Antibacterial Activities Test of the Curcuminoid Compound in the Endophytic Bacteria of *Curcuma zanthorrhiza* Roxb.

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Article Info

Article history:

Received Jul 12th, 2017
Revised Aug 20th, 2017
Accepted Oct 26th, 2017

Keyword:

Curcuminoid
TLC
Contact bioautography
Antibacterial
Endophytic bacteria

ABSTRACT

This study aims to determine the active compound curcuminoid contained in secondary metabolites 2 endophytic bacteria of temulawak (*Curcuma zanthorrhiza* Roxb.) rhizome BT2 and PD2 potentially as antibacterial compounds. TLC method used for separation of curcuminoid compounds followed by contact bioautography test to know the ability of compound activity which has been separated through TLC in inhibiting the growth of bacteria. The results of curcuminoid compound separation by TLC method are curcumin can be detected in BT2 and PD2 endophytic bacteria in the 4th spot with the same values of Rf 0.43. The desmetoxicurcumin fraction can be detected in BT2 and PD2 in the 3rd spot with the values of Rf 0.39 and 0.38 respectively. While bisdesmetoksikurkumin fraction can be detected in BT2 and PD2 in the 1st and 2nd spot with the value of Rf 0.25 and 0.32 respectively. Testing of contact bioautography on TLC plate containing separation of curcuminoid compounds produced by BT2 and PD2 doesn't show any antibacterial activity.

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1. INTRODUCTION

Infectious diseases in humans by bacteria and fungi in developing countries including Indonesia are still a big problem. The World Health Organization (WHO) estimates infectious diseases such as diarrhea (18%), pneumonia (14%) and measles (5%) are among the causes of 161,000 deaths among children under five in Indonesia in 2005 [19]. The high demand for antimicrobial ingredients for the treatment of infectious diseases, the weakening of the rupiah has caused the price of antimicrobial medicines to be expensive, causes a decline in people's purchasing power. On the other hand, the emergence of pathogenic microorganisms that are resistant to existing antimicrobials has been a clinical and public health problem to date [8] [12]. Research on alternative antibiotic materials that are sourced from natural resources is important to do, so that gradually antibiotic needs in Indonesia can be fulfilled.

Indonesia has a high diversity of medical plants, one of them is from the genus Curcuma. This genus is widely used as an antimicrobial because the active compound content is able to prevent microbial growth. Based on the results of Adila *et al*., (2013) [1] studying the extract of 6 species of *Curcuma* spp. Rhizome, which has the greatest potential in inhibiting test microbes is *Curcuma zanthorrhiza* Roxb. (temulawak). The extract of the temulawak rhizome had the highest inhibition to the *Candida albicans* (13.07 mm), *Staphylococcus aureus* (15,75 mm) and *Escherichia coli* (31,56 mm).

Some microorganisms that are pathogenic to humans are *Escherichia coli* and *Staphylococcus aureus. Escherichia coli* can cause diarrhea and contamination of *Staphylococcus aureus* enterotoxins as a cause of food poisoning. Both are commonly found in society and are commonly used antibacterial test microorganisms [1].

The rhizome of temulawak contains starch, fiber, curcuminoid (curcumin and desmetoxycurcumin) and essential oils (phelandren, camphor, tumerol, sineol and xanthorrhizol) [2] [16]. Benefits of curcuminoid include

acne medication, increased appetite, antioxidants, cancer prevention and antimicrobials. [11] The spectrum of antibacterial activity from curcuminoid is wide which is active against various types of gram-positive and gram-negative bacteria, antiviral and induced cell apoptosis (anti-tumor) [3].

The rhizome of temulawak besides containing curcuminoid compounds also have endophytic microbes that live in a mutualism symbiosis with host plants. Prior research by Imawati (2015) [5] has successfully isolated endophytic bacteria from the rhizome of temulawak. Endophytic microbes live in plant tissue to form colonies without endangering the host [4]. Endophytic microbes in the fermentation medium will produce similar compounds with the plant host [15].

The curcuminoid compound of temulawak rhizome has been known to have antibacterial activity so it is necessary to research the potential of curcuminoid as antibacterial that produced by endophytic bacteria from temulawak rhizome with TLC method and bioautography. The separation of chemical compounds by TLC only takes a short time, is easy to do, requires absorbents and samples with small amounts and produces better separation [13]. TLC results still need to be combined with bioautography methods, in order to provide information on the antibacterial active components of microbial metabolites from endophytic microbes of temulawak rhizome [18]. The type of bioautography used in this research is a contact bioautography because it is easy to do and the results can be seen clearly without using a dye solution such as MTT (Methyl Thiazole Tetrazolium).

