EVALUATION OF BENZYLIDENE-ACETONE ANALOGUES OF CURCUMIN AS ANTITUBERCULOSIS

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ABSTRACT

Objective: The objective of this research is to evaluate the effect of benzylideneacetone analog of curcumin against *Mycobacterium tuberculosis* (MTB) H37Rv.

Method: The activity of benzylideneacetone analog of curcumin is evaluated using mycobacteria growth indicator tube (MGIT) method and microplate alamar blue assay (MABA) method. The compound of minimum inhibitory concentration (MIC) is defined as the minimum concentration of drugs which really inhibit the growth of MTB.

Result: The MIC compound of 1,5-bis(3,4-dichlorophenyl)-1,4-pentadiene-3-one (C9) and 1,5-bis(3-chlorophenyl)-1,4-pentadiene-3-one (C10) can inhibit the bacteria of MTB at the concentration of 500 μg/ml using MGIT method. Based on MABA method, it can be obtained similar MIC value of C9 compound and C10 compound that are 187.5 μg/ml.

Conclusion: C9 and C10 have antituberculosis activity. Benzylideneacetone analog of curcumin which has chlor moiety has a better activity in inhibiting MTB, but it still needs further research to make it become a potent antituberculosis drug.

Key words: Tuberculosis, Curcumin analog, Mycobacteria growth indicator tube, Microplate alamar blue assay.

INTRODUCTION

Tuberculosis (TB) is one of the infectious diseases which are caused by MTB and it happens in most of developing countries in the world. In 2015, based on the World Health Organization, the case of TB in the world was about 10.4 million on the new TB case. 5.9 million (56%) in men and 3.5 million (34%) in women. Whereas, HIV patients with new TB case were only 1.2 million (11%) [1]. The most identified countries of 60% of new TB case were India, Indonesia, China, Nigeria, Pakistan, and South Africa [1].

TB case is still being a big burden in Indonesia since the TB case amount is always increasing with the incident rate was about 300.000 in 2014. TB patients should obey the TB treatment to get an effective treatment and to avoid the existence of anti-TB drugs resistance. The failure of treatment can cause resistance of drugs which is usually known as multidrug resistance (MDR-TB) and extensively drug resistance (XDR-TB). MDR-TB infected patients who were resistant against the first line of TB drugs such as isoniazid and rifampicin, while XDR-TB happened when the bacteria were resistant against the first line and the second line of TB drugs such as the group of fluoroquinolones [2].

Turmeric is known as traditional ingredient and it is used as traditional medicine or traditional drinks. The yellow pigment of *Curcuma longa* was known as curcuminoid which was found as active substance in curcuma rhizome [3]. Curcuminoid consists of curcumin, dimetoxycurcumin, and bis-dimethoxycurcumin which are found in the rhizome of turmeric and it is widely used in traditional medicine [4,5]. Curcumin has an activity of antioxidant and anti-inflammation [6,7], antidiabetic [8], antivirus [9,10], antiallergy [11], antihyperlipidemic [12], anti-infective [13], anticancer [14], and antibacteria against *Escherichia coli*, *Bacillus subtilis*, *Helicobacter pylori*, and MTB [15,16]. Curcumin was reported as non-toxic compound against mice, rabbits, monkeys, and human volunteers [17].

Curcumin had been reported as potent anti-TB drugs even though the mechanism of anti-TB was certainly not known yet [16]. The previous studies showed that curcumin could inhibit inflammation, oxidation, and it could induce apoptosis, and it also could inhibit the expression of gene and the function of toll-like receptor 2 which might occur through oxidation process. It indicated the ability of curcumin which had a role in macrophage apoptosis which was caused by MTB [16,18]. Hence, the objective of this research is to know the antimycobacterium activity in benzylideneacetone analog of curcumin.

