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THE EFFECTIVENESS OF FLAVONOID AND TERPENOID ISOLATE SARANG SEMUT (*MYRMECODIA PENDENS* MERR and PERRY) AGAINST *PHORPHYROMONAS GINGIVALIS* ATCC 33277

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ABSTRACT

Ethyl acetate fraction of sarang semut plants (*Myrmecodia pendens* Merr and Perry) were reportedto contain flavonoids and terpenoids which have antibacterial activity against dental cariesbacteria. The aim of this study is to isolate flavonoids and terpenoids from *M. pendens* tubers and to test their effectiveness against *Porphyromonas gingivalis* ATCC 33277bacteria which are the main cause of periodentitis. Flavonoid and terpenoid were isolated using chromatographic methods. Characterization of flavonoids and terpenoids is done using UV, IR, NMR, and MS spectroscopy. Minimum inhibitory concentration (MIC) and minimum bacterioside concentration (MBC) were tested using micro dilution method. The result showed that flavonoids and terpenoids isolated from *M. pendans* effectively inhibited the growth of these periodontitis phatogens at a minimum inhibitory concentration (MIC) of19.57 and 39.06 µg/mL respectively. These compounds also killed the periodontitis phatogens at a minimum bactericidal concentration (MBC) of 312.5 and 625 µg /mL respectively. Our study concluded that flavonoids and terpenoids isolated from *M. pendens* were effective antibacterial agents against *P. Gingivalis* ATCC 33277.

INTRODUCTION

Sarang semut (*Myrmecodia pendens* Merr and Perry) are endemic plants found in Papua, an area located in eastern part of Indonesia. *Myrmecodia pendens* are widely used by the people in West Papua as medicinal plants. These plants are believed to cure various diseases such as diabetes, cancer, tumors, gout, diarrhea, fever etc. The plants can be found across Malay Peninsula, the Philippines, Cambodia, Sumatra, Java, Papua, and the Solomom island. *Myrmecodia pendens* belong to *Rubiaceae* family plants with five genus. Out of these five genus, only two genus are inhabited by ants: *Myrmecodia* (which has 45 species) and *Hypnophytum* (which has 26 species). Among these species, only *H. formicarum*, *M. pendens*, and *M. tuberosa* that have medicinal values (Engida *et al*., 2013; Roslizawati *et al*., 2013).

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The results of phytochemicals analysis show that *M. pendens* extracts contain flavonoids, tannins, phenolic, glucosides, and terpenoids. Prior research conducted by Engida *et al*., (2013) has successfully analyzed five flavonoid compounds from methanol extracts of *M. pendens* using high performance liquid chromatography (HPLC). Similar research to investigate the antibacterial activity of flavonoids from *M. pendens*, has also been conducted by Roslizawaty et al., (2013). The results show that ethanol extracts and the stew of *Myrmecodia* sp. have antibacterial activity against *Escherichia coli*. Other studies also reported that ethyl acetate extracts from *M. pendens* can inhibit the growth of *Streptococcus* and *Enterococus* bacteria (Dharsono, 2014). *Porphyromonas gingivalis* bacteria are the main cause of chronic periodontitis. Besides scaling and root planning, treatments to cure periodontitis also include the use of mouthwash (chlorhexidine) and systemic antibiotics (Li and Christine, 1997; Park *et al*., 2008; Villinsky *et al*., 2014). The use of

systemic antibiotics has deficiency because the *P. gingivalis* bacterium may become resistant to this drug. This requires researchers to find another alternative treatment from plants that can cure periodontitis without any side effects (Katsura*et al*., 2001; Singh and Pandeya., 2011). *Myrmecodia pendens* is one of the plants that has potential as an antibacterial. Its potential needs to be further explored by isolating the flavonoid and terpenoid, and test their effectiveness against *P. gingivalis* ATCC 33 277 bacteria.

MATERIALS AND METHODS

Materials

The research specimen is *M. pendens* tuber collected from Ayawasi village, South Sorong district, West Papua province, Indonesia. The chemicals used in this research were ethyl acetate, *n*-hexane, acetone, methanol, distilled water, silica gel G60 (70-320 mesh), thin layer chromatography (TLC) silica plate, octadecylsilane (ODS) RP-18, 10% H2SO4 in ethanol, alcohol 70%, chlorhexidine 1000 ppm, and Mueller-Hinton agar.

Extraction and Pre-Purification

Dried ground leaves of *M. pendens* tuber cutted into small pieces (with a diameter of ± 1 cm) as much as 1.5 kg. As much as 300 g sample extracted during 2x6 hours used soxhlet flask 5 L. Extracion repeatedly 5 times using ethyl acetate as solvent with soxletation methods. The soluted filtered and then evaporated by rotary evaporator at a temperature of 40°C to give a residu. Concentrate of ethyl acetate extract obtained as much as 15 g.

