

ANTIBACTERIAL ACTIVITY OF *DRACONTOMELON DAO* EXTRACTS ON METHICILLIN-RESISTANT *S. AUREUS* (MRSA) AND *E. COLI* MULTIPLE DRUG RESISTANCE (MDR)

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Article History

Received: March. 15, 2017.

Revised Received: July. 23, 2017

Accepted: Aug. 23, 2017

Published Online: March. 07, 2018

Abstract

Background: *Staphylococcus aureus*, methicillin-resistant and *Escherichia coli*, multidrug-resistant included in the list of antibiotic-resistant priority pathogens from WHO. As multidrug-resistant bacteria problem is increasing, it is necessary to probe new sources for identifying antimicrobial compounds. Medicinal plants represent a rich source of antimicrobial agents. One of the potential plants for further examined as antibacterial is *Dracontomelon dao* (Blanco) Merr. & Rolfe. The present study designed to find the antibacterial activity of *D. dao* stem bark extracts on Methicillin-resistant *S. aureus* (MRSA) and *E. coli* Multiple Drug Resistance (MDR), followed by determined secondary metabolites with antibacterial activity and determined the value of MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration).

Materials and Methods: *D. dao* stem bark extracted using 60% ethanol. Disc diffusion test methods used to find the antibacterial activity, following by microdilution methods to find the value of MIC and MBC. Secondary metabolites with antibacterial activity determined by bioautography using TLC (thin layer chromatography) methods.

Results: *D. dao* stem bark extracts are sensitive to MSSA, MRSA and *E.coli* MDR bacteria. The inhibition zone is 16.0 mm in MSSA, 11.7 mm in MRSA and 10.7 mm in *E. coli* MDR. The entire MBC/MIC ratios for MSSA, MRSA and *E.coli* MDR is lower than 4. The ratio showed bactericidal effects of *D. dao* stem bark extracts. In TLC results, colorless bands found to be secondary metabolites with antibacterial activity.

Conclusion: *D. dao* stem bark extracts are potential to develop as antibacterial agent especially against MRSA and *E. coli* MDR strain.

Key-words: Antibacterial, *Dracontomelon dao*, MRSA, *E. coli* MDR

Introduction

World Health Organization (WHO) published its first ever list of antibiotic-resistant priority pathogens on February 2017. There were twelve families of bacteria that pose the greatest threat to human health. They include *Staphylococcus aureus*, methicillin-resistant and *Escherichia coli*, multidrug-resistant (WHO, 2017).

S. aureus is a major cause of both hospital- and community-acquired infections, in developed and developing countries. Treatment of *S. aureus* infections is becoming increasingly more complicated due to emerging of various types of antimicrobial resistance worldwide. Methicillin-resistant *S. aureus* (MRSA) strains are the most concern since these are resistant to all beta-lactam antibiotics and in many cases to other groups of antimicrobials as well, especially in the hospital setting. Overall, rates of MRSA in Southeast Asia ranged from 7% in the Philippines to 25% in Malaysia and 39% in Singapore (Lestari et al., 2012).

E. coli exist as normal flora in originally susceptible to many antimicrobial agents. However, selective pressure by repeated exposure to antibiotics has led to the development of resistance. In Southeast Asia, many studies have assessed antimicrobial resistance among *E. coli*. The overall prevalence of ampicillin resistance was $\geq 50\%$, higher than other countries of the world. The fluoroquinolone resistance among gram-negative rod is, however, a phenomenon is seen worldwide (Severin et al., 2012).

WHO priority pathogen list was drawn up in a bid to guide and promote research and development of new antibiotics, as part of WHO's efforts to discuss growing global resistance to antimicrobial medicines. New antibiotic targeting this priority list of pathogens will help to reduce deaths due to resistant infections around the world (WHO, 2017).

Nature has been a source of medicinal agents for thousands of years and about 80% of the world's population relies on traditional medicines for their primary health care. Plant materials remain an important resource to combat serious diseases in the world. Due to the most important bioactive constituents which are alkaloids, tannin, flavonoids and phenolic compounds. Advances in identifying new sources of natural products with antimicrobial activities and expanding antibiotic chemical diversity are providing chemical leads for the new antibiotic (Kamath et al., 2016).

As the multidrug-resistant bacteria are increasing, it is necessary to probe new sources for identifying antimicrobial compounds. Medicinal plants represent a rich source of antimicrobial agents. Plants used medicinally in different countries and a source of many potent, powerful drugs. The antibacterial compounds found in plants may prevent bacterial infections by different mechanisms than the commercial antibiotics and may have clinical value in treating resistant microorganisms (Ganapathy and Karpagam, 2016).

