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Antimycobacterial Activity of Benzylidene Acetone Analogues on Curcumin Against Resistant And Sensitive Mycobacterium Tuberculosis

Cikra Ikhda Nur Hamidah Safitri[1,2], Ritmaleni[1], Ning Rintiswati[3], Sardjiman[1], Takushi Kaneko[4]

[1]*Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia*
[2]*Academy of Pharmacy Mitra Sehat Mandiri Sidoarjo, Indonesia*
[3]*Faculty of Medicine, Universitas Gadjah Mada, Indonesia*
[4]*Tuberculosis Alliance, New York, USA*
**Corresponding author:Ritmaleni[1]*
[*]*Email: ritmaleni@ugm.ac.id*

Abstract:*Curcumin is a compound possessing many pharmacological activities, but it has bad bioavailability and stability. Therefore modification should be conducted in curcumin structure. One of the results of curcumin analog modification is curcumin analog of Benzylidene acetone which is known as Gamavutton (GVT) series. The aim of this research is to determine the antituberculosis activity of curcumin compound analog on Benzylidene acetoneand Mycobacterium tuberculosis on sensitive strain (H37Rv). As well as determining the resistant strain (HE and SR). The anti-tuberculosis test used a method of agar dilution. The concentration used 0,125; 0,250; 0,500 and 1mg/mL; and the observation is done from the first week to the eighth week. Based on the test done, it can be seen that GVT-10 compound has an activity in inhibiting the M. Tuberculosis growth of HE and SR strains with the concentration of the media is 0,125 mg/mL.*

Keywords:*Antituberculosis, Mycobacterium tuberculosis, Curcumin analogs, GVT*

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I. Introduction

Tuberculosis (TB) is a direct disease transmission caused by M. Tuberculosis which attacks some human organs especially lungs organ. According to WHO, there is an increase of TB patients each year. In 2013, about 9 million people in the world were infected with TB and the fatality number reaches about 1,5 million people. Thirteen percents of TB patients were also infected with HIV and MDR-TB as a new case was about 3,5 percents. Sixty percents cases and mortality caused by TB occurred men, yet there are a number of women inflicted with this particular disease. Three million and three hundred thousand of women and 550.000 children were estimated infected with TB. The number of the mortality was about 510.000 women and 80.000 children. TB had been a burden in almost all countries in the world. Nevertheless, 80 percents of TB case which was reportedly attacked 22 countries in the world. In 2013, 56 percents of new TB case occurred in Southeast Asia and West Pacific[1]. Five countries with high TB case in 2014 were India, China, Indonesia, South Africa, and Nigeria[2]. TB treatment is given in form antituberculosis drugs combination (OAT) such as Isoniazid (INH), Rifampicin, Pyrazinamide, Ethambutol, Streptomycin; it is aimed at improving better effectiveness and avoiding the resistance and monotherapy. Antituberculosis drugs combination (later on called as OAT) is given for 6 months. Therapy durationreceived by TB patients often increase the side effect of the drugs, namely hepatotoxicity, nauseous, vomiting, anorexia, spasmodic, headache, urination disorder, color blind, peripheral nerve disorder, and etc. Hepatotoxicity caused by OAT occurred in 2 – 27,7% of the patients who undergone TB treatment[3].

The combination of TB drugs regimen nowadays has a high level of success. However, the use of this regimen depends on the drugs compliance. Noncompliance in using the prescribed medication often evokes TB strain which is resistant to some or even all first and second line of antibiotics[4]. The resistant strain is called as Multiple Drug Resistance (MDR- TB), Extensively Drug Resistance (XDR-TB) and Totally Drug Resistance (TDR-TB) which would exacerbate the disease[5]. The emergence of XDR-TB has been already confirmed in Indonesia[6].Therefore people compliance is very needed to inhibit the XDR-TB rate since it is very dangerous and possess high mortality rate. World Health Organization (WHO) estimated 6.800 cases of new MDR-TB per year in Indonesia. It was estimated 2% of new TB case and 12% of TB case which should take another treatment[7]. New drugs is needed for increasing TB disease control, especially in developing countries. The high burden caused by TB cases - which needs therapy for a long time, attacks productive age population and also impacts the increasing the case of Human Immunodeficiency Infection and Acquired Immune Deficiency

Syndrome (HIV/AIDS) - which could increase TB patient who is either sensitive or resistant to standard medicine/treatment. Therefore exploration towards new drugs is required[8].

