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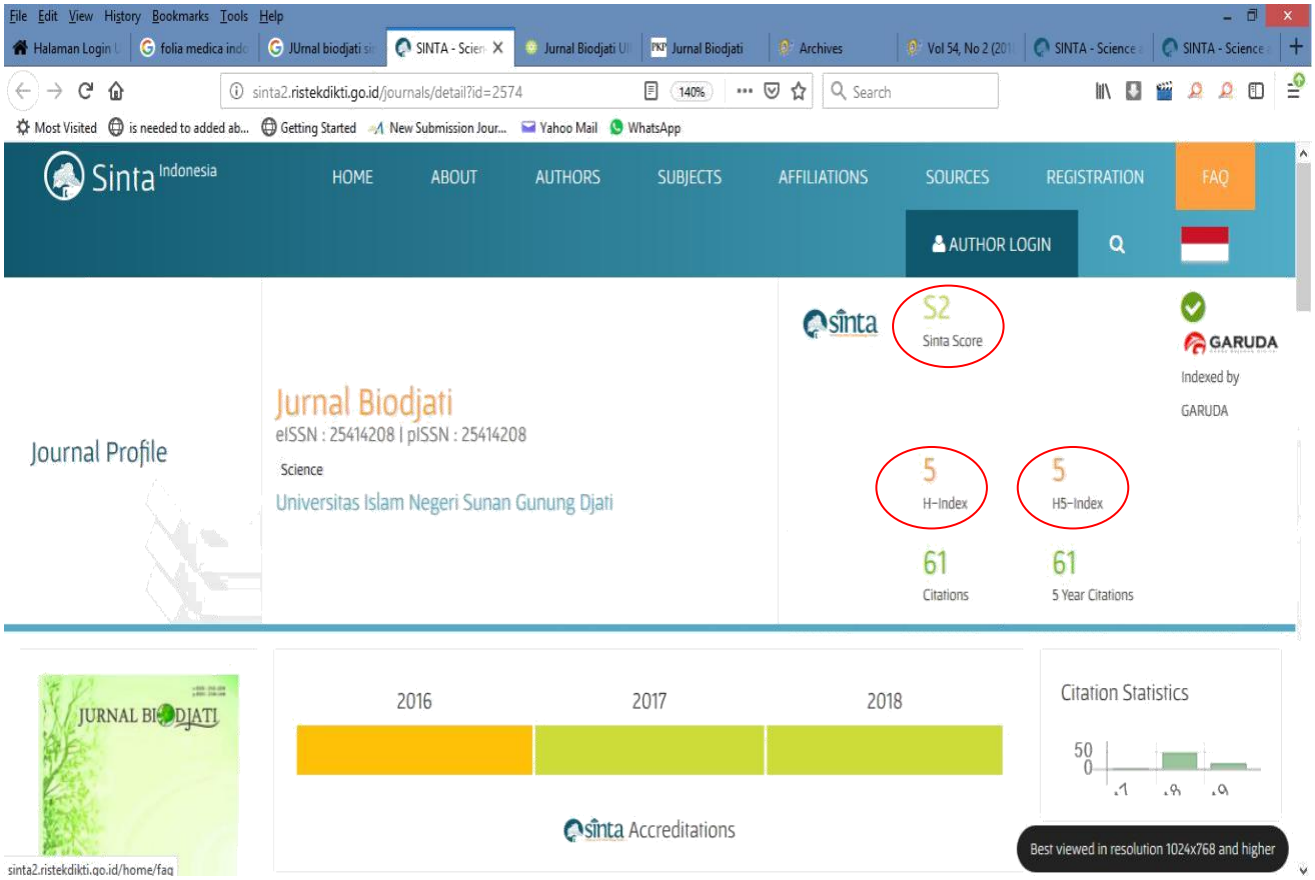
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Table of Contents
Articles

Indexing By :

PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF *Acorus calamus* L. EXTRACTS

