability barrier due to the presence of lipopolysaccharide. Other than that, the electronegativity of halogen also could be attributed to the antibacterial activity of the tested compounds. Furthermore, the inclusion of alanine plays significant role in the antibacterial activity of thiourea derivatives added that incorporation of α-alanine could also improve the antibacterial activity of thiourea compounds as compared to β-alanine (Marcone et al., 2019; Mohammed et al., 2014). This can be seen from the current study where 4Fα strongly inhibited the growth of bacteria compared to 4Fβ. This is also applied to Bα which showed greater inhibition to the growth of bacteria compared to Bβ. Overall, 4Fα showed greatest antibacterial activity to gram-negative and gram-positive bacteria in the range of 14.0-18.2 mm.

3.2 MIC and MBC analysis

All compounds with the inhibition zone of more than 12 mm were further analysed for MIC and MBC. Table 3 shows MIC and MBC values and for the synthesized compounds in a range of 7.8 – 62.5 mg/mL.

Table 3: MIC and MBC values of benzoyl and halobenzoyl thiourea bearing α-alanine and β-alanine.

Bacteria	Compounds	MIC value (mg/mL)	MBC value (mg/mL)
S. aureus	benzoylthiourea α-alanine	62.5	62.5
	benzoylthiourea β-alanine	62.5	62.5
	4-fluorobenzoylthiourea α-alanine	31.3	62.5
	4-bromobenzoylthiourea β-alanine	31.3	62.5
	4-chlorobenzoylthiourea β-alanine	31.3	62.5
B. subtilis	benzoylthiourea α-alanine	31.3	62.5
	4-fluorobenzoylthiourea α-alanine	15.6	31.3
	4-bromobenzoylthiourea β-alanine	15.6	31.3
	4-chlorobenzoylthiourea β-alanine	31.3	62.5
E. coli	4-fluorobenzoylthiourea α-alanine	7.8	15.6

4. Conclusion

In conclusion, this study has achieved its overall objective, which was to evaluate the antibacterial activity of benzoyl and halobenzoyl thiourea bearing α-alanine and β-alanine. Compound 4Fα exhibited the most active antibacterial properties against gram-negative bacteria and gram-positive bacteria with inhibition zone of 14.0 mm to 18.2 mm and 15.6 mg/mL of minimum inhibition concentration against S. aureus and B. subtilis. This compound is the most potent antibacterial agent due to inclusion of fluorine at para position of benzoylthiourea. Fluorine is the most electronegative molecule which is the main reason for its ability to penetrate the cell. The presence of α-alanine also becomes the main contributor for the enhancement of its antibacterial activity.

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