Evaluation of Antibacterial and Antioxidant of Thiosemicarbazide Derivatives: A Preliminary Study on Antitubercular Activities

Reyza Fadly Maghfiroh, Adi Setyo Purnomoa), Fahimah Martak, Mardi Santoso

*Department of Chemistry, Institut Teknologi Sepuluh Nopember (ITS), Kampus ITS Sukolilo, Surabaya 60111, Indonesia*

a) Corresponding author: [adi\_setyo@its.ac.id](mailto:adi_setyo@its.ac.id)

**Abstract.** Tuberculosis (TB) has been classified as a global problem by the WHO. TB is initiated by the bacterium *Mycobacterium tuberculosis*, with high infectious power. Indonesia ranks fourth in Southeast Asia with an increase in cases by 2022 of 63.41%. Heterocyclic compounds have broad bioactivity, making them good as pharmacological agents. Furanoyl (furan-containing carbonyl) combined in the thiosemicarbazide structure has activity in inhibiting the growth of *M. tuberculosis*, but its performance is still in the moderate category. The lipophilic character plays an important role for TB drugs, as this character helps the transfer of drugs into body cells and bacteria. TB drugs do not only depend on lipophilic character, but the penetration rate of drugs into bacterial cells needs to be considered. Efforts to increase the penetration rate of drugs into *M. tuberculosis* cells can be made by adding furan-2-carboxamide groups to the structure of drug compounds. Qualitative antibacterial activity tests were carried out for compounds **(10)**, **(12)**, **(13)**, and **(14)**, and antioxidant tests were carried out using the DPPH assay. The results showed that all test compounds were specifically active on *P. aeruginosa*, which is a Gram-negative bacterium, with inhibition zones of 0, 7.5, 11.5, and 9 mm, respectively. In the approach of antitubercular activity, the test compounds should be more active in inhibiting Gram-positive bacteria. Compounds **(13)** and **(14)** were used as comparators to confirm the correlation between antibacterial and antitubercular. The results obtained were different; compounds **(13)** and **(14)** have been tested for antitubercular activity (*M. tuberculosis* H37rv) with satisfactory results. The tested compounds didn’t show any potential for antioxidant activity because they have different structures than antioxidant-active compounds. In the future, thiosemicarbazide could be combined with a hydroxyl group to enhance its antitubercular activity. Antitubercular screening was carried out on the next step by *in silico* and *in vitro* study.

Keyword: Antibacterial, Antitubercular, Furan, Lipophilicity

# INTRODUCTION

Tuberculosis (TB) is a disease initiated by the bacterium *Mycobacterium tuberculosis* [1]. WHO has designated TB as a global problem with a high mortality rate and has claimed up to 1.7 million lives in 2016 [2]. The Southeast Asian region accounts for the largest number of TB cases on a global scale, with a percentage of 45.6%. Indonesia is ranked fourth in Southeast Asia with 9.2% of cases. The Indonesian Ministry of Health presented statistical data on TB patients increasing significantly, with a percentage of 63.41% in 2022 [3].

Isoniazid (INH) **(1)**, rifampicin (RIF) **(2)**, ethambutol (EMB) **(3)**, and pyrazinamide (PZA) **(4)** are first-generation TB drugs with a consumption period of 6-12 months [4-6]. INH **(1)**, RIF **(2)**, and EMB **(3)** have a minimum inhibitory ability against *M. tuberculosis* of 0.73, 0.24, and 7.64 μM, respectively, while PZA **(4)** still cannot be determined precisely because it is highly dependent on pH and target proteins [7]. INH **(1)** and RIF **(2)** are the most effective drugs in treating TB. INH **(1)** has a mechanism to inhibit the formation of mycolic acid, which is the core substance in *M. tuberculosis* cell wall biosynthesis. That drug requires activity by the enzyme catalase (**Fig. 1**) [8,9], so that in the process it can undergo mutations, causing resistant effects [10]. Several other TB drugs have been developed, such as bedaquilline (BED) **(5)**, delamanid (DEL) **(6)**, pretomanid (PRE) **(7)**, and metronidazole (MET) **(8)**. BED **(5)** and DEL **(6)** have been approved as MDR-TB drugs by the European Medicines Agency. PRE **(7)** is under development in America, while MET **(8)** could accelerate the healing process but causes neurotoxic side effects, so its use has been limited [2]. The presence of these problems encourages the development of new compounds that do not cause resistance effects. Structure of Tuberculosis Drug first generation can be seen in **Fig. 2**.