Bayyinatul Muchtaromah*[1], Alfiah Hayati[2], Erna Agustina[3]

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[1,3]Department of Biology, Faculty of Science and Technology, Universitas Islam Negeri (UIN) Maulana Malik Ibrahim Malang, Indonesia. Jl. Gajaya-na 50, Malang, 65144, Jawa Timur, Indonesia. Phone: +62341558933, +6281231842316, Fax: +62341558933

[2]Department of Biology, Faculty of Science and Technology, Universi-tas Airlangga, Kampus C, Mulyorejo, Surabaya, 60115, Phone 031-5936501, Fax : 031-5936502.

e-mail:
* [1]bayyinatul@bio.uin-malang.ac.id
[2]alfiah-h@fst.unair.ac.id
[3]ernaagustina78@yahoo.com

Corresponding author

Abstract. *Staphylococcus aureus and Escherichia coli are among the most common species of gram-positive and gram-negative bacteria, which cause vaginitis, in infertile women. The Calamus rhizome (Acorus calamus L.) is an Indonesian plant that has antibacterial properties that can be used to treat vaginitis and increase fertility. The aim of this study was to determine the phytochemical and antibacterial activity of the calamus rhizoma in polar, semi-polar and non-polar solvents in the growth of S. aureus and E. coli. The antibacterial activity test was in the form of inhibitory test using the Kirby-Bauer, Minimum Inhibi-tion Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by microdilution method with multilevel dilution (concentra-tions 50; 25; 12.5; 6.25; 3.13; 1.56; 0.78; and 0.39%). The screening results showed that ethanol and n-hexane extract contained alkaloids and triterpenoids, while chloroform extract was only triterpenoid. Chloroform extract produced the largest inhibition zone diameter of S. aureus and E. coli (7.26 and 3.28 mm), followed by ethanol extract (5.90 and 3.07 mm) and n-hexane extract (5.33 and 2.95 mm). The concentrations of 0.39 and 0.78% were the values of MIC and MBC for all three extracts, indicating that the extract of the calamus rhizome with several solvents in this study had the same antibacterial activity.*

Keywords: *Acorus calamus, antibacterial activity, phytochemical screening*

Citation
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INTRODUCTION

Staphylococcus aureus is one of the normal flora in the female reproductive tract. *Escherichia coli* can be a pathogen when it reaches tissues outside the digestive tract. Both contribute to reproductive tract infections. Under certain conditions, the normal flora can cause disease or infection, when the substrate changes or bacteria move into suitable habitat (Conway & Cohen, 2015). Pino et al. (2019) and Bhandari & Prabha (2015) report that predominant vaginal normal flora is lactobacilli (95%), besides that there is also a small amount (5%) of wide variations of *S. aureus* and *E. coli*. If the Lactobacillus population decrease, the population of other bacteria such as *S. aureus* and *E. coli* will increase and can become pathogens in the reproductive tract.

One infection caused by *S. aureus* and *E. coli* is vaginitis. Vaginitis is the contamination of the female reproductive tract. The number of bacteria in the normal vaginal ecosystem is 10^5 to 10^6/gram vaginal secretions,

but in reproductive tract infections increas-es 10^9-10^{10}/gram which can cause infertility (Orji, 2015). Orji (2015) reported that *S. au-reus* had a prevalence rate (18.6%), whereas Isibor et al. (2011) revealed that *E. coli* had a prevalence rate of 13.1% in cases of vaginitis.

Acorus calamus L. is one type of medicinal plant with many benefits. One of commercial herbal products that have properties to improve fertility and overcome infertility problems is "Subur Kandungan" herbs. The main ingredient of this product is calamus rhizome, containing approximately around 12% and other components up to 100%.

Anisah et al. (2014) reported ethanol and water extracts of calamus rhizoma con-taining alkaloids, flavonoids and polyphe-nols. Methanolic extract of *Acorus calamus* showed the presence of glycosides, carbohy-drates, phenolic compounds, saponins, alka-loids, flavonoids, tannins, saponins, steroids and triterpenoids (Mamta & Jyoti, 2012). The antimicrobial activity of *A. calamus* is relat-ed to A- and B-asarones contained in rhizome and leaf extracts (Devi & Ganjewala, 2009).