MATERIALS AND METHODS

Materials

The benzyliene-acetone analogues of curcumin were obtained from Curcumin Research Center (CRC) Faculty of Pharmacy, Universitas Gadjah Mada. Dimethyl sulfoxide (DMSO) (Sigma Aldrich), Middlebrook 7H9 broth (Difco Laboratories), Oleic acid/Albumin/Dextrose/Catalase enrichment (OADC) (Becton Dickinson and Company), Polymyxin B, Amphotericin B, Nalidixic acid, Trimetroprim, and Azlocillin (PANTA) (Becton Dickinson and Company), Tween 80 (Sigma Aldrich), Alamar Blue Reagent (Sigma Aldrich), Isoniazid (Sigma Aldrich), Rifampicin (Sigma Aldrich), Kit mycobacteria growth indicator tube (MGIT) (Becton Dickinson and Company), Mycobacterium tuberculosis H37Rv from Mirobiology Laboratory, Faculty of Medicine, Universitas Gadjah Mada.

Preparation of MTB

MTB H37Rv was grown in Middlebrook 7H9 broth supplemented with 10% OADC and 0.05% (v/v) Tween 80 to the log phase at 37°C for 4–5 weeks on shaking incubation of 120 r/min.

Preparation sample and drug of TB

The first-line therapy for TB drug is isoniazid and rifampicin. Isoniazid and rifampicin were dissolved in water and dimethylsulfoxide 1% with

concentration of 10 μg/mL, respectively. Then, they were filtered using 0.45 μm membrane syringe filter before use and stored at −80°C deep-freezer until use. Sample was dissolved in dimethylsulfoxide 1% with concentration of 1000, 750, 500, 375, 250, 187.5, and 125 μg/ml.

Drug susceptibility testing of MTB

It is used to determine the ability of compound curcumin analogs to inhibit MTB using the kit MGIT BD Biosciences. MGIT tube contains the growth medium. 500 μL of OADC and 1000 μL of PANTA were added to each MGIT tube. Next, 1000 μL of drug or sample solution with various concentrations was added to the MGIT tube. Then, 1000 μL bacterial suspension in medium Middlebrook 7H9 was added and incubated at a temperature 370°C for 2 days. The bacterial cells were adjusted accord McFarland standard. The MGIT tubes were bar scanned and transferred to the Bactec 960 system for 14 days. The growth of MTB detected in these instruments (Bactec 960 System). The instrument monitors the entered susceptibility test set. Once the test is complete (14 days), the instrument will indicate that the results are ready. The results are qualitative such as susceptible (S), resistant (R), or indeterminate (X). The instrument interprets results at the time when the growth unit (GU) in growth control reaches 400. At this point, the GU values of the drug vial are evaluated. Susceptible when the GU of the drug tube is <1000 and resistant when the GU of the drug tube is 1000 or more. Further, X results when certain conditions occur which may affect the test, such as GU of the control reaches >400 in <4 days.

Microplate alamar blue assay (MABA)

This test was used alamar blue reagents and performed 96-well microplates. The wells on 96-well microplates are illustrated with rows A-H and columns 1-12. The outer wells are the lines A1 - H1, A12 - H12, A1 - H12, and H1 - H12 filled with 200 μL sterile water. 100 μL of 7H9GC media was added to all experimental wells (in addition to the outer wells). 200 μL of the sample solution was added to the well on line B in columns 2, 3, 4, and 5 (B2, B3, B4, and B5). Using a micropipette, 100 μL was transplanted to line C and homogenized then from line C of 100 μL transplanted to line D. The dilution system was continued until line G. Line B6-G6 was administered 100 μL isoniazid 10 μg/mL and line B7-G7 was administered 100μL rifampicin 10 μg/mL, both compounds were used as positive controls. Line B8-G8 was used as a controlled solvent which was administered as 100 μL DMSO 1% and administered 100 μL of bacteria in line B9-G10 used as a sample free control (inoculum). Suspension of MTB (100 μL) is added to the test well. The plates were sealed with parafilm and incubated at 37°C for 5 days. On the 6th day after incubation, 50 μL alamar blue reagent (1:1 mixture of alamar blue and 10% Tween 8) was added to B11 and incubation for 24 h. Observed the change color in B11 which when turned into pink then the alamar blue reagent is added to the all well test but if B11 remains blue, so apply the reagent on B12 (and same treatment as B11) incubation for an additional 24 h at 37°C, and color change recorded. Pink color is interpreted as the ability of compounds in inhibiting bacteria minimum inhibitory concentration (MIC) and blue color is a described of the growth of bacteria and inactive compounds in bacterial inhibition.

