Isolation of Artemisinin as Antimalarial Drugs from Artemisia annua L. Cultivated in Indonesia

Deliana Dahnum, Haznan Abimanyu and Ahmad Senjaya

***Abstract*-- Malaria disease is endemic in developing countries like Indonesia. This disease is caused by protozoa of the genus Plasmodium infection that is easily recognizable from the symptoms of prolonged fever. Some of the methods taken to stop spread of this disease. Artemisinin is a sesquiterpene lactone found in the leaves and flowers of plants Artemisia annua L and have different chemical structures and higher efficacy than others. Artemisia annua L. is originated from subtropical and can be introduced into the tropics such as Indonesia. The content of artemisinin itself is very small at around 0.01 to 1.4% of dry weight of plants. The first step in this research is solvent extraction using methanol. Step followed by partition using hexane and column chromatographic separation process with ethyl acetate / hexane as eluent. Isolates were characterized using TLC, FTIR, UV spectrophotometer, and HNMR spectroscopy. Through the process is obtained S4 as a result of isolation hexane fraction which has a character similar to artemisinin with 2.0 mg (0.016% w / w)**

***Index Term*-- artemisinin, Artemisia annua L., malaria, methanol extraction**

I. INTRODUCTION

Malaria is one of the infectious diseases that are endemic in Indonesia. This disease is common in the tropics and subtropics that is a serious health problem for society and complex world. An estimated 300-500 million people worldwide are infected with malaria and about 1-1,5 million people die every year according to the WHO in 2003[1]. In Indonesia, malaria is still not able to solve and be a health problem that needs attention from the government and society. Until 2009, approximately 80% of districts / cities are still categorized as endemic malaria and 45% of the population lives in areas at risk of contracting malaria.

Many ways are used to against malaria disease. At the first, it is using pesticides such as DDT for mosquito eradication. This approach was discontinued because of the effect of mosquitoes resistant to DDT and some effects are not expected to break the food chain due to the extinction of one of the animals. Another way is utilizing of quinine and chloroquinine for protection against disease in patients [2]. Some are there using sulfadoxine/pyrimethamine [3]. This leads to protozoan resistance against drugs. Now, WHO recommended some medicine, i.e ACTs (Artemisinin-based combination treatments) to solve that problem.

Artemisia annua L. is the plants from temperate regions, but can be developed in the tropics through breeding (hybridization selection and adaptation). In Indonesia, demand for artemisinin is very large and all are imported, therefore the development of cultivation of Artemisia annua L. in Indonesia is a pretty big opportunity [4]. The potential cultivation of this crop is in Tawangmangu area, Central Java and Ciwidey, West Java, Indonesia.

Approximately 42% of the total artemisinin compounds found in upper of leave [5]. The highest artemisinin compounds found in plant A. annua aged 12 to 13 weeks [6]. The total amount of artemisinin is found in different varieties of A. annua which is about 0.01 to 1.4% by weight based on weight of dry leaves.

Artemisinin is a new antimalarial drugs that have different chemical structures and has a higher efficacy when compared with other conventional antimalarial drugs which have been resistant to Plasmodium falciparum. Artemisinin was isolated from Artemisia annua L., in China known as Qinghaosu. This plant is used as a traditional medicine that is consumed by the public and after analyzed the compound, artemisinin and its derivatives can inhibit the growth of P. falciparum both in vitro and in vivo [7].

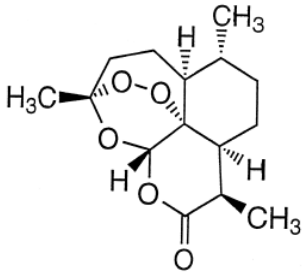

Fig. 1. Chemical structure of Artemisinin

Artemisinin, a sesquiterpenoid lactone peroxide, was shown to be effective in killing malaria parasites. Besides artemisinin itself, some derivatives such as artemether,dihydroartemisinin, arteether, and artesunate have ability to against Plasmodium falciparum [3]. These compounds can be obtained through chemical synthesis [8], recombinant DNA techniques [9], and isolation from natural materials. Method of isolation of natural material is the most economical way to get artemisinin than through chemical synthesis and recombinant DNA techniques.