Previous research, ethanol extract of calamus rhizoma with a concentration of 100% inhibited the growth of *S. aureus* (2.75 cm/very strong) and *E. coli* (2.98 cm/very strong), while water extract inhibited *S. au-reus* (1.53 cm/medium) and *E. coli* (1.03 cm/ medium) (Anisah et al., 2014). Similar stud-ies but carried out on other plants also pro-duce varying antibacterial activity. Rahman et al. (2012) showed that chloroform extract of *Phyllanthus niruri* produced antibacterial activity against *S. aureus* and *E. coli* higher than ethyl acetate extract which is 26 mm and 6.6 mm respectively. At last, Ningsih et al. (2016) stated that chloroform solvents in soursop leaves had a higher ability to inhibit *E. coli* than n-hexane (8.34 and 3.45 mm).

According to the background above, it was essential to look for phytochemical content and the best organic solvent of calamus rhizoma extract in inhibiting the growth of *S. aureus* and *E. coli* considering that these two bacteria were normal flora in the reproductive and digestive tract with a high prevalence in cases of vaginitis. The results of this study can be trigger for the calamus rhizoma certification process, as one of component of Madurese herbal medicine which can overcome infertility. Therefore, traditional medicine product could be accepted in the modern medical system for enhancing public health

MATERIALS AND METHODS

The type of research was experimental research. Extraction of the active component of calamus rhizoma using maceration method with three different kinds of solvents which were different in polarity, including ethanol, chloroform and n-hexane. Antibacterial activity test of *S. aureus* and *E. coli* using the Kirby Bauer method, to determine the diameter of bacterial inhibition and liquid microdilution to determine Minimum Inhibition Concentra-tion (MIC) and Minimum Bactericidal Concentration (MBC).

Materials came from calamus rhizoma (*A. calamus*) which obtained from the Balai Materia Medika, Indonesia. All chemicals from Merck Ltd and the culture of bacteria from the Microbiology Laboratory, Faculty of Medicine, Universitas Brawijaya, Indonesia.

Making extracts using 100 g of calamus rhizoma powder mixed with ethanol (polar), chloroform (semi-polar) and n-hexane (non-polar) solvents of about 400 ml (1:4) respectively. The object was soaked and stirred until smooth, after that shaked for 24 hours, 120 rpm. The filtrate was obtained by filtra-tion, and then the pulp was macerated again with the same solvent and concentrated using

a rotary evaporator at a temperature of 50°C (Muchtaromah et al., 2011).

Phytochemical screening (alkaloids, flavonoids, terpenoids, saponins, tannins, steroid) based on the method of Tiwari et al. (2011) and Ibironke et al. (2010). The bacterial inhibitory zone test used the Kirby Bauer method with 100% calamus rhizome extract. MIC and MBC tests used a series of dilutions of 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39%. The pure culture of bacteria (stock) was inoculated in 5 ml Agar Nutrient media then incubated for 24 h in an incubator at 37°C. The formed colonies showed bacterial growth. Bacteria that have been recultured were taken one streak and added 0.9% sterile NaCl as much as 5 mL (Vineetha et al., 2015). The bacteria solution was compared with the McFarland 0.5 solution. If the turbidity of the bacterial suspension test was the same as turbidity in McFarland 0.5 solution, the concentration of bacteri-al suspension is 10_8 CFU/mL (Fatisa, 2013).