One of the potential plants for further examined as antibacterial is *Dracontomelon dao* (Blanco) Merr. & Rolfe. *D. dao* belongs to the *Anacardiaceae* family. *D. dao* local name is Pacific Walnut, in Malay known as sengkayang and in Indonesia known as dahu. *D. dao* is a large tree up to 45-55 meters tall. *D. dao* tree could be found in Cambodia, China, India, Indonesia, Malaysia, Myanmar, Papua New Guinea, Philippines, Solomon Islands, and Thailand. As medicinal plants, the bark used against dysentery, leaves, and flowers are also employed in traditional medicine (Orwa et al., 2009). As a traditional Chinese medicinal material with a regional feature, it has been widely used to treat various infectious diseases, such as decubitus and skin ulcers (Li et al., 2017). *D. dao* stem bark used as medicinal plants for diarrhea by Dayak Benuaq people in East Kalimantan, Indonesia (Falah et al., 2013).

Liu et al. (2014) showed an anti-bacterial effect of ethyl acetate extracts from leaves of *D. dao* on *E. coli*. Zhao et al. (2015) also showed an anti-bacterial effect of ethyl acetate extracts from leaves of *D. dao* on *S. aureus*. Ethanol extracts of the leaves of *D. dao* showed anti-Staphylococcus aureus activities. Based on those studies, extracts from the leaves of *D. dao* have possessed anti-infectious potential (Li et al., 2017).

Considering the promising therapeutic potential, our study designed to find antibacterial activity of *D. dao* stem bark extracts on Methicillin-resistant *S. aureus* (MRSA) and *E. coli* Multiple Drug Resistance (MDR), followed by determined secondary metabolites with antibacterial activity and determined the value of MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration).

Material and Methods

Collection and identification of plant material

The stem bark of *D. dao* collected from Kutai Kartanegara, East Kalimantan, Indonesia. Identification and voucher specimen deposition of this plant performed at the Dendrology Laboratory, Faculty of Forestry, Mulawarman University, Samarinda, Indonesia with voucher specimen (number: UM 532016).

Processing of plant material and preparation of extracts

The stem bark of *D. dao* washed with distilled water. The stem bark shed dried, followed by drying in the oven. The sample powdered using a mechanical grinder. Simplicia of stem bark carried in cleaned airtight bottles for maceration in 60% ethanol for 3 days. All bottles whipped by orbital shaker every day at 2 rpm for 10 minutes and repeated three times. The extracts filtered with micro fibreglass filter in the vacuum pump, followed by freeze-drying process. Plant extracts obtained stored at 4°C.

Bacteria strains

Bacterial strains used in this study are MRSA ATCC 25923, MSSA ATCC 43300 and local isolates of *E. coli* MDR. All these strains cultured in brain heart infusion broth at 37°C for 24 hours. Concentrations for all cultures were measured by spectrophotometry.

Antibacterial activity test with disc diffusion method

Antibacterial activity tested with disc diffusion method as described by Ifesan et al. (2010). Ten microliters of the extracts dissolved in ethanol added to sterile filter paper discs. The discs dried at 70°C overnight. The plates of Mueller-Hinton agar applied with 200 uL culture of bacteria. The discs contained extracts seeded on those plates. Ampicillin and oxacillin used as positive controls. The plates then incubated at 37°C for 18 hours. The experiments performed in duplicate and the means of the diameters of the inhibition zones calculated.

Evaluation of MIC and MBC

Evaluation MIC by standardized broth microdilution method using serially diluted plant extracts, as described by Shaheen et al. (2015). The plates incubated at 37°C for 18 hours. MIC showed the lowest concentration of highest dilution of plant extracts that did not give any visible bacterial growth in the microdilution wells. The absence of bacterial growth indicated the MBC for the respective bacteria. The experiments performed in three times duplication.

Bioautography with TLC (thin layer chromatography)

Bioautography was carried out by using TLC methods described by Suleiman et al. (2010). Plant extracts loaded on TLC plates in a narrow band and eluted using methanol. The developed plates dried using Laminar flow cabinet. After overnight, the plates mounted on bacteria cultures grown on Mueller-Hinton agar. Colorless bands showed secondary metabolites that inhibited the growth of tested organisms. Visualization is usually carried out by spraying plates with MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), as described by Choma and Jesionek (2015).

Data analysis

Data subjected to statistical analysis for mean and SD at significant level $p < 0.05$ using SigmaPlot version 12.5.

