Phytochemical Analyses of Rosemary (*Rosmarinus Officinalis*) and Its Effects on the Growth of *Propionibacterium Acnes* in Mueller Hinton Broth (Mhb) Media

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Abstract. *Rosemary* (*Rosmarinus officinalis*) is a plant originating from the Mediterranean region and indicated as having antibacterial activity. The purpose of this study was to evaluate the potential phytochemical substances of *rosemary* in inhibiting the activity of *Propionibacterium acnes* bacteria using microdilution method and the identification of compounds that have activity as antibacterial use bioautography method. Every extract and fraction tested using microdilution method with a concentration of $1024^{\mu g}/_{mL}$ against *Propionibacterium acnes*. Results showed that the extracts contain phytochemical substances known as alkaloids, flavonoids, saponin, phenol, and triterpenoids. The value of the minimum inhibitory concentration (MIC) against *Propionibacterium acne* bacteria contained in the ethyl acetate fraction with MIC $512^{\mu g}/_{ml}$. Thin layer chromatography (TLC) monitoring with silica gel stationary phase GF254 and chloroform phase methanol was done in the proportion of 9:1. The antibacterial activity test by bioautography method showed that there were spots on chromatogram of TLC resulting in inhibition zone with Rf value of 0.2, and with the appearance of $AlCl_3$ showed patches of greenish yellow color in a λ366 nm UV lamp on the Rf. Presumably the antibacterial active compound for *Propionibacterium acne* from the ethyl acetate fraction is a flavonoid group compound. The result of non-parametric statistic test of Kruskal-Wallis followed by Post Hoc test of Mann Whitney obtained conclusion that there was no significant difference between control and ethyl acetate fraction at concentration of 512 acetate fraction at concentration of $512^{\mu g}/_{ml}$

Keywords: *Rosmarinus officinalis;* phytochemicals *Propionibacterium acnes*; microdilution; minimum inhibitory concentration (MIC)

1 Introduction

Originally grown in Mediterranean, Rosemary (*Rosmarinus officinalis*) has been used traditionally as stimulant and mild analgesics. Raskovic *et al.* (2014) reported the uses of this plant to cure headache, circulation problems, menstrual cycle problem, and inflammation. One of bacteria that causes acne is *Propionibacterium* acnes, the anaerobic positive gram bacteria which is tolerant to the air (Brook, *et al.*, 2007). New study of Rosemary had been

UNISET 2021, December 02, Kuningan, Indonesia

DOI 10.4108/eai.2-12-2021.2320281

focused on antibacterial, antifungal, anticancer, antioxidant and insecticide (Jiang, *et al.*, 2011).

Phytochemicals are natural substances derived from plants which worked against diseases or specifically protect ones from diseases (Singh, *et al.* 2014). The analyses of phytochemicals of rosemary therefore is necessary to be done to reveal their effects on the growth of *Propionibacterium* acnes.

The aims of this study were to analyze phytochemical substances in rosemary (*Rosmarinus officinalis*) extracts and to determine potential substances that inhibit the growth of *Propionibacterium acnes*. The hypothesis was stated as there is phytochemical compound of *Rosmarinus officinalis* that inhibits the growth of *Propionibacterium acnes*

2 Methodology

This is an experimental qualitative study using rosemary extracts prepared from processes included extraction, maceration, fltration, and dilution. Fractionation yields three fractions (water fractions, ethyl acetate fractions, and n-hexana fractions. Experimental solutions was made based on (b/v) by diluting each thick extract in DMSO 1% solution (CLSI., 2009).

The next procedures are preparing Mueller Hinton Broth (MHB) media, McFarland 0.5 standard, and bacterial suspension. Positive tetracycline control was made by weighing 10.24 mg of tetracycline which ten be diluted in 1 ml DMSO and added by 4 ml aqua pro injection.

Phytochemical analyses were done for alkaloids, flavonoids, tannins. saponins, triterpenoids, steroids, and phenols. Antibacterial activity was tested for its minimum inhibitory concentration by using broth microdilution method (Swanson, 2003).

