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Antibacterial Activity of *Melaleuca alternifolia* Extract from Different Extraction Method

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Abstract. *Melaleuca alternifolia* (Tea tree/*MA*) is a powerful antimicrobial agent that could be one of the solutions to antimicrobial resistance. The important benefit of this plant comes from its volatile oil compounds named tea tree oil (TTO). On the other hand, studies related to the use of tea tree leaf extract as antimicrobial were still limited. Therefore, an evaluation of the active compound content and antimicrobial activity of tea tree extract obtained from different extraction methods will be carried out. Thin-layer chromatography (TLC) was performed to analysis the chemical profile, the antibacterial activity of the extracts was evaluated using the Kirby Bauer method against *Staphylococcus aureus* and *Escherichia coli*, whereas the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determined by broth dilution method. Based on the chromatographic profile of the extract indicated that cardiac glycosides and terpenoids could be present in maceration and soxhletation extracts. This study suggests that maceration and soxhletation yielded different bioactive compounds from *MA*. Extracts of *MA* from both method have an excellent activity to inhibit the *S. aureus*, and *E. coli* in a dose-dependent manner. Maceration extract of *MA* has a stronger effect against *E. coli* meanwhile the soxhletation extract of *MA* reveals to have stronger antimicrobial activity against *S. aureus*. The both extract even obtained from different extraction method yielded the same MIC and MBC values namely = 0.1875% against *E. coli*. In contrast MBC of *S. aureus* range from two to fourfold of the MIC, and the maceration seem to have the highest MBC value (MBC = >12%).

Keywords: antibacterial activity, bioactive compounds, maceration, *Melaleuca alternifolia*, soxhletation.

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INTRODUCTION

Antimicrobial resistance is currently a global health problem due to the improper usage of antibiotics for the patient and extensive usage of antibiotics in various industries such as food, beverage, and animal (Gupta and Birdi, 2017). Therefore, it is necessary to conduct intensive studies to find new therapeutic agents that have no implication to antimicrobial resistance effects. *Melaleuca alternifolia* (tea tree) essential oil demonstrated to have antimicrobial activity (Yasin et al., 2021). A study revealed no resistance effect in the tested strains of *S. epidermis, S. aureus*, and *E. coli* after exposure to tea tree oil (TTO) (Hammer et al., 2012).

M. alternifolia (Figure 1.) popular known as the tea tree is native to Australia and belongs to the Myrtaceae family (Sharifi-Rad et al., 2017). This plant is a shrub that grows up to 7 m tall with layered papery bark. Inflorescence a terminal or at the ends of branches or twigs after the plant is 3 years old or more, spike fairly densely flower, white, greenish white or creamy, and appear in October or November (Malathi et al., 2020). In general, planting of *M. alternifolia* begins with sowing seedlings in a greenhouse and then transplanting them into the field or garden. The growth rate and harvest time or production period of *M. alternifolia* are strongly influenced by climate change, which varies from one to three years. Harvesting is done by cutting the twigs and upper branches of the plant (Rodney et al., 2015).

Essential oils produced by plants generally have antimicrobial activity. *M. alternifolia* produce essential oil known as tea tree oil (TTO) which devired by distillation method. The TTO widely used as a topical antiseptic such as for herpes, abscess, blisters acne, cold sores, burns, insect bites, dandruff, and also used to reduce oily skin (Mertas et al., 2015; Ali et al., 2015). TTO has a wide spectrum of antimicrobial activity against a variety of bacteria, viruses, and fungi. The TTO also could be used to treat *S. aureus*

infections of the oral cavity, urinary tract, and the pharynx; vaginitis, and respiratory tract diseases such as tuberculosis, cough, bronchitis, asthma, whooping cough, and catarrh (de Assis et al., 2020).

Refer to the broad spectrum of TTO properties, *M. alternifolia* could be an excellent antimicrobial agent. Essential oil of the *M. alternifolia* leaves derived from steam distillation mostly use as the powerful antimicrobial agent (Sankara Malathi et al., 2020). However, the distillation process using the evaporation method from boiling water (hydrolysis, cyclization), may cause changes and damage to various chemical compounds (Asbahani et al., 2015). Therefore, the compound profile of the distillation product compared to the original material has a difference in the resulting isolate (Jajaei et al., 2010). Oral antimicrobial preparations from tea tree oil have limitations in product design, and the development of oral antimicrobial preparations from *M. alternifolia* extracts is very promising. Ethanol extraction could be considered as applicable way of antimicrobial production of tea tree in commercial scale (Li et al., 2021).