Results

Table 1 showed the antibacterial activity of *D. dao* stem bark extracts. The plant extracts are sensitive to MSSA, MRSA and *E.coli* MDR bacteria, especially in disc concentration of 3 mg plant extracts. The inhibition zone is 16.0 mm in MSSA, 11.7 mm in MRSA and 10.7 mm in *E. coli* MDR. Table 2 showed MIC and MBC activity of *D. dao* stem bark extracts. MBC/MIC ratio for MSSA is 1.5, MRSA is 1.67 and *E. coli* MDR is 1.37. The entire MBC/MIC ratios for MSSA, MRSA and *E.coli* MDR are lower than 4. The ratio shows bactericidal effects of *D. dao* stem bark extracts. Figure 1 shows there are secondary metabolites with antibacterial activity of *D. dao* stem bark extracts. Colorless bands show of secondary metabolites that inhibited the growth of tested organisms.

Table 1: Antibacterial activity of *D. dao* stem bark extracts

Disc Concentration	Inhibition Zone (mm)		
	MSSA	MRSA	ECMDR
	Mean ± SD	Mean ± SD	Mean ± SD
0.5 mg	9.0 ± 0.8	7.7 ± 0.5	6.3 ± 0.5
1 mg	11.7 ± 1.2	8.7 ± 0.5	6.7 ± 0.5
2 mg	14.0 ± 0.8	10.0 ± 0.8	7.7 ± 0.5
3 mg	16.0 ± 0.8	11.7 ± 0.5	10.7 ± 0.5
Ampicillin 10 µg	18.7 ± 0.9	6.2 ± 0.2	6.0 ± 0.0
Oxacillin 1 µg	-	6.2 ± 0.2	-

Note: MSSA = Methicillin-sensitive *Staphylococcus aureus*, MRSA = Methicillin-resistant *Staphylococcus aureus*, ECMDR = *Escherichia coli* Multiple Drug Resistance

Table 2: MIC and MBC activity of *D. dao* stem bark extracts

Methods	Extracts Concentration (mg)		
	MSSA	MRSA	ECMDR
	Mean ± SD	Mean ± SD	Mean ± SD
MIC	2.2 ± 1.0	1.8 ± 0.4	2.7 ± 1.0
MBC	3.3 ± 1.0	3.0 ± 1.1	3.7 ± 0.8

Note: MIC = Minimum Inhibitory Concentration, MBC = Minimum Bactericidal Concentration, SSA = Methicillin-sensitive *Staphylococcus aureus*, MRSA = Methicillin-resistant *Staphylococcus aureus*, ECMDR = *Escherichia coli* Multiple Drug Resistance

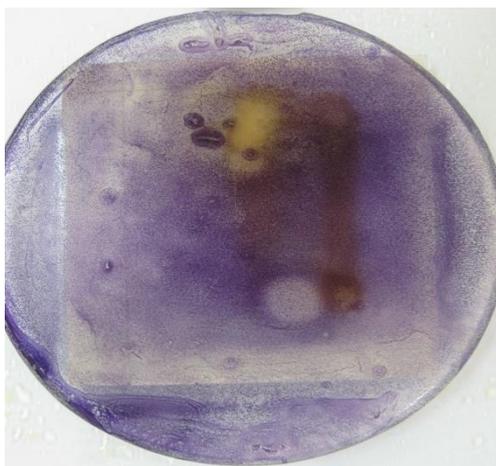


Figure 1: TLC result of *D. dao* stem bark extracts on MRSA with MTT

Note: colorless bands show secondary metabolites that inhibited the growth of MRSA; TLC = thin layer chromatography, MH = Mueller-Hinton, MTT = 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, MRSA = Methicillin-resistant *Staphylococcus aureus*



Figure 2. TLC result of *D. dao* stem bark extracts on MSSA with MTT

Note: colorless bands show secondary metabolites that inhibited the growth of MSSA; TLC = thin layer chromatography, MH = Mueller-Hinton, MTT = 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, MSSA = Methicillin-sensitive *Staphylococcus aureus*

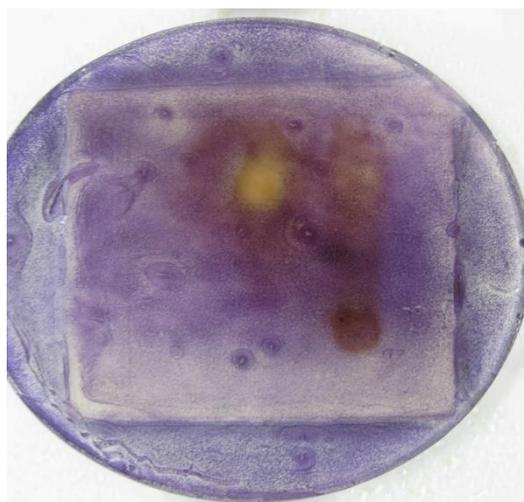


Figure 3: TLC result of *D. dao* stem bark extracts on ECMDR with MTT

Note: colorless bands show secondary metabolites that inhibited the growth of ECMDR; TLC = thin layer chromatography, MH = Mueller-Hinton, MTT = 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, ECMDR = *Escherichia coli* multiple drug resistance

