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Abstract – Tobacco plays an important role in international trade as one of the export commodities. Indonesia is one of the good quality export contributors of tobacco leaves in the world. Nevertheless, tobacco is used only as a raw material for the cigarette industries, and the rise on anti-cigarette regulations prompted the exploration of alternative product from tobacco plants. The content of alkaloids, flavonoids, terpenoids and steroids in tobacco leaves were reported in literatures as antibacterial. Therefore, this study proposed in vitro assay of the ethanolic heat reflux extract (EHRE) of *Nicotiana tabacum* var. Virginia against nosocomial bacteria pathogen ((*Pseudomonas aeruginosa* (ATCC 27853), *Eschericia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212)). Kirby-bauer diffusion method was used for this assay. The concentration of the EHRE for Kirby-bauer assay were 20; 40; 60; 80; and 100%. The presence of clear zones on Kirby-bauer test, against the growth of each nosocomial bacteria pathogen show that tobacco extract has antibacterial effect. Statistical analysis result showed that each extract concentration had significant difference value ($p < 0,05$). This study indicated that the content (alkaloids, flavonoids, terpenoids and steroids) of tobacco leaf extracts (*N. tabacum*) has potential as antibacterial against nosocomial bacteria pathogen. Nevertheless, optimization of tobacco leaf extract to obtain maximum active ingredient still needs to be done. This study is important for further development of the tobacco leaf extract as antibacterial

1 Introduction

Indonesia is one of the world's largest biodiversity nations. There are approximately 30,000 species are spread throughout Indonesia, and about 300 plant species in Indonesia have been used as traditional medicinal materials by the traditional medicine industry.[1] One of the many medicinal plants in Indonesia is tobacco, whose processing is used as raw materials for cigarettes. Tobacco contains 2,500 chemical components. After burning, as many as 1,400 components will be unravel. The unravel component will react and form 4,800 chemical components in cigarette smoke.[2]

The rise of anti-cigarette regulations encouraged necessitate exploration of alternative product of tobacco. Tobacco in Indonesia has an important role, not only in the economic aspects but also in work employment.[3] Besides as raw materials of cigarettes, tobacco was also able as antibacterial,

antifungal and bioinsektisida.[4,5,6] Tobacco leaf extract (*Nicotiana tabacum* L.) could inhibited the growth *Bacillus amyloiquefaciens*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*.[4] But the optimization of antimicrobial compound composition in tobacco extract still require in demand. The antimicrobial inhibition were caused by secondary metabolite chemical compounds like, alkaloids, flavonoids, terpenoids and steroids which are bioactive compounds that act as antibacterial.[7] In addition, tobacco bio-oil pyrolysis extract has been used as a mosquito repellent for humans, since it does not contain DEET (N, N-diethyl-m-toluamide).[7] Excessive exposure of DEET in humans can cause sensory and motor disturbances, and DEET is not found in tobacco extracts.[8] This tobacco potential makes it a potential feedstock for both topical medicines or as a disinfectant in humans.

addition, although the potency of tobacco extract as antibacterial has been tested many times, but the isolation of extract is mostly done by maceration and socletation extraction method, while the isolation by reflux method has not been studied for antibacterial potential.[4,5] The difference of isolation technique lead to different composition of active substance. This led to the importance of an antibacterial test on *Nicotiana tabacum* L. var Virginia tobacco extract by reflux method.

Nosocomial infection will cause the cost of treatment, the stay in the hospital will prolonged or endanger the patient's life. A survey of the prevalence of WHO-managed nosocomial infections, in 55 hospitals in 14 countries divided into 4 regions, namely Europe, Eastern Mediterranean, Southeast Asia and the Western Pacific, showed that approximately 8.7% of hospital patients had nosocomial infections, other surveys suggest about 1.4 million patients worldwide have nosocomial infections. The infection late operation (ILO) could established from nosocomial infections, in the presence of pus, pain, and redness. Pus culture showed various nosocomial bacteria as the cause of infection, both Gram positive and Gram negative. Several researchers have reported the incidence of ILO with 3 types the most bacterial infection, for example in Bangladeshi hospital bacteria identified were *Pseudomonas* sp., *Bacteroides fragilis* and *Escherichia coli*.[14] In RS M. Djamil Padang Indonesia obtained *Klebsiella* sp., *Staphylococcus aureus*, and *Enterobacter aglomerans*, whereas in RS Moewardi Surakarta Indonesia caused by *Enterobacter* sp., *Pseudomonas* sp., and *Proteus* sp.[10] Anaerobic bacteria *Bacteroides fragilis* is also one cause of wound infection.[11]