Method of isolation from natural materials that practical and economical is extraction technique. Research done by using solvent extraction hexane then partitioned with acetonitrile

Deliana Dahnum, Haznan Abimanyu and Ahmad Senjaya are working in Research Center for Chemistry – Indonesian Institute of Sciences –LIPI, Kawasan PUSPIPTEK Serpong,
Tangerang Selatan 15314 Indonesia, Ph. : +62 21 7560929, Fax: +62 21 7560549
Pharmacy Faculty, University of Indonesia

and the obtained yield of 0.12% [10]. Another organic solvent is also use in the research. Another method is supercritical carbon dioxide extraction, which is one of method to obtain artemisinin from plants that have the efficiency and selectivity of extraction better than others. Nevertheless, both the extraction of artemisinin has not been obtained pure. Crystallization process is a necessary process for purifying artemisinin [11]. Based on economically two methods of extraction, the extraction using an organic solvent extraction is best done as a minimum in terms of cost and energy.

In this present research, artemisinin compounds was isolated using organic solvent extraction. The solvent used was methanol and then partitioned with hexane and purified using column chromatography. Results isolates were analyzed using TLC, FTIR, UV spectrophotometer, and HNMR spectroscopy.

II. MATERIALS AND METHODS

II.1 MATERIALS

The raw material used is plant Artemisia annua L. The plant was dried and mashed into powder. The chemicals used are methanol, hexane, and ethyl acetate.

II.2 METHODS

Extraction

100 grams of powder herb Artemisia annua L. macerated using methanol solvent. Maceration performed in erlenmeyer with a magnetic Stirrer speed of 700 rpm for 1 hour. This process is done many times until the methanol layer is colorless. The extract then was evaporated using a rotavapor vacuum at a temperature of 40 ° C until the extract volume to 100 ml.

Partition of Extract

Extract solution is partitioned using hexane 50 ml. Partitioning done many times until a colorless hexane layer. Two layers got from this process, hexane extracts (non-polar fraction) and methanol extracts. Furthermore, the methanol extract obtained was added 10 ml of distilled water and partitioned again using 50 ml of ethyl acetate. Partitioning done many times and stopped until the ethyl acetate layer is colorless. By this process was got the ethyl acetate extract (semipolar fraction) and methanol-water extract (polar fraction). Each extract was concentrated using a rotavapor at a temperature of 40 ^{o}C.

Fractionation of Extract

The most viscous extract containing artemisinin was fractionated by column chromatography. In column chromatography, used a silica gel 60 as the stationary phase and for mobile phase used a mixture of ethyl acetate-hexane with increasing polarity (gradient elution). Each fraction was collected, and identified the presence of artemisinin by a thin-layer chromatography (TLC) with standard artemisinin comparison. In thin layer chromatography, the stationary phase used 60 F254 silica gel and mobile phase is ethyl acetate: hexane (3:97). Fractions containing artemisinin separated. Fractions with the same profile spots combined and for the next concentrated then re-chromatography (preparative TLC) to obtain a fraction with a single spot containing artemisinin.

Artemisinin Content Analysis

IR spectrophotometer

2 mg isolates were crushed and mixed with 98 mg of KBr which had been dried for 24 hours at temperatures of 105 ^{o}C. Isolates were analyzed at wave number 4000 cm^{-1} to 400 cm^{-1}. Baseline used was KBr. The resulting spectrum of isolates compared with the spectrum of standard artemisinin.

UV spectrophotometer

1 mg isolates were dissolved using 10 ml methanol and analyzed at λ 200-400 nm, the resulting spectrum of isolates compared with the spectrum of standard artemisinin.