Discussion and Conclusion

As traditional medicine material in South and Southeast Asia, *D. dao* has been widely used to treat various infectious diseases. *D. dao* extracts reported to show antimicrobial properties. The synonyms of *D. dao* are *Comeurya cumingiana* Baill., *Dracontomelon brachyphyllum* Ridl., *Dracontomelon celebicum* Koord., *Dracontomelon cumingianum* (Baill.) Baill., *Dracontomelon edule* (Blanco) Skeels, *Dracontomelon edule* Merr., *Dracontomelon lamiyo* (Blanco) Merr., *Dracontomelon laxum* K.Schum., *Dracontomelon mangiferum* (Blume) Blume, *Dracontomelon puberulum* Miq., and *Dracontomelon sylvestre* Blume (The Plant List, 2013).

The results of the study showed an antibacterial activity of *D. dao* stem bark extracts. The plant extracts are sensitive to MSSA, MRSA and *E. coli* MDR bacteria. To the best of our knowledge, this is the first report of antibacterial activity of *D. dao* stem bark extracts to MRSA, MSSA, and *E.coli* MDR. The results for *S. aureus* similar to study using the ethyl acetate extracted fraction of *D. dao* leaves. Ethyl acetate fraction had the highest total flavonoid content (41.86%), expressed the strongest anti-*S. aureus* effect with half-inhibitory concentration (IC₅₀) of 83.93 µg/mL (Zhao et al., 2015). The results for *E. coli* similar to study using the ethyl acetate extracted fraction of *D. dao* leaves. Ethyl acetate fraction observed to have the strongest anti-*E. coli* activity with half-inhibitory concentration IC₅₀ of 98.5 µg/mL (Liu et al., 2014).

To the best of our knowledge, this is the first report of MIC and MBC of *D. dao* stem bark extracts. The results of the study showed MIC and MBC activity of *D. dao* stem bark extracts. MBC/MIC ratios for MSSA, MRSA and *E.coli* MDR is lower than 4. The ratio shows bactericidal effects of *D. dao* stem bark extracts. A sample is bactericidal when the ratio MBC/MIC ≤ 4 and bacteriostatic when this ratio is > 4. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC. The MBC is complementary to the MIC since the MIC test demonstrates the lowest level of antimicrobial agent that inhibits growth; the MBC demonstrates the lowest level of antimicrobial agent that results in microbial death (Djeussi et al., 2013).

The results of the study showed secondary metabolites with antibacterial activity of *D. dao* stem bark extracts. Colorless bands show secondary metabolites that inhibited the growth of tested organisms. Results obtained are similar to the study searching for secondary metabolites of *D. dao*. The study by Ragasa et al. (2016) reported isolate of anacardic acid, β-sitosteryl-3β-glucopyranoside-6'-*O*-fatty acid esters, β-sitosterol, phytol, a mixture of phytol fatty acid esters and β-sitosteryl fatty acid esters, chlorophyll a, squalene, long-chain fatty alcohols, and long-chain hydrocarbons from the leaves of *D. dao*.

Further study of the main components of ethyl acetate fraction of extracts *D. dao* leaves which are flavonoids, including Cianidanol, L-Epicatechin, Quercetin, and Luteolin. The result showed that the antibacterial activity of four flavonoids is Luteolin > Cianidanol > Quercetin > L-Epicatechin from the result of the single-factor analysis, which indicated that Luteolin and Cianidanol are very important factors in their antibacterial mechanism. Of note, Quercetin and L-Epicatechin are not the major factors of the antibacterial activity of the extract of the leaves of *D. dao* (Li et al., 2017).

As the conclusion, *D. dao* stem bark extracts are potential to develop as antibacterial agent especially against MRSA and *E. coli* MDR strain due to the result of the present study. The further in vivo research and mode of its action need to shed light the antibacterial effect.

Competing interest: The authors affirm that there are no competing interests with this study.

Acknowledgements

We would like to acknowledge Ministry of Health of the Republic of Indonesia for providing funding for this research by RISBINIPTEKDOK Grants in 2011.

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