Isolation of Flavonoid and Terpenoid

Concentrated ethyl acetate extract (15.0 g) was separated using open column liquid chromatography with silica gel G60 (70-230 mesh) as stationary phase, and using a gradient solvent system composed of n-hexane and ethyl acetate with polarity increase of 10% (v/v). The total volume collected for each fraction was 500 mL. The separation of ethyl acetate extract using the column chromatography resulted in 11 fraction (A-K). The separation of stain spot analysis performed using thin layer chromatography (TLC) with silica gel G60 and a gradient solvent composed of n-hexane and ethyl acetate (7:3).

Table 1. Inhibition zone values of flavonoids and terpenoids against *P. gingivalis* ATCC 33 277

Compounds	Inhibition zone (mm) at concention (ppm)								
	10000			5000			1000		
	Ke-1	Ke-2	Rata-rata	Ke-1	Ke-2	Rata-rata	Ke-1	Ke-2	Rata-rata
flavonoid	9,3	9,1	9,2	8,5	8,4	8,5	5,6	5,7	5,7
terpenoid	10,4	10,5	10,5	10,1	10,2	10,2	9,0	9,8	9,4
klorheksidin*	nt	Td	td	Td	td	td	10,2	10,0	10,1

*) positif control nd) not tested

Table 2. Determination MIC values of flavonoid against *P. gingivalis* ATCC 33277

	Hole	Consentrasi (ppm)											
		5000	2500	1250	625	312,5	156,25	78,12	39,06	19,53	9,76	4,88	2,44
A	M+S	2.068	1.069	0.380	0.221	0.160	0.130	0.107	0.685	0.079	0.093	0.059	0.055
		1.944	1.212	0.514	0.267	0.187	0.148	0.119	0.695	0.079	0.186	0.062	0.066
B	M+P	0.139	0.104	0.132	0.123	0.089	0.099	0.066	0.074	0.080	0.106	0.049	0.047
		0.100	0.095	0.085	0.070	0.087	0.106	0.061	0.054	0.076	0.102	0.056	0.069
C	M+S+B	1.616	1.084	0.497	0.311	0.287	0.152	0.165	0.203	0.104	0.327	0.087	0.062
		1.719	0.947	0.526	0.315	0.209	0.167	0.179	0.216	0.130	0.312	0.078	0.057
D	M+P+B	0.093	0.095	0.100	0.089	0.085	0.085	0.066	0.068	0.047	0.340	0.082	0.047
		0.101	0.105	0.086	0.102	0.089	0.083	0.056	0.051	0.051	0.357	0.072	0.058

Table 3. Determination MIC values of terpenoid against *P. gingivalis* ATCC 33277

	Hole	Concentrasi (ppm)											
		5000	2500	1250	625	312,5	156,25	78,12	39,06	19,53	9,76	4,88	2,44
A	M+S	0.977	1.167	1.536	1.321	1.059	0.532	0.201	0.121	0.090	0.070	0.117	0.205
		0.921	1.158	1.528	1.331	1.047	0.447	0.180	0.117	0.088	0.062	0.142	0.202
B	M+P	0.054	0.052	0.050	0.050	0.051	0.055	0.052	0.049	0.048	0.052	0.059	0.054
		0.058	0.057	0.050	0.050	0.049	0.053	0.057	0.055	0.052	0.049	0.053	0.059
C	M+S+B	0.963	1.170	1.497	1.348	1.006	0.439	0.242	0.161	0.208	0.212	0.220	0.193
		0.939	1.225	1.472	1.396	0.989	0.547	0.188	0.178	0.210	0.214	0.218	0.185
D	M+P+B	0.150	0.070	0.187	0.126	0.167	0.223	0.204	0.181	0.185	0.188	0.179	0.172
		0.158	0.075	0.189	0.141	0.178	0.225	0.216	0.204	0.205	0.200	0.211	0.231

Instrumentation

Spectrum measurements were performed using a variety of spectroscopy tools. Ultraviolet (UV) and infrared (IR) spectra were measured with Shimadzu FTIR, 1H and 13C-NMR spectra were measured using JEOL JNM A-500 which works at 500 MHz (for 1H-NMR spectrum) and at 125 MHz (for 13C-NMR spectrum) with TMS as an internal standard, and ES-MS spectrometry (UPLC MS/MS TQD type Waters), Laminar air flow, Bunsen lamp, Memmert incubator, autoclave HVE-50 Hirayama, and Diagnostic Automation ELISA reader.

Factions G and J were further purified using various composition of the solvent system with normal-phase and reversed phase column chromatography.