2. RESEARCH METHOD

Fermentation of Endophytic Bacteria and Extraction of Fermented Supernatant

A piece of the colony of endophytic bacteria that had been incubated in an NA (*Nutrient agar)* medium for 24-48 hours under the temperature of 37 ^{0}C was taken and moved into 5 ml MHB (*Muller-Hinton Broth*) medium. One ml of the suspension of the endophytic bacteria colony was moved into 9 ml MHB medium in an 12 ml ependof tube, incubated under temperature of 30^{0}C using an inkubator shaker with 130 rpm for 16 hours (isolate BT2) nd 18 hours (isolate PD2). Then it was centrifubalized with the speed of 5000 rpm at 4˚C for 20 minutes [20]. The obtained supernatan was extracted twice with the solvent of ethyl acetate (1:1 v/v) placed in a tube for 20 minutes. The obtained extract was then evaporated by adding N_2 for the next tests [6].

Separation of Curcuminoid Compounds with Thin Layer Chromatography (TLC)

The separation of the curcuminoid compound from extract of the ethyl acetate was performed by Thin Layer Chromatography (TLC) method on 60F_{254} silica gel plate as stationary phase [9]. TLC plates are cut in length of 10 cm and 1 cm wide.The bottom of the plate is marked with a distance of 1 cm to the lower limit and 1 cm for the upper limit using a pencil. The samples were applied on the plates with a total 15 dots in each spots using a capillary pipe. After the samples have been applied on the plates, each plate was then intered into the TLC containing eluent/ mobile phase (chloroform: benzene: 98% ethanol (45:45:10)) for some minutes until it reaches the upper level of (1 cm from the lower and upper ends), then it was dried and observed under the UV 254 nm and 365 nm. The patterns of the spots were observed and drawn, and then the Rf value was calculated [14]. All data obtained in the form of Rf values were analyzed descriptively.

Bioautography

The test was made using a contact method by placing the plates from the TLC results on the surface of the NA medium that had been mixed with the 5 µl bacterial species tested suspension (*Escherichia coli* and *Staphylococcus aureus*) and was kept for 60 minutes. Then the plates incubated in temperature of 37 ˚C for 24 hours. The antibacterial activities were indicated by the formation of the inhibiting zone around spotsfrom the TLC results [21]. The results are arranged in the form of images then were analyzed descriptively.

3. RESULTS AND ANALYSIS

Separation of Curcuminoid Compounds with Thin Layer Chromatography (TLC)

The result of compound separation with TLC can be seen in table 1 and image 1. BT2 endophytic isolate bacteria extract in ethyl acetate obtained 7 spots on 254 nm UV visualization and 6 spots on 365 nm visualization, whereas in PD2 endophytic bacteria obtained 9 spots on 254 nm UV visualization and 7 spotted on 365 nm visualization with the values of Rf presented in Table 1. The Rf values are the average of twice the TLC measurement.

Table 1. TLC result of endophytic bacterial secondary metabolite ethyl acetate extract with chloroform: benzene: 98% ethanol (45:45:10) eluent

Bacteria Isolates	Rf (UV_{254})	Rf (UV_{365})	Color (UV_{365})	Rf Standard of Curcuminoid (Stahl, 1985)
BT2	0,025	0,25	Blue	0,40-0,45 (Curcumin)
	0,06	0,32	Purple	
	0,14	0,39	Blue	
	0,23	0,43	Greenish orange	
	0,30	0,51	Pale gray	
	0,40	0,57	Pale gray	0,35-0,40 (Desmetoxycurcumin)
	0,45			
PD2	0,037	0,25	Greenish blue	
	0,075	0,32	Purple	
	0,16	0,38	Blue	
	0,24	0,43	Greenish orange	0,25-0,35 (Bisdesmetoxycurcumin)
	0,31	0,46	Blue	
	0,36	0,51	Pale gray	
	0,40			
	0,45	0,56	Pale gray	
	0,51			

The identification of the compound on the spots of the separation product by TLC can be compared with the standard Rf values, as in literature [17] which separates the compound of temulawak by using TLC silica gel G in the same eluent solution ie chloroform: benzene: 98% ethanol (45 : 45: 10) and spots were visualized under 365 nm UV light, with the results listed in Table 1. Based on the data in Table 1, the curcumin fraction from BT2 and PD2 endophytic bacteria can be detected on the 4th spot with the same Rf value 0.43. Desmetoxycurcumin from BT2 and PD2 endophytic bacteria were detected in the 3rd spot with Rf values 0.39 and 0.38, respectively, while bisdesmetoxycurcumin from BT2 endophytic bacteria visualized in the 1st spot and from PD2 in the 2nd spot with the values of Rf 0.25 and 0.32 respectively. In the results of studies indicating the presence of bisdesmetoxycurcumin is possible because of the curcuminoid biotransformation. Biotransformation is the conversion of a compound into its derivative compounds whose structure is different from its original compound due to the metabolic activity of a microorganism [10].