Kirby Bauer method used paper discs (6 mm diameter). 200 µL of bacterial suspension was put into sterile Petri dishes, then 20 mL of Mueller Hinton Agar (MHA) media was inserted, and allowed to solidify. The media was divided into three areas for laying paper discs and did the replication three times. A sterile disc was soaked with 100% extracts, for one hour. A positive control using clindamycin and negative control using DMSO. The disc paper was placed on the surface of bacterial media and incubated at 37°C for 24 h. After 24 h, the presence of clear zones around the paper disk was observed and measured using a caliper. The liquid microdilution method used 30 microplate wells for each type of bac-teria (three wells for material control, three wells for microbial control and 24 wells for test treatment) (Vineetha et al., 2015).

MIC was a minimal concentration of calamus rhizoma extract which could inhibit the growth of test bacteria, while MBC was a minimum concentration capable of killing bacteria, which was indicated by the absence of colonies or the number of colonies <0.1% of the Original Inoculum (OI) (Winarsih et al., 2011).

Bacterial suspension of 10_6 CFU/mL was incubated in Nutrient media for 24 h, then the turbidity of each level was observed. MIC values were determined visually from each concentration which had clarity compared to bacterial control. Then, confirmed the amount of MBC by planting each concentration in solid media to find out the number of colonies (Vineetha et al., 2015). Counting of bacteria number used the stereomicroscope with APD colony counter. The data obtained in the form of inhibitory zone diameter, MIC value, MBC value and total bacterial colonies were analyzed by descriptive qualitatively.

RESULTS AND DISCUSSION

The Yield of Calamus Rhizoma Extract The extraction results of several organic solvents produced different yield, colors, and textures (Table 1).

Ethanol produced the highest yield (7.8 g) followed by chloroform (3.3 g) and n-hexane (2.4 g). The reason due to ethanol was a polar solvent, and perhaps the calamus rhizoma contained more polar compounds than non-polar compounds. Polarity level of different solvents produced varied colors and textures. Vargas et al. (2016) stated that the yield data for each solution showed a variety of colors, textures and quantities, because of the differences in the active compounds that were successfully extracted, even though the extracted samples came from the same material.

Jurnal Biodjati 4(1):68-78, May 2019

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Table 1. Yield, color, texture and weight of calamus rhizoma extract

No	Solvent	Color	Texture	Extract Weight (g)	Yield (%) (w/w)
1.	Ethanol p.a	Greenish brown	Porridge	7.8	7.8
2.	Chloroform p.a	Reddish brown	Porridge	3.3	3.3
3.	N--Hexane p.a	Blackish brown	Concentrated liquid	2.4	2.4

Qualitative Phytochemical Screening

Phytochemical testing of the extract was the first step that gave an overview of classes of secondary metabolite compounds contained in each extract (Table 2).

The phytochemical test showed that ethanol and n-hexane extract contained alkaloid and triterpenoid, the extract n-hexane contained relatively high triterpenoid and the chloroform extract contained triterpenoids only. According to Imam et al. (2013), photochemical studies have reported that etha-nol extract of calamus rizhome comprised of glycosides, flavonoids, saponins, tannins, polyphenolic compounds, mucilage, volatile oil and bitter principle. This plant has been reported for the presence of glucoside, alkaloid and essential oil containing calamen, clamenol, calameon, asarone and sesquiterpenes. It also contained a bitter glycoside named acorine along with eugenol, pinene and camphene in various solvents (Chandra & Prasad, 2017).

Ethanol was a polar solvent that attract most of the active polar compounds and cannot draw semi-polar and non-polar active compounds. Anisah et al. (2014) reported that the ethanol extract of calamus rhizoma gave positive results for alkaloid compounds, fla-vonoids and polyphenols. Chloroform was a semi-polar solvent that can extract phenol, terpenoid, alkaloid, aglycone and glycoside compounds (Akhtar et al., 2015). The n-hex-ane solvent was non-polar which attracted to most non-polar compounds. Hartati (2012) reported that the calamus rhizome n-hexane extract contained essential oils with beta-asa-rone main compounds.

