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SCREENING OF ACTIVE COMPOUNDS FROM *Artemisia annua* USING HFC-134A SUBCRITIC EXTRACTION SYSTEM

Arthur Lelono*, Sabar Simanungkalit, Ibnu Umarudin, Herlan Herdiawan

Research Center for Chemistry, Indonesian Institute of Science, Kawasan Puspiptek Serpong - Tangerang Selatan
*Corresponding author: aalelono@gmail.com

ABSTRACT

Artemisia annua is known as the source of artemisinin, a sesqueterpene lactone possessing endoperoxide moeity with antiplasmodium activity. Sub-critical extraction of artemisinin from *Artemisia annua* using R134a solvent was conducted. A close system cycle process was constructed to investigate the ability of R134a to extract artemisinin together with other active compounds. Artemisinin and other isolated compouds yield was investigated at 30°C and presure process at 10 barr, with variation of process time. The extracted product was analysed as artemisinin as the target compounds with several isolated compounds as by product. Artemisinin content was reported ranging up to 0,5%, and the sub critic system was reported to be more efficient and effective comparing to other conventional extraction methods. The phytonics HFC-134a sub critics process can be used for extraction in selected pharmacologically active products from herbal plants.

Keywords: *Artemisia annua, HFC-134a sub critic, extraction*

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INTRODUCTION

The quality of natural extracts and consequently their biological activity is related to their composition, and majorly dependent on the extraction procedure, the type of solvent, origin of the raw materials, its storage condition and the pre-treatment applied (Moure, 2001 & Louili, 2004) Conventional extraction, such as soxhlet and maceration methods, often requires high quantity of organic solvents, long periods, and temperature higher than ambient, which can degrade some bioactive compounds (Pessoa, 2015). Therefore, alternative methods to extract natural bioactive substances, such as supercritical (SE) and subcritical fluid extraction (SFE), have been studied. Supercritical fluid extraction demonstrates effective method yet high in investment cost and high level of safety process limitation. In view of the economic, safety and environmental needs, subcritical extractions can be used as alternative to

SFE, thereby allowing alternative design in the process with more reasonable costs. There are several reports regarding the extraction of natural products by subcritical water (Babu, 2014). However, reports on the R134a refrigerants as solvent in the subcritical extraction are sparse. R134a (1,1,1,2-tetrafluoroethane) is non toxic, non reactive, non flammable, and non ozone depleting. It has high volatility and boiling point at atmospheric pressure is -25.9°C, which mean it leaves negligible solvent residue in the products. R134a is in a gas form at room temperature, also stabile to aqueous acids and bases, and immiscible with water and sparingly soluble in water (1500ppm at 20°C). It is normally handled as a compressed gas under pressure in liquid form and has a liquid density of about 1.3kg/l (Corr, 2002). There is growing interest in the use of lower boiling fluids, particularly R134a, as liquid solvents across a range of applications. The extracts obtained by R134a are generally low in color, low in inert lipid content and high in desired impact or active species with good recovery of the active ingredients from the raw material. Extraction could be conducted anywhere within the liquid temperature range of R134a but is normally conducted around or below room temperature.

In the 1970s, from the plant *Artemisia annua*, artemisinin was discovered and extracted as a pure drug; since then the exploration and chemical synthesis of its derivates and studies on other biological activities has been carried out. The small yield of artemisinin in the plants and heat sensitive properties gives a challenging effort in the isolation process. Conventional extraction method has been reported to have high toxicity and environment issues, with non economical yield (Choursi, 2017). Many of the traditional organic solvents are under increasing regulatory pressure due to residual toxicity, whereas conventional steam distillation could affect the character of certain products through hydrolysis, thermolysis and loss of volatile compounds from the extract. Recent studies showed that R134a has potent to replace other conventional solvents in the extraction of natural flavors and pharmaceutical active compounds from plants, which also has been reported to give good result in extracting artemisin from *Artemisia annua* (Wilde, 2001). The process is advantageous in that the solvents can be customized: by using modified solvents with HFC134a, the process can be made highly selective in extracting a specific class of phytoconstituents. Similarly, other modified solvents can be used to extract a broader spectrum of components (Kumar, 2014). The biological products made by this process have extremely low residual solvent. The residuals are invariably less than 20 parts per billion and are frequently below levels of detection. These solvents are neither acidic nor alkaline and, therefore, have only minimal potential reaction effects on the botanical materials (Emmen, 2000). In this study, subcritical R134a extraction using 10L modified SS316 close system vessel was evaluated as alternative solid liquid extraction method of artemisinin and other compounds from *Artemisia annua*.

MATERIALS AND METHOD

Plant Samples.