Data were collected and analyzed by using Kruskal Wallis Nonparametric test (Stephanie, 2014). After that, Post Hoc test Mann Whitney was used according to Lund and Lund (2018). The data then was processed by SPSS 23.0 for Windows (Stephanie, 2014).

3 Result and Discussion

Table 1. Characterization of *Rosmarinus officinalis*

Characterization	*Rosmarinus officinalis*
Dried shrink	**10,2%**
Water content	**9,5 %**

Water content determination is used to give minimum limitation of water content in samples. According to MMI standard, the water content should be less than 10% (Ministry of Health Dept. of the Republic of Indonesia, 1995). Water content measured in this study was less than 10%, therefor it is acceptable according to the standard of MMI. Dried shrink determination is used to know the number of water loss through evaporation.

Table 2. Phytochemical screening in *Rosmarinus officinalis*

Group of Substances	Results in Samples
Alkaloids	+
Flavonoids	+
Tannins	-
Saponins	+
Triterpenoids	+
Steroids	-
Phenols	+

Note:
+ : contains the tested substances
- : absents the tested substances

Phytochemical screening (Farnsworth, 1966) was done to determine groups of substances in rosemary extracts. Extraction method was used by using maceration. Maceration was done using 96% of ethanol in 3X24 hour at room temperature. The filtrate then was evaporated at 50 0C using a rotary evaporator. This temperature is suitable to be used to avoid destruction or chemical modification of simplicial compounds. Based on the work of Intan *et al*. (2017), extraction conditions are important to maximize extraction yields and enrich the phenolic components. Several factors need to be considered when employing extraction techniques including the solvent types and ratios, extraction temperatures, extraction times, and solid to liquid ratios to ensure a complete extraction of the compounds of interest, while avoiding chemical modification (Intan et al., 2017).

Evaporation was done to yield thick extracts which then be weighed. Ethanol 96% was chosen for nontoxic property and for having boiling point which is lower than water. This leads to faster extraction. Ethanol has good extraction capability for almost all chemicals which has low molecular weight such as secondary metabolites (CLSI, 2009). Maceration was done for 3X24 hours at room temperature. The filtrate was evaporated using rotary evaporator at 50 °C, and finally redissolved in ethanol for further analyses. Thick extract as final products were then weighed. It can be seen that the extracts contain alkaloids, flavonoids, saponins, triterpenoids, and phenols.

Evaporation yields 16.5% *Rosmarinus officinalis* crude extracts, while fractions result is shown in Table 3 below.

Table 3. Fractions Result

Fractions	Results (%)
N-Hexane	3,25
Ethyl Acetate	2,60
Water	21,54

The crude extract must be processed (by purification and fractionation). Fractionation was done by using liquid-liquid extract method (CLSI, 2009). The substances were separated based on their polarity. This was supported by the work of Letricia *et al.* (2013) that fractionation and purification is needed to analyze bioactive compounds in crude extracts of samples.

Antibacterial activity test was done using microdilution method. The concept of this method is based on the work of Jorgensen *et al*. (2011) which used two different laboratory standard tests to ensure that the antibiotics exceed standards for purity and effectiveness against bacteria. Broth microdilution method, the most popular method, is used because of its accuracy and clear results. Broth microdilution test involves the use of small volumes of broth dispensed in sterile microdilution plates with conical bottom wells. Each well should contain 0.1 ml of broth (Jorgensen *et al*., 2011).

Microdilution technique was used to determine minimum inhibitory concentration antibacterial in *Rosmarinus officinalis* extracts, n-hexane, ethyl acetate, and water fractions using antibiotics comparison, tetracycline, with its concentration of 1024 µg/(ml). This was done on sterile microplates for each bacteria examined to avoid contamination among them. MHB media was used for its specific in bacterial isolation which increases aerobic bacterial growth or facultative organisms. This is supported by Sagar (2018) which stated that Mueller Hinton Broth is used for determining minimal inhibitory concentrations (MICs) of bacterial growth.