Table 2. Phytochemical screening of calamus rhizome extract

Phytochemical	Reagen test	Extracts		
		Ethanol	Chloroform	n-hexane
Alkaloid	Dragendorff	+	-	+
	Mayer	+	-	+
Flavonoid	Wilstater	-	-	-
Triterpenoid	Lieberman-Burchard	+	+	+++
Steroid	Lieberman-Burchard	-	-	-
Saponin	Forth	-	-	-
Tannin	$FeCl_3$	-	-	-

(+++) very highly present, (++) highly present, (+) present, (-) not present

Antibacterial Activity of Calamus Rhizoma Extract in Several Solvents

Inhibitory Zone Diameter of Calamus Rhizoma Extract

The results showed that calamus rhizoma extract had inhibitory zone activity against *S. aureus* and *E. coli*. The presence of a clear zone around the disc paper showed inhibitory activity (Figures 1 & 2).

This research showed that the type of solvent extracted from the calamus rhizoma had an influence on the average diameter of the inhibition zone both for *S. aureus* and *E. coli.* The highest results of *S. aureus* inhibition zone diameter were chloroform extract (7.26 ± 1.45 mm/strong), followed by ethanol extract (5.90 ± 1.11 mm/medium) and n-hexane extract (5.33 ± 0.44 mm/medium). The results of the inhibition zone diameter on *E. coli* from the highest were chloroform extract (3.28 ± 0.15 mm/medium), ethanol extract (3.07 ± 0.37 mm/medium) and n-hexane extract (2.95 ± 0.22 mm/weak) (Table 3).

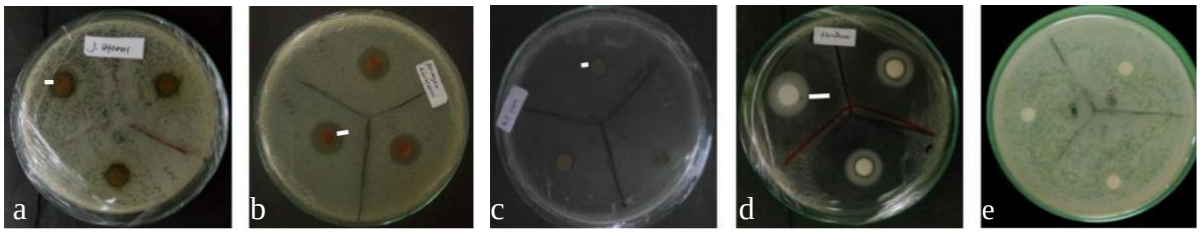

Figure 1. Results of inhibitory zone diameter for *Staphylococcus aureus*, (a) ethanol extract; (b) chloroform extract; (c) n-hexane extract; (d) clindamycin; and (e) DMSO

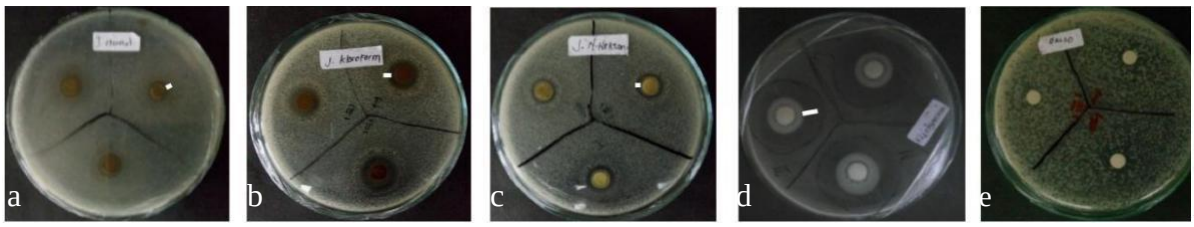

Figure 2. Results of inhibitory zone diameter on *Escherichia coli* (a) ethanol extract; (b) chloroform extract; (c) n-hexane extract; (d) clindamycin; and (e) DMSO

Table 3. The diameter of the inhibition zone of calamus rhizoma extracts against *S. aureus* and *E. coli*