The strategy in developing the drugs was conducted by modifying the compound structure from nature through synthesizing to produce a compound with high potential and more specific activity. One of the natural compounds which have been isolated and applied structure modification onto it was curcumin [1,7- bis(4-hydroxy-3-methoxy) heptadiene-3,5-dion]. It has been extracted from Curcuma Longa plant[9]. Curcumin has antioxidant activities[10], anti inflammation[11,12], anti cholesterol[13], anti infection[14], anti cancer[15], anti viral[16,17], and antibacterial towards *Escherichia coli, Bacillus Subtilis, Helicobacter pylori,* and *Mycobacterium tuberculosis (M. tuberculosis)*[18,19]. Curcumin is reported as a potential anti-tuberculosis drug[20,] even though most of the mechanism of anti-tuberculosis is still unknown [21,22]. The previous study exhibited that curcumin could inhibit the inflammation and oxidation, induct the apoptosis cell [23,24,25]and also inhibit the gene expression and functions as Tool Like Receptor 2 (TLR2) possibly through an oxidation process. It shows that the curcumin ability which has a role in macrophage apoptosis induced by *Mycobacterium tuberculosis*[26].

The research about modification of curcumin structure mostly has already been done. Sardjiman (1997) modified the curcumin structure into three groups compound which was dibenzylidin cyclohexanone (A series), dibenzylidin cyclopentanone (B series) dan 1,5-diphenyl-1,4-pentadiena-3-on (C series). The other three compounds of curcumin analogue from those three series are HGV-6 [2,6-bis(31,51-dichlor-41-hydroxybenzylidin) cyclohexanone], PGV-6 [2,5-bis(31,51-dichlor-41-hydroxybenzylidin) cyclopentanone] and GVT-6 [1,5-bis(31,51-dichlor-41-hydroxyphenyl)-1,4-pentadiene-3-ones] which has anti bacterial activity towards *E.coli, Staphylococcus aureus, Bacillus subtilis, Streptococcus pneumonia* and *Candida albicans*. These three compounds are oxidizing compounds,therefore, it becomes the most potential antibacterial compounds[27]. Hydroxyl cluster has an important part in inhibiting bacteria activity potentially and the phenolic cluster can be potential denaturant on the cell wall of the bacteria[12]. *Streptococcus pneumonia* is a bacteria which causes pneumonia disease. Pneumonia and tuberculosis are the diseases which often attack the lower part of the respiratory tract. This research was conducted to see activity ofbenzylidine acetone of curcumin analogues as anti tuberculosis against*Mycobacterium tuberculosis*.

II. Material And Method

2.1Material

The materials used in this research iscurcumin analog compound of Benzylidene acetone which was obtained from Curcumin Research Center, Faculty of Pharmacy in Gajah Mada University. Purified water (aquadest), TB medium Löwenstein-Jensen (LJ), glycerin, duck eggs, rifampicin, isoniazid, ethambutol, streptomycin, dimethylsulfoxide.

2.2Bacteria

Bacteria used for a sensitive strain of Mycobacterium tuberculosis H37Rv test, HE, and SR strains which were resistant. These were obtained from Balai Pengembangan Laboratorium Kesehatan (Agency for Development of Medical Laboratory) in Surabaya.

2.3Antitubercular Assay

Bacteriaproduction as media for the test compound were at concentration of 0,125; 0,250; 0,500 dan 1 mg/mL. The drugs used as a comparisonwere rifampicin (40 µg/mL), isoniazid (0,2 µg/mL), ethambutol (2,0 µg/mL), and streptomycin (4,0 µg/mL). LJ media was added in a tube filled with test solution or drug comparison until the total volume reached up to 5ml, then mixed it until it became homogeneous. The tube was tilted, then put into an oven at 85°C for about an hour so that the media became solid.