Table 4. Microdilution of Fractions

K(-)	K(+)											
1	**2**	**3** $2^{\mu g}/_{mL}$	**4** $4^{\mu g}/_{mL}$	**5** $8^{\mu g}/_{mL}$	**6** $16^{\mu g}/_{mL}$	**7** $32^{\mu g}/_{mL}$	**8** $64^{\mu g}/_{mL}$	**9** $128^{\mu g}/_{mL}$	**10** $256^{\mu g}/_{mL}$	**11** $512^{\mu g}/_{mL}$	**12** $1024^{\mu g}/_{mL}$	
-	+	+	+	+	+	+	+	+	+	+	+	**A**
-	+	+	+	+	+	+	+	+	+	+	+	**B**
-	+	+	+	+	+	+	+	+	+	-	-	**C**
-	+	+	+	+	+	+	+	+	+	-	-	**D**
-	+	+	+	+	+	+	+	+	+	+	+	**E**
-	+	+	+	+	+	+	+	+	+	+	+	**F**
-	+	-	-	-	-	-	-	-	-	-	-	**G**
-	+	-	-	-	-	-	-	-	-	-	-	**H**

Note:
A and B: N-Hexane Fractions; C and D: Ethyl Acetate Fractions; E and F: Water Fractions; G and H; Tetracycline (control).
K (-) = negative control
K (+) = positive control
(-) = clear (no growth of bacteria)
(+) = cloudy (positive growth of bacteria)
Column 1: MHB Media as anegative control
Column 2: MHB Media + Bacteria as positive control
Column 3-12: MHB Media+Bacteria+Fractions

Mueller Hinton medium is recommended by FDA (FDA. 2001), World Health Organization and NCCLS for testing most commonly encountered aerobic and facultative anaerobic bacteria in food and clinical material. The medium shows good batch-to-batch reproducibility, it is low in sulfonamide, trimethoprim, and tetracycline inhibitors and yields satisfactory growth of most non-fastidious pathogens. Beef infusion and Casein provide nitrogenous compounds, vitamins, carbon, sulphur and amino acids in Mueller Hinton media. Starch is added to absorb any toxic metabolites produced. This method was supported by Márió *et al.* (2017) that broth microdilution is a method currently available for the identification and antimicrobial susceptibility testing.

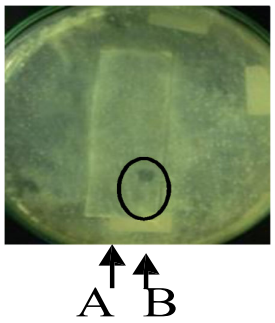
Table 5. Microdilution result of *Rosmarinus officinalis* fractions

Fraction	MIC (µg⁄ml.)
N-heksan	> 1024
Etil asetat	512
Water	> 1024

Note: MIC: Minimum Inhibitory Concentratio

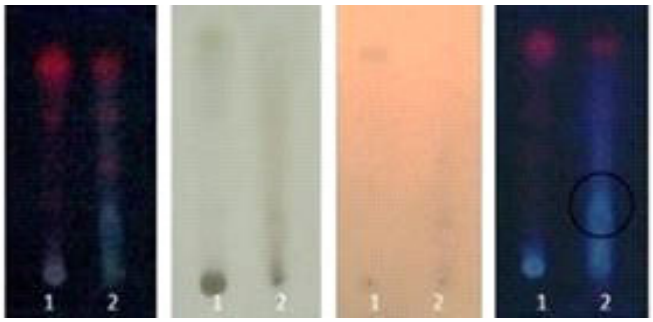
The results of antibacterial test by microdilution method showed that rosemary extracts have minimum inhibitory concentration of 512 µg/ml towards *Propionibacterium acnes*. MHB has been endorsed by the Clinical and Laboratory Standards Institute (CLSI), the global nonprofit organization that ensures quality in healthcare testing, as the appropriate media for routine bacterial antibiotic susceptibility determination, with updated cutoff standards (CLSI, 2015; Victor, 2017).