Type of Extract	Bacterial Species			
	Staphylococcus aureus		*Escherichia coli*	
	Diameter (mm) ± SD	Category (Pan et al., 2009)	Diameter (mm) ± SD	Category (Pan et al., 2009)
Ethanol (T1)	5.90 ± 1.11	Moderate	3.07 ± 0.37	Moderate
Chloroform (T2)	7.26 ± 1.45	Strong	3.28 ± 0.15	Moderate
n-Heksana (T3)	5.33 ± 0.44	Moderate	2.95 ± 0.22	Weak
Clindamycin (C+)	37.08 ± 0.50	Strong	30.29 ± 1.78	Strong
DMSO (C-)	0	-	0	-

These results indicated that the diameter of *S. aureus* inhibition zone was higher than *E. coli*. Clindamycin as a positive control resulted in a greater inhibition zone compared to calamus rhizome extract, namely *S. aureus* (37.08 ± 0.50 mm/strong) and *E. coli* (30.29 ± 1.78 mm/strong). DMSO as a negative control did not indicate the clear zone around the disc paper, proving that DMSO 100% solvent had no antibacterial activity.

Unlike the previous studies, the inhibition zone test for *Candida albicans* produced the highest value on ethanol extract (3.72 mm/moderate) followed by n-hexane (3.32 mm/medium) and chloroform (2.22 mm/weak) (Muchtaromah et al., 2017). According to Chandra & Prasad (2017), differences in solvents could affect the results of phytochemical compounds drawn during extraction while the number and type of active compounds influenced the biological activity.

In contrast to the study of Shreelaxmi et al. (2018), reported that ethyl acetate as the best solvent for the extraction of active ingredients (A- and B-asarone) from the rhizomes and leaves of *A. calamus* compared to other waters. Solutions such as methanol, ethanol and hexane used in most previous studies were appropriate for the extraction of active ingredients. A- and B-asarones found in leaf, root and rhizome tissues were responsible for antimicrobial activity of *A. calamus*.

Here are some studies that use several kind of solvents and their antibacterial activity. Singh et al. (2011) study revealed that methanol extract from *A. calamus* showed inhibitory action against strains of *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiel-la pneumoniae* and *Staphylococcus aureus*. The third fraction of crude methanol extract showed the highest inhibition of *S. aureus*, *E. coli* and the fraction was confirmed as B-asarone. B-asarone compounds from *A. calamus* have the highest inhibitory effect on *E. coli* strains at various concentrations. Ethanol and water extracts from the *A. calamus* also showed inhibitory effects on the above organ-isms (Manikandan et al., 2010).

According to Balakumbahan et al. (2010), the crude methanol (polar) extract of the *A. calamus* showed a low inhibitory activity against *S. aureus* and *E. coli*. Devi & Ganjewala (2009) reported that the ethyl acetate (semi-polar) extract of calamus rhizome did not affect *S. aureus* while in this research ethanol and methanol extracts had anti-bacterial effect on this bacterium. It was suggested that this difference originated from the location where the plant had grown and resulted in gaps in the profile of secondary metabolites that have antibacterial properties (Singh et al., 2011). In addition, geographical, year and season differences also affected the results of extraction, active compounds and radical scavenging activities (Bonilla et al., 2013).

Calamus rhizome recorded a zone of in-hibition of the 12.5 mm Mucur species, while the calamus rhizome oil recorded an inhibito-ry region of 11.5 and 10.3 mm for *Aspergillus* and *Penicillium* species. Srividya et al. (2014) reported similar findings.

The triterpenoid group was an antibacterial compound, which worked by reacting with transmembrane proteins (porin) on the outer membrane of the bacterial cell wall and forming strong polymeric bonds that caused damage to the porin Tiwari et al. (2011). Alkaloids worked as antibacterial by disrupting the constituent components of peptidoglycan in bacterial cells, therefore the bacterial cell wall layer was not formed intact and caused bacterial cell death (Cushnie et al., 2014).