Traditional medicine has grown globally and its benefits are known to some extent. It expected improve the health care in Indonesia. Here, we tested antibacterial effectivity of tobacco leaf extract (*Nicotiana tabacum* L. var *Virginia*) isolated by reflux method, against some pathogenic nosocomial bacteria with Kirby-bauer diffusion method.

2. Material And Methods

2.1. Time and Place

The time of the research was conducted in March-October 2017. The research was conducted in Microbiology Laboratory of Medical Faculty of National Development University "Veteran" Jakarta. Tobacco extraction was carried out at the Chemical Engineering Department, Faculty of Engineering, and Universitas Indonesia.

2.2. Experiment Design

The type of research used in this study is experimental research with true experiment research design. The research was conducted in laboratory, by giving treatment in various concentration (20%, 40%, 60%, 80% and 100%) tobacco leaf extract (*Nicotiana tabacum* L.var Virginia) and control (aquadest) comparison group. Diffusion method Kirby-Bauer were used to calculate Minimal Inhibition Concentration (MIC). The test group used were *Pseudomonas aeruginosa* (ATCC 27853), *Eschericia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Enterococcus faecalis* (ATCC 29212).

2.3. Ethanolic Heat Reflux Extract of Nicotiana tabacum L. var Virginia

Tobacco leaves (*Nicotiana tabacum* L.var Virginia) obtained from Ponorogo, East Java. The taxonomy of *Nicotiana tabacum* L.var Virginia was confirmed by the Center for Plant Botanic Gardens, Indonesian Institute of Sciences, Indonesia. To make the simplicia, tobacco leaves were

cleaned and washed with water until clean. They were then dried under the sun. In addition for completely drying, the leaves were inserted into an oven with a temperature of 120 ° C for 2 hours. Dried leaf powder is obtained from the grinding of simplicia which is then sieved, to obtain fine powder. The extraction was performed by heat reflux method. A 50 g of the tobacco leaf powder was dissolved into 250 ml of ethyl alcohol. This solution was then placed in a three-neck flask with connected to the Allihn condenser and heated to 80°C. The solution is continuously stirred automatically by a 150 rpm stirrer hotplate for 6 hours. The three neck flask containing tobacco leaf powder and solvent soaked in water bath for heating process to take place evenly. On the side of the Allihn condenser there is a hose connecting the condenser with the cooler (chiller) at 4^0C, so condensation process proceed. The vapor formed by heating condensation, and returned to the flask. The mixtures were filtrated under vacuum condition to separate the filtrate from tobacco waste. The filtrate was then evaporated by vacuum evaporator to get concentrated extract.

2.4 Measurement of Antibacterial Activity

Measurement of antibacterial activity was carried out at the laboratory of microbiology FKUPN "Veteran" Jakarta. Kirby-bauer diffusion method was used in this study. The filter paper containing tobacco leaf extract is placed on the surface of a solid medium that previously inoculated by test bacteria on its surface. After 24 hours incubation, the inhibition zone diameter around the disc were measured. Formed around the disc paper was used to measure the resistance strength of extract against the pathogen bacteria.[12]

2.5 Data analysis

One-Way ANOVA test (parametric test) were used to compare the 5 group concentration (0%, 20%, 40%, 60%, 80% and 100%). The data distribution should be normal and the data variance must be the same, to meet the One-Way ANOVA test requirements. Saphiro-Wilk were used to test data normality, while Levene's test were used to test data homogenity. Hypothesis were accepted when a p value <0.05. The Post Hoc analysis test is conducted to determine which groups have significant differences. If the One-Way ANOVA test is not eligible, then the Kruskal-Wallis (non-parametric) alternative test is used. [13]