2.4Inoculation Bacteria

Inoculation and inoculation observation was done towards three strains of M. tuberculosis, the sensitive strain of H37Rv as well as two resistant strains, HE strain (resistant to isoniazid and ethambutol) and SR (resistant to streptomycin dan rifampicin). Each bacterial strains was suspended into NaCl 0,9 % therefore whole 10-3 and 10-5 McF were obtained. Each 0,1 mLbacterial suspension was inoculated on the surface of the media which contained extract, drug comparison, or solution. Each tube was incubated at 37°C. Bacterial colony growth is observed every week starting from 4^{th} week to 8^{th} week.

III. Result

During initial phase of research, the compound was used to determine melting point. It was conducted to determine the compound's physicochemical identity. The test result indicated that the compound type was appropriate to its characteristics which are described in Table 1.

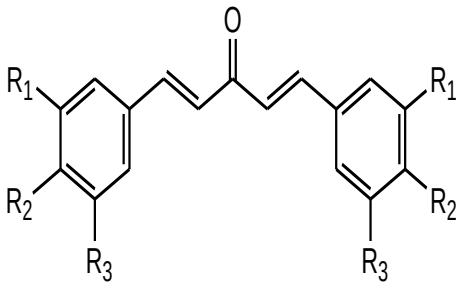

Figure 1. Structure of 1,5-diphenyl-1,4-pentadiene-3-one

Table 1. Characteristic and Chemical Name of Benzylidine Acetone Analogues of Curcumin

Compound	**R1**	**R2**	**R3**	**Chemical Name**	**Melting Point (^{0}C)**
GVT-0	H	OH	H	1,5-Bis(4-Hydroxyphenyl)-1,4-Pentadiene-3-One	243-245
GVT-1	OCH_3	OH	H	1,5-Bis(4-Hydroxy-3-Methoxyphenyl)-1,4-Pentadiene-3-One	98-99
GVT-2	H	H	H	1,5-Diphenyl-1,4-Pentadiene-3-One	109-110
GVT-6	Cl	OH	Cl	1,5-Bis(3,4-Dichloro-4-Hydroxyphenyl)-1,4-Pentadiene-3-One	198-200
GVT-10	Cl	H	H	1,5-Bis(3,4-Dichlorophenyl)-1,4-Pentadiene-3-One	255-256

Tuberculosis activity examination in vitro began with resistance test towards anti-tuberculosis drug compounds. Those compounds were rifampicin (40 µg / mL), isoniazid (0.2 µg / mL), ethambutol (2.0 µg / mL), and streptomycin (4.0 µg / mL).

Table 2. Drug-Resistant Assay after 8 weeks

Drugs	H37Rv	HE	SR
Isoniazid	S	R	R
Rifampicin	S	S	S
Ethambutol	S	R	R
Streptomycin	S	R	R

S = Sensitive R = Resistant

The resistance test on anti-tuberculosis drug compounds was conducted on two strains of bacteria. It consisted of one sensitive strain (H37Rv) and two resistant strains (HE and SR). The H37Rv strain was still sensitive and could be decimated by the first option of rifampicin and isoniazid. HE strain was already resistant to isoniazid and ethambutol, while SR strain was resistant to streptomycin and rifampicin.

Table 3. Drug-Resistant Assay against Mycobacterium tuberculosis H37Rv after 8 weeks

Compound	Conc. (µg/mL)	H37Rv (Week)							
		I	II	III	IV	V	VI	VII	VIII
GVT-0	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-1	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-2	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-6	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-10	125	S	S	S	S	S	S	S	S
	250	S	S	S	S	S	S	S	S
	500	S	S	S	S	S	S	S	S
	1000	S	S	S	S	S	S	S	S

S = Sensitive R = Resistant

Table 4. *Drug-Resistant Assay against Mycobacterium tuberculosis HE after 8 weeks*

Code	Conc. (µg/mL)	HE (Week)							
		I	II	III	IV	V	VI	VII	VIII
GVT-0	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-1	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-2	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-6	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-10	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R

