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Research Article

ANTI-*MYCOBACTERIUM TUBERCULOSIS* STRAIN H_{37}RV AND IRON CHELATION ACTIVITY OF SAPPAN WOOD EXTRACT (*CAESALPINIA SAPPAN* L.) *IN VITRO*

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ABSTRACT

Objective: The objective of this study is to determine anti-*Mycobacterium tuberculosis* (MTB) strain H_{37}Rv and iron chelation activities of sappan wood extract (SWE).

Methods: The evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by proportion methods. Whereas the iron chelate in Lowenstein–Jensen (LJ) medium as the indicator of Mycobacterium growth and SWE effect.

Results: The SWE has bacteriocidal to MTB of 10^{-3} and 10^{-5} dilutions in of all concentrations (250, 500, 750, 1000, 2000, 4000, 8000, and 16000 part per millions [ppm]) also bacteriostatic in concentration 50 and 100 ppm.

Conclusion: The SWE at 100 ppm could inhibit 87% of the MTB in 10^{-3} and 10^{-5} dilutions, respectively, also to reduce to growth the colony of MTB, and has chelating effects of iron expression of LJ medium and MTB.

Keywords: Iron chelation, *Mycobacterium tuberculosis, Caesalpinia sappan* L.

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INTRODUCTION

Iron is a pre-requisite for *in vitro* growth of mycobacteria and an obligate cofactor for at least 40 different enzymes encoded in the *Mycobacterium tuberculosis* (MTB) genome [1]. Iron helping cell in oxygen transport, proliferation, and ATP generate. It has the role as the coenzyme of ribonucleotide reductase and DNA synthesis [2]. The MTB is the main agent in the pathogenesis of TB. The iron reported as the virulence factor of MTB to survive in the human lung [3]. On the TB patient with anemia history that indicates low iron intake [4]. . It is therefore very important to develop potential iron chelators, to inhibit the growth of MTB for use as a substitute, or to strengthen treatment of TB by main antibiotics.

The sappan wood extract (SWE) in the Latin language called *Caesalpinia sappan L.* is used in the traditional medicines of various Asiatic countries including in Indonesia. Empirically, the sappan wood of heartwood dried has often been used as herbal remedy for the locals and as a traditional ingredient like in food or beverages. In Indonesia, its material has long been used in Indonesia folk medicine to treat TB, diarrhea, dysentery, skin infections, anemia, chelator, detoxifying, treating syphilis, stop the bleeding antiseptic, as well as pain due to blood circulation disorders [5]. Sireeratawong *et al*. have evaluated that the SWE is safe and did not produce any acute or subacute toxicity in both male and female rats and has antioxidative and as an iron chelation [6]. Sappan wood contains various structural types of phenolic components including xanthone, coumarin, chalcones, flavones, flavonoids, and brazilin as the major compound that proved has the ability as antioxidative and iron chelation [7].

Phenolics are described as multifunctional antioxidants with chain-breaking and metal-chelating activities in the same molecule. Flavonoids also exhibit the properties of the metal, such as quercetin that can bind strong copper and iron [8]. In flavones, the presence of a 3', 4' dihydroxy group is essential for metal binding and specifically the four position is very important for the metal dyeing activity, In flavones, the presence of a 3', 4' dihydroxy group is essential for metal binding to formation metal solid. It is suspected that there is cooperation between the four carbonyl groups with the 3 or 5 dihydroxy groups to be able to dissolve the copper ions, and Whereas, the catechol group of flavonoid Brazilin has been binding of heavy metal [9]. In microbes based on the metal-chelating group, there are three major classes of microbial siderophores, catecholate, hydroxy-carboxylate, and the hydroxamate class [10]. The third substances have exhibit highest affinity for iron bind and hold it with three bidentate bonds [11].

The SWE was assayed as the antimicrobial anti positive gram such as *Staphylococcus aureus* and *Bacillus subtilis* and negative gram such as *Klebsiella pneumoniae*, *Escherichia coli,* and *Proteus vulgaris* [5]. Seo *et al*. reported that the 3-deoxysappanchalcone isolated from the heartwood of *C. sappan* Linn. possessed the antitubercular activity of both drug susceptible and drug resistant of MTB strain H_{37}Rv [12]. Based on these facts, this study was conducted to determine anti-MTB strain H_{37}Rv and iron chelation activities of SWE.

METHODS

Materials

This research has approved with the ethical clearance No.639/UN6. C1.3.2./KEPK/PN/2016 issued by Faculty of Medicine, Universitas Padjajaran Bandung, Indonesia. In this study, we are used to the sappan woods (*C. sappan* L.) in the various concentration (50, 100, 250, 500, 750, 1000, 2000, 4000, 8000, and 16000 part per millions [ppm]) as the material anti-MTB that compared the first-line antibiotic (main

antibiotic) (rifampicin 40 ppm, isoniazid 0,2 ppm, ethambutol 2 ppm, and streptomycin 4 ppm).

