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Improvement of Nicotine Yield by Ethanolic Heat Reflux Extraction of *Nicotiana tabacum* var. Virginia Origin of Ponorogo

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Abstract

Nicotine is known as an active compound contained in tobacco, which can be used for many purposes including pharmaceutical substances. Current extraction methods reported yields between 0.3 to 3.6 wt% of nicotine per dry weight of tobacco leaves. This study proposed an ethanolic heat reflux extraction (EHRE) method with temperature at chiller system 0°C and an ultrasonic applied before an EHRE process. The extraction was carried out at low temperature and moderate extraction time to increase the yield of nicotine from *Nicotiana tabacum* var. Virginia origin of Ponorogo. The highest yield of nicotine (6.3 wt%) was achieved at 6 hours. The yield was characterized using thin-layer chromatography and ultra-performance liquid chromatography. Optimum reflux in EHRE increased the nicotine yield to 60% more than the highest yield reported in the literatures. This significant improvement is important for further purification in nicotine production. The EHRE method in this study has a potential to be increased to a larger scale due to its ability to produce a high quantity of nicotine.

Keywords: *Nicotiana tabacum* var. Virginia origin of Ponorogo; tobacco leaves; ethanolic heat reflux extraction; nicotine improvement

INTRODUCTION

Nicotiana tabacum var. Virginia is one of the most widespread varieties of tobacco plants in the world. This variant is planted on about 1.8-2.0 million hectares. Currently, there are 1.5-2 million hectares of tobacco planted area around the world [1,2].

Studies have reported that *N. tabacum* var. Virginia contains more than 4000 chemical compounds. At least 200 chemicals in this tobacco variant are hazardous, including nicotine [3]. Nicotine (a colourless compound and hygroscopic oily form) is the major alkaloid compound that found in tobacco. As much as 95% of tobacco alkaloids are nicotine [4-6]; however, despite its hazards, nicotine can be used as pharmaceutical product at measured doses, for example, as a remedy to restore a person's memory. This is due to its capacity to stimulate memory receptors in the brain [7].

Nicotine can also be used as Nicotine Replacement Therapy, which is a patch medicine through the skin [8]; in this formulation, it is used for the treatment of addicts, especially as a remedy for active smokers' tobacco dependence [9], and also Schizophrenia and Alzheimer's disease [10]. An in vitro study reported the potential use of nicotine from *N. tabacum* extract for treating hyperglycemia. The results showed that the extract can inhibit α-amylase (IC_{50} 5.70 $mg.ml^{-1}$) and α-glucosidase (IC_{50} 4.50 $mg.ml^{-1}$). This result suggests that nicotine in the extracts has the potential to control diabetes mellitus [11].

Nicotine is synthesized in roots of tobacco plants along with associated pyridine alkaloids, such as nornicotine, anabasine and anatabine. They are then transported by the xylem through aerial parts with capillary system and mostly stored in vacuoles [12]. The vacuoles as a cell organelle are commonly found in stomata guard cells, especially at magnoliophyta division plants including tobacco. In the middle of stomata guard cells, there are the holes namely stomata (plural of stoma) that found on the surface layer of plants leaf. Nicotine

as a defensive toxin in vacuoles can be excreted through stomata against herbivorous insects [13,14].

Nicotine as a tobacco alkaloid in vacuoles can be extracted by heating under reflux with assisted by sonication process before the extraction. Reflux involves heating is required to open the pores around stomata on surface layer of tobacco leaves. Ultrasonic waves that generated in the sonicator can physically damage the cells (cells lysis) in tobacco leaf. Thus, ethanol as a solvent can be easily absorbed into the lysis stomata guard cells; and deeply permeated into the membrane layer (tonoplast) as outer layer of the vacuole; and then entering the vacuoles to extract nicotine and other polaric compounds [15,16]. Ethanol can be minimized from volume loss due to the evaporation process during reflux extraction using a condenser. The optimal yield of the extraction is also determined by a constantly sustained solvent. Therefore, the choice a type of condenser and its cooling agent are important in the reflux extraction. A type of condenser that has been widely used in heat reflux extraction is allihn condenser. The surface area of the condensed vapor components can be increased by using this type of condenser; it is due to a series of bulbs which is designed inside the condenser [17].