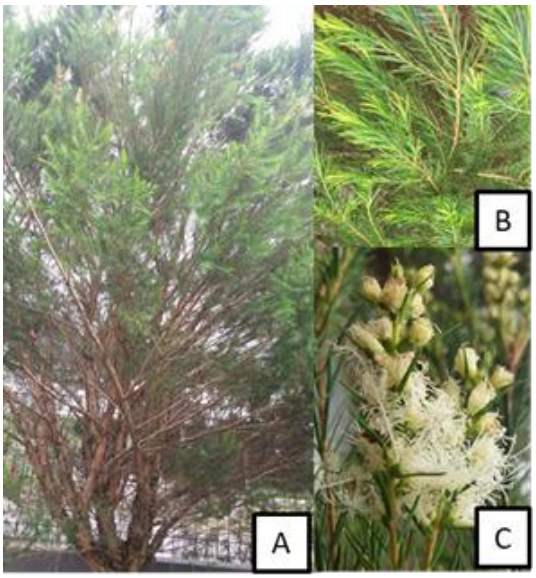

Figure 1. The picture of *Melaleuca alternifolia* tree grown at Tlogodlingo Research Station of Medicinal Plant and Traditional Medicine Research and Development Center Tawangmangu; A. Tree; B. Leaves; C. Flower.

The influence of other extraction method of *M. alternifolia* on the chemicals compound profile and the bioactivity, have not studied intensively. The two simple extraction methods such as maceration and soxhletation could be applied in developing the new therapeutic product from *M. alternifolia*. The methods are also easy to be performed and cheaper than encapsulation of essential oil, which may reduce the cost of *M. alternifolia* production for more affordable utilization. The objective of the study was to evaluate the different methods of extraction namely soxhletation and maceration on the chemical profile and the antimicrobial activity of *M. alternifolia*.

METHODS

Plants extraction

M. alternifolia leaves were collected from research station of Medicinal Plant and Traditional Medicine Research and Development Center *(*B2P2TOOT) in Tlogodlingo, with the altitude of 1.800 m above sea level. The leaves were dried in the oven with 40^{o}C of temperature for 3x24 hours, and then pulverized with 40 mesh of size powder. For the maceration method of extraction, 100g of sample was macerated with ethanol 96% at room temperature for 3 x 24 hours. The mixture was then filtered and evaporated with a rotary evaporator. The soxhletation was carried out by take 25g powder of sample, and then wrapped with filter paper to form a lead according to soxhlet size, sample is inserted in soxhlet tube, and pump for circulation the condenser is turned on, the 150 mL of ethanol 96% were added into the extraction column from the top of the condenser. The Soxhlet apparatus was heated to 350°C, and the process was done until the color of the solvent is clear. The extract was then evaporated with a rotary evaporator.

Chromatography profiling

The chemical compounds of extract from different extraction methods were identified qualitatively with Thin Layer Chromatography, using silica gel GF254 as stationary phase and n-hexane: ethyl acetate (6:4) as mobile phase. Visualization of the chromatogram was performed by UV in 254 and 366 nm wavelength, as well as H2SO4 10% within methanol.

Antibacterial activity determination

Antibacterial activity of the maceration and soxhletation extract was determined by the Kirby Bauer method and broth dilution method. The samples were tested against *E. coli* and *S. aureus* (Hudzicki, 2016).

Determination of antibacterial activity by Kirby Bauer method

Concentrations of soxhletation and maceration extract that were used for the Kirby Bauer test are 12.5%, 25%, 37.5%, and 50%. The dry extract was

carefully weighed as much as 500 mg and added 1 mL of DMSO, as stock with a concentration of 50%. Furthermore, to increase the homogeneity of the solution, sonication was carried out. From the stock solution, a concentration series of 12.5%, 25% and 37.5% was made, respectively. Bacteria namely *E.coli* ATCC 25922 and *S. aureus* ATCC 25923 (Thermo scientific culti-loops) were taken from agar slant into 9 mL saline solution using inoculation loop and grown in a 35°C incubators for 24 hours or until the turbidity of 2.0 McFarland standard was reached.