**FIGURE 1**. Mechanism activation and deactivation of INH

Heterocyclic compounds have a major role in the design of medicinal compounds. Heterocyclic-containing compounds such as furan and pyridine have a broad spectrum of bioactivity, such as antimicrobial, antitumor, analgesic, antihypertensive, and anti-inflammatory [11]. In their original form, furan and pyridine have a lack of lipophilic character, so they need to be modified by adding carboxamide and thiosemicarbazide groups [12,13]. Lipophilic character has an important role in increasing the penetration rate of drug compounds into bacterial cells. In this work, the antibacterial and antioxidant activities of compounds **(9-12)** were evaluated, along with compounds **(1, 13, 14)** (**Fig. 3**), as a preliminary study on antitubercular activity.



**FIGURE 2**. Structure of tuberculosis drug first generation



**FIGURE 3**. Structure of tested compounds

# MATERIALS AND METHOD

**Materials and Equipment**

Compounds **(9-15)** were obtained from the Natural Product and Synthesis Laboratory, Dept. Chemistry, Institut Teknologi Sepuluh Nopember (ITS), Indonesia. Stocks of the bacteria *Pseudomonas aeruginosa* and *Bacillus subtilis* were obtained from the Microbial Chemistry Laboratory, Dept. Chemistry, ITS, Indonesia. Dimethyl sulfoxide (DMSO) Merck and methanol Merck were used as an organic solvent. Antibacterial assay materials included nutrient agar (NA) (13 g/L) Merck, nutrient broth (NB) (20 g/L) Himedia, chloramphenicol as a positive control for Gram-positive and Gram-negative bacteria, and distilled water [14-15]. All chemicals used had a purity >98%. The antioxidant assay used was the DPPH assay, which also included ascorbic acid Merck and gallic acid Merck as positive controls [16-19].

# Bacteria Culture Preparation

Bacteria culture preparation was performed using a method described in the literature [20], had a minor modification. NB (0.26 g) was dissolved with 20 mL of distilled water and sterilized using an autoclave at 121 °C for 15 min. Each bacterium (P. aeruginosa and B. subtilis) was added into NB solution using an inoculating loop and incubated at 37°C for 24 h using a shaker incubator. Those bacterial suspensions were diluted with NB until their optical density (OD) reached 0.4 (1 × 104 CFU/mL). OD was measured using a UV-Vis spectrophotometer Genesys 10 at a wavelength of 600 nm.

# Antibacterial Assay

The antibacterial assay was performed using a method described in the literature [21]. Antibacterial activity was using well diffusion in accordance with a resulting inhibition zone. NA (1.20 g) was dissolved with 70 mL of distilled water and sterilized using an autoclave at 121 °C for 15 min. The NA solution was quenched by air until it cooled down (37 °C). Each bacterial suspension was added into the NA solution within a ratio of 1:5 (v/v). The mixture was homogenized by vortex in a minute, then poured into a petri dish, and we waited until it set. Each petri dish was divided into 6 zones; in each zone, the well (d = 7 mm) was created. The samples were prepared by dissolving 1 mg into 1 mL DMSO until the concentration reached 1000 g/mL. A 50 L sample was added to each well and incubated at 37 °C for 41 h. The inhibition zone was measured after the incubation process as shown in Eq. 1 (with d was the well hole’s diameter). Chloramphenicol was used as a positive control, and DMSO was used as a negative control.