RESULTS

Antituberculosis susceptibility using MGIT

The ability of curcumin analogs inhibits the growth of MTB determined MGIT test. The result is described in Table 1. Based on data, it has been shown that the curcumin analogs C9 and C10 have significant TB activities when they are compared to isoniazid and rifampicin. The susceptibility of MTB test results showed that H37Rv strain is sensitive to isoniazid and rifampicin.

Inhibition MTB with MABA

The MIC of antituberculosis assay of curcumin analogues against MTB H37Rv are shown in Table 2. The results showed that C9, and C10 inhibited MTB H37Rv with MIC 187.5 μg/mL. Isoniazid and rifampicin were used as positive control in determining MIC of curcumin analogues.

DISCUSSION

TB is the main cause of morbidity and mortality against one-third of population in the world. The failure of treatment is often occurred in this transmitted disease, especially in developing countries, so it can cause human health problems. MDR-TB and XDR-TB can increase the serious threat against human health which is caused by the failure of TB treatment and impracticality TB treatment. The treatment regimen for TB consists of four drugs with different mechanism of treatment. Most of new generations of drugs are developed on clinical therapy, so we need the new drugs candidate that has different mechanism and new target. Therefore, new drugs are needed to shorten the duration of TB treatment in both of new TB case and MDR-TB case to minimize the infection and mortality.

Curcumin has many beneficial pharmacological activities, but the instability of the compound can be a serious problem. The stability of curcumin is affected by sunlight ray and pH of the compound. The increasing of pH more than 7 can cause curcumin decomposition and it becomes curcumin decomposition products. They are feruloylmethane and ferulic acid from the condensation of retroaldol to vanillin and acetone at a rapid tempo. Then, the curcumin decomposition caused by sunlight ray also produced ferulic aldehid, ferulic acid, products from dihydroxynaphthalene, 4-vinylguaiacol, vanillin, and vanillic acid [11]. The active part of methylene is one of the factors of the instability of curcumin (Fig. 1). The modification of the structure is made to overcome the problem of the compound instability by substituting other compounds such as 1,4-pentadiene-3-one, cyclopentanone, and cyclohexanone in the middle part of curcumin (Fig. 2) structure [18].

Table 1: Antituberculosis activity results of curcumin analogs using MGIT method

No	Code	Concentration (μg/ml)						
		1000	750	500	375	250	187.5	125
1	C0	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
2	C1	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
3	C2	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
4	C3	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
5	C4	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
6	C5	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
7	C6	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
8	C7	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
9	C9	Sensitive	Sensitive	Sensitive	Resisten	Resisten	Resisten	Resisten
10	C10	Sensitive	Sensitive	Sensitive	Resisten	Resisten	Resisten	Resisten
11	C11	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
12	C15	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
13	C16	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten	Resisten
14	Isoniazid 10 μg/Ml							Sensitive
15	Rifampicin 10 μg/Ml							Sensitive

Table 2: MICs of curcumin analogs against MTB H37Rv

No	Code	Concentration (µg/ml)						
		1000	750	500	375	250	187.5	125
1	C0	Not active	Not active	Not active	Not active	Not active	Not active	Not active
2	C1	Not active	Not active	Not active	Not active	Not active	Not active	Not active
3	C2	Not active	Not active	Not active	Not active	Not active	Not active	Not active
4	C3	Not active	Not active	Not active	Not active	Not active	Not active	Not active
5	C4	Not active	Not active	Not active	Not active	Not active	Not active	Not active
6	C5	Not active	Not active	Not active	Not active	Not active	Not active	Not active
7	C6	Not active	Not active	Not active	Not active	Not active	Not active	Not active
8	C7	Not active	Not active	Not active	Not active	Not active	Not active	Not active
9	C9	Active	Active	Active	Not active	Not active	Not active	Not active
10	C10	Active	Active	Active	Not active	Not active	Not active	Not active
11	C11	Not active	Not active	Not active	Not active	Not active	Not active	Not active
12	C15	Not active	Not active	Not active	Not active	Not active	Not active	Not active
13	C16	Not active	Not active	Not active	Not active	Not active	Not active	Not active
14	Isoniazid 10µg/Ml							Active
15	Rifampicin 10µg/Ml							Active

