Original Article

Isolation and Identification of Active Compounds from Ethanol Extract *Piper crocatum* Leaves as Antituberculosis Candidate

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ABSTRACT

Background

Treatment of tuberculosis is still a problem in Indonesia or in the world. There needs to be a new alternative drug for tuberculosis therapy.

Objective

The purpose of this study was to isolate and identify the active compounds from ethanol extract of red betel vine (*Piper crocatum*) leaves as an antituberculosis candidate.

Methods

The chloroform fraction of 70% ethanol extract was further purified, tested antimycobacterial activity against *M. tuberculosis* and identified its chemical structure using the method of Infra Red (FTIR) Spectrophotometer, UV-Vis Spectrophotometer and H/C-Nuclear Magnetic Resonance Spectrometer.

Results

Chloroform fraction identified 29 compounds.Compound 1 (S1) with Rf 0.96 has the best activity against *M. tuberculosis* with a minimum bactericidal concentration (MBC) of 5 µg/mL. The analysis of FTIR spectra and UV-Vis spectra, it is proposed that S1 compound is a triterpenoid compound which is hydroxylated and has more than one aliphatic alkene group.

Conclusion

The isolated compound with the best antimycobacterial activity against M. tuberculosis is suggested to be a hydroxylated triterpenoid containing multiple aliphatic alkene groups.

Keywords

isolation; identification; *Piper crocatum*; antituberculosis; triterpenoid

INTRODUCTION

Tuberculosis (TB) is a major global health problem. In some countries, it has become a resurgent infectious disease.[1] Indonesia is the second-largest country of TB patients in the world[2]. Treatment of tuberculosis still requires a long time (at least 6 months), the method of therapy that applies with multi-drug (more than 1 type of drug) and the side effects that occur are very unpleasant for patients.

The problem is further complicated by the emergence of bacterial resistance. To standard antituberculosis drugs called MDR (Multiple Drug Resistance). MDR cases are increasing, namely the presence of *M. tuberculosis* which is resistant to 2 or more first-line drugs, especially rifampicin and INH. MDR is a particular complication in the treatment of tuberculosis. Another problem arises with the existence of XDR (Extremely Drug Resistance). XDR is *Mycobacterium tuberculosis* MDR which is also resistant to one of the fluoroquinolone class drugs, and at least one of the aminoglycosides. XDR TB has been confirmed in Indonesia[3].

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XDR TB is highly dangerous, often leading to rapid mortality, is swift. The problem of TB disease is not comparable to the emergence of new drugs.

The latest discovery of antituberculosis drugs is rifampicin (1965). Since then, there was almost no significant antituberculosis discovery. On the other hand, Indonesia is a country rich in natural resources. One of them is red betel vine (*Piper crocatum*). Therefore, in this study, the potential extract of red bete vine was tested as antituberculosis agent. Chloroform fraction of red betel vine leaf ethanol extract has better antimicobacterium activity against *Mycobacterium tuberculosis* than ethyl acetate and methanol fraction[4].

1. Tuberculosis

Tuberculosis is still a problem today. A total of 1.5 million people died because of TB in 2018. Worldwide, TB is one of the top 10 causes of death. An estimated 10 million people fell ill with tuberculosis (TB) worldwide. There where cases in all countries and age groups, but TB is curable and preventable. In developing countries, this death constitutes 25% of the deaths of diseases which can be prevented. It is estimated that 95% of TB patients are in developing countries. With the emergence of the HIV/AIDS epidemic in the world, the number of TB sufferers is increasing[5]. The WHO report in 2015, noted that Indonesia ranked second in the world after India.

Tuberculosis is caused by *M. tuberculosis* which is an extremely successful pathogen that adapts to survive within the host[6]. The *optimum temperature* for *growth* is 35-*37*°C. Grows at *pH* 4.5–7.0 with *optimum growth* at *pH* 6.5–7.0[8]. *M. tuberculosis* can enter some *macrophages* and can *survive and multiply*[8,9].

2. Red betel vine (*Piper crocatum*)

Red betel vine (*P. crocatum*) *have* much *potential* to treat *various diseases*[10].