Fluorescence is evident in visualization under 365 nm UV lamp. The results of visualization can be seen in image 1. The resulting fluorescence appears purple, blue, greenish orange until gray. This is reinforced by the literature [17] which states that in the analysis of the temulawak compounds separation using TLC with the same eluent in this study, ie chloroform: benzene: 98% ethanol (45:45:10) under 365 nm UV light, obtained fluorescent yellow-pale gray. Pale gray color is suspected because the levels of compounds in the extract (sample) are too minimal, so that in the separation process with TLC also produces thin patches.

Bioautography

Contact bioautography was performed to determine the antibacterial capability of curcuminoid compounds present in the TLC plates. Antibacterial compounds are detected by the presence of clear zone that are not overgrown with bacteria [7]. The results of bioautography test can be seen in image 2. The results in image 2 show that there were no clear zone formed as inhibition zone of bacterial growth of *Escherichia coli* and *Streptococcus aureus*. These results indicate that antibacterial compounds that have been separated in the form of spotted in TLC plates are not able to inhibit the growth of bacterial spesies tested. This is because the transfer of bioactive from endophytic bacteria is less than optimal, it is suspected that the chromatogram plate does not stick well to the surface of the agar medium. It is also suspected that the bioactive compounds in the TLC plates are too slight and produce very thin zones that are difficult to recognized. Some compounds may bind to the chromatogram plate matrix, especially the silica-based matrix so that it can't diffuse in the agar medium [7].

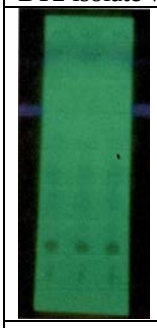
BT2 isolate with eluent chloroform:benzene:98% ethanol (45:45:10)	
	
254 nm UV visualization	365 nm UV visualization
PD2 isolate with eluent chloroform:benzene:98% ethanol (45:45:10)	
	
254 nm UV visualization	365 nm UV visualization

Image 1. TLC Test Results of Endophytic Bacteria Isolates

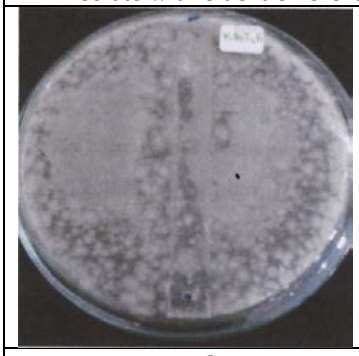
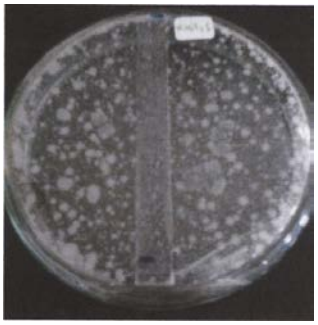
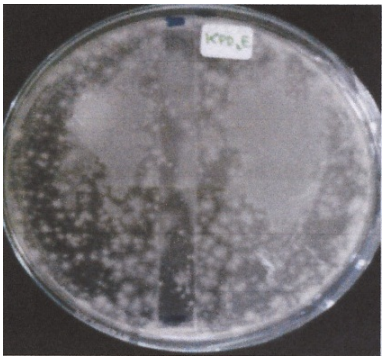
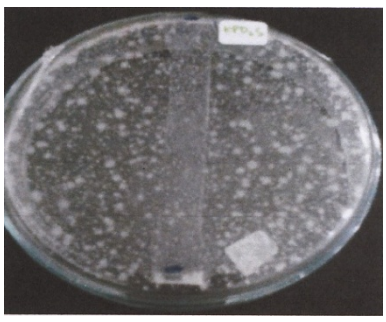
BT2 isolate with eluent chloroform:benzene:98% ethanol (45:45:10)	
	
254 nm UV visualization	365 nm UV visualization
PD2 isolate with eluent chloroform:benzene:98% ethanol (45:45:10)	
	
254 nm UV visualization	365 nm UV visualization

Image 2. Contact Bioautography Test of Endophytic Bacteria Isolates

4. CONCLUSION

The result of separation of curcuminoid compound through TLC method, curcumin fraction can be detected in BT2 and PD2 endophytic bacteria in the 4th spot with the same Rf values 0.43. The desmetoxicurcumin fraction can be detected in BT2 and PD2 isolates in the 3rd spot with the values of Rf 0.39 and 0.38 respectively. While bisdesmetoxycurcumin fraction from BT2 and PD2 can be visualized in the 1st and 2nd spot with the values of Rf 0.25 and 0.32 respectively. Furthermore, contact bioautography test on TLC plates containing separation of curcuminoid compounds produced by BT2 and PD2 endophytic bacteria did not show any antibacterial activity.

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