(1)

# Antioxidant Assay

Antioxidant assay was performed using a method described in literature [22]. DPPH (2.4 mg) was dissolved with 100 mL methanol and stirred vigorously without light exposure for 30 min. On the other hand, the samples were prepared by dissolving 1 mg into 1 mL DMSO until the concentration reached 1000 mg/mL. Each sample (33.3 μL) was taken into a PCR tube of 1.5 mL, then DPPH solution (1 mL) was added and incubated at 37 °C for 30 min in a darkened room. Each variant was done in triplicate. Absorbance of those mixtures was measured by UV-Vis spectrophotometer at a wavelength of 517 nm. DPPH solution was used as a positive control. If the radical scavenging activity (%RSA) at this concentration was so high, within >50%, the samples were diluted in series (100, 10, 1, and 0.1 g/mL). The scavenger ability was supposed to focus on the percentage of DPPH radical scavenger as a succeeding comparison (Eq. 2).

(2)

# RESULTS AND DISCUSSION

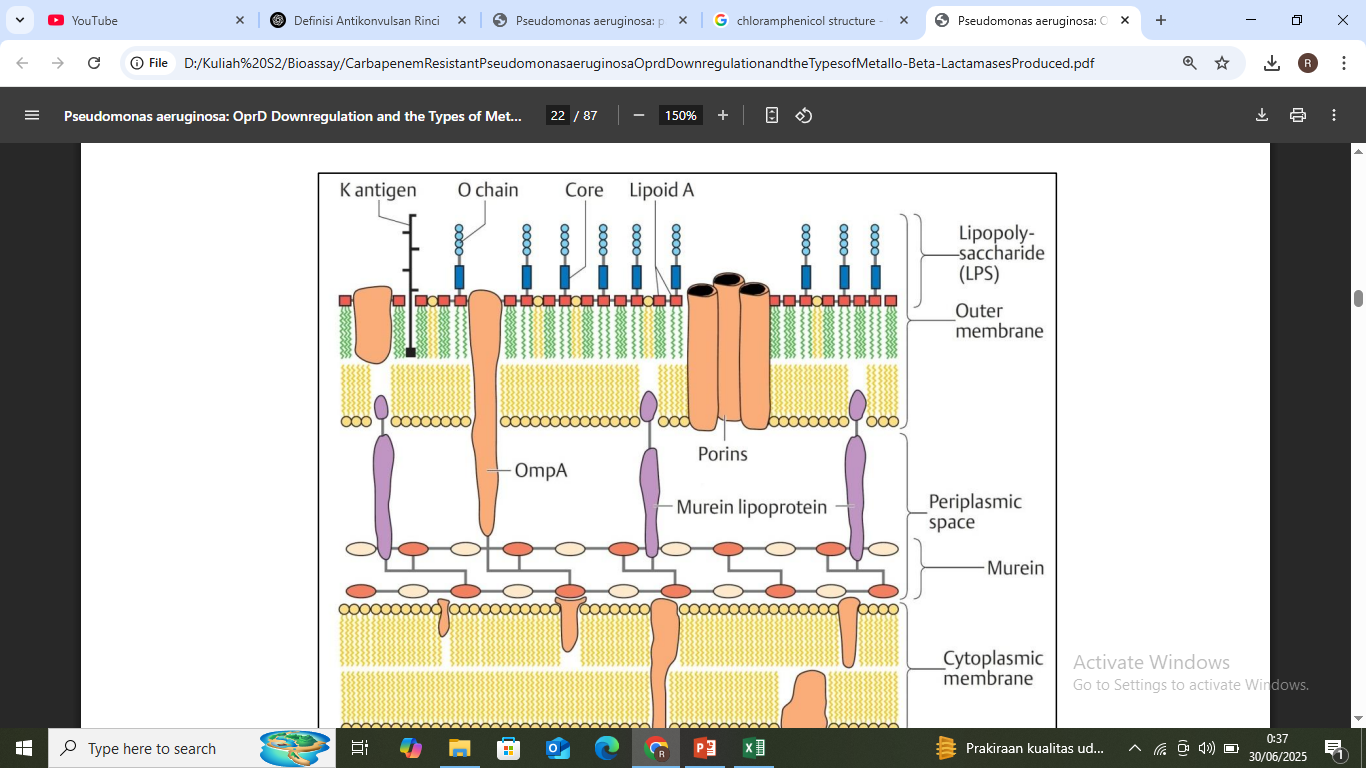
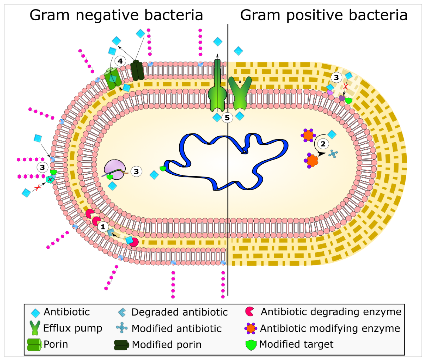
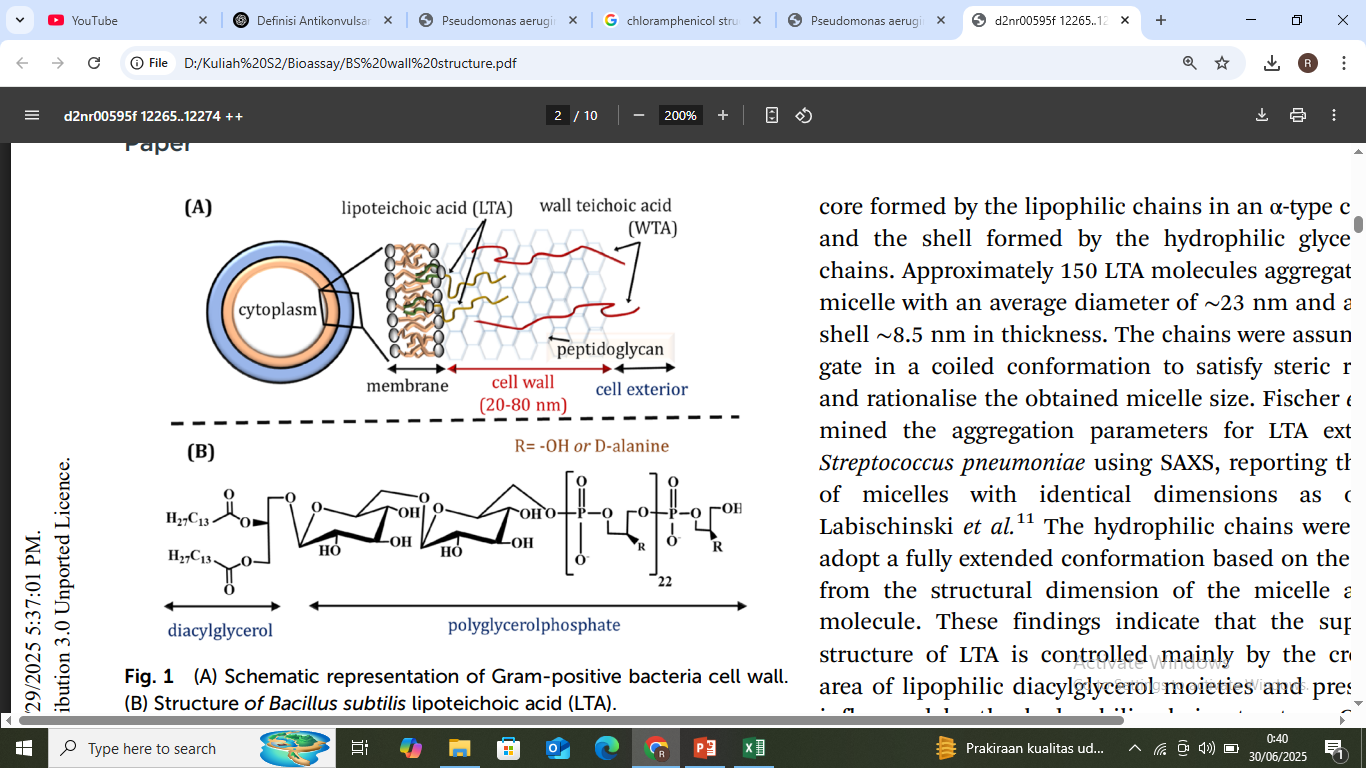
Antibacterial assay by well diffusion resulted in the samples exhibiting a potent activity for inhibiting *P. aeruginosa.* **Table 1.** Presenting inhibition activities of the samples. *P. aeruginosa* represented Gram-negative bacteria, while *B. subtilis* represented Gram-positive bacteria.