Characteristic of this tropical plant, the trunk is purplish-green round and does not flower. The leaves are heart-stemmed form and tapered top. Shiny and uneven leaf surface. Red betel vine plants also grow in the fence or tree. The leaves taste bitter shakes but smell more fragrant than green betel. When torn, red betel vine leaves will slim down. Red betel vine plants like shade, cool weather and morning sunshine. Red betel vine plants flourish and are good in mountainous areas. When growing in hot areas, direct sunlight, the stem dries quickly. In addition, the red colour of the leaves will fade whereas the possibility of its efficacy lies in the chemical compounds contained in the red colour of the leaves[13].

3. Compounds contained in red betel vine

Based on research conducted by Puspitasari, red betel vine chromatography contains alkaloids, flavonoids,

Figure 1. Red betel vine plant (*P. crocatum*). The green leaves with silvery colour. The colour below the red leaves.[11,12]

polyphenolic compounds, tannins and essential oils[10]. Red betel vine also contains saponins[14]. The identity compound of red betel vine ethanol extract is Trimethoxyallylbenzen[15].

a. Alkaloids

The benefits of alkaloids include analgesic, antibacterial, antitussive, malaria medicine and cancer drugs[16]. Alkaloids can act as antibacterials by changing the constituent components of peptidoglycan in bacterial cells so that the cell wall layer is not formed intact and causes cell death[17].

b. Flavonoids

Flavonoids have been identified as polyphenolic compounds capable of exerting *antibacterial* activities[18]. *Flavonoids can bind and form complex compounds with extracellular proteins*[19]. Chalcone and chalcone compounds which are included in flavonoids have been studied as antimycobacterial. It can inhibit the activity of *M. tuberculosis* H37Rv[20]. Several compounds can inhibit de novo fatty acid biosynthesis in mycobacteria[21].

c. Tanin

Tannins have been found to inhibit bacterial growth using different mechanisms of action including iron chelation, inhibition of cell wall synthesis, disruption of the cell membrane, and inhibition of fatty acid biosynthetic pathways[22].

d. Essential oil

Essential oils can act as antibacterials by interfering with the formation of membranes or cell walls[23]. Essential oils that are active as antibacterials generally contain hydroxyl (-OH) functional groups and carbonyl. Phenol derivatives interact with bacterial cells through an adsorption process involving hydrogen bonds. At low levels, phenol protein complexes are formed which are weakly bonded and immediately undergo decomposition, followed by penetration of phenol into the cell and cause precipitation and protein denaturation. At high levels, phenol causes protein coagulation, and membrane cells undergo lysis[24]. Essential oils in red betel vine contain 29 components. The five largest components contained in red betel vine are Sabinene, beta-Myrcene, L-Linalool, Beta-Caryophyllene and Germacrene[25].

e. Saponin

Saponins can have antimicrobial properties[26]. Saponin compounds will damage the cytoplasmic membrane and kill cells[27]. Research conducted by Utami *et al*.[28] proved that saponins can inhibit the growth of *M. tuberculosis*. How the mechanism cannot be explained.

METHODS AND MATERIALS

This research is explorative research to isolate and identify the active compounds of ethanol extract of red betel vine leaves that function as antimycobacterial against *M. tuberculosis.* This research was conducted at the Integrated Laboratory of the Universitas Islam Indonesia and the Tuberculosis Laboratory at Universitas Gadjah Mada.

1. Tools used

The main tools used in this study were units of Vacuum Liquid Chromatography (VLC), Column Chromatography, Thin Layer Chromatography GF 254 0.25 mm x 20 cm x 20 cm, UV-Vis Spectrophotometer, FTIR Spectrophotometer, Gas Chromatography-Mass Spectrometer (GC-MS), Liquid Chromatography-Mass Spectrometer, glassware, micropipette, yellow and blue tip, incubator, biosafety cabinet level 2 (BSC 2), bunsen, ose, test tubes, vortex, scales.