The difference in active compounds drawn in ethanol, chloroform and n-hexane extracts will affect the ability of the inhibitory zone on *S. aureus* and *E. coli*. The diameter of

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the *S. aureus* inhibition zone was much higher than *E. coli*. The results of this research indicated that calamus extract could inhibit *S. aureus* better than *E. coli* (Table 3). The difference in cell wall structure in both types of bacteria caused differences in inhibitory zone activity of calamus rhizome extract (Jawetz et al., 2005), such as peptidoglycan, the number of lipids, crosslinking,enzyme activity, determine penetration, binding and antibacterial activity.

According to Jawetz et al. (2005), the structure of cell walls of gram-positive bacteria was simpler than gram-negative. The construction of single-layered gram-positive cell walls with low lipid levels (1-4%) made easier for bioactive materials to enter the cell, while the cell wall structure of gram-negative bacteria was more complicated, which con-sisted of three layers. The outer and middle layer included of lipoprotein and lipopolysac-charide which acted as a barrier to the entry of antibacterial bioactive material, while the inner layer consisted of peptidoglycan with high lipid content (11-12%). The difference in the structure and components of the cell wall caused *E. coli* to be more resistant than *S.au-reus*.

Value of Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Calamus Rhizoma Extracts

In this study, it was necessary to know the minimum concentration of calamus rhizoma extract that could inhibit both test bacteria. MIC was the lowest concentration that inhib-its bacterial growth, marked by no turbidity after incubation for 18-24 hours and observed visually (Table 4).

The administration of calamus rhizoma extract started at 0.39% concentration and 0.78% produced clear color (no turbidity) which meant that at this concentration *S. au-reus* and *E. coli* bacteria did not grow (Table 4).

Direct observation of the level of turbidity visually (liquid dilution test) for determining MIC is confirmed using the drop plate method (Table 5).

Original inoculum (microbial control) containing the highest number of bacteria compared to the others, namely *S. aureus* (1.59 x 10_{14}/ mL), whereas in *E. coli* (1.67 x 10_{18}/mL). As the increasing of treatment concentration using calamus rhizoma extract was given, the number of bacteria decreased (Table 5).

Table 4. The diameter of the inhibition zone of calamus rhizoma extracts against *S. aureus* and *E. coli*

Treatment (Extract Concentration, %)	Turbidity					
	Staphylococcus aureus			*Escherichia coli*		
	Ethanol	Cloroform	n-Heksana	Ethanol	Cloroform	n-Heksana
Extract Control (100)	+++	+++	+++	+++	+++	+++
50	+++	+++	+++	+++	+++	+++
25	+++	+++	+++	+++	+++	+++
12.5	+++	+++	+++	+++	+++	++
6.25	++	++	++	+++	+++	+
3.13	++	++	++	++	++	+
1.56	+	+	+	+	+	+
0.78	+	+	-	+	+	-
0.39	+	+	-	+	+	-
Microbial Control (0)	+++	+++	+++	+++	+++	+++

Description: +++: very cloudy, ++: cloudy, +: somewhat cloudy, -: clear

Jurnal Biodjati 4(1):68-78, May 2019

JURNAL BIODJATI

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Table 5. The diameter of the inhibition zone of calamus rhizoma extracts against *S. aureus* and *E. coli*

Treatment (Extract Concentration, %)	Average Number of Colonies (CFU/mL)					
	Staphylococcus aureus			*Escherichia coli*		
	Ethanol	Cloroform	n-Hexana	Ethanol	Cloroform	n-Hexana
Microbial Control (O)	1.59×10^{14}	1.59×10^{14}	1.59×10^{14}	1.67×10^{18}	1.67×10^{18}	1.67×10^{18}
0.39*	8.00×10^{10}	7.70×10^{10}	7.30×10^{10}	15.9×10^{10}	16.1×10^{10}	13.9×10^{10}
0.78**	0	0	0	0	0	0
1.56	0	0	0	0	0	0
3.13	0	0	0	0	0	0
6.25	0	0	0	0	0	0
12.50	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
Extract Control	0	0	0	0	0	0