Bacterial suspension (200 mL) was placed on nutrient agar, and 10µL of the samples was transferred to blank discs. The negative control used in this study was DMSO and the positive control on nutrient agar grown with *E. coli* was Neomycin disc, while positive control of samples on nutrient agar grown with *S. aureus* was chloramphenicol disc. One petri dish contain of 6 discs consisting of 4 discs for concentrations tested, 1 disc for positive control, and 1 disc for negative control. The treatment was repeated for five times and it was incubated at 35°C for 24 hours. Interscience Scan 1200 was used to calculate the zone of inhibitions.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Determination of MIC and MBC of *M. alternifolia* extract from maceration and soxhletation extraction method was carried out based on Seyyednejad et al. procedure (Seyyednejad et al., 2014). Bacterial isolates were made in a tube containing 5 mL of nutrient broth by transferring the bacteria from the agar slant using an inoculation loop, and then the suspension was mixed using a vortex. Incubate the suspension at 37°C for 24 hours. Make serial dilutions from 0.1875% to 12% of both types of extracts, using each of these sample solvents. A total of 30µL of pre-grown bacterial suspension was added to 1 ml of nutrient broth and 1 ml of sample. The test tubes were then incubated at 37°C for 24 hours. MBC was carried out by coating 100µL test tubes without any visible growth of bacteria into nutrient agar which was then incubated at 37°C for 24 hours. The negative control used in the treatment were DMSO and methanol.

RESULTS AND DISCUSSION

Phytochemical evaluation

The antimicrobial activity of *M. alternifolia* extract from different extraction methods is evaluated based on the chemical profile among them. The different extraction methods may affect the different activity due to the differences in chemical compounds which are able to eliminate microbial contamination. This study suggests that different extraction methods of *M. alternifolia* could affect the bioactive compound obtained and the antimicrobial activity of the extracts. Different extraction methods and adjustment of mobile phase during thin layer chromatography were used to test the selectivity and presence of bioactive compounds in each extract. The compounds contain in maceration and soxhlet extracts were different as shown by different numbers and intensities of spots which represent compounds observed in 254 and 366 nm UV light, and also by chemical visualization (Figure 2).

Figure 2 demonstrated that different extraction method for *MA* leaves gives the different profiles of the chromatogram. This result reveal that difference extraction methods causes differences in the number and types of compounds dissolved in each type of extract, which is influenced to the antimicrobial activities. This finding is in line with a previous study conducted by Paz et al. (Paz et al., 2017) which revealed the different antimicrobial activity and bioactive compounds of *H. patens* extract obtained by maceration, percolation, and soxhletation method. Moreover, the other study conducted by Abah and Egwari (2011) found a similar result which is stated that there were differences in the activity and chemical profile of the maceration and soxhletion extracts of *Anchomanes difformis* (Abah and Egwari, 2011).

The chromatogram profile of the results of the elution of the two types of *M. alternifolia* extract using the mobile phase of n-hexane:ethyl acetate (6:4) showed the difference in the number of spots detected by UV light and reagent solution (5% H2SO4 in methanol) (Figure 2). Spray reagents using a 5% solution of sulfuric acid in methanol are generally used to detect the presence of cardiac glycosides, terpenoids and lignans compounds (Wagner and Bladt, 1996). Thus, the appearance of spots A and B after spraying the sulfuric acid reagent may indicate the presence of glycosides, terpenoids and lignans in both extracts. However, the lignans should also be detectable with UV light at 254 nm, because these compounds contain an aromatic ring with conjugated double bonds which can reduce the layer fluorescence. The presence of lignan compounds is indicated as a dark band on a light green background (Sherma, 2005), which was not detected in this study, as seen in Figure 2.