2. Materials used

The main ingredients needed in this study were extracts of red betel vine leaf chloroform fraction, Silica Gel 60 for column (60-70) mesh, organic solvents (methanol, ethyl acetate, dichloromethane, chloroform, n-hexane), shear reagent (methanol, sodium hydroxide, aluminium chloride (AlCl3), hydrochloric acid (HCl), sodium acetate (NaOAc), boric acid (H3BO3) standard *M. tuberculosis* (H37Rv) isolates, middlebrook 7H9 media, Lowenstein Jensen media, Isoniazid, Ringer Lactate (RL).

3. The Procedure of Research

a. Determination and Making of Betel Red Ethanol Leaf Extract

Red betel vine was identified according to the literature and was determined in the Faculty of Biology, Gadjah Mada University. The red betel vine which is used is red betel vine which grows in the Sleman regency and Yogyakarta kodya. Making ethanol extract was carried out by standard with maceration method using 70% ethanol. The extraction process was carried our in the integrated laboratory of the Universitas Islam Indonesia.

b. Fractionation and isolation of red betel vine leaf ethanol extract

The extract obtained was removed from the solvent using a rotary evaporator to obtain the crude. Crude is fractionated using Vacuum Liquid Chromatography (VLC) with various kinds of organic solvents based on polar (n-hexane, chloroform) gradients. A total of 3 grams of crude ethanol extract of red betel leaf was fractionated with Vacuum Liquid Chromatography (VLC) using a polarity (hexane-chloroform) gradient elution method. Each eluate was collected in Erlenmeyer and tested by thin-layer chromatography using the best eluent results of the determination by TLC. Furthermore, the chloroform fraction as the best activity was removed from the solvent to obtain crude chloroform extract. The components of the chloroform extract compound were separated by column chromatography using eluents that were previously known. Each fraction in the bottle was tested for purity by TLC. A single spot shows a single compound. The fraction with a single spot is then removed from the solvent and purified using the recrystallization method. Single compounds were tested in vitro on 7H9 media and cultured on Lowenstein Jensen media to find out the most active compounds. The compounds obtained then identified their chemical structure using spectroscopic methods (LC-MS/GC-MS, UV-Vis spectrophotometer with shear reagent, FTIR spectrophotometer.

c. Antituberculosis Activity Assay

The process of bacterial preparation is carried out in BSC 2 plus by default (using personal protective equipment). Isolates of *M. tuberculosis* aged between 3-4 weeks were taken 2-4 colonies, inserted into a tube containing a sterile glass beat to middlebrook 7H9 (liquid) 1 mL media. A bacterial suspension with a density of 10^8 was made. Then the bacterial suspension was made to a density of 10^4-10^5 and presented with fractionation/active compounds for 2 days and planted in Lowenstein Jensen media for 4-8 weeks. The results obtained are then analyzed.

RESULTS

In this study, the yield of ethanol extract was 12.03%. After the crude chloroform fraction was separated using preparative Thin Layer Chromatography (TLC) with silica gel GF 254. stationary phase, the ethyl acetate and n-hexane used were 2: 3. Separate compounds in TLC are then taken (scraped) together with the silica gel and placed in different vials. Chloroform is added to each vial to dissolve the compound and separate with silica gel and then evaporated to obtain pure compounds for each vial. From the results of chloroform fractionation obtained yield of 10.45% (4.1 grams of crude chloroform fraction of 39.2 grams of ethanol extract).

DISCUSSION

1. Separation of Chloroform Faction Composite Compounds

The compound separation was carried out by column chromatography using the silica gel 60 stationary phase for the column and mobile phase of a mixture of ethyl acetate: n-hexane (3:2). Eluat is collected in small bottles of 10 mL volume. Fraction in each bottle was evaluated using TLC GF 254 to determine the separation pattern based on the R_f value and the number of spots formed. The elaborate TLC profile is presented in Figure 2.