**TABLE 1**. Well diffusion inhibition zone result

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Bacteria | Petri Dish  Code | Sample  (1000 g/mL) | Inhibition  Zone (mm) | Category | Log P |
| *P. aeruginosa* | A | Compound **(9)** | 8.5 | Strong | 0.85 |
| Compound **(10)** | - | - | 1.81 |
| Compound **(11)** | 10.0 | Strong | 0.01 |
| Compound **(12)** | 7.5 | Strong | 1.29 |
| Chloramphenicol | 8.0 | Strong | 3.26 |
| DMSO | - | - | - |
| B | Compound **(1)** | 8.5 | Strong | -0.35 |
| Compound **(13)** | 11.5 | Strong | 3.48 |
| Compound **(14)** | 9.0 | Strong | 2.77 |
| Chloramphenicol | 12.5 | Strong | 3.26 |
| DMSO | - | - | - |
| *B. subtilis* | C | Compound **(9)** | - | - | 0.85 |
| Compound **(10)** | - | - | 1.81 |
| Compound **(11)** | - | - | 0.01 |
| Compound **(12)** | - | - | 1.29 |
| Chloramphenicol | 21.5 | Strong | 3.26 |
| DMSO | - | - | - |
| D | Compound **(1)** | - | - | -0.35 |
| Compound **(13)** | 4.0 | Moderate | 3.48 |
| Compound **(14)** | 3.0 | Low | 2.77 |
| Chloramphenicol | 20.5 | Strong | 3.26 |
| DMSO | - | - | - |

\*Log P was obtained from ADME website

As a comparison, aztreonam and polymyxin B are antibiotics that exhibit strong inhibitory activity against Gram-negative bacteria, while gentamicin primarily targets Gram-negative bacteria. The existing –NH group in the samples, which also contain this functional group in their structure, is likely to interact favorably with a target receptor. The –NH group can arrange itself into a positive charge, allowing it to interact effectively with lipopolysaccharide, which forms the first layer of the gram-negative bacteria cell wall [23-25]. As can be seen in **Fig. 4**, Gram-negative bacteria have a thinner layer of peptidoglycan (2-7 nm) than Gram-positive bacteria (20-80 nm) in cell walls [26-28]. The first layer of Gram-negative bacteria was lipopolysaccharide (LSA), which has a negative charge characteristic due to the existence of a saccharide group. In addition, Gram-positive bacteria have lipoteichoic acid (LTA) as the outer layer, along with a matrix of peptidoglycan. The structure prevents large molecules from penetrating into the cells, unlike in Gram-negative bacteria. Consequently, this layer contributes to the positive charge of gram-positive bacteria. LSA was hydrophobic, which means only compounds with a lower log P can penetrate, since LTA is more hydrophilic, so it's vice versa. Those explained why chloramphenicol was active on both bacteria; chloramphenicol has a log P around 2.24, which is categorized as mid-range log P. This aligns with findings from studies on the role of lipophilicity (log P) in bacterial cell penetration, where moderate log P values are often optimal for Gram-negative activity, while higher log P values may be required for Gram-positive bacteria.