Extraction and fractionation of *C. sappan* L.

The extraction and fractionation methods were adopted by Safitri *et al.* [7]. The sappan woods (*C. sappan* L.) were dried in the open air and sheltered from direct sunlight. Once dried, the bulbs were crushed using blender to obtain fine powder. Sappan wood powder was then weighed as much as 1 kg, placed in a Buchner funnel, then was macerated using 15 l of technical methanol solvent for 24 h, and was repeated up to 3 times. The macerate was filtered using Whatman filter paper No. 2, and then, it was concentrated using a rotary evaporator at 60°C to obtain dry extract. To remove the oil (non-polar compounds) fluids, liquid extraction was conducted using 500 ml of technical n-hexane solvent, and then, it was evaporated.

Medium and inoculum preparation

The preparation of Lowenstein–Jensen (LJ) medium and inoculum (MTB) was used as the principal of Gupta *et al*. [13] and Health Ministry of Indonesia standard [14]. The medium prepared first to the homogenization of duck eggs, the eggs soaked in alcohol 70% 15 min, then solved to homogenize on 1000 rpm, and later on, filtered in 1 L. Afterward will prepare the LJ medium with dissolved in all materials of medium potassium dihydrogen phosphate 2.5 g, magnesium sulfate heptahydrate 0.24 g, tri-magnesium dicitrate 14-hydrate 0.6 g, and L-asparagine. The mixtures will go down in aquades 600 mL, pH 6.8–7. After on, added glycerol 12 ml and 20 ml malachite green 2% solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Hereafter sterilized 15 min at 121°C and cooled in room temperature also homogenized and stirred slowly in McCartney bottle 7 ml and loose-closed, hereupon, placed and heated at 85°C 45 min, as well as a bottle cap, is tightened. Next, MTB H37Rv standardized by McFarland 0.5 (1×10^{-8} CFU/ml) incubate for 28– 42 days in this bottle.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay

The assessment of MIC and MBC of MTB used to the 10^{-3} and 10^{-5} dilutions was counted based on colony growth that approached by proportion method [13,14]. The concentrations of SWE were particular scale in ppm (50, 100, 250, 500, 750, 1000, 2000, 4000, 8000, and 16000) as the material treatment and positive control used to Rifampicin 40 ppm, Isoniazid 0, 2 ppm, Ethambutol 2 ppm, and Streptomycin 4 ppm. The treatment uses 100 µl MTB synchronized by McFarland 0.5 in *C. sappan L.* extract. The inoculum is to insert in LJ medium and antibiotic with three independent experiments. The next step procedure will repeat the inoculum preparation. The SWE effect on the MTB growth compared with antibiotic anti-TB. The negative (−) called MBC (absence of MTB) and the positive (+) is MIC (20–100 colonies). The standard of indicator of colony is 500 ≥ 4+ (confluent); 200–500= 3+ (almost confluent); 100–200= 2+; 20–100= 1+; 1–19= no growth= negative (−).

Measurement of iron chelate level

The iron chelate level was measured by atomic absorption spectrophotometer (AAS) (Shimadzu Europa GmbH, Duisburg, Germany) that adopted by Hu *et al*. [15]. The iron titer was measured on the LJ medium and LJ medium plus SWE in the concentration of MIC and MBC that cultured the MTB. All of the samples were destructed by HNO^{3-} to remove the iron content. Later on, the iron level was analyzed based on absorbance. The chelating level of iron was measured by the formula of iron titer (result of [AAS] [mg/L] × dilution) to weight of samples (gram).

Statistical analysis

The MIC and MBC effect were analyzed by the iron level of SWE, significant ($p<0.05$ and $p<0.01$) and Pearson correlation ($r=1$).

RESULTS AND DISCUSSION

MIC and MBC of MTB

Hafidh *et al*. reported that MIC is defined as the minimum concentration of a drug to inhibit the growth of pathogens and amount the inoculum as one of the references [16] According to the Health Ministry of Indoensia, MIC value has similarity with proportion method that is characterized by the lowest concentration which shows the number of MTB colonies between 20 and 100 [17]. In our research was conducted the lowest SWE concentration on LJ medium is shown absence MTB. MBC is influenced by several factors, including the strains of microorganisms, concentrations of antimicrobial agents, the amount of inoculum, and temperature [18]. MIC and MBC data of SWE against MTB during 8 weeks observation are presented in Table 1, where MIC of SWE against MTB at both inoculum dilutions was at 100 ppm concentration which showed the ability to inhibit MTB growth in 6th week. Pitaloka and Sukandar suggested that the ursolic acid has MIC activities in 25–150 µg/mL on the MTB strain H_{37}RV [19].