Air dried *Artemisia annua* leaves were collected from Cibodas Botanical Garden, Cipanas West Java. The dried leaves were ground to 200mesh, and kept in a seal compartment prior to extraction. The leaves were stored in storage room and extracted no more than 7 days.

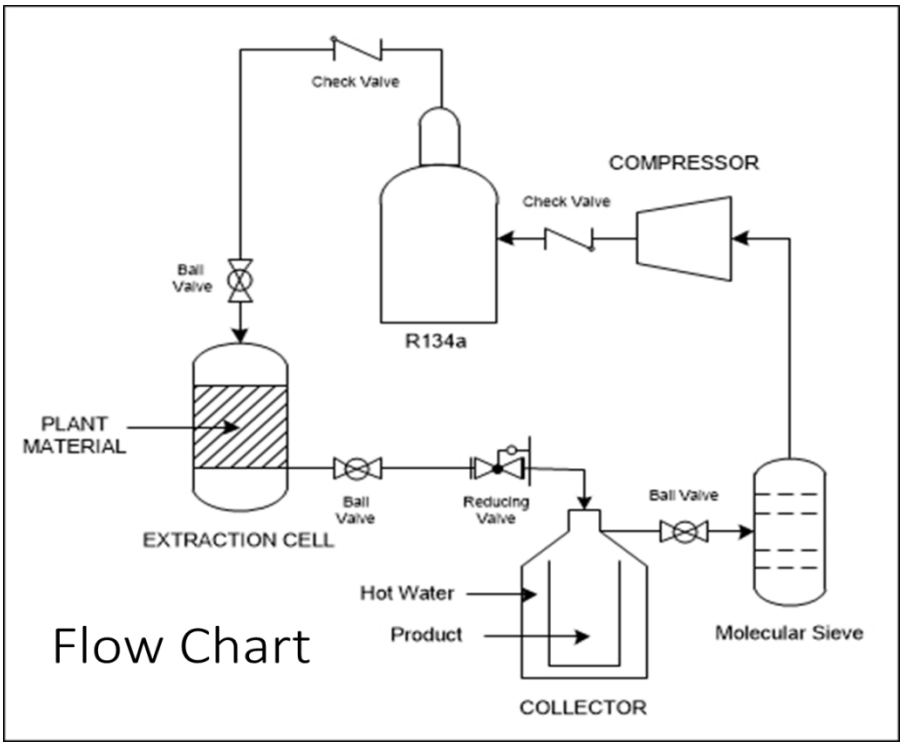

Figure 1 Schematic diagram of the HFC-134a subcritical extraction unit

Figure 2 Subcritical HFC-134a extraction unit system

Subcritical R134a Extraction of *Artemisia annua*.

All of the extraction experiments were conducted on a self-designed instrument. The schematic diagram of the HFC-134a subcritical extraction early state unit used in the present study and pictorial view are shown in Figure 1 and Figure 2 respectively. The unit consists of a 5L extraction vessel, a 20L of collector vessel, compressor and heat exchanger unit, and 1 filtration system. All vessels are constructed with SS316, and designed to hold pressure up to 40barr. Water bath heating mantle was constructed to obtained adequate temperature in the collector vessel to maximize the HFC-134a transfer rate of liquid to gas state.

Artemisia annua ground air dried leaves measuring 250 gram were subjected to HFC-134a subcritical extraction under room temperature. The unit was operated under various operating conditions to determine the effects parameter such as pressure of solvent during process, time process and ratio between HFC-134a and plants samples on yield percentage of the products.

Characterization of extraction products

Chromatography method was conducted prior to the characterizations of products obtained from HFC-134a subcritical extraction of *Artemisia annua*. TLC and HPLC analysis of crude extract product was conducted for preliminary analysis. Comparison of artemisinin standard was used as a guided fractionation. Purification was conducted using flash column chromatography, and isolated compounds molecular weight were analyzed using LC-MS.

RESULT AND DISCUSSION

Subcritical R134a Extraction of *Artemisia annua*.

Extraction of 250 gram air dried *Artemisia annua* leaves using HFC-134a subcritical system gives 1,525 gram of products. The product was observed having a dark yellow to green paste with needle like crystalline characteristic (Figure 3), is different from conventionally used organic solvent maceration method product which give dark green paste consistency. HPLC analysis and TLC chromatogram profiles indicate the presence of artemisinin in the products (Figure 4), a sesquiterpene lactone having peroxide bridge moiety as one of the target compound from *Artemisia annua*. Study from Lapkin (2006), reported that artemisinin has been successfully isolated from *Artemisia annua* under HFC-134a subcritical system.

