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To cite this article: A P Asmara *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **251** 012016

IOP Conf. Series: Earth and Environmental Science **251** (2019) 012016 doi:10.1088/1755-1315/251/1/012016

Antibacterial Bioactivity of *n*-Hexane Extract from Mahogany (*Swietenia humilis* Zucc.) Seed and Its Fatty Acid Compound Identification

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Abstract. An investigation of antimicrobial bioactivity followed by GCMS analysis for the fatty acid composition has been conducted to the crude *n*-hexane extract of *Swietenia humilis* Zucc. seed. The goal of this research to determine the inhibition zone diameter for antimicrobial activity and the percentage of fatty acid contained in the extract. The seed of *S. humilis* Zucc. have been extracted by soxhletation with *n*-hexane as the solvent. The extract has been tested through in vitro antimicrobial bioassay toward *Staphylococcus aureus* and *Escherichia coli* by paper disc diffusion method. The fatty acid composition has been identified by fatty acid methyl esters method through gas chromatography coupled with mass spectroscopy. The result shows that the extract is more effective to inhibit the *S. aureus* activity with diameter range from 10 to 17 mm while *E. coli* tends to be resistance. The extract contains palmitic acid 15.49%; palmitoleic acid 0.28%; octadecanoic acid 14.04%; oleic acid 37.02 %; linoleic acid 24.70%; linolenic acid 7.33%; arachidic acid 0.84%; and tricosanoic acid 0.33%. The research confirms that *S. humilis* Zucc. seed oil contains polyunsaturated fatty acid and can be used as an antimicrobial agent.

1. Introduction

Currently, people's interest to consume traditional medicine is increasing, partly may happen because of considering the side effects and high prices of chemical drugs today. Various medicinal plants have been used to prevent or cure a lot of disorders and also used as a natural source of antibacterial agent [1,2]. Furthermore, World Health Organization (WHO) published that medicinal plants are the sources of bioactive compounds against bacterial infections used by as many as 80% of people in developing countries [3]. One variety of medicinal herbs is *Swietenia humilis* Zucc. which is also known as Mahogany or Venadillo from Meliaceae family [4]. The plant's part which contains most of the secondary metabolite compounds is the seed [5].

Antimicrobial constituents from the plants which are safer to the human body with few side effects are unsaturated fatty acids [6]. The mahogany seed contains variant secondary metabolites which are found as essential unsaturated fatty acids (Polyunsaturated Fatty Acid-PUFA) and as potentially antibiotic agent [7]. *S. humilis* Zucc. seed also consists of many PUFA compounds with different composition [4] which are potential as reducing agent of cholesterol and blood triglyceride levels. However, there is a lack of information about its bioactivity against micro bacterium.

The antimicrobial bioactivity towards *E. coli* and *S. aureus* of acetonic, methanolic, and ethanolic extract of the seed of *S. humilis* Zucc. has already been revealed that all of them are effective for controlling the number of the microorganisms [8]. Furthermore, ethanol–ozonolyzed extract of *S. humilis* Zucc. seed is also capable of inhibiting the growth of these bacteria effectively [9]. While several extracts of the botanical part taken out by polar solvent are confirmed as an antibiotic, further investigation is needed to analyze the ability of non-polar extract regarding bacteria inhibition activity. Therefore, in the context of the fundamental importance of this bioactivity, in this paper, we report the bioactivity of *n*-hexane extract of *S. humilis* Zucc. seed versus *E. coli* and *S. aureus* bacteria using paper disc diffusion. In addition, the fatty acids composition were also investigated by using gas chromatography coupled with mass spectroscopy.

2. Materials and Methods

2.1. Equipment and Chemicals

The study was conducted by using a set of soxhlet extraction tools (Borosil, Lucknow), jars, scissors, strainer, separating funnel, blender (Miyako), rotary evaporator (RII0 Buchi, Switzerland) with vacuum pump, oven (GCA Corporation, USA), measuring cylinders (Pyrex), Müller-Hinton agar media (Acumedia, USA), cellulose discs (6 mm sterile diameter Whatman cellulose filters number 5, Germany), Agilent 6890/capillary column HP-5 gas chromatography (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, California, US), and UV-Vis spectrophotometer (517 nm on a Cary-50-Bio Varian).