Description: *Minimum Inhibition Concentration (MIC) **Minimum Bactericidal Concentration (MBC)

The MIC value of the calamus rhizo-ma extract against *S. aureus* and *E. coli* was 0.39%, with the number of colonies were not significantly difference between the solvents namely ethanol extract at 8.00×10^{10} and 15.9×10^{10}, chloroform extract 7.70×10^{10} and 16.1×10^{10} and n-Hexane extract at 7.30×10^{10} and 13.9×10^{10}. These results showed that cala-mus rhizoma extract at 0.39% concentration was able to inhibit *S. aureus* bacteria bet-ter than *E. coli*. It was proven by a decrease number of *S. aureus* colonies than *E. coli*. The level of 0.78% was the MBC value of all types of extract for both test bacteria, charac-terized by the absence of bacterial growth at all (colonies <0.1% of the original inoculum). Previous studies showed that concentrations of 0.39% and 0.78% were also the MIC and MBC values of calamus rhizoma extract against *Candida albicans* (Muchtaromah, 2017). Moreover it was known that the cala-mus rhizome extract effectively inhibited both gram positive and negative bacteria (broad spectrum) and fungi. Marliani (2012) study of the MIC and MBC tests of bangle rhizomes (*Zingiber cassumnar* Roxb. against *S. aureus* and *E. coli* showed minimum inhibitory con-centrations at a concentration of 1.56% while the minimum bactericidal level was at a level of 3.125%, indicating the calamus rhizome extract was more effective in inhibiting *S. aureus* and *E. coli* than the bangle rhizome.

Furthermore, Devi & Ganjewala (2009) reported the antimicrobial activity of rhizome and leaf extracts of *A. calamus* with different solvents, namely petroleum ether, chloro-form, hexane, and ethyl acetate. Ethyl acetate extract of rhizomes and leaves produced the most considerable antifungal activity with in-hibition zone diameters ranging between 20-28 and 18-25 mm, and anti-yeast action with inhibition zone diameters ranging between 22-25 and 20-23 mm respectively. The MIC of rhizome and leaf extract for antifungal activi-ty was 2-4 mg/mL, except for *P. chrysogenum* whereas for yeast it was relatively higher, 4-5 and 6-8 mg/mL. The MIC value for antibacte-rial activity was relatively very high ~ 16-42 mg/mL. A-asarone and B-asarone produced robust antimicrobial activity against fungi and yeast compared to rhizome and leaf ex-tracts. This study informed that rhizome and leaf extracts of *A. calamus* must have active compounds A- and B-asarones which were

responsible for the antimicrobial activity. The rhizome and leaf extract in this study had no antibacterial activity except for *E. coli*.

Available reports showed that the antimicrobial properties of plant parts vary depend on solvent type. Dichloromethane and ethanol extracts of *A. calamus* rhizome had been reported to show substantial antifungal activity. The ethanol extract of *A. calamus* inhibited clinical isolates of *C. albicans*. Based on some previous studies it was known that the difference in effectiveness (MIC value) was partly due to the type of solvent used for extraction, parts of plants and differenc-es in climate and geography. Differences in morphology and constitution of cell walls of microorganisms affected the sensitivity to extract (Balakumbahan et al., 2010).

Based on the results, it can be concluded that ethanol and n-hexane extract contained alkaloids and triterpenoids, while chloroform extract was only triterpenoid. The calamus rhizome extract had antibacterial activity against *S. aureus* and *E. coli*. Chloroform extract produced the highest inhibition zone diameters (7.26 mm and 3.28 mm), followed by ethanol extract (5.90 mm and 3.07 mm) and n-hexane extract (5.33 mm and 2.95 mm). The concentration of MIC and MBC were 0.39% and 0.78% for all three extracts, indicating the extracts of *Acorus calamus* rhi-zomes with several solvents had almost the same antibacterial activity.

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