Table 3: Structure and chemical name of 1,5-diphenyl-1,4-pentadiene-3-one (Series C) analogs of curcumin

No.	R1	R2	R3	Chemical name	Code
1	H	OH	H	1,5-bis (4-hydroxyphenyl)-1,4-pentadiene-3-one	C0
2	OCH_3	OH	H	1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one	C1
3	H	H	H	1,5-diphenyl-1,4-pentadiene-3-one	C2
4	H	Cl	H	1,5-bis-(p-chlorophenyl)-1,4-pentadiene-3-one	C3
5	H	OCH_3	H	1,5-bis (4-methoxyphenyl)-1,4-pentadiene-3-one	C4
6	H	CH_3	H	1,5-bis (4-methylphenyl)-1,4-pentadiene-3-one	C5
7	H	$t\text{-}C_4H_9$	H	1,5-bis (4-tert-butylphenyl)-1,4-pentadiene-3-one	C6
8	H	CF_3	H	1,5-bis (4-trifluoromethylphenyl)-1,4-pentadiene-3-one	C7
9	H	$N (CH_3)_2$	H	1,5-bis (4-dimethylphenyl)-1,4-pentadiene-3-one	C8
10	Cl	Cl	H	1,5-bis (3,4-dichlorophenyl)-1,4-pentadiene-3-one	C9
11	Cl	H	H	1,5-bis (3-chlorophenyl)-1,4-pentadiene-3-one	C10
12	CH_3	OH	CH_3	1,5-bis (4-hydroxy-3,5-dimethylphenyl)-1,4-pentadiene-3-one	C11
13	OCH_3	OH	OCH_3	1,5-bis (4-hydroxy-3,5-dimethoxyphenyl)-1,4-pentadiene-3-one	C15
14	Cl	OH	Cl	1,5-bis (3,4-dichloro-4-hydroxyphenyl)-1,4-pentadiene-3-one	C16

In addition, the modification can also increase the stability of curcumin which can obtain better stability (Table 3), more potent and secure activity. Some curcumin analog compound was reported that they had better pharmacological effect as antioxidant, anti-inflammation, and antibacteria. They were pentagamavunon-1 (PGV-1) and PGV-2 [19]. PGV-0 was also a result from modification of monoketone curcumin structure which had an activity as anti-inflammation, anticancer, and antioxidant [20,21], but it still had bioavailability problem like what curcumin had [22]. GVT-0, especially for lower dosage (10 mg/kg), showed the action of hepatoprotective on mice as the model [23]. Tetrahydrocurcumin was the main metabolism of curcumin which had 3 times better activity as antioxidant than curcumin *in vitro* [24]. Other compounds which are resulted from the modification of curcumin structure are benzylideneacetone analogs of curcumin. Modification of structure was done in the middle part of curcumin by substituting aliphatic β-diketone part to acetone. The compound of benzylideneacetone analog of curcumin has better activity as antibacteria against *Streptococcus pneumoniae* which can cause respiratory tract infection.

The activity of antimycobacterial from benzylideneacetone analog of curcumin is evaluated using MGIT method and MABA method. The concentrations of benzylideneacetone analog of curcumin which are used here are 1000, 750, 500, 375, 250, 187, 5, and 125 µg/mL and the positive control group used here is the first-line anti-TB drugs, i.e. isoniazid and rifampicin (10 µg/mL).

The result shows that 1,5-bis(3,4-dichlorophenyl)-1,4-pentadiene-3-one (C9) and 1,5-bis (3-chlorophenyl)-1,4-pentadiene-3-one (C10) are the compounds which are the better inhibitor against the growth of MTB than other compounds in benzylideneacetone analog of curcumin using both MGIT method and MABA method. C9 and C10 compounds have lower MIC value than other compounds. These two compounds contain chlor moiety at para position. C9 has two chlor moieties both at para and at ortho position with hydrogen moiety inside the phenyl ring, while C10 has only one chlor moiety inside the phenyl ring and it has two hydrogen moieties. It shows that C9 has more halogen moiety as the electron withdrawing group. Hence, C9 is more active than C10 compound. Meanwhile, C16 compound which has two chlor moieties and one hydroxyl moiety at ortho position does not have inhibitory activity against MTB. Besides, C3 compound which has chlor moiety at ortho position and which is in the middle of two hydrogen moieties does not show inhibitory activity too against the growth of bacteria. It shows clearly that the existence of moiety as the electron withdrawing group and the position of the moiety as the electron withdrawing group have an important role here.