Nicotine can be extracted from *N. tabacum* leaves via several methods as literatures extraction yield, such as ultrasonic extraction [4,18]; ionic liquid extraction [19]; pyrolysis with fluidized bed extraction [20]; supercritical fluid extraction [21]; stirred vessel maceration [22]; and mechanical shaker extraction [23,24]. Nevertheless, the yield from those methods only ranges between 0.3 to 3.6 wt%. Nicotine content in the major cultivated tobacco such as *N. tabacum* and *N. rustica* has been reported theoretically; it ranges between 0.5 to 8 wt% [3,8,25]. Therefore, effort is still required to increase nicotine yield.

The main compounds of nicotine, phenolic and diterpene were reported in previous studies. However, the compositions of *N. tabacum* extract may vary according to the species, place of origins, type of extraction methods and/or solvents used [3,23,26]. Therefore, this study examines the development of the ethanolic heat reflux extraction (EHRE) method to increase nicotine yield from *N. tabacum* var. Virginia origin of Ponorogo. High yield of nicotine in the extract would be as a purpose in this study.

MATERIALS AND METHODS

Materials

N. tabacum var. Virginia leaves were taken from Ponorogo District, East Java Province, Indonesia. The leaves were dried at 40°C in an oven for 4 hours and ground until they reached 200-mesh fineness. The nicotine standard for UPLC and TLC analysis was purchased from Sigma-Aldrich, Germany. Ethyl alcohol p.a (Merck, Germany) was used as the solvent.

Ethanolic Heat Reflux Extraction (EHRE)

A sonicator bath (Powersonic 410, Hwashin Technology Company, Gyeonggi, Korea) was used for 30 minutes at each treatment prior to the extraction. The sonication process was carried out for each four 500-ml Erlenmeyer containing 250 ml of ethyl alcohol and 50 g of dried tobacco powder. Internal dimension of sonicator bath is 30 x 24 x 15 cm, with frequency of 40 kHz and operated at room temperature. Four 500-ml three-neck rounded flasks were prepared for ethanolic heat reflux extraction (EHRE). A mixture of tobacco powder and solvent in each erlenmeyer after completion of the sonication process was then poured into the flask. The EHRE durations for the different flasks were 2, 4, 6, and 12 hours. The temperature of all flasks was set at the solvent's boiling point (75–78°C), and agitation was set at 150 rpm. This step was carried out in triplicate; it was then followed by the recovery process.

Ethylene glycol as a condenser coolant (with cooling temperature to 0°C) was used to condense vapor compounds back to the flask. Ethylene glycol with the formula $(CH_2OH)_2$ is known as a condenser coolant that can be applied in EHRE chiller system. Pure ethylene glycol has a freezing point of about -12°C; it is lower than water that is only 0°C [27]. This will certainly have implications on the cooling results. The ethyl alcohol that evaporates with volatile compounds therein will be more easily condensed and flows back into liquid form.

Recovery processes

The extracts from the previous step were filtrated under vacuum conditions to separate the liquid from solid products. The filtrate was then evaporated using a vacuum evaporator (Laborota 4000, Heidolph, Germany) until a quarter of the amount of initial liquid content was reached (V = ¼Vo). *N. tabacum* concentrated extract was obtained after the evaporation process using water bath concentrator.

Determination of yield

Extraction yield was obtained by dividing the mass of the extract by the dry weight of *N. tabacum* leaves (% w/w). The nicotine yield was determined by dividing the mass of nicotine that obtained from concentration of nicotine in the extract by the dry weight of *N. tabacum* leaves (% w/w). Nicotine concentrations can be calculated on the basis of peaks that found in the chromatograms of ultra-performance liquid chromatography (UPLC). The calculation of extract yield and nicotine yield are shown in the following equations.

$$\text{Yield of extract} = \frac{\text{Weight of extract}}{\text{Dry weight of tobacco leaves}} \text{ x } 100\% \quad (1)$$

$$\text{Yield of nicotine} = \frac{\text{Weight of nicotine}}{\text{Dry weight of tobacco leaves}} \text{ x } 100\% \quad (2)$$

$$\text{Weight of nicotine} = \text{Concentration of nicotine x Volume extract} \quad (3)$$

Statistical evaluation

Each experiment was performed in triplicate and the result was expressed as mean ± standard deviation of three replications to assure the accuracy of the experimental data. P value < 0.05 was regarded as significant.