3. Result And Discusssion

3.1. The tobacco extract showed inhibitory effect on all concentration against all 4 bacteria

Based on the results, the effect of tobacco leaf extract (*Nicotiana tabacum* L.) against all four bacterial nosocomial pathogen (*P.aeruginosa* ATCC 27853, *Eschericia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212). The inhibitory power shown by the formation of clear zones around disk paper in experiments on 0%, 20%, 40%, 60%, 80% and 100% extract concentration. Any data in this method was obtained by four-replications (Figure 1.).

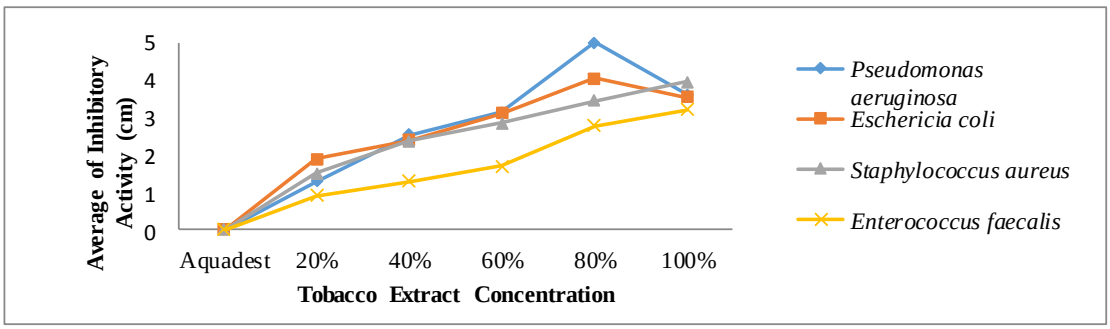

Figure 1. Average Inhibitory Activity of Ethanolic Heat Reflux Extract of *Nicotiana tabacum* L. var Virginia Against Nosocomial Bacteria Pathogen

This study showed that tobacco leaf extract (*Nicotiana tabacum* L.) has an antibacterial effect on *P.aeruginosa* ATCC 27853, *E.coli* ATCC 25922, *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212). All result against 4 bacteria, were included in the weak category.

3.2. Statistical analysis showed significance difference on all extract concentration with some exception

Table 1. Statistical analysis MIC result of different concentration tobacco extract

Tobacco concentration	**extract**	***P. aeruginosa***	***E. coli***	***S. aureus***	***E. faecalis***
		Significance	**Significance**	**Significance**	**Significance**
20%	40%	0,000	0,001	0,021	**0,083**
	60%	0,000	0,000	0,021	0,020
	80%	0,000	0,000	0,021	0,021
	100%	0,000	0,000	0,021	0,021
40%	20%	0,000	0,001	0,021	**0,083**
	60%	0,000	0,000	0,021	0,020
	80%	0,000	0,000	0,021	0,021
	100%	0,000	0,000	0,021	0,021
60%	20%	0,000	0,000	0,021	0,020
	40%	0,000	0,000	0,021	0,020
	80%	0,000	0,000	0,021	0,020
	100%	0,007	0,004	0,021	0,020
80%	20%	0,000	0,000	0,021	0,021
	40%	0,000	0,000	0,021	0,021
	60%	0,000	0,000	0,021	0,020
	100%	0,000	0,001	**0,248**	0,021
100%	20%	0,000	0,000	0,021	0,021
	40%	0,000	0,000	0,021	0,021
	60%	0,007	0,004	0,021	0,020
	80%	0,000	0,001	**0,248**	0,021