TLC one-way motion contrast with the Phase 2

1 mg isolates were dissolved using 5 ml of ethyl acetate. Isolates were spotted with a solution using the capillary tube on a Thin Plate Chromatography (TLC) silica gel 60 F254. The mobile phase used was ethyl acetate: hexane (3:97) and ethyl acetate: hexane (7:93).

Liquid Chromatography - Mass Spectrometry (LC - MS)

Artemisinin content analysis performed using HPLC-MS system Mariner Biospectrometry with ESI (Electrospray Ionisation) Positive Ion Mode is equipped with Hitachi LC 6200 pump. Sample of 1 mg dissolved in 1 ml of methanol and then injected as much as 20 µl and eluted using a methanol: water (9:1). With a flowrate of 1 ml / min, the separation is done through C18 column (RP 18) Supelco column length = 150 mm, ID = 2 mm, and particle size = 5 µm. The samples will be analyzed separately according to their retention times.

HNMR spectroscopy

The results were analyzed using NMR Brand insulation type JEOL JNM ECA 500 with 500 MHz magnetic fields. Analysis of these isolates using 1HNMR spectroscopy using $CDCl_3$ solvent and tetramethylsilane reference compounds. The spectrum of isolates compared with the spectrum of standard artemisinin.

III. RESULT AND DISCUSSIONS

The advantages artemisinin as an antimalarial drug encouraged to do research considering the very small levels of artemisinin is about 0.01 to 1.4%. Several methods of the study was conducted to obtain the maximum yield from the plant Artemisia annua L.

Artemisia powder used is a dark green powder dry <0.50 µm (figure 2). Particle measurements conduct to ensure any particle size.

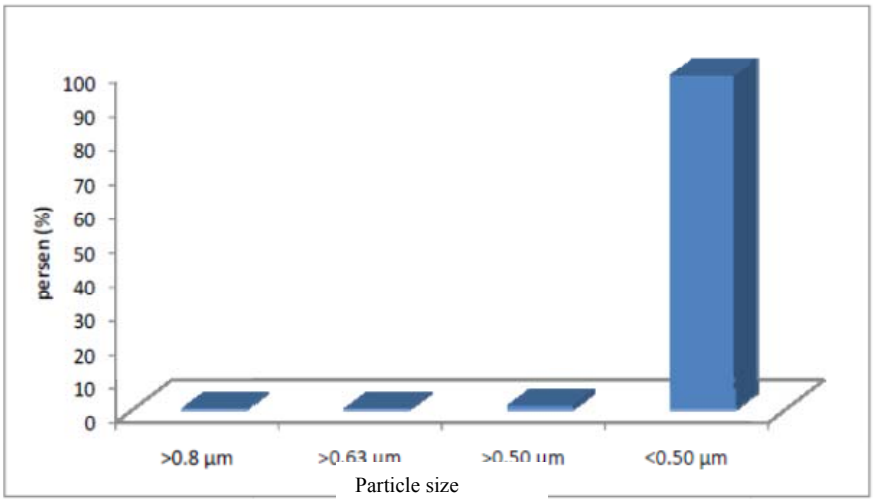

Fig. 2. Powder particle size of Artemisia annua L.

Powder particle size used is determine the maximum artemisinin extracted from plant cells. Glandular trichoma, ie the cells in Artemisia annua L. is the place to keep artemisinin as secondary metabolites. With the small size of the powder particles, the surface area for contact with the solvent becomes larger. This will facilitate the solvent extracting artemisinin out of his cell.

Maceration extraction performed assisted with Stirrer magnetic stirring. The solvent used was methanol which was distilled. Extraction is better done artemisinin maceration because the unstable nature of the high temperatures in solution. At estimated 20% of artemisinin will be broken at high temperatures in solution [12]. Maceration has done many times by adding a new solvent into the extraction. Each cycle takes 1 hour and the methanol used was 300 ml every once maceration.

Artemisinin is extracted with methanol until a clear supernatant solution. For 103 grams of powder of Artemisia annua L. required approximately 18 times extraction cycles, or about 5400 ml of methanol. Liquid extract obtained greenish-black. Before partition, the liquid extract was evaporated until the volume remaining approximately 100 ml using a vaccum rotavapor at a temperature of 40 ^{o}C. In this process, it is resulting dark greenish-black extracts.