S = Sensitive R = Resistant

Table 2 exhibited that all compounds of Anti-tuberculosis first line and second line were able to inhibit the growth of M. tuberculosis H37Rv strain. Nevertheless, only rifampicin was still sensitive to HE and SR strains. Based on the results of anti-TB activity test on curcumin analog of benzylidene acetone, the only GVT-10 compound could inhibit the growth of M. tuberculosis H37Rv strain was the lowest concentration tested (125 µg / mL). The HE and SR strains were still sensitive towards GVT-10, therefore, the two strains' growth could be inhibited by GVT-10. Even the bacteria has already grown on it in the 6^{th} week as exhibited in Table 3, 4 and 5.

Table 5. Drug-Resistant Assay against *Mycobacterium tuberculosis* SR after 8 weeks

Code	Conc. (µg/mL)	SR (Week)							
		I	II	III	IV	V	VI	VII	VIII
GVT-0	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-1	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-2	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-6	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R
GVT-10	125	R	R	R	R	R	R	R	R
	250	R	R	R	R	R	R	R	R
	500	R	R	R	R	R	R	R	R
	1000	R	R	R	R	R	R	R	R

S = Sensitive R = Resistant

IV. Discussion

The benzylidene acetone's Curcumin analog had been synthesized to improve its pharmacodynamics characteristic and pharmacokinetics characteristic. Sardjiman et al., synthesized the curcumin analog series which consisted of benzylidene cyclohexanone, benzylidene cyclopentanone,benzylidene acetone to improve curcumin stability. Curcumin bad stability will easily be degraded at higher pH value and will be exposed to the sunlight[28]. Benzylidene acetone's Curcumin analog used in this research was only its some series that were

GVT-0, GVT-1, GVT-2, GVT-6, and GVT-10 in order to know the activity towards the inhibition of Mycobacterium tuberculosis bacteria of H37Rv, HE and SR strains.

Anti-tuberculosis activity examinationwas conducted by using a proportional method. This method used two concentrations of bacteria inoculum at 10^{-5} and 10^{-3} McF respectively which contained around 10^2 and 10^4 cell/mL. This was conducted in order to obtain the total amount of the colony which could be calculated (50-100 colonies) in one of the concentration. Therefore total amount of observed colony could be determined[29].The GVT-10 compound exhibited higher of the test compound concentration and better sensitivity level on H37Rv strain when compared to other compounds. However, the inhibition activity of GVT-10 towards the M. tuberculosis strain MDR growth could not be determined. During the observation, the inhibition activity of GVT-6 in all concentration had a tendency to be decreased in the fourth week; while in the fifth week until the end of the observation, the inhibition activity exhibited constant result which was resistant or inactive. The compound was categorized as active anti tuberculosis should it resulted in more or similar inhibition to 90%. Referring to this concept, GVT-10 was determined to possess anti-tuberculosis activity in the 0,125 mg/mL concentration.There were two clusters of chlor on para position which resulted in more active GVT-10 than GVT-6 which had two clusters of chlor by clamping hydroxyl cluster. The existence of chlor cluster has an important role on tuberculosis activity[30]. Even though the existence of hydroxyl cluster also had an effect in inhibiting the growth of the bacteria[31] as in GVT-6 which had low activity. Mycobacterium tuberculosis bacteria was lipophil and it had a high density of cell wall with microplate acid, therefore, cell wall penetration process was relatively difficult. In this research, most of the curcumin analogs could not inhibit Mycobacterium tuberculosis on sensitive strain (H37Rv), HE, and SR strains which were resistant. It needed higher concentration to inhibit Mycobacterium tuberculosis H37Rv. This phenomenon was possibly caused by those compounds which had different mechanism/ target from existing anti-tuberculosis[32].

V. Conclusion

Mycobacterium tuberculosis of H37Rv strain can be inhibited by GVT-10 on 0,125 mg/mL concentration. But HE strain (resistant to isoniazid and ethambutol) and SR strain (resistant streptomycin and rifampicin) cannot be inhibited by all curcumin analog compounds of benzylidene acetone.

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