The effect of extraction process time demonstrated that the product increased as time dependent. After 24 hours of extraction process, the yield product increased up to 1,63% (dry weight) from 250gram of *Artemisia annua* (Table 1). These results showed that the increase time contact between HFC-134a as the extraction solvent with the plant material gives direct result on how much material extracted in the extraction vessel. For further application the time process will selected at 1 hour reaction due to non effective and time consuming effort in the 24 hours process. The replication extraction at 1 hour process will meet up with the amount of product collected in 24 hours of process.

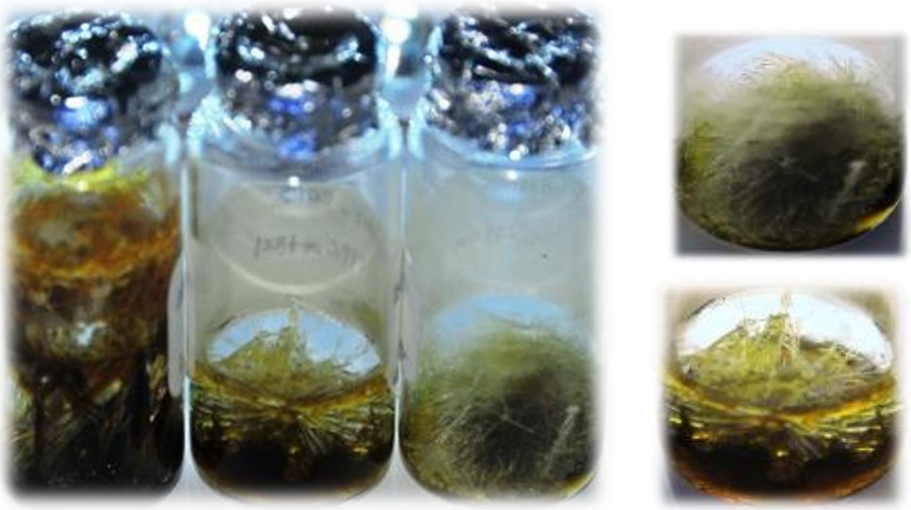

Figure 3. Product collected form extraction of *Artemisia annua* using HFC-134a subcritical system

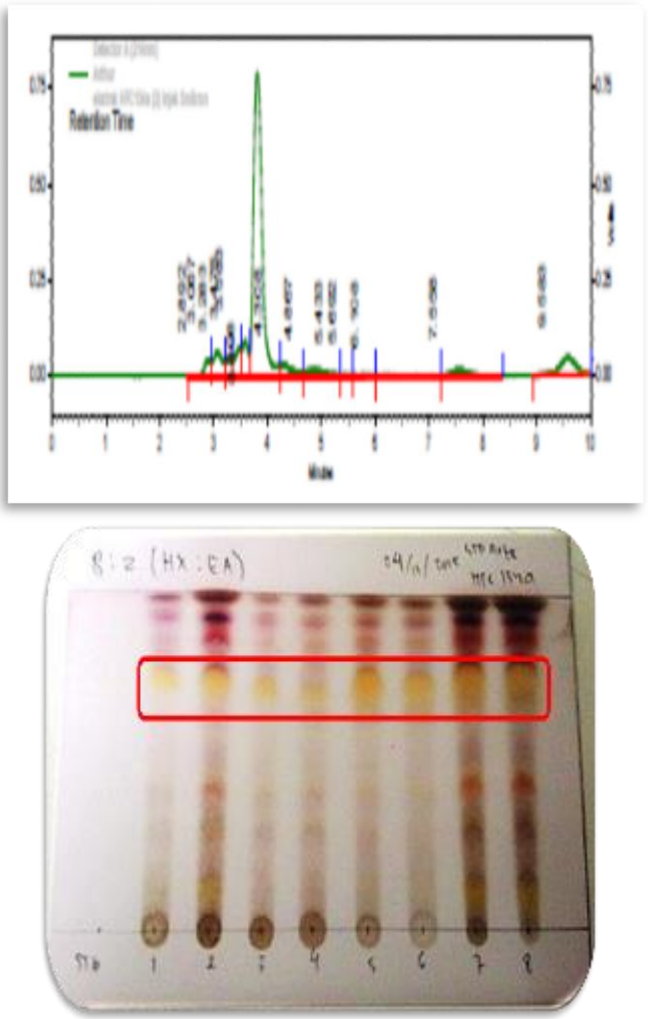

Figure 4. HPLC and TLC chromatogram from the product collected form extraction of *Artemisia annua* using HFC-134a subcritical system

Table 1. Effect of process time on yield product collected form extraction of Artemisia annua using HFC-134a subcritical system

n	Time (minutes)	Plant material (gram)	Product Extract Weight (gram)	Product Yield (D.W) (%)
1	90	250	1.525	0,61
2	180	250	2.025	0,81
3	360	250	2.529	1,01
4	720	250	3.072	1,23
5	1440	250	4.076	1,63

