HAYATI
Journal of Biosciences

Vol. 29 No. 4, July 2022 515-525
DOI:10.4308/hjb.29.4.515-525
ISSN: 1978-3019
EISSN: 2086-4094

Nicotiana tabacum var. Virginia Bio Oil-based Pyrolysis Extraction Have Prominence Antimicrobial Potential Compared to Ethanol Heat Reflux Extraction (EHRE)

Andri Pramesyanti Pramono[1], Basra Ahmad Amru[1], Halimah Anggi Rahmani[1], Sheila Azelya Fernanda[1], Yudhi Nugraha[3], Muhammad Yusuf Arya Ramadhan[2], Andre Fahriz Perdana Harahap[2], Ahmad Fauzantoro[3], Nasihin Saud Irsyad[1], Meiskha Bahar[1], Oktania Sandra Puspita[1], Fajriati Zulfa[1], Kori Yati[4,5], Mahdi Jufri[5], Misri Gozan[2*]

[1]*Faculty of Medicine, Universitas Pembangunan Nasional Veteran Jakarta, Jakarta, Indonesia*
[2]*Chemical Engineering Department, Faculty of Engineering, Universitas Indonesia, Depok, Indonesia*
[3]*National Research and Innovation Agency Republic of Indonesia, Jakarta, Indonesia*
[4]*Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. DR. HAMKA, Jakarta, Indonesia*
[5]*Faculty of Pharmacy, Universitas Indonesia, Depok, Indonesia*

ARTICLE INFO

Article history:
Received March 2, 2021
Received in revised form September 24, 2021
Accepted October 5, 2021

KEYWORDS:
bacterial growth inhibition,
Enterococcus faecalis,
Escherichia coli,
Pseudomonas aeruginosa,
Staphylococcus aureus,
tobacco leaves from Ponorogo Indonesia

ABSTRACT

Tobacco leaf contains antibacterial secondary metabolite compounds, such as phenol, alkaloids, and essential oils. This study compares the potential antibacterial effects of Indonesian tobacco leaf extracted using the heat reflux method (producing an extract) and pyrolysis method (providing a bio-oil). The tobacco leaf extract was challenged against *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853. The bio-oil from the pyrolysis method showed inhibitory Kirby Bauer zones higher than those of the extract from heat reflux method, with the maximum results in the pyrolysis method indicating zones of 6.35 mm (*S. aureus*), 5.90 mm (*E. faecalis*), 3.97 mm (*E. coli*), and 5.025 mm (*P. aeruginosa*). Further study analyzed the effectiveness of the disc and well diffusion antibacterial test methods for measuring the antibacterial effect of bio-oils against *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The bio-oil used in the well diffusion test method showed the most significant antibacterial effectiveness. It showed the biggest inhibition zone, with a maximum of 11.65 mm and 8.90 mm for *E. coli* and *P. aeruginosa*. Our results showed *Nicotiana tabacum* var. Virginia Bio Oil from Ponorogo (Indonesia) is a strong potential antimicrobial, especially using well diffusion test.

1. Introduction

The risk of nosocomial infection is highest approximately 48 hours after admission to a hospital environment and 30 days after hospital care ends (Suleyman *et al.* 2018). Such infections can be of exogenous origins, such as from epidemic diseases, person-to-person contact, and contact with devices (Suleyman *et al.* 2018). Antibiotic resistance caused by inappropriate treatments and therapies can increase the risk of nosocomial infections in patients in hospitals (Gentile *et al.* 2020). *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* are the most common bacteria causing nosocomial infections (Kudo *et al.* 2014; Iseppi *et al.* 2020; Jean *et al.* 2020; Kolar *et al.* 2020).

The use of antimicrobials from plants for nosocomial infection can reduce the side effects of excessive antibiotics and antibiotic resistance (Cheesman *et al.* 2017). The Indonesian government's limitation of cigarette production makes it important to look for alternative products, one of which is health products. In addition to social and