**FIGURE 4**. Comparison of Gram-negative bacteria and Gram-positive bacteria cell walls

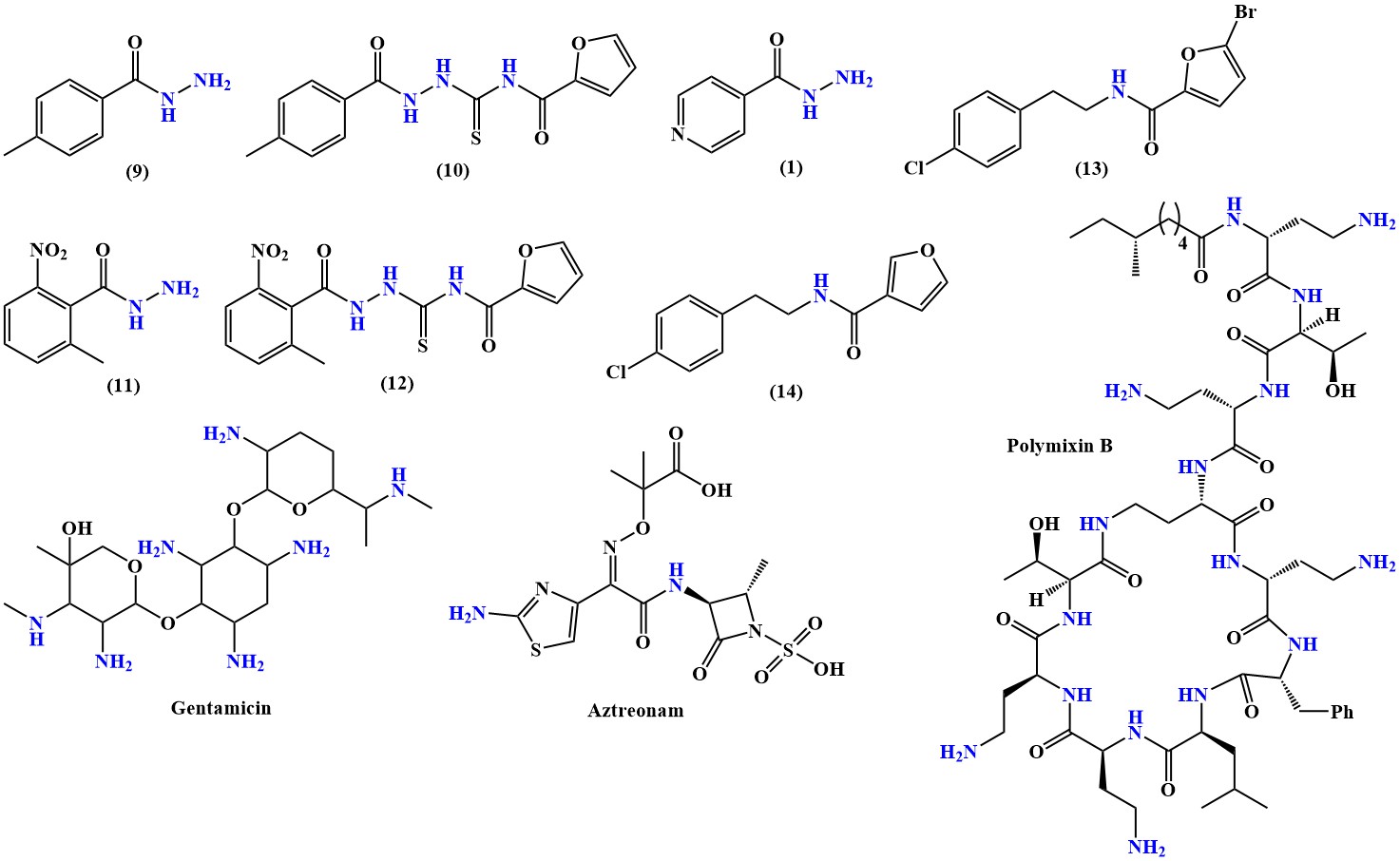
Structure Activity and Relationship (SAR) according to **Fig. 5** indicates that compounds with a nitro (-NO₂) group and halogen (-Cl and -Br) group have potent antibacterial effects. Compound **(10)** didn’t show antibacterial activity due to lack of solubility, so it crystallized on agar. Compound **(13)** was active on both bacteria due to its halogen group on the furoyl and phenyl structures. The transformation of hydrazide into thiosemicarbazone can increase the log P; otherwise, antibacterial activity is enhanced. Compounds **(1)**, **(13)**, and **(14)** were primarily targeted at gram-negative bacteria, while in other experiments, they showed antitubercular activity. M. tuberculosis as an object of antitubercular study was Gram-positive bacteria, so its antibacterial activity doesn’t have any correlation with antitubercular activity [29]. This discrepancy may arise from the unique cell wall structure of M. tuberculosis, which contains mycolic acids and arabinogalactan, differing significantly from typical Gram-positive bacteria. The lipophilic character of the compounds, as indicated by their log P values (3.48 and 2.77, respectively). It caused penetration through the lipid-rich mycobacterial cell wall, a critical factor for antitubercular activity. Further studies could explore modifications to enhance specificity for mycobacterial targets.