The dark greenish-black extracts is a crude extract which the other components dissolved therein. Extracts were separated into fractions less soluble components so it can easier in the separation process of chromatography column. Liquid-liquid extraction (partition) is done by using hexane as solvent. Each cycle takes about 50 ml hexane. Partitioning done many times until hexane layer is clear. For 100 ml of methanol extract partition takes 60 cycles or about 3000 ml hexane. Both of layer was evaporated with a vacuum rotavapor at temperature of 40 ^{o}C. Thick extracts from hexane layer is non-polar fraction while thick extracts from methanol layer is polar fraction.

Both fractions were analyzed by LC-MS spectroscopy. From the analysis, artemisinin content was found on non-polar extracts. Extract polar is not contain artemisinin relatively. Peaks at M +1 (283), M +23 (305), 2M+1 (565), and 2M+23 (586), showed the presence of artemisinin in the extract hexane (figure 3). From 103 grams of powder Artemisia, obtained viscous non-polar fraction (hexane) as much as 6 grams (5.82% w / w).

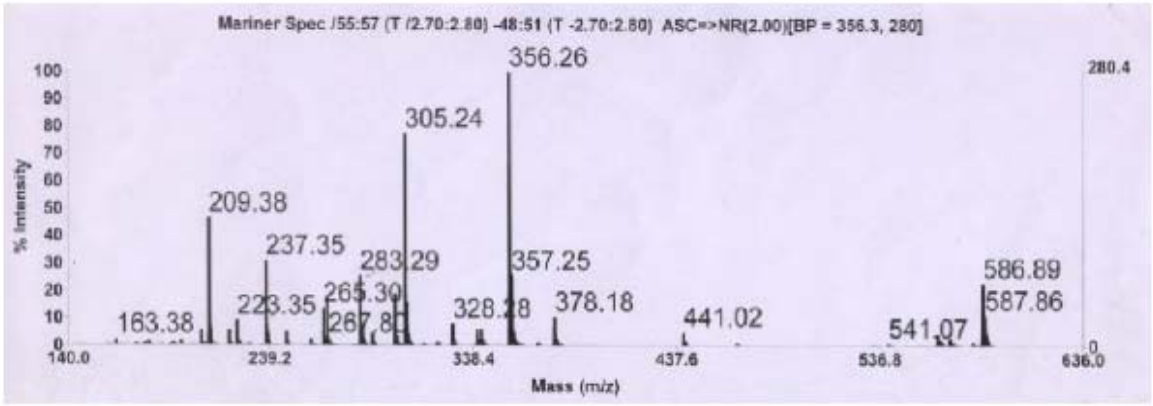

Fig. 3. LC-MS Result of Non Polar Fraction

Extracts of the constituent components hexane monitored using thin layer chromatography (TLC). It aims to obtain a suitable mobile phase so that at the time of separation in the chromatography column, artemisinin is obtained separately

from the other components. Extract non-polar fraction and polar fraction extract spotted on silica gel 60 F254, artemisinin standard for comparison. The mobile phase used hexane respectively 100%, ethyl acetate: hexane (5:95); ethyl acetate: hexane (10:90): ethyl acetate: hexane (15:85): ethyl acetate: hexane (20:80) and ethyl acetate: hexane (25:75). Artemisinin spots on non-polar fraction extract (extract hexane) is eluted (Rf = 0) by using a mobile phase hexane 100%. Ever increasing amount of ethyl acetate used in the mobile phase, the greater the value of Rf artemisinin spots, but the closer distance with other artemisinin spot. The same thing was also done in polar fraction extract but the methanol fraction did not show an artemisinin spots.