The materials were *n*-hexane (Merck), H_2SO_4 (Merck), $BaCl_2$ (Merck), chloroform (Merck), dimethylsulfoxide/ DMSO (Merck), anhydrous Na_2SO_4 (Merck), *Staphylococcus aureus* ATCC 2921 (USA), *Escherichia coli* ATCC 700609, DPPH free radical solution (2.9 mL, 60 μM, in methanol, Sigma-Aldrich), standard antibiotics Ampicillin as a positive control while DMSO as negative control. All tests were done in duplicate training.

2.2. Procedure for Sample Preparation and Extraction

S. humilis Zucc. seeds were collected from Wonosari, Gunungkidul, Yogyakarta Special Region Province, Indonesia. The sample was identified by the Center for Plant Conservation Botanic Gardens–LIPI Bogor with specimen number B-525. The seeds were washed and dried at room temperature and kept away from direct sunlight. The cotyledon then was taken out and cut into 0.5 to 1 cm in diameter size and dried for a few days. The cotyledon pieces were ground into 40–60 mesh then dried at 40 °C for 24 hours. As much as 450 g of sample powder was extracted through soxhletation using *n*-hexane at 60–70 °C for 7–8 hours. The *n*-hexane extract then was dehydrated over anhydrous Na_2SO_4, filtered, and concentrated through the rotary evaporator. The yield (w/w%) was calculated and then stored in a dark-glass bottle at 4 °C.

2.3. Preparation for McFarland Turbidity Standard

A total of 9.95 mL of 1% H_2SO_4 was mixed with 0.05 mL $BaCl_2$ 1% in a reaction tube and stirred until homogeneous. This standard suspension is used to determine the suspension concentration of tested bacteria (> 3 × 108 CFU/mL) to be made by comparison of bacterial suspension turbidity with standard suspension McFarland.

2.4. Preparation of Bacterial Suspension

S. aureus ATCC 29213 and *E. coli* ATCC 700609 are suspended by adding 0.9% of NaCl solution in different tubes. Turbidity is adjusted to McFarland 0.5 turbidity standard to obtain bacteria with a concentration of 108 CFU/mL. Turbidity is determined by taking a small amount of suspension into a smaller reaction tube, filling it into a hole in the nephelometer, and looking at its turbidity number. If it is less cloudy, the suspension is added to the colony whereas the cloudier one will be added by 0.9% NaCl.

2.5. Preparation for Sample Prime Liquor

Each sample of an extract of *n*-hexane *S. humilis* Zucc. seed was taken as 100 mg then dissolved in 10 mL of solvent (9 ml H_2O + 1 ml DMSO). The prime liquor was diluted to 10, 20, 50, 100, and 1000 µg/mL, then stored in the freezer for subsequent antibacterial trials.

2.6. Preparation of MHA (Müller-Hinton Agar)

A total of 9.5 grams of Müller-Hinton Agar/ MHA (38 g/ L) with medium composition (beef infusion 300 gram, casamino acid 17.5 gram, starch 1.5 gram, and agar) is dissolved in 250 mL distilled water and heated to reach the boiling point and then sterilized in autoclave for 20 minutes in 1 atm of air pressure and 121 ºC of temperature.

2.7. Inhibitory Test of n-hexane S. humilis Zucc. *Seed Extract against S. aureus and E. coli*

The antibacterial test was performed by Kirby-Bauer method of paper disc diffusion [10]. The results are based on the measurement of the inhibitory diameter (DDH) of the bacterial growth formed around the disc paper. The *n*-hexane extract of *S. humilis* Zucc. seed was taken as much as 20 µL and dripped onto sterilized disc paper and waited until it becomes saturated. Media for sterile Müller-Hinton was poured into a petri dish with ± 0.5 cm thickness allowed to solidify at room temperature. Then the sterile stick cotton was immersed in the suspension of the tested bacteria and then inoculated in a flattened MHA medium. Wait a few minutes to dry, then place the saturated disc paper with the *n*-hexane extract. Both negative and positive control were also placed on the paper disc for both bacteria. The test was performed with two replications. Furthermore, it was incubated at 35-37 °C for 24 hours. After 24 h, the DDH formed around the disc is observed using SCAN 500® analyzer version 6.3.8.0.