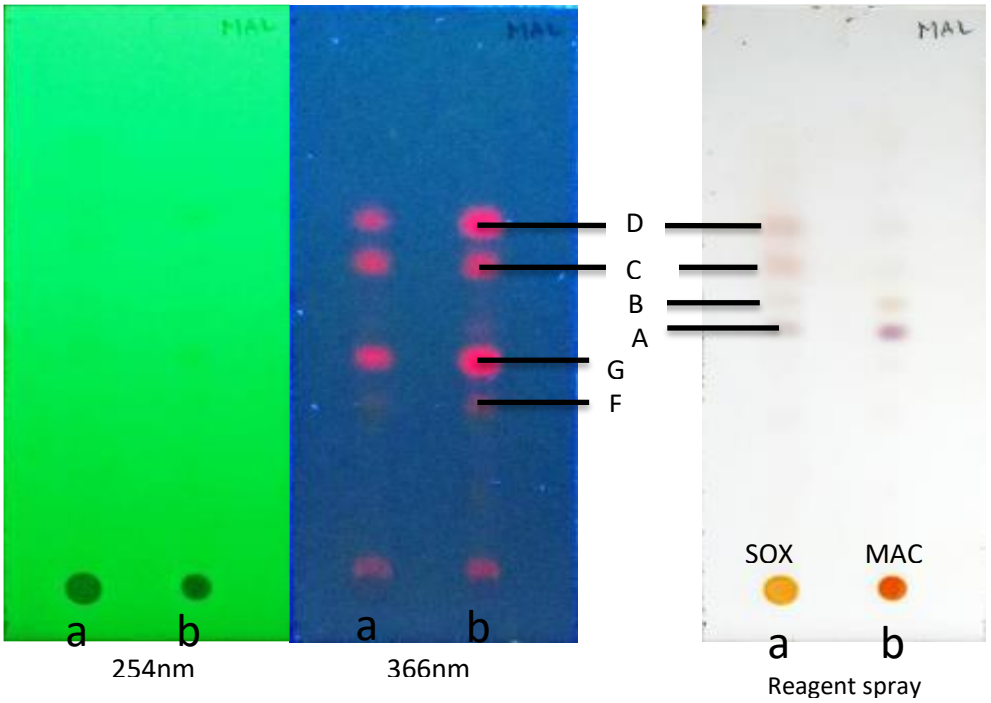

Figure 2. Chromatographic profile of M. alternifolia extracts from different extraction methods using Thin Layer Chromatography, I. Visualized by UV 254 nm (left); II. Visualized by 366 nm (middle), and III. Visualized by spray reagent (H2SO4 5% within methanol) (right), a. Soxhletation method; b. Maceration method.

Furthermore, cardiac glycosides could not be detected in 366 nm UV light; this confirms that compound A and B are actually compounds from the cardiac glycosides group. In addition, the same finding is obtained by the phytochemical test conducted by previous experiment, which indicated the contain of cardiac glycosides in *M. alternifolia* (Afiqah and Priya, 2016). UV light visualization in 366 nm of wavelength could be detected of cardiac glycosides, terpenoids, coumarins, and flavonoids compounds which could not be detected by 254nm UV light as such present in Figure 2 (Syarifah et al., 2019). To determine which compound has a strongest antimicrobial activity in *M. alternifolia* extract, it is necessary to identify by further study named bioautographic studies.

Antibacterial Activity

Zone of Inhibition (ZoI) Determination

This experiment exhibited that the both *M. alternifolia* extracts have moderate antimicrobial activity against the tested strains of bacteria as shown in Table 1. Interestingly, contrast results were obtained in the experiments of Kirby Bauer and broth dilution for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Three out of five data that were obtained from five times repetition was used to determine the zone of inhibition of each concentration tested. Extract obtained from soxhletation method has the best antimicrobial activity against *E. coli*, as shown by the largest zone of inhibition which is observed in this study (Table 1).

The zone of inhibition (ZoI) is a clear zone formed by the rate of diffusion of phytochemical active compounds through the agar, furthermore the Zone of Inhibition should be correlated well with MIC value (Szewczyk and Wisniewski, 2007). Referring to the observed result as mentioned in Table 1, it can be concluded that the extract of *M. alternifolia* coming from maceration and soxhelatin method is strongly affecting the inhibition of *S, aureus,* and *E. coli* in dose-dependent manner. Maceration extract of *M. alternifolia* has stronger effect against *E.coli* meanwhile the soxhelation extract of *M. alternifolia* reveals to have stronger antimicrobial activity against *S.aureus*. According to Bian et al. (2015), the antibacterial activity of a substance or compound is stated to be very strong if the diameter of the inhibition zone is more than 20 mm, strong if it is 10-20 mm, moderate if the inhibition zone is 5-10 mm and weak if <5 mm.

he presence of terpene alcohol detected in tea tree essential oil (TTO) has been previously revealed in many other studies on *M. alternifolia*

Table 1. Effect of concentrations of tea tree extract from different extraction methods to the inhibition of *E. coli* and *S. aureus* grows

Extraction	Concentration (%)	*E. coli* (mm)	*S. aureus* (mm)
Soxhletation	12.5	9.5 [c]	10.27 [b]
	25	11.27 [b]	11.73 [b]
	37.5	13.17 [b]	12.73 [b]
	50	13.47 [b]	13.50 [b]
	Control +	17.77 [a]	24.20 [a]
Maceration	12.5	13.63 [b]	9.20 [c]
	25	13.33 [b]	10.73 [b]
	37.5	14.20 [b]	11.53 [b]
	50	16.03 [ab]	12.20 [b]
	Control +	19.77 [a]	22.30 [a]

Note: The numbers within the column follow with the same alphabet are not significantly different from the LSD test of 5% level