From concentration of mobile phase used above, the best separation obtained by the mobile phase composition of ethyl acetate: hexane (5:95) with Rf value of 0.44. Re-optimization of the mobile phase was done too for another concentration. There are phase ethyl acetate: hexane (2:98); ethyl acetate: hexane (3:97), and ethyl acetate: hexane (4:96). The best separation which artemisinin spots well separated from other spots on the concentration of ethyl acetate: hexane (3:97) with Rf value of 0.22.

TABLE I
Comparison of Rf values of artemisinin in the optimization phase

Mobile Phase	Rf
Hexane	0
Ethyl Acetate-Hexane (2:98)	0.13
Ethyl Acetate-Hexane (3:97)	0.22
Ethyl Acetate-Hexane (4:96)	0.27
Ethyl Acetate-Hexane (5:95)	0.44
Ethyl Acetate-Hexane (10:90)	0.58
Ethyl Acetate-Hexane (15:85)	0.87
Ethyl Acetate-Hexane (20:80)	0.96
Ethyl Acetate-Hexane (25:75)	1.00

Column chromatography has 3.5 cm in diameter and 50 cm long with a slow column type. Ratio of extracts and condensed silica used was 1:30. 5 grams of viscous hexane extract (non-polar fraction) was crushed and added acetone become powder. Slow column provides better separation than the quick column. Results of separation are taken in vial 10 ml. At vial 302-325 there is a crystal after the mobile phase is evaporated. One spot with the same standard artemisinin (Rf value 0.22) was obtained by single TLC, except in the vial 302, 303, and 304. Vial containing the same spot was combined. The results of these samples have weighed 172.4 mg (0.17% w / w) and then called So. So has melting range 148-151 ^{o}C. Melting range of S0 was different from the previous studies. There are 156-157 °C, 153-154 ^{o}C [12], 150-152 ^{O}C [13], 154 ^{o}C [10]. The difference is because S0 still has impurities.

Examination of IR spectra S0 was done by comparing the absorption bands that appear on S0 and artemisinin as a default (figure 4). IR spectrum of S0 largely still contains impurities when compared with standard artemisinin, which the artemisinin S0 absorption overlap with the absorption of impurities.