After testing antibacterial activity, bioautography test was followed to predict substances in the fraction extracts. Observation to identify substances in the rosemary extracts and the fractions (Rozman and Jersèk, 2009) was done initially to stick a plate on an agar surface using stationary phase of silica gel GF254 and moving chloroform-methanol ratio of 9:1.



Picture 1. Antibacterial activity test using bioautography;
A: Rosemary extracts; B: Ethyl acetate fractions

The result shows that ethyl acetate fraction has antibacterial activity for *Propionibacterium acnes*. Rf value in clear zone of ethyl acetate fractions toward the bacteria is 0.2. This Rf value then was matched with ethyl acetate observation using Thin Layer Chromatography (TLC) method, continued by spot identification using 10% of H_2SO_4 in methanol, 10% of $FeCl_3$ and 5% of $AlCl_3$ as spots viewer. This was done to determine the active substances as antibacterial from the fractions. 10% of H_2SO_4 is used as universal spot viewer that can show all components of substances. By using 5% of $AlCl_3$ under UV rays of λ 366 nm it can be seen that the spots fluorescens in yellow-green color which shows the flavonoids content. Meanwhile, the plate that has been sprayed by 10% of $FeCl_3$ shows black spots that indicate phenolic compounds.



(a) (b) (c) (d)

Picture 2. Chromatogram with stationary silica F254 chloroform enhancer-methanol (9:1); (1) Rosmarinus officinalis extract (2) ethyl acetate fractions of rosemary. Spots viewer; (a) λ366 nm of UVrays (b) H2SO4 10% (c) FeCl310% (d) AlCl3 5

The Rf values from bioautography is then compared with Rf values from the observations and spot viewer spraying. Active compounds on ethyl acetate fractions towards Propionibacterium acnes have an Rf value of 0.2 and is almost similar with compound spots which is positive to $AlCl_3$. From the results it can be determined that the antibacterial active compounds toward Propionibacterium acnes in ethyl acetate fractions is flavonoids (Yang, et al., 2009). Secondary metabolites activity such as flavonoids, tannins, alkaloids, terpenoids

and phenolic compounds have antibacterial activities toward gram positive bacteria (Cushine and Lamb, 2005).

Table 6. Kruskal-Wallis Test Result

Test Statistics[a,b]

	konsentrasi
Chi-Square	6.136
df	2
Asymp. Sig.	.047

Non-parametric test of Kruskal-Wallis in Table 5 has done to determine if there are statistically significant differences between two or more groups of an independent variable on continuous or ordinal dependent variable. It can be seen from Table 5 that non-parametric statistical test reveals p value of 0.047 ($p<0.05$). This means that there is a significant difference among three groups of fractions obtained from rosemary extracts.

Table 7. Mann-Whitney Post Hoc Test Result

Test Statistics[a]

	konsentrasi
Mann-Whitney U	.000
Wilcoxon W	1.000
Z	-1.225
Asymp. Sig. (2-tailed)	.221
Exact Sig. [2*(1-tailed Sig.)]	.667[b]

Mann Whitney post hoc test in Table 6 has done. The test is used to compare whether there is a difference in the dependent variable for two independent groups. Table 6 shows Asymp.sig. 0.221 (> 0.05). This means that there is no significant difference between ethyl acetate fractions and control in minimum inhibitory concentration of 512 µg/ml. The fact that ethyl acetate is able to control bacterial growth is supported by Jean *et al* (2012) which clearly mentioned that the ethyl acetate extract possesses antioxidant and antimicrobial principles. These results provide promising baseline information for the potential use of rosemary extracts in the treatment of infections associated with the studied microorganisms.

4 Conclusion

It can be concluded from the study that:

1. Rosmarinus officinalis extracts have capability to inhibit Propionibacterium acnes. This inhibitory was found in ethyl acetate fractions with minimum inhibitory concentration is 512µg⁄ml.
2. Bioautography of ethyl acetate actively inhibits Propionibacterium acnes with stationary phase of silica gel G F254 and moving phase of chloroform-methanol

with Rf value of 0.2 on spots viewer of AlCl3. It can be assumed that active antibacterial phytochemical compounds toward ethyl acetate fractions are flavonoids

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