(Shah and Baghel, 2017). This chemical compound is most likely responsible for the antimicrobial activity of *M. alternifolia* essential oil (TTO), which has also been exhibited in many previous studies. On the other hand, despite the absence of terpene alcohol content in the extract of *M. alternifolia* obtained from maceration and soxhletation method, both extracts showed antibacterial activity. This study implies that the compounds present in the macerated and soxhletated extracts, such as flavonoids, cardiac glycosides, terpenoids, and possibly other compounds, also have antibacterial activity on the bacterial strains tested. Terpenes and terpenoids contained in essential oil showed excellent antibactericidal activity against both *S. aureus* and *E. coli* (Guimarães et al., 2019). Moreover, methanol extract from *Daemonorops draco* derived from maceration also demonstrated to have antibacterial activity against *E. coli* and *S. aureus* since the extract rich in terpennoid compound (Wahyuni et al., 2018).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrtion (MBC)

For selection of the most effective concentration of macerated and soxhletated extract of *M. alternifolia* for *E. coli* and *S. aureus* bacteria, the smallest concentration tested on MIC and MBC was 0.1875%. Although the lowest concentrations of all extracts appeared to inhibit the growth of *S. aureus*, they were not able to kill all the bacteria tested. Different MBC values were observed from all types of extracts, the extract resulting from soxhletation had the lowest MBC at a concentration of 0.375% while the macerated extract had the highest MBC value at a concentration of more than 12% (Table 2).

The previous study using tea tree oil, it was revealed that the same value of MIC and MBC for *E. coli*, which was 0.25%, slightly higher than the previous study observed in this experiment that was only 0.1875% for maceration and soxhletation extract (Mumu and Hossain, 2018). However, the different values of MIC and MBC for *S. aureus* has been reported by other studies conducted by Falcil et al. (Falci et al., 2015). Base on the previous study, it could be concluded that to eliminate *S. aureus*, a higher concentration of *M. alternifolia* extract was needed than the use of its essential oil. The MBC value of an antibacterial agent or compound is usually equal to the MIC value, or generally not more than four times the MIC (Levison and Levison, 2009). Thus, the same MIC and MBC values of the *M. alternifolia* extract derived from soxhletation and maceration method for *E. coli*, indicated that both types of *M. alternifolia* extract were bactericidal against *E. coli*. These results were supported by observations

Table 2. Value of MIC and MBC of *M. alternifolia* extract on *E. coli* and *S. aureus*

Extract of *M. alternifolia*	*E. coli*		*S. aureus*	
	MIC (%)	MBC (%)	MIC (%)	MBC (%)
Maceration extract	0.1875	0.1875	0.1875	>12
Soxhletation extract	0.1875	0.1875	0.1875%	0.375%

which showed that the MBC values of both types of extracts were lower for *S. aureus*. However, the classification of an antibacterial agent as bacteriostatic and bactericidal in vitro, sometimes does not have a significant clinically effect on in vivo experiment (Nemeth et al., 2015).

In general, tea tree oil was more often used for antibacterial activity assay compared to *M. alternifolia* leaf extract. Tea tree oil was extracted from the leaves of *M. alternifolia* by the common technique of steam or water distillation (Sankara Malathi et al., 2020). The use of high temperatures in the soxhletation and hydrodistillation extraction methods may increase the solubility of several bioactive compounds which be able to improve their activity (Lenise et al., 2015). Therefore, extract from the soxhletation technique may have stronger bacteriostatic and made the extract have more therapeutic effects that similar to bactericidal agents. The essential oil (EO) or crushed leaves of *M. alternifolia* are traditionally used to treat coughs and colds, and as an antiseptic for wound and skin treatment or throat infections (Wright et al., 2015). Then, the EO of *M. altermifolia* were extensively studied for antimicrobial properties by a number of scientists. However, essential oils are very difficult to be developed in oral preparations. The results of this study are expected to provide basic information for the use of *M. alternifolia* extract as an ingredient for developing herbal preparations for antimicrobials that can be administrated orally.

CONCLUSION

Extracts of *M. alternifolia* from maceration and soxhelatin method have an excellent activity to inhibit the *S. aureus*, and *E. coli* in a dose-dependent manner. Maceration extract of *M. alternifolia* has a stronger effect against *E. coli* meanwhile the soxhelation extract of *M. alternifolia* reveals to have stronger antimicrobial activity against *S. aureus*. The same MIC and MBC values for all extracts (Maceration = 0.1875%; Soxhletation = 0.1875%) were observed from *E. coli*. MBC of *S. aureus* is two to more than fourfold of the MIC, with maceration having the highest MBC (MBC = >12%). The different extraction method of *M. alternifolia* gives the different profile of chromatogram revealing that both extracts has different chemicals contained. Moreover**,** these extract also possess similar antibacterial activity to those observed in the tea tree oil.

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