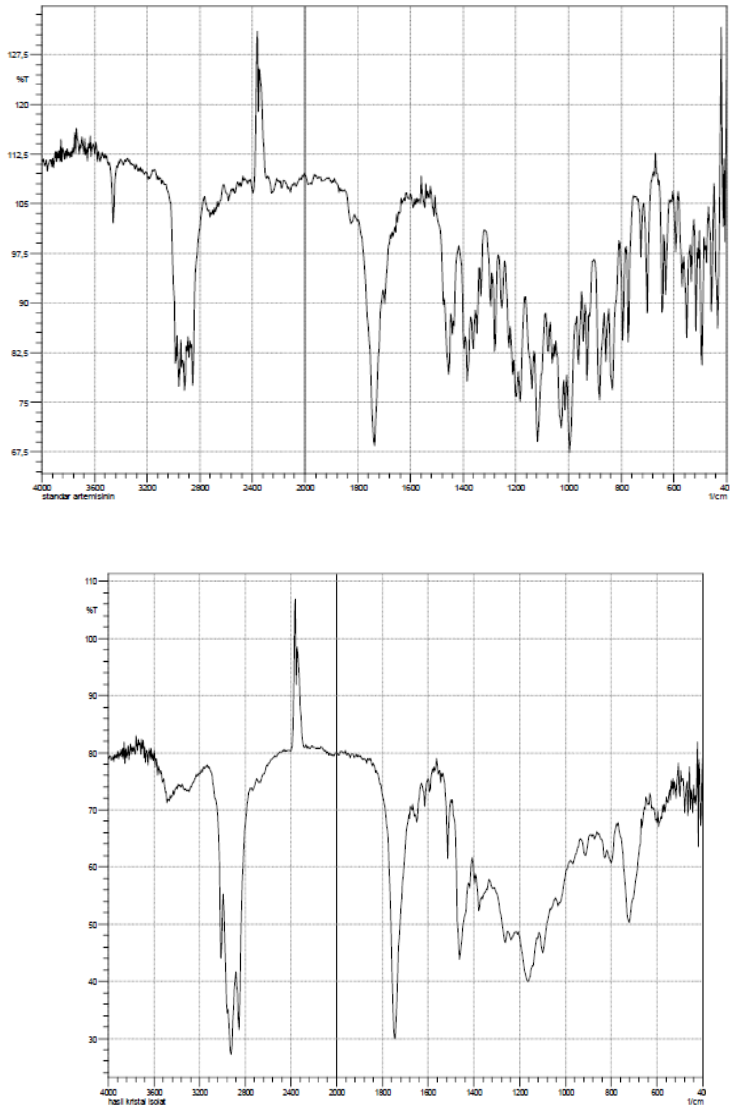

Fig. 4. Artemisinin Standard Spectrum IR (left) dan S0 Isolate (right)

Checking of S0 is also performed by using a UV spectrophotometer. The solvent used was methanol with a wavelength of 200-400 nm

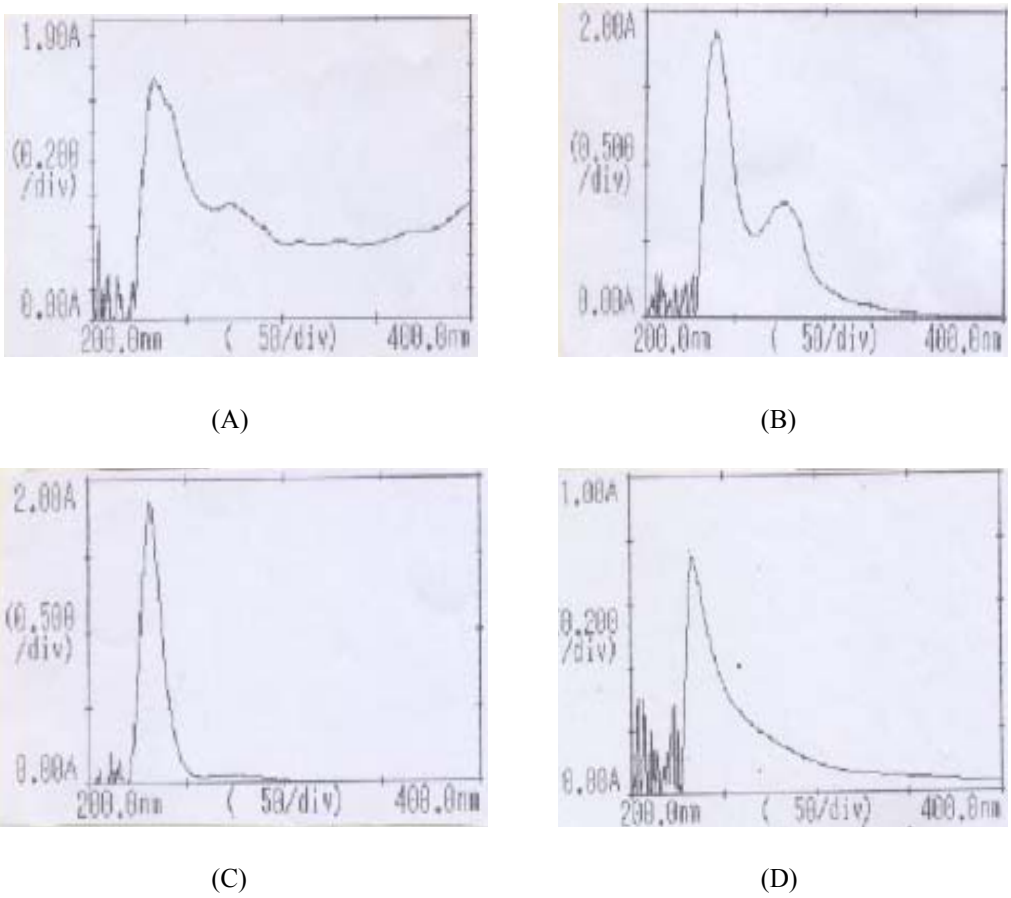

Fig. 5. UV spectrums of hexane extract (A), S0 isolate (B), S4 isolate (C), and standard artemisinin (D)

Standard artemisinin (D) gave maximum peak absorption at wavelength 232 nm. When compared with So, So had two peaks that provided maximum absorption at a wavelength of 232 nm and 276 nm. Like the IR spectrum analysis, it was possible there were impurities on S0 which was able to provide maximum absorption at a wavelength of 276 nm.