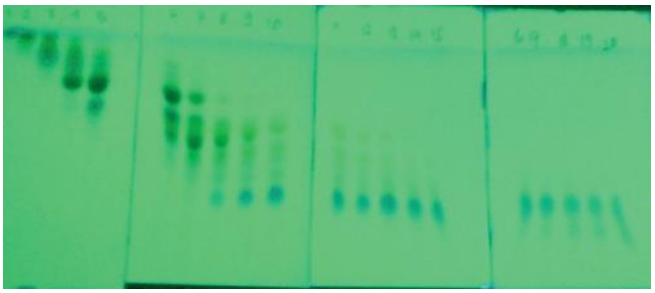

Figure 2. TLC profile of chloroform fraction of red betel vine leaf extract

Figure 2. Shows that pure (1 spot) compounds are isolated in bottle number 1 with Rf 0.96. Furthermore, the compound at Rf 0.96 was given the Compound 1 (S1). Bottles 15 and 16 show the same Rf value (0.32) and are pure so that they can be combined. Furthermore, compounds with Rf 0.32 were given the Compound 2 (S2). In the next stage, the two compounds were tested for antituberculosis/antimycobacterial activity against *M. tuberculosis.*

2. Antimycobacterial Activity Assay to *M. tuberculosis*

The two pure compounds were then tested for their antimycobacterial activity against *M. tuberculosis* using standard bacteria H37Rv. In compound 1 (S1) obtained 17.2 mg. The concentration used is 10; 5; 2.50; 1.25; 0.625; and 0.313 µg / mL. For control of standard antituberculosis drugs, Isoniazid is used. In the test, compound 2 (S2) is Rf 0.32, and all tubes 1-6 have

growth of M. tuberculosis. For compound 1 (Rf 0.96) tubes 1 and 2, there was no growth of *M. tuberculosis* while tube 3-6 contained growth/proliferation of bacteria (Figure 3).

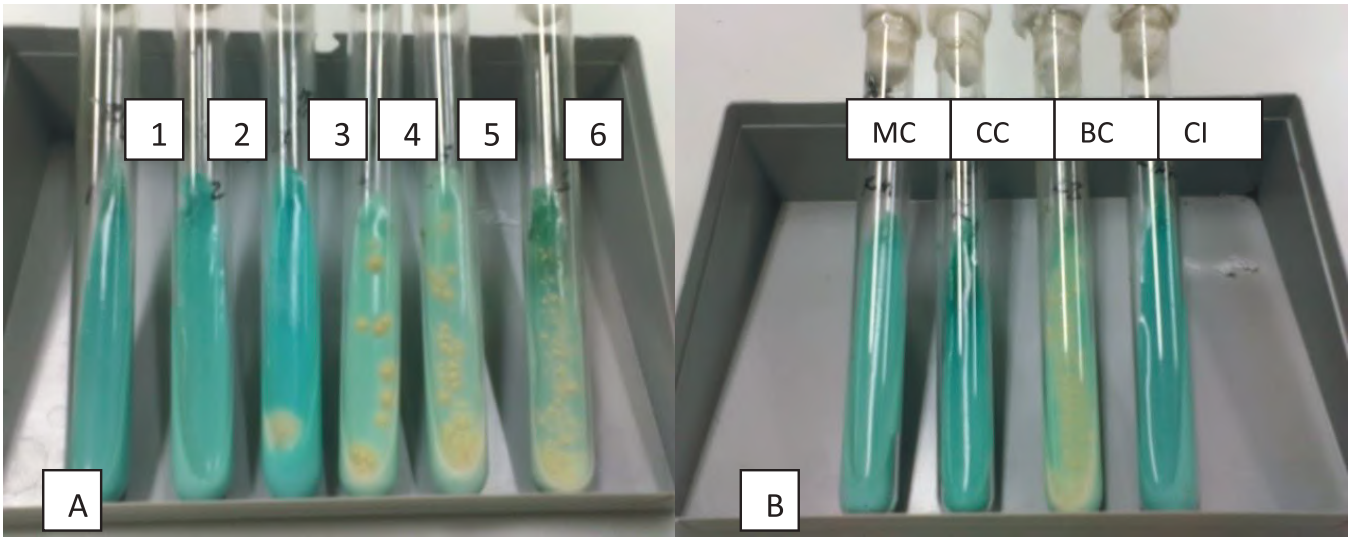

Figure 3. A. Test results for compound 1 (S1). Tube 1: concentration of 10 µg/mL, 2: 5 µg/mL, 3: 2.5, 4: 1.25, 5: 0.625 and 6: 0.313 µg/mL (tubes 1 and 2 have no growth). MC: media control, CC: compound control, BC: bacterial control and CI: control Isoniazid. Only control bacteria that have bacterial growth.