Characterizations by UPLC and TLC

Nicotine content was characterized by UPLC (ACQUITY UPLC H-Class System, Waters, USA). The UPLC used is a new generation of HPLC that is able to detect and isolate of nicotine sensitively from mixtures of organic compounds in tobacco extract with a short runtime. A 100% methanol (HPLC grade) as an isocratic mobile phase and an ACQUITY UPLC BEH C_{18} Column (1.7 µm; 2.1 x 50 mm) were used for UPLC to examine the nicotine content. The following conditions were employed: flow rate of 0.6 ml.minute^{-1}, injection volume of 10 µl, pressure for each 7-minute injection of 4.1x10^3 kPa and ultraviolet (UV) 260 nm as a detector. The nicotine standard was prepared at five different concentrations; namely 200 ppm, 400 ppm, 600 ppm, 800 ppm and 1000 ppm, to make the standard curve. Thin-layer chromatography (TLC) was conducted using 100% methanol as a mobile phase (eluent). Silica gel 60G F_{254} TLC plates (Merck Co., Germany) were used to examine nicotine in the extract qualitatively. The elution process was carried out using TLC chamber (CAMAG, Switzerland). TLC cabinet unit (CAMAG UV Cabinet 4, Switzerland) with UV lamps (2 x 8 W, 254 and 366 nm) was used also to detect spots on TLC plate as elution results.

Morphological comparison by Scanning Electron Microscope (SEM)

The SEM (JSM-6510 LA, JEOL Ltd., Japan) was used to compare the morphological differences among dried tobacco leaf samples before and after EHRE process. This comparison is intended to examine action mechanism of the extraction. Both of dried tobacco leaf samples (before and after EHRE) were coated with platinum under high vacuum conditions. The accelerated voltages and samples diameter observed were 20 kV and 1 µm; with 2000x magnification. Observation was carried out at around of stoma area of tobacco leaf samples.

RESULTS AND DISCUSSION

Characterization results by TLC and UPLC

Table 1 shows the result of TLC plate chromatogram for the *N. tabacum* extract that obtained from the EHRE process. The extract was compared with nicotine standard as the control. UV 254 nm was also applied as a detector in TLC cabinet. There were three spots in the chromatogram, as follows: nicotine standard as a control (S) and two visualizations of the extract (1 and 2).

Table 1: The TLC chromatogram result

Spots in the chromatogram	Retention factor (R_f)
S	0.62
1	0.60
2	0.60

S is nicotine standard. 1 and 2 are two visualizations of the extract.

The retention factor (R_f) values that calculated of the TLC result for the elution spots of S, 1 and 2 were: 0.62, 0.60 and 0.60, respectively. These R_f values were obtained as the quotient between elution distance of the spots with elution distance of the eluent. The elution spots of 1 and 2 had similar R_f values to that of S; this means that the nicotine compound was detected qualitatively in elution spots of 1 and 2, as well as in S.

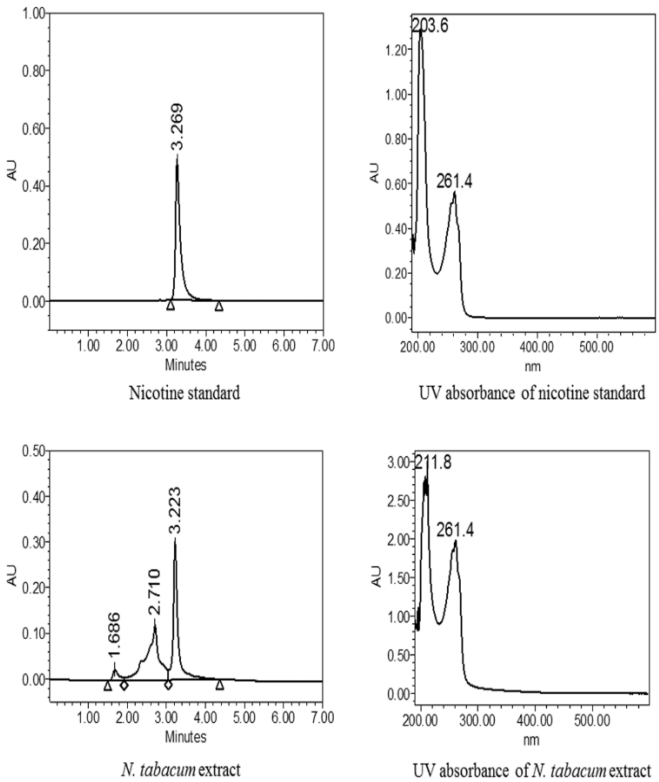

Figure 1: Ultra-performance liquid chromatography (UPLC) chromatograms and ultraviolet (UV) absorbance of the nicotine standard and *N. tabacum* extract for 6 hours of extraction.