Table 2. Effect of HFC-134a and pressure on yield product collected form extraction of Artemisia annua using HFC-134a subcritical system

n	Plant material (gram)	HFC-134a Used (gram)	Release Pressure (barr)	Product Weight Extract (gram)
1	250	2500	1	1,612
2	250	2500	1	1,232
1	250	2500	2	1,411
2	250	2500	2	1,012
1	250	2500	4	1,711
2	250	2500	4	1,143
1	250	1250	1	0,411
2	250	1250	1	0,387
1	250	2500	1	1,611
2	250	2500	1	1,287
1	250	5000	1	2,021
2	250	5000	1	1,215

The yield content of the product is highly influenced by the amount of HFC-134a ratio to the ground *Artemisia annua* leaves samples, as described in Table 2. The ratio of 1:20 demonstrated to give maximum yield content compared to ratio 1:5. The higher ratio of HFC-134a is related with more fresh solvents in the extraction vessel to interact and acting as carrier solvents to extract selected compounds from *Artemisia annua*, therefore giving more yields in the extraction product. The pressure variation during the extraction process did not show significant difference in the overall yield content. Subcritical extraction release process of 1 barr pressure yields an average of 1,422gram of product compared to 1,427gram under 4 barr pressures, therefore it is suitable to use the 1 barr pressure for further extraction process due to safety procedure in the extraction process. This low pressure give more advantages in handling with liquefy HFC-134a gas and safety in constructing the pilot scale unit extractors. This is the first report of process parameters in the subcritical extraction of *Artemisia annua*, using the equipment described in Figure 2. Babu *et. al* in 2014 reported that the increased level of pressure up to 11 barr resulted in 91% yield of turmeric oil subcritical extraction.

Characterization of extraction products

Flash column chromatography was used for further purification of the product, yielding artemisinin and other isolated compounds. A total amount of 0,6051 gram of artemisinin crystal was isolated from 250 gram of *Artemisia annua* dried leaves, and was confirmed by LC-MS comparison of artemisinin commercial standard. Three other fractions above artemisinin TLC spot was collected and purified weighing at (Fraction A1 = 64mg), (Fraction A2 = 95,1mg), and (Fraction A3 = 328 mg), respectively. Each fraction were subjected for LC-MS analysis to determine their molecular weight using C-8 column [150mm x4.6mm] coupled with 5µL injection and 0.2ml/min flow rate of methanol. LCMS chromatogram in Figure 5, showed the peak area, whereas in Figure 6, showed the mass fragmentation of each fraction, respectively.

Fraction A1, showed major peak at Rt 2,92 min, with some impurities still observed in the chromatogram. From the mass spectrum analysis, it showed most intense peak are m/z 245,43 $[M+H]^+$ (Figure. 6) Fraction A2, showed two major peak area at Rt 2.88 minutes and 3.27 minutes, respectively. Peak at Rt 2.88 minutes gives base peak molecular weight m/z 267,31, $[M+H]^+$ and it was confirmed by addition of sodium ion m/z 289,29 $[M+Na]^+$, which is also similar to the most intense peak m/z 267,29 $[M+H]^+$ in Fraction A3. From the mass spectrum analysis, the most intense peak in fraction A3, was confirmed with addition of sodium ion m/z 289,29 $[M+Na]^+$ and m/z 559,69 $[2M+Na]^+$.The peak which appears at Rt 3.27minutes of Fraction A2 gives a molecular weight m/z 235,45 $[M+H]^+$, and it was confirmed by addition of Na^+ which gives 257,48 $[M+Na]^+$ and 491,92 $[2M+Na]^+$. TLC colorization using anisaldehyde-sulphuric acid indicates the presence of steroids and terpenes compounds. Further identification of isolated compounds is needed to identify the isolated compounds, since this the first report of the subcritical isolation from *Artemisia annua* besides artemisinin as selected compound. Bhakuni, et.al reported in 2001 several compounds has been successfully isolated form *Artemisia annua*, specially its essential oils, and characterized a large number of monoterpenoids.

CONCLUSION

The extraction unit designed and constructed based on HFC-134a subcritical has successfully isolate artemisinin from *Artemisia annua*. Time process, pressure release and ratio between samples and HFC-134a prove to gives significant difference in the yield product. As a byproduct of artemisinin isolation, three fractions were isolated and mass spectrum analysis showed Fraction A1 m/z 245,43 $[M+H]^+$, Fraction A2 m/z 235,45 $[M+H]^+$, and Fraction A3 m/z 267,29 $[M+H]^+$. Further identification of isolated compounds is needed to identify the isolated compounds.

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