The MTB growth on LJ medium that was added with SWE showed slower growth than the one on LJ medium without SWE addition, which grew since the 3rd week of the experiment. This was probably due to the limited availability of iron in the LJ medium containing SWE. Iron is the main mineral component as a source for MTB growth. When the iron is limited in the substrate, the growth becomes stunted and MTB performed dormancy as a defense against unfavorable environmental

Table 1: Effects of SWE on the MIC and MBC of MTB

Assay material	MIC and MBC of MTB (weeks)															
	I		II		III		IV		V		VI		VII		VIII	
	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}	10^{-3}	10^{-5}
50 ppm	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
100 ppm	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
250 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
500 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
750 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1000 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2000 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4000 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8000 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16000 ppm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rifampicin (40 ppm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isoniazid (0.2 ppm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethambutol (2 ppm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptomycin (4 ppm)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

MIC: +, MBC: −, 10^{-3}: 3rd dilution, 10^{-5}: 5th dilution, ppm: Part per millions. MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, MTB: *Mycobacterium tuberculosis*, SWE: Sappan wood extract

conditions [20]. Walter (2016) reported that during this phase, MTB maintains the integrity of the cell wall, cell membrane potential, as well as protecting the DNA structure to survive during extreme conditions without losing its pathogenicity [21]. MTB that grown on LJ medium with the addition SWE could only began to show their growth in the 6th week. It also probably due to the competition between MTB and SWE in binding iron on LJ medium was impacted to do a reduction in iron levels on LJ medium.

Percentage of MTB growth

The MTB growth depends on the availability of mineral components on the substrate. The MTB growth depends on iron that important role play in the cell metabolism [22]. The MTB requires iron for its biosynthesis of siderophores. Siderophores is an iron chelating compounds with high affinity secreted by microorganisms [23]. In addition, apart from MTB siderophores biosynthesis, iron is an essential element that acts as a cofactor of enzymes involved in cellular respiration and DNA replication of the bacterial cell [24].

The antibacterial was given the effect of the MTB to binding iron [25]. The SWE phenolic can inhibit intake of the iron chelate of MTB [7]. In this research, the SWE addition as antimicrobial agents into LJ medium also affects the growth of MTB where the number of colonies was less than the control. The MTB growth reduction is 10^{-5} dilution for 8 weeks as presented in Fig. 1, where SWE at 100 ppm concentration in 10^{-5} dilution started to inhibit MTB growth on the 6th week as the percentage of MTB reduction population was 87%. Table 1 shows that since from the 6th to the 8th weeks, SWE at 100 ppm concentration in 10^{-5} inoculum dilution was able to reduce the MTB population by 70.4%. The MBC concentration (250 ppm) in both 10^{-3} and 10^{-5} of inoculum dilution showed the absence of MTB growth, and thus, SWE at 250 ppm was able to kill the MTB population by 92%. The greater the dilution of inoculum resulting less number of colonies MTB contained in a growth medium; therefore, the growth of MTB became higher in the 10^{-3} compared to 10^{-5} inoculum dilution (Table 1).

The results of anti-MTB activity of SWE were then compared with the MIC and MBC on first-line anti-TB drugs (ATBD). Our results showed that four types of first-line drugs (rifampicin, kanamycin, streptomycin, and ethambutol) that were used as a comparison with SWE had better sensitivity. First-line ATBDs were able to kill MTB with lower concentrations compared to SWE. The concentration of the first-line ATBDs that were used as a comparison with SWE in this study was 40, 0.2, 2, and 4 ppm for rifampicin, isoniazid, ethambutol, and streptomycin, respectively. Palomino and Martin give expression that one of the first types of ATBD lines, isoniazid, capable of inhibiting the synthesis of mycolic acid, which is the main constituent of MTB cell wall [26]. Although the first ATBD has good activity against MTB, these drugs still cause several side effects to the TB patients [27].

The SWE concentrations to inhibit and kill the growth of MTB were higher than ATBD, and SWE has no adverse effects because in SWE contains phenolic compounds, tannins, and saponins. This result is in coherence with Saravanakumar and Chandra who observe that these compounds do not cause any side effects except the benefits of natural antimicrobial and antioxidant agent [28]. In addition, similar to isoniazid, one of SWE phenolic compounds, coumarin, was able to inhibit the synthesis of mycolic acid. According to Stanley *et al*.,, coumarin inhibits the synthesis of enzymes that are needed for mycolic acid biosynthetic [29].