Figure 1 shows UPLC chromatogram of *N. tabacum* extract that compared with nicotine standard. Nicotine yield in each

N. tabacum extract was quantitatively determined based on the peaks that found in the chromatograms for each time of the extraction using nicotine standard as a control. The result shows that three adjacent peaks of *N. tabacum* extract that obtained from 6 hours extraction were detected by UPLC, with 1.7, 2.7 and 3.2 minutes of retention time. The 3.2-minute peak had a similar retention time to the peak of the nicotine standard. It was also in accordance with the TLC data. The peaks also had similar R_f values with spot S as a nicotine standard for each elution spot on the TLC plate (see Table 1). There was similar UV absorbance in both the nicotine standard and *N. tabacum* extract; especially at a wavelength (λ) of 261.4 nm, it was observed that nicotine was a dominant compound in the extract. The chromatograms of each extract were then calculated to determine each nicotine yield in the extract. All of the calculated results were shown in Figure 2.

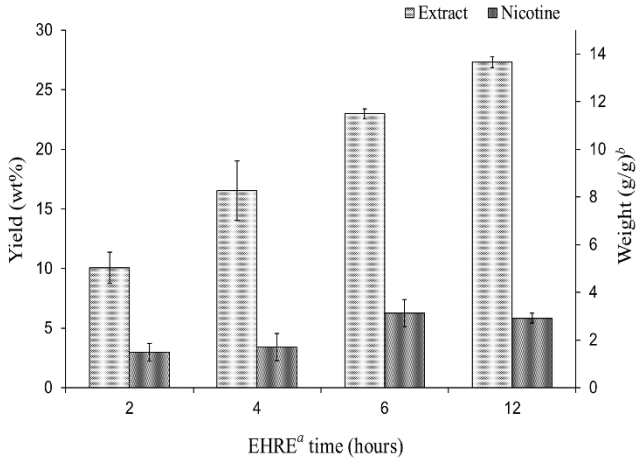

Figure 2: Results for the extract and its nicotine content (mean ± SD; n = 3). [a]Weight of the extract and nicotine were obtained based on 50 g of dried tobacco leaves (g/g).

Figure 2 showed that extract yields were significantly increased along with the extraction times. These yields (in wt%) were 10.1±1.3; 16.5±2.5; 23.0±0.4; and 27.3±0.5, at 2; 4; 6; and 12 hours of extraction time, respectively. The nicotine yields were also determined from each of the extract. They were (in wt%) 3.0±0.7; 3.4±1.1; 6.3±1.1; and 5.8±0.4, at 2; 4; 6; and 12 hours of extraction time, respectively. The nicotine yields that obtained from each of the extract were also significantly increased over 2–6 hours of extraction; they then slightly decreased at 12 hours of extraction. They were 1.5±0.4 g; 1.7±0.6 g; 3.1±0.6 g; and 2.9±0.2 g, at 2; 4; 6; and 12 hours of extraction time, respectively (based on 50 g of dried tobacco leaves, w/w). The decline in the nicotine yield after 6 hours of extraction showed that 6 hours is the optimum time for EHRE of *N. tabacum* var. Virginia origin of Ponorogo. The resulting graph agrees with the findings reported previous studies [28-30]. In the extraction of organic compounds, whether from tobacco or other plants, 6 hours has been identified as the optimal time. This is due to organic compounds have a faster decomposition time than inorganic compounds do, so that when the extraction time is more than 6 hours, many organic compounds have already decomposed, include nicotine [31,32].

The scanning electron microscope (SEM) was used to show morphological differences between stomata guard cells on dried tobacco leaf before EHRE as a control and after EHRE. There were significantly different between control and after EHRE process (see Figure 3).