S0 was purified using preparative silica gel plates 60 F254. After eluted with ethyl acetate: hexane (7:93), S0 well separated into 4 spots, S1, S2, S3, S4. By comparation with standard artemisinin known that S3 and S4 have spots with Rf similar to artemisinin. Analysis using UV spectrum (Fig. 5), S4 showed similar results with standard artemisinin and is located at 232 nm. S4 was obtained 2.0 mg (0.016% w / w).

Analysis of S4 was also conducted using HNMR spectroscopy. The solvent used was $CDCl_3$ and the reference compound was tetramethylsilane (TMS). Results of S4 will be compared with standard artemisinin. It can be seen in Figure 6, S4 have the same spectrum with the spectrum of standard artemisinin. S4 has a peak at (δ: 0.86-0.89 / triplet), (δ: 0.91-0.99 / multiplet), (δ: 1.25 / singlet), (δ: 7.04 / singlet), and (δ: 7.44 / singlet). It looks a little different with standard artemisinin. It is possible since the fat or the presence of impurities of hydrocarbons located in the up field region (δ: 0.00 - 2.00) and aromatic compounds are located in the regions δ: 6.00 to 9.00. These results indicate that S4 was artemisinin and still contains impurities.

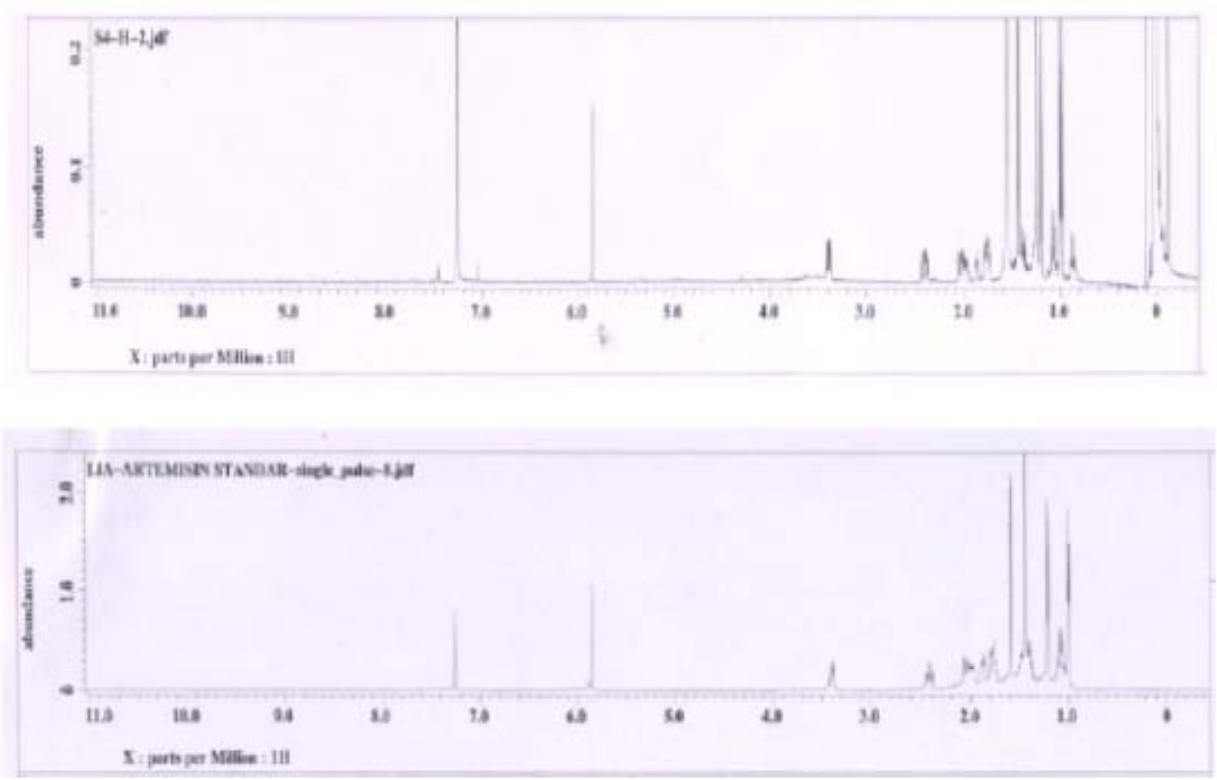

Fig. 6. Spectrum HNMR of S4 isolate (above) and Artemisinin Standard (Below)

IV. RESULTS

Based on the research done, artemisinin compound was obtained 2.0 mg (0.016% w / w) by methanol extraction and column chromatography methods.

ACKNOWLEDGMENTS

Thanks to the Ministry for Research and Technology of Indonesia for funding the research project through Insentif Program in 2010.

REFERENCES

1. Els Marchand, Magnus A. Atemnkeng, Stijn Vanermen, Jacqueline Plaizier Vercammen. *Development and validation of a simple thin layer chromatographic method for the analysis of artemisinin inArtemisia annua L. plant extracts*. Biomed. Chromatogr. 2008. p. 454–459