One-Way ANOVA test of *P.aeruginosa* ATCC 27853 and *E.coli* ATCC 25922, showed that significance value of p <0,05 meaning tobacco leaf extract (*Nicotiana tabacum* L.) had inhibitory effect as antibacterial. Post-Hoc Test Results (LSD) showed all groups in *P. aeruginosae* and *E. coli*, had significant inhibitory differences (p<0,05) on both bacterium (Table 1.). The extract concentration of 80% yielded the largest mean inhibitory zone diameter in *P.aeruginosa* ATCC 27853 and *E.coli* ATCC 25922. However, when tested with 100% extract concentration, the mean value of the resulting inhibit zone decreased. From these results, it can be seen that the concentration of tobacco leaf extract is most effective in inhibiting the test bacteria is the concentration of 80% extract. At 80% extract concentration, high active ingredient content can be absorbed into paper and media disc to optimally and effectively, so that the drag zone diameter is large (Table 1). This phenomenon happened due to the high concentrated and high consistency of tobacco extract. The diffusion process of extracts on paper discs and media would be less effective, when the consistency of tobacco extract almost solid[14]

Our result is in line with previous research, that concentration of 20%, 40%, 60%, 80% and 100% tobacco extract against *Staphylococcus aureus* and *Escherichia coli* bacteria, has an antibacterial power.[4,7,11] The highest inhibitory were showed in 100% concentration on both *S. aureus* and *E. faecalis* (Gram positive), which was different from *P. aeruginosae* and *E.coli* (Gram negative), which was on 80% concentration (Figure 1.). Probably, this is due to the complexity of bacterial plasma membrane of Gram positive bacteria group, which was more moderate that Gram negative bacteria group.

Post Hoc test on *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212) with Kruskal-Wallis showed that there were difference of various tobacco leaf extract

concentration, except concentration 80% with 100% against *S. aureus*, and 20% with 40% against *E. faecalis*.

3.3. Phytochemical result of ethanolic heat reflux tobacco extract

The activity of inhibitory power of tobacco leaf extract (*Nicotiana tabacum* L.) to pathogen bacteria were caused by phytochemical content of tobacco extract, that acts as antibacterial, such as alkaloids, flavonoids, terpenoids and steroids.[5] The phytochemistry result of our ethanolic heat reflux extract showed that the extract composed of alkaloid, saponin, tanin, fenolik, flavonoid, tritefenoid and glycoside (Table 2.).

Table 2. Qualitative phytochemistry result test of EHRE of *Nicotiana tabacum* L. var Virginia

Type of testing	Result	Type of testing	Result
Alkaloid	+	Flavonoid	+
Saponin	+	Tritefenoid	+
Tanin	+	Steroid	-
Fenolik	+	Glycoside.	+

The phytochemical content in the tobacco leaf extract gives antibacterial effect to each of the test bacteria with different mechanism. *Aeruginosa* and *E.coli* bacteria were included in Gram-negative bacteria, whereas *S. aureus* and *E. faecalis* were included Gram-positive bacteria. Gram-negative bacteria have a layered cell wall structure, consisting of lipopolysaccharides, lipoproteins and peptidoglycan. Alkaloid content can interfere with peptidoglycan components in bacterial cell walls. The disruption of peptidoglycan formation makes the lining of the cell wall unintegrated resulting in cell death.[15] In addition, the aromatic compounds contained in the alkaloids cause the formation of bonds with bacterial DNA so that bacterial DNA synthesis is disrupted.[42] The flavonoid content of the tobacco leaf extract may bind to proteins cell membranes that cause cell membrane damage. The destruction of the cell membrane results in disruption of the integrity of the cell membrane of the bacteria resulting in the disturbance of bacterial cell growth and bacterial death.[11] The content of steroids and terpenoids, have an antibacterial ability by inhibiting the action of enzymes involved in energy production and changing the composition of constituents cells due to the accumulation of lipophilic components, thus disrupting the formation of bacterial cell walls. The tobacco extract also contained phenol. High levels of phenol can cause protein denaturation and cause bacterial cell lysis.

This study showed that tobacco leaf extract (*Nicotiana tabacum* L.) isolated by this reflux method still resulted in small MIC effect, compared to previous studies that used tobacco leaf extract (*Nicotiana tabacum* L.) [5,16] The extract improvement still need to be done to recover high amount of antibacterial active compound, to improve growth inhibition on nosocomial bacteria.

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