**TABLE 2**. Antioxidant activity

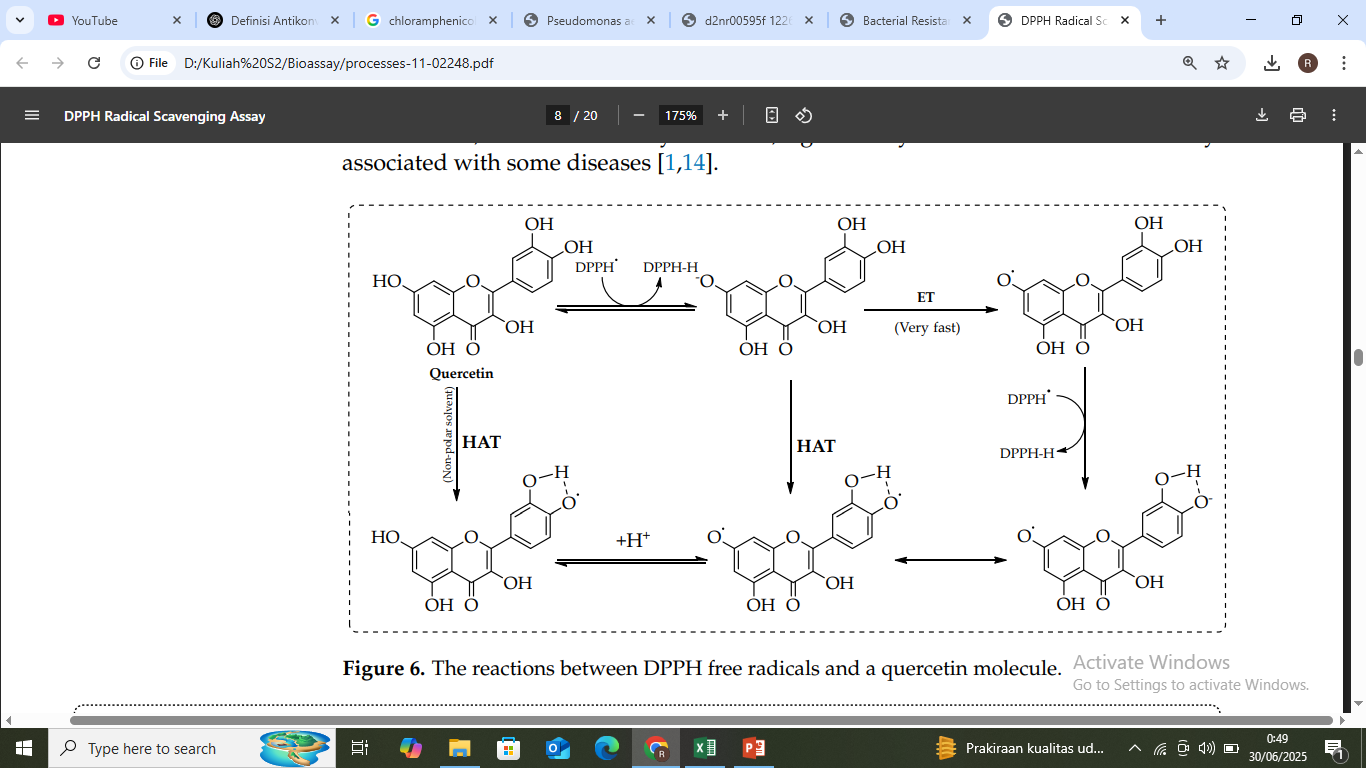
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample**  **(1000 g/mL)** | **%RSA** | | | **Mean** |
| **1** | **2** | **3** |
| Compound **(10)** | 7.20 | 8.35 | 7.03 | 7.52 ± 0.72 |
| Compound **(12)** | 15.60 | 13.63 | 12.47 | 13.90 ± 1.58 |
| Compound **(13)** | 12.44 | 11.64 | 11.38 | 11.82 ± 0.56 |
| Compound **(14)** | 12.00 | 9.60 | 8.18 | 9.92 ± 1.93 |
| Gallic Acid | 96.52 | 93.84 | 95.43 | 95.26 ± 1.35 |
| Ascorbic Acid | 97.62 | 97.71 | 97.22 | 97.52 ± 0.26 |
| Quercetin\* | N.d. | N.d. | N.d. | 96.34 ± 0.01 |