***C. sappan* L. ability to iron chelate**

The *C. sappan L.* The SWE is used in the anti-MTB assay, because the phenolic compounds of SWE have the ability to inhibit the MTB growth [30]. In this study, measurement of iron content in the LJ medium was conducted to determine the iron chelation ability of SWE (Fig. 2). The iron chelation by SWE at 100 ppm on LJ medium was 50.6% and decrease in 250 ppm. Phenolic compounds such as xanthones, coumarin, and chalcone, as well as brazilin, which imply the highest compound in SWE, are able to bind iron because it has a catechol group. Catechol group is organic substances contained in plant extracts and has a role as an iron chelator [31].

The most organisms, including MTB, require iron as an essential element for their growth [32]. The iron dependency by MTB will lead to competition with SWE to bind the iron contained in LJ medium. The iron level in LJ medium that was inoculated with MTB at 10^{-3} inoculum dilution appeared to be lower than the 10^{-5} inoculum dilution. Hypothetically, the iron chelation of MTB is also higher MTB population at 10^{-3} dilution compared to 10^{-5} that influenced by atomic absorption.

The SWE 100 and 250 ppm was inhibited the expression of the iron level of MTB and higher removal iron in LJ medium. The concentration of SWE was influenced by iron chelate (low expression is high, mainly in 250 ppm) both 10^{-3} and 10^{-5} dilutions. These results assumed that the SWE phenolic compounds have capable to inhibit the growth of MTB. It has related that the SWE has good ability to chelate iron and thus the high levels of iron were bound by MTB. The SWE and MTB have the ability to chelating of iron that leads to competition in binding iron on LJ medium. This potential SWE to bind iron and to reduce the MTB growth can be used as a herbal remedy for overcoming TB in the future.

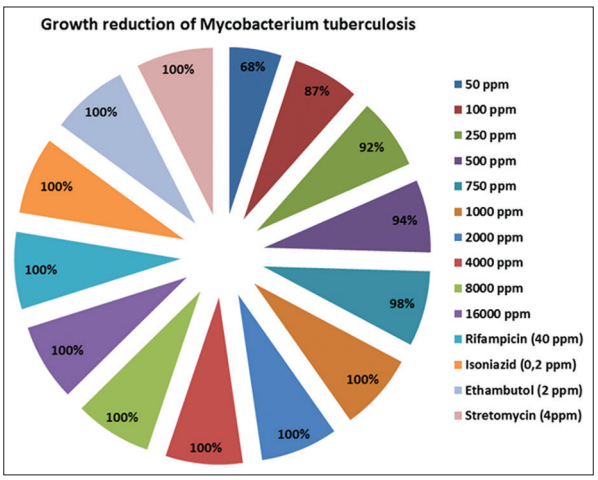

Fig. 1: Average of growth percentage of *Mycobacterium tuberculosis* after treated by sappan wood extract and antibiotics. The data conducted in 1–8 weeks, triple, and 5th dilution (10^{-5})

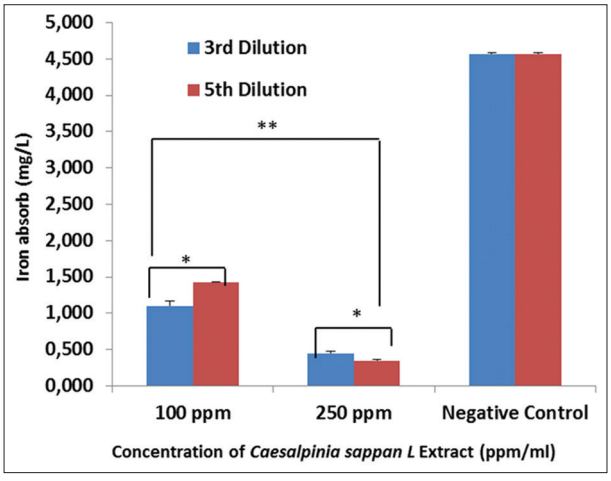

Fig. 2: Iron chelate in Lowenstein–Jensen medium after interacted *Mycobacterium tuberculosis* with sappan wood extract. Bar (average iron expression), Error bar (deviation standard); **=p<0.05 (concentration) and *=0.01 (dilution). The data were analyzed by t-test independent *p<0.01 (1.5000±0.52223); **p>0.05 (0.8557±0.444437)

CONCLUSION

The MIC of SWE was caused the MTB development and can reduce the colony growth of MTB 10^{-3} and 10^{-5} in 50 ppm (68%) and 100 ppm (87%) and MBC in concentration 250–1600 ppm. Furthermore, the SWE has the potential effect to induce the iron chelate in LJ medium and MTB.

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AUTHORS' CONTRIBUTION

RS carried out the conception, extraction, and purification of SWE and analyses of iron chelate also drafted the manuscript. II, MAAS, RP, and MG have been given the research references and design of research. BAG has arranged the manuscript, statistical analysis, correcting the manuscript, and corresponding author. All of the authors were read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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