In the control tube, only a bacterial control tube contained growth/development of *M. tuberculosis* bacteria, while 3 other controls were negative. Thus, it can be said that this experiment is in accordance with what it should be. The minimum kill rate of compound 1 is 5 µg/mL.

3. Analysis of Active Compound Structure

In this study, structural analysis of active compounds was carried out using an FTIR spectrophotometer (Thermo Nicolet Avatar 360) to determine the functional groups contained in the active compound S1.

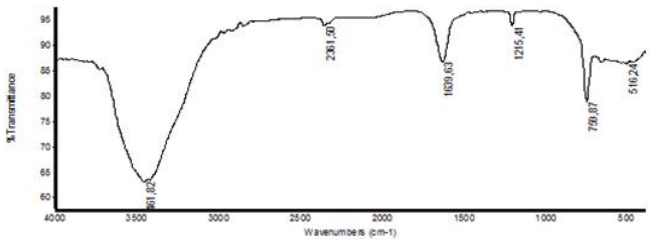

Figure 4. FTIR spectra of S1 compounds

The FTIR spectra shown in Figure 4 it can be analyzed that the absorption of 3461.82 cm-1 indicates the uptake of hydroxyl groups (-OH). Absorption at 1639.63 cm-1 indicates the presence of aliphatic alkene groups (not aromatic). The appearance of absorption at 758.87 cm-1 which indicates the C-Cl bond range may be caused by the presence of residual chloroform (CH3Cl) in the analyzed sample.

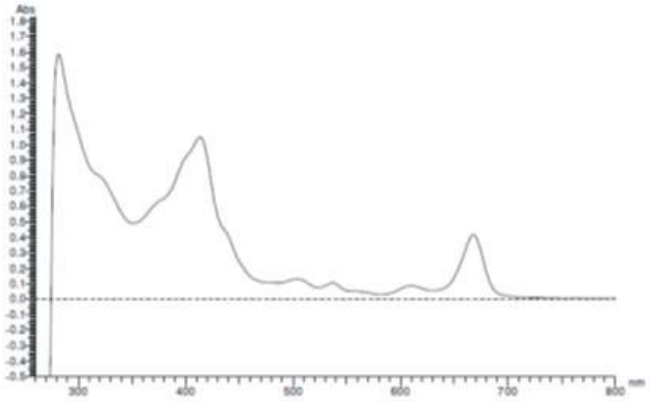

Figure 5. UV-Vis spectra of S1 compounds

Figure 5 reinforces the presumption of aliphatic alkene groups shown in the previous FTIR spectra. The number of peaks that appear on the UV-Vis spectra shows that the alkene group in the Compound 1 (S1) is more than one. From FTIR spectra analysis and UV-Vis spectra, it can be proposed that Compound 1 (S1) are triterpenoid compounds which are hydroxylated and contain more

than one aliphatic alkene group. This is accordance with the screening results on compound 1 (S1), TLC spots positively show as triterpenoid compounds. These compounds are in accordance with previous studies that isolate and identify active compounds of red betel which have cytotoxic activity. In this study, the active compound was identified as β-Sitosterol, which is a hydroxylated triterpenoid compound[29]. Based on previous studies, triterpenoids isolated from the stem bark and sap of *Staudtia kamerunensis Warb*. (*Myristicaceae*) are known to have good antibacterial activity.[30]

CONCLUSION

The active compound which was isolated from the chloroform fraction of ethanol extract of red betel vine leaf *(P. crocatum)* and had antimycobacterial activity against *M. tuberculosis* was a pure compound with Rf 0.96. The compound is thought to be a triterpenoid compound which is hydroxylated and has more than one aliphatic alkene group.

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Conflict of Interest

No conflict

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Authors' Contribution

1. Farida Juliantina Rachmawaty: overall responsibility, antibacterial activity test, and manuscript.
2. Tatang Shabur Julianto: analysis of the structure of the active compound.
3. Hady Anshory Tamhid: preparation and fractionation.

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