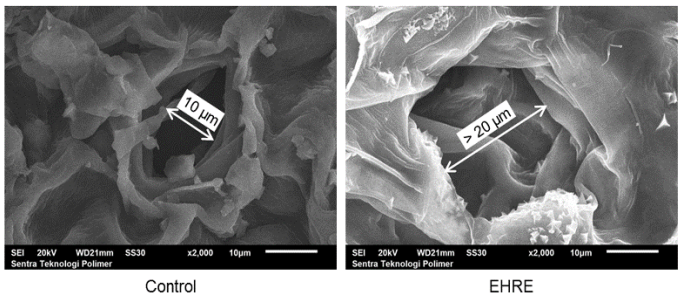

Figure 3: The morphological differences by scanning electron microscope (SEM) among dried tobacco leaf samples before EHRE as a control (left) and after EHRE process (right).

The physically damaged cell (cell lysis) is shown by a stoma guard cell after EHRE process. A wider cracked-hole in stoma after EHRE (> 20 µm) than the control (10 µm) indicates that opened the pore on the leaf surface with cell lysis is occurred around stoma guard cell as the effect of sonication and heating process of EHRE for 6 hours extraction. The vacuole membrane inside the cell is also broken. Hence, ethyl alcohol as a solvent that used in the EHRE can be easily absorbed into the vacuoles through the lysed membrane (lysed tonoplast) to extract nicotine and other polaric compounds in the vacuoles.

Table 2: Nicotine separation from tobacco leaves

Methods	Solvents	Operating Conditions	Yield (wt%)	Ref.
Packed bed extraction (PBE)	Ethanol	Room temperature, 3 ml.min^{-1}, 0.45 mm	0.2	[33][a]
Ionic liquid extraction (ILsE)	1-Butyl-3-methyl-imidazolium chloride	60°C, 1 atm, 1 h	0.3	[19]
Pyrolysis with fluidised bed extraction (PFlBE)	N_2 gas	565°C, 10°C.min^{-1}	1.3	[20]
Supercritical Fluid Extraction (SFE)	Metanol/KH_2PO_4 (2/3, v/v) and CO_2 gas	100°C, 135.5 atm, 2x10 min	2.3	[21]
Stirred vessel maceration (SVM)	Methanol/dichloromethane (1/1, v/v)	Room temperature, 1 atm, 1 h, 500 rpm	2.7	[22]
Mechanical shaker extraction (MSE)	tert-butil metil eter (TBME)	Room temperature, 1 atm, 1 h shaken	2.7	[24]
Ultrasonic extraction (UE)	0.05% NaOH and dichloromethane	Room temperature, 1 atm, 15 min sonicated	2.9	[18]
Mechanical shaker extraction (MSE)	Dichloromethane/methanol (3/1, v/v)	Room temperature, 1 atm, 30 min shaken	3.2	[23]
Ultrasonic extraction (UE)	Methanol	Room temperature, 1 atm, 30 min sonicated	3.6	[4]
Digestion extraction (DE)	Ethanol	50°C, 1 atm, 2 h, 150 rpm	3.9	[34][a]
Ethanolic heat reflux extraction (EHRE)	Ethanol	Ethanol bp, 1 atm, 6 h, 150 rpm	6.3	This research

[a]Our preliminary experiment

Table 2 lists selected studies on nicotine production from tobacco leaves. Nicotine yields of greater than 3 wt% can be produced by some methods, such as mechanical shaker extraction (MSE; 3.2 wt%) [23]; ultrasonic extraction (UE; 3.6 wt%) [4]; and also our preliminary experiment, digestion extraction (DE; 3.9 wt%) [34]. However, our EHRE method gives a higher nicotine yield than the other methods do. The optimum reflux in EHRE increased the nicotine yield up to 6.3 wt%. This is approximately 60% higher than the highest yield found in other studies.

Ethanolic heat reflux extraction (EHRE) is known as one of the extraction method which is used due to its simplicity and it can easily be developed to a larger scale. The reflux extraction method is also superior to the other extraction approaches, as it provides higher extraction yields with reduced energy consumption and CO_2 emission [35,36].

CONCLUSION

The ethanolic heat reflux extraction (EHRE) method produces the highest nicotine from *N. tabacum* var Virginia origin of Ponorogo. Our work showed an improvement of 60% in comparison with other study results. The optimum time for tobacco extraction by EHRE method was 6 hours, with a nicotine yield of 6.3 wt%. The EHRE method in this study has a potential to be developed on a larger scale in order to produce a high nicotine quantity from tobacco plants, which will be beneficial for pharmaceutical and agricultural industries.

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