The position of chlor moiety at para and ortho position has stronger activity of anti-TB than those at para and meta position. Hence, the MIC value on C9 compound becomes lower than C10 and C16 compound. C9 and C10 compound are the most potent compounds in inhibiting MTB. The previous studies stated that the existence of electron withdrawing group could increase the activity of anti-TB efficiently. According to QSAR studies, it was stated that 2-chlor-, 3-chlor-, 4-methyl-, and 2-chlor-5-substituent could increase the activity of TB [23]. The existence of electron withdrawing group such as pyridine, nitro, and chlor inside the phenyl ring can also produce strong activity of antimycobacterium. Meanwhile, the electron-donating group, such as hydroxyl, methoxy, and amino, shows weaker activity. Then, based
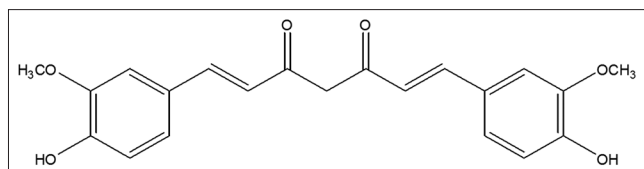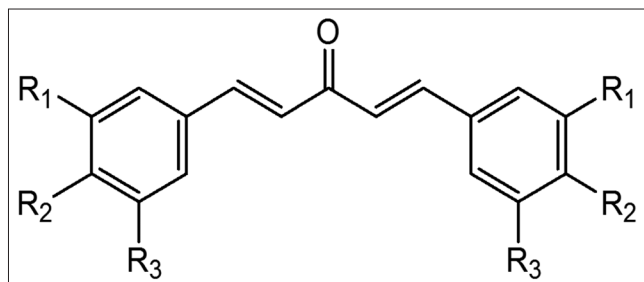
Fig. 1: Structure of curcumin

Fig. 2: Structure of benzylideneacetone analogs of curcumin (1,5-diphenyl-1,4-pentadiene-3-one)

on QSAR studies, it was clearly shown that the existence of electron withdrawing group inside the phenyl ring was very important for antimycobacterium activity [25].

The effect of anti-TB activity from C9 and C10 compound depended on the existence of the substituent at para and/or meta position in aromatic ring which could inhibit the growth of MTB [16,18]. In this research, the compound with chlor moiety at meta position can inhibit the growth of MTB, while the compound without electron withdrawing moiety has weaker activity as anti-TB.

This research shows the importance of aromatic ring which contains chlor moiety at para position as the potent anti-TB. The previous research showed the importance of bromo and fluoro moieties at para position since those two compounds could increase the activity of anti-TB, while the electron donating moiety could decrease the activity of anti-TB [26]. The mechanism from anti-TB on the compound which has electron-withdrawing moiety is estimated has an interaction with protein binding on the enzyme of Inh-A which has been activated. The mechanism on the activated enzyme of Inh-A is really affected by any kinds of chemical binding and the polarity. The halogen compound is lipophilic since it has electronegative characteristic, so the halogen compound affects the activity of anti-TB [27]. The lipophilic characteristic is really needed since the MTB is a bacteria contained mycolic acid. Mycolic acid is the component which forms MTB cell wall that has lipophilicity. Therefore, it was needed a compound of drug which has lipophilicity, so it could break the biosynthesis of mycolic acid in the cell wall and it could cause cellular malformation which resulted in cell death [28].

CONCLUSION

The compound 1,5-bis(3,4-dichlorophenyl)-1,4-pentadiene-3-one(C9) and 1,5-bis(3-chlorophenyl)-1,4-pentadiene-3-one (C10) have inhibitory activity against MTB.

ACKNOWLEDGMENTS

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