\*N.d. was not determine, mean was obtained from literature [30] in concentration 159.7 g/mL

Compounds **(10)**, **(12)**, **(13)**, and **(14)** did not show potent antioxidant activity. Their structures lacked hydroxyl groups or anything like flavonoids or phenolic compounds. As is known, the positive controls are flavonoid and phenolic class compounds. They have a radical scavenging mechanism, as shown in **Fig. 6**. The radical from DPPH attacks the proton on the phenol structure so that the radical can be stabilized and quercetin becomes a stable radical without harm [31]. According to the sample structure, the proton on the -NH group is thought to be attacked by the radical. Phenolic groups can be easily attacked by radicals because their oxygen atoms have a positive charge due to the influence of the mesomerism phenomenon. This limitation is inspiring the future design for antitubercular drug design. Future designs could incorporate phenolic moieties to combine antitubercular and antioxidant properties.



**FIGURE 5**. Comparison structure with antibiotics



**FIGURE 6**. Radical Scavenging Mechanism by Quercetin

# CONCLUSION

Qualitative antibacterial activity tests were carried out for compounds **(10)**, **(12)**, **(13)**, and **(14)**, and antioxidant tests were carried out using the DPPH assay. The results showed that all test compounds were specifically active on *P. aeruginosa*, which is a gram-negative bacterium with inhibition zones of 0, 7.5, 11.5, and 9 mm, respectively. In the approach of antitubercular activity, the test compounds should be more active in inhibiting Gram-positive bacteria. Compounds **(13)** and **(14)** were used as comparators to confirm the correlation between antibacterial and antitubercular. The results obtained were different; compounds **(13)** and **(14)** have been tested for antitubercular activity (*M. tuberculosis* H37rv) with satisfactory results. The tested compounds (compounds **(10)**, **(12)**, **(13)**, and **(14)**) didn’t show any potential for good antioxidant activity due to the different structure of antioxidant-active compounds. The tested compound could be improved by adding an OH group on thiosemicarbazide scaffold. Further work held *in silico* study by molecular docking and pharmacokinetics prediction (ADME) for tested compound and *in vitro*.

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