Sensitivity test of Flavonoids and Terpenoid against *P. gingivalis* ATCC 33 277

The sensitivity test was conducted using the Kirby-Bauer method, wherein the bacterial growth inhibition zone was used as a parameter to determine the antibacterial activity. Bacteria that have grown on solid media were given a test compound solution on a paper disk with various concentrations: 1000,

5000, and 10000 µg/ml. Chlorhexidine was used as a positive control at a concentration of 1000 µg/ml in the solvent water, and methanol/water are used as negative controls (3:1). After the incubation for 72 hours at a temperature of 35-37 °C in anaerobic, clear zone around the paper disk which has been given a test solution (test compound, positive control and negative control), was observed and measured using calipers (Thomas et al., 2011; Villinsky et al., 2014). This clear zone indicates the bacterial growth inhibition zone produced by the test compound.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against *P. Gingivalis* ATCC 33277

The micro-dilution method was used in this study to determine the MIC and MBC values of flavonoid and terpenoid. A liquid medium for bacterial growth (Mueller-Hinton agar) was placed into each of the 96 wells of the microplate (8 wells/line and 12 wells/column) (Souza et al., 2008; Park et al., 2008). The initial concentration of flavonoid and terpenoid was 10,000 µg/ml. After performing a series of dilutions using methanol/water (75:25) we obtained test compounds with the following concentrations: 5,000; 2,500; 1,250; 625; 312.5; 156.2; 78.1; 39; 19.5; 9.7; 4.8 and 2.44 µg/ml which we placed into the microplate wells in columns 1-12 respectively. After all solutions properly mixed, we closed the microplate and incubated it at 37 °C for 72 hours. We then determined the MICs using the values given by a spectrophotometer, ELISA microplate reader, which was operated at wavelength of 610 nm. Next, broth aliquot from the wells were placed on the nutrient agar and incubated at 37 °C for 72 hours. We determined the MBCs by identifying the lowest concentration at which the bacteria failed to grow.

RESULTS AND DISCUSSION

Isolation and Characterization of Isolates

The *M. pendens*tuber is extracted by soxhletation methods using etyl acetate solvent. Soxhletation method is choosen because the time used more quickly and the result obtained is over. It is based on several repetitions in the isolation of target compounds and compared with extraction by maceration.Mass of ethyl acetate extract tubers *M. pendens* gained as much as 20 g. From the ethyl acetate extract *of M. pendens* were isolated two compounds with a mass of 25 mg and isolates **2** with a mass of 130 mg. Furthermore, the individual isolates were characterized by spectroscopy UV, IR, 1D-nuclear magnetic resonance (NMR) and 2D-NMR and mass spectroscopy (MS). Isolate **1** obtained yellow colour, solid in methanol soluble. Based on the mass spectrum, isolate **1** molecular weight of 525.2507. These molecular weights indicated to calculation the molecular weight of the alleged number of atoms isolate **1** consisting of 30 carbon atoms, 20 hydrogen atoms and nine oxygen atoms with molecular formula $C_{30}H_{20}O_9$. Based on the range of chemical shift emerging, suggesting that compound isolate**1**indicated flavonoid biflavon (Villinsky et al., 2014). Isolate **2** indicated as yellow oilic, completely soluble in methanol. Based on the results of mass spectroscopy measurements, isolate **2** has a molecular weight m/z 469.99 indicating molecular formula $C_{31}H_{50}O_3$. The results of elucidation and comparisons structure with reference, compounds isolate **2**leading to terpenoid type labdane diterpenes (Patra *et al*., 2015; Dong *et al*., 2008). Chemical structure of flavonoid and terpenoid isolates from*M. pendens* are shown in Figure **1** below:

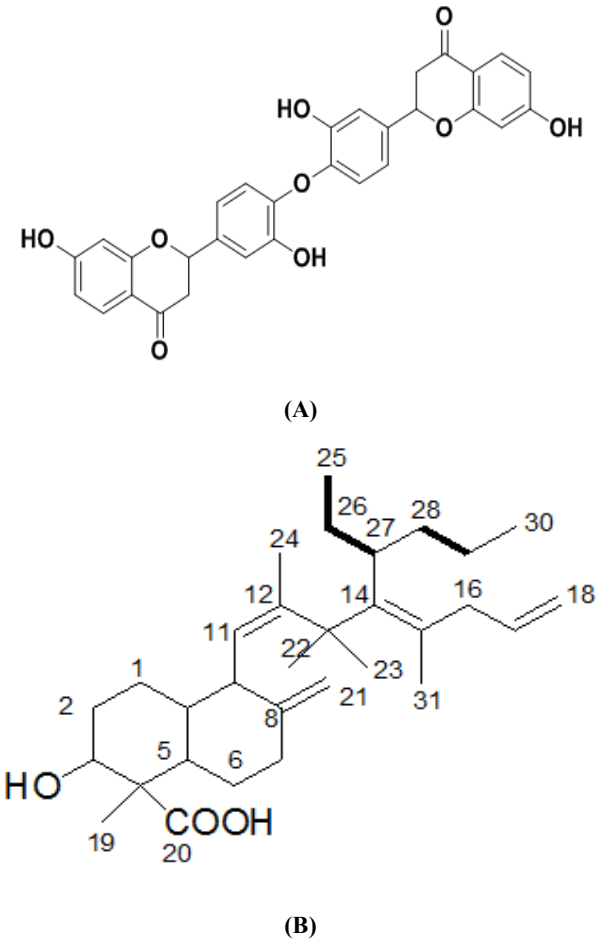

Figure 1. Structure of flavonoid (a) and terpenoid (b) isolate from *M. pendens*

Sensitivity testing Flavonoids and Terpenoid against *P. gingivalis* ATCC 33 277

The results of antibacterial sensitivity testing of flavonoids and terpenoids isolates *M. pendens* based on the inhibition zone of both compounds on bacterial growth of *P. gingivalis* ATCC 33 277 is shown in Table 1. At all concentrations, both of these compounds (flavonoids and terpenoids) have a inhibition zone against *P. gingivalis* ATCC 33 277.

Determination Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Flavonoids and Terpenoid of *P. gingivalis* ATCC 33 277

Determination of MIC value seen with comparison of the absorbance value on line C (medium, sample, and bacteria) with line D (media, solvents and bacteria), determination of MIC flavonoids shown in Table 2. Absorbance value of the solution in the well marked with yellow lines and shows the same relative value. The result shows amount of bacteria that grow on lines C and D alike. While the value of absorbance of the solution in the marked well with a red line there are differences value that indicate differences in the number of bacteria that grow on the C and D (Souza *et a*l., 2008; Park *et al*., 2008). The MIC value for each compound is the concentration of the test which is circled in red. MIC flavonoid 19.53 ppm. The determining value of the test were taken by looking for the lowest concentration of test compound that can

kill bacteria. After the MIC value is obtained, then the value is a reference to determine the value of MBC (Filho *et al*., 2002; Huang *et al*., 2006). The lowest concentration of flavonoids used in MBC ranges from 19.53 to 5000 ppm. The results showed that flavonoid MBC value is 312.5 ppm due to the medium at a concentration of more test compounds have demonstrated the presence of bacterial growth. The same treatment was done to terpenoids for determining the MIC and MBC value. The MIC flavonoids of 39.06 ppm. The MIC measurement results using spektroscopy ELISA reader is shown in the following table:

Determining the value of teaching is taken by looking for the lowest concentration of test compound that can kill bacteria. After the MIC value is obtained, then the value is a reference to determine the MBC value. Terpenoids lowest concentrations used in MBC ranging from 39.06 to 5000 ppm. The results showed KBM value of terpenoids is 625 ppm for the medium with concentrations of test compound has demonstrated the presence of bacterial growth. Based on the result antibacterial in vitro data, flavonoids and terpenoids provide inhibitory zone as well as the positive control (chlorhexidine 0.2%). Furthermore flavonoids and terpenoids using micro-dilution method MIC values obtained 19.53 and 39.06 ppm respectively. The MBC value of flavonoids and terpenoids are 312.5 and 625 ppm respectively. Ability of flavonoid compound as antibacterial relation with flavonoid structure can inhibit DNA nucleat acid synthesis. The result of Chusnie *et al* (2005) shown that flavonoid can inhibit DNA synthesis of *Proteus vulgaris*, and on *Staphylococcus Aureus* effect in RNA synthesis. Ring B of flavonoid has fuction in intercalation or hydrogen bounding with basic boundingof nucleat acid which describe action for inhibition of synthesis DNA and RNA. Terpenoids compounds including a group of diterpenoid compounds. Some literature explaining that the group of diterpenoid compounds and their derivatives have antibacterial activity (Urzua *et al*., 2008). In addition, terpenoid type diterpenoid compound isolated from oleoresin diesel tree by Souza *et al* (2011) was obtained as (-) - copalik acid and antibacterial activity with MIC values of 3.10 µg ml^{-1}. Flavonoids and terpenoids isolates *M. pendens* efektive as an antibacterial agent against *P. gingivalis* ATCC 33277.

Conclusion

In this research we successfully isolated flavonoid and terpenoid from *M. pendens* and tested their effectiveness against the main cause of periodontitis which is *Porphyromonas gingivalis* (*P. gingivalis*) ATCC 33277 bacteria. The flavonoid and terpenoid effectively inhibited the growth of the bacteria with MIC values of 19.57 µg/ml and 39.06 µg/ml respectively, and killed the bacteria with MBC values of 312.5 µg/ml and 625 µg/ml respectively. We concluded that flavonoid and terpenoid isolated from M. pendans are effective antibacterial agents against *P. gingivalis* ATCC 33 277.

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