2. Bhakuni, R. S.; Jain, D. C.; Sharma, R. P. In *Artemisia*; Wright, C.W., Ed.; Taylor & Francis: London, 2002
3. Mingfu Wang, Chungheon Park, Qingli Wu, and James E. Simon, *Analysis of Artemisinin In Artemisia Annua L. By LC-MS with Selected Ion Monitoring*, J. Agric. Food Chem. 2005, 53, 7010-7013
4. Gusmaini dan Hera Nurhayati. 2007. Potensi Pengembangan Budidaya Artemisia annua L. di Indonesia. Perspektif Vol 6 No 2 (2007): 57-67.
5. Alexei A. Lapkin, *Reviews Comparative Assessment of Technologies for Extraction of Artemisinin* J. Nat. Prod. 2006, 69, 1653-1664
6. H. Bouwmeester Et Al, Research To Improve Artemisinin Production For Use In The Preparation Of Anti-Malarial Drugs, *Medicinal and Aromatic Plants 2006, pp.* 275-290.
7. Casteel, DA. (1997). *Antimalarial Agents*. Burger's Medicinal Chemistry and Drug Discovery. Vol. 5, Ed.5, Bab 59.
8. Marcel Hommel. (2008). *The Future of Artemisinins: Natural, Synthetic or Recombinant?.* Journal of Biology. 7:38.
9. Keasling JD, et al. (2006). *Production of The Antimalarial Drug Precursor Artemisinic Acid in Engineered Yeast*. Nature 2006. 440: 940-943.
10. ElSohly HN, et al. (1990). *A Large Scale Extraction Technique of Artemisinin From Artemisia Annua.* Journal of Natural Products. 53(6):1560-1564.
11. Yan Liu, Huisheng lu, Fei Pang. *Solubility of Artemisinin in Seven Different Pure Solvents from (283.15 to 323.15) K*. J. Chem. Eng. Data 54. 2009. p. 762–764
12. Klayman DL, AJ Lin, et al (1984). *Isolation of Artemisinin (qinghaosu) From Artemisia Annua Growing in United States.* Journal of Natural Products. 47(4): 715-717.
13. Acton N, et al. (1993). *Synthesis and Antimalarial Activity of Some 9-Subtituted Artemisinin Derivatives.* J. Med. Chem. 36: 2552-2557.