2.8. Analysis of Saturated and Unsaturated Fatty Acids

Fatty acid analysis and fatty acid methylation were carried out by following procedures from AOAC [11] at the Integrated Research and Testing Laboratory-Universitas Gadjah Mada (LPPT-UGM). A sample of 5 mg is added by 10 mL of NaOH 0.5 M; 12 mL of BF_3 14%; and 4 mL of *n*-heptane and then refluxed for 5-10 min at 60 °C. After reaching the room temperature, the mixture is extracted with a saturated NaCl solution to form two layers. The top layer is taken out and then transferred to a reaction tube containing anhydrous Na_2SO_4 then filtered with a glass of fiber. The filtrate is placed into Eppendorf, and a total of 0.1 µL of sample is injected in Agilent 6890 gas-spectroscopy mass chromatography equipped with flame ionization detector and HP-5 silica capillary column (30 m × 0.25 mm × 0.25 µm). The injectors and detectors are set at 250 °C and helium are used as carrier gas (3 mL/ min). The identification of fatty acid compounds is performed by comparing the spectral characteristics of sample components with standard data of C4-C24 methyl esters for 37 fatty acids [4].

3. Results and Discussion

3.1. Antibacterial Activity of n-hexane S. humilis Zucc *Seed Extract*

Antibacterial bioactivity analysis of *n*-hexane extract of *S. humilis* Zucc. seed was tested *in vitro* by paper disc diffusion method through which it was expressed in the inhibitory diameter (mm) as shown in Table 1. The inhibitory test was carried out under varied concentration. It was found that *n*-hexane extract of *S. humilis* Zucc seed inhibited the gram-positive and negative bacteria actively. As shown in the inhibitory diameter, this seed extract is more active against *S. aureus* bacteria with an inhibitory diameter of 10.4 mm at 10 µg/mL, 15.2 mm at 500 µg/mL and 18.3 mm at 1000 µg/mL.

S. aureus bacteria belong to gram-positive bacteria that are sensitive to secondary metabolite compounds from the seed of mahogany species based on several antimicrobial bioactivity assays of various mahogany seed extracts. This matter is regarding the physicochemical factor and the bacterial anatomy that is appropriate to the action mechanism of bioactive compounds from mahogany seed. The gram-positive bacterial cell wall is known to consist of peptidoglycan and teichoic or teichuronic acid. The bioactive compounds of the mahogany seed are predicted to bind the penicillin-binding protein to the transpeptidase enzyme in the bacterial cell wall which causes the inhibition of the

transpeptidation reaction. This leads to inactivation and loss of inhibitors of the autolytic enzymes in the bacterial cell wall so that the wall might undergo lysis [12].

Table 1. Bacteria inhibitory of *n*-hexane *S. humilis* Zucc seed extract.

Tested Substance	Concentration (µg/mL)	Bacteria	Inhibitory Diameter (mm)		
			Result 1	Result 2	Average Result [a]
n-hexane extract of *S. humilis Zucc* seed	100	*E. coli*	0.12 ± 0.4	0.22 ± 0.5	0.17 ± 0.2
	500	*E. coli*	2.55 ± 0.7	2.8 ± 0.3	2.68 ± 0.4
	1000	*E. coli*	4.3 ± 0.3	4.02 ± 0.5	4.66 ± 0.6
Positive Control	1000	*E. coli*	28.2 ± 0.7	26.7 ± 0.4	27.45 ± 0.1
Negative Control		*E. coli*	–	–	–
n-hexane extract of *S. humilis Zucc* seed	100	*S. aureus*	10.6 ± 0.2	10.2 ± 0.4	10.4 ± 0.2
	500	*S. aureus*	13.3 ± 0.1	13.1 ± 0.5	13.2 ± 0.4
	1000	*S. aureus*	17.4 ± 0.5	17.2 ± 0.2	17.3 ± 0.2
Positive Control [b]	1000	*S. aureus*	31.5 ± 0.9	31.1 ± 0.3	31.3 ± 0.4
Negative Control [c]		*S. aureus*	–	–	–

[a] Data values determined by mean ± standard deviation with two repetitions (n=2). [b] Ampicillin 1 g/mL. [c] DMSO.

3.2. Fatty Acid Composition of n-hexane S. humilis Zucc. *Seed Extract*

The fatty acid composition of the extract was investigated through GC-MS spectroscopy analysis. The result obtained by fatty acid methyl esters (FAME) method is shown in Figure 2. The chromatogram shows eight peaks that appeared based on comparative analysis of the fatty acid retention time in the sample with a mixture of 37 FAME standard C4-C24 under the same conditions. The composition of those eight fatty acid compounds of this analysis as presented in Table 4, sequentially shows the percentage of saturated, monounsaturated, and polyunsaturated fatty acids were 30.98%, 37.02%, and 32.03%. The saturated fatty acid compounds that found at the higher percentage are palmitic acid (C16:0) 15.49% and octadecanoic (stearic) acid (C18:0) 14.04%. Those contents are close to a report of diethyl ether and n-hexane extract of *S. macrophylla* King seed in which the level is 14.62% and 15.47% of palmitic acid, 16.75% and 17.65% of stearic acid and 0.59% and 0.54% of palmitoleic acid [6]. Similar results are also reported that the content of saturated fatty acids in *S. mahagoni* Jacq. are 14.32% of stearic acid and 12.97% of palmitic acid [7].

Consumption of palmitic and stearic acid is reported not to increase the blood fat levels due to its solid state at body temperature (37 °C) and insoluble in blood plasma [4]. Consumption of palmitic acid can increase the levels of high-density lipoprotein (HDL) which may reduce the risk of coronary heart disease as well [13].

Furthermore, the *n*-hexane extract consists of monounsaturated fatty acid (MUFA) that contains 37.02 % of oleic acid (C18:1 cis-9). It means that this is also composed of more oleic acid than the same seed in Mexico which is only 29.27% of the acid [4]. Several studies to the seed of two other types of mahogany, *S. macrophylla* King and *S. mahagoni* Jacq., report that both species contain oleic acid with different amounts depending on their habitat [6,7,14]. As a result, the presence of these compounds indicates that the seed of *S. humilis* Zucc. is potential for a natural herbal remedy to reduce the risk of coronary heart disease [15].

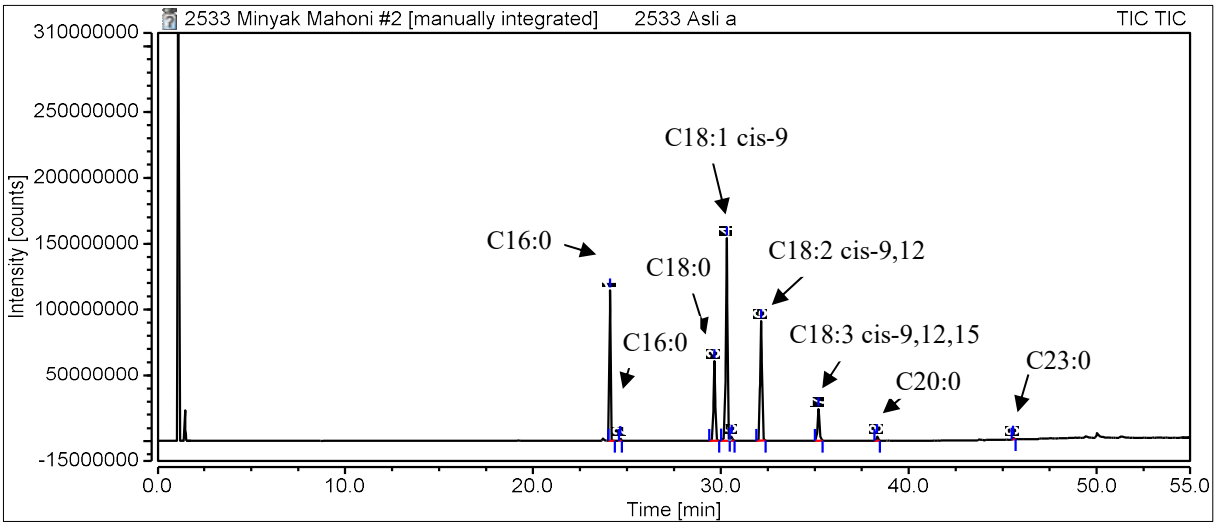

Figure 1. Gases Chromatogram of Fatty Acid Compound from *n*-hexane *S.humilis* Zucc. Seed Extract.

Table 2. Fatty acid composition of *n*-hexane *S. humilis* Zucc seed extract.

Fatty Acid Compound	Type of Fatty Acid	Composition Percentage (%)
Palmitic Acid	Saturated	15.49
Palmitoleic Acid	Saturated	0.28
Octadecanoic Acid	Saturated	14.04
Oleic Acid	Monounsaturated	37.02
Linoleic Acid	Polyunsaturated	24.70
Linolenic Acid	Polyunsaturated	7.33
Arachidic Acid	Saturated	0.84
Tricosanoic Acid	Saturated	0.33

Meanwhile, 32.3 % of PUFA in this extract includes 24.70 % of linoleic acid (C18: 2 cis-9,12) and 7.33% of linolenic acid (C18: 3 cis-9,12,15). These fatty acids are very important for human's health because both of them are precursors for the other essential fatty acids such as DHA (docosahexaenoic acid, C22:6, omega 3) and EPA (eicosapentaenoic acid, C20:5, omega 3) from linolenic acid and arachidonic acid (C20: 4, omega 6) of linoleic acid [4]. Previous research states that both of these compounds are PUFA finding in the seed of the three species of mahogany in the world. Both of them are also associated with the antibacterial bioactivity of *S. macrophylla* seed against *S. aureus, Salmonella typhimurium*, and *Pseudomonas aeruginosa* because the diethyl ether and *n*-hexane extracts of the seed provide a competitive inhibitory zone compared to Streptomycin as its positive control [5].

PUFA compounds in mahogany seed effectively inhibit gram-positive bacteria such as *S. aureus* whereas gram-negative bacteria such as E. coli tend to be more resistant to hydrophobic groups in these fatty acids [2]. In this case, *S. aureus* is inhibited by a termination of the transpeptidase reaction within its cell wall. In addition, the reaction is activated by a transpeptidase enzyme. The enzyme is a receptor for the penicillin drug compound which acts via a bonding interaction between the hydroxyl group in the serine residue and C carbonyl group in the β-lactam ring. Subsequently, it is a nucleophilic addition reaction between a carbonyl group and an ammonium group in the lysine residue which is incorporating carboxylic ions with cations [2]. The bonding of this penicillin establishes a blockade of peptidoglycan that should be bound to the active side (serine and lysine) of the receptor. Based on a literature review about the structure and its activity relationship, the presence of the C=C

double bond of the *cis* isomer and the carboxylic group in PUFA is analogous to the penicillin structure so that the action mechanism in the transpeptidase enzyme inhibition may also occur toward PUFA in mahogany [2,16].

4. Conclusion

In summary, we examined the activity of *n*-hexane extract of the seed of *S. humilis* Zucc. which is potential as a natural antibacterial source against *S. aureus*. The analysis of its fatty acid compounds showed that the seed contains 15.49 % of palmitic acid, 0.28 % of palmitoleic acid, 14.04 % of octadecanoic acid, 37.02 % of oleic acid, 24.70 % of linoleic acid, 7.33 % of linolenic acid, 0.84 % of arachidic acid and 0.33 % of tricosanoic acid. Further research is needed to determine the action mechanism of *S. aureus* inhibition activity between unsaturated fatty acid compounds toward transpeptidase enzyme through computational chemical-based molecular modeling.

5. Acknowledgments

The authors are grateful to the Directorate of Islamic Higher Education of the Ministry of Religious Affairs of the Republic of Indonesia which has fully funded this research through the Quality Assurance Scheme of Basic Development Research based on the Decree of Dirjen Pendis number 4653 in 2017 and Unit for Research and Technology of Natural Product of Indonesia Science Institute (BPTBA-LIPI) Gunungkidul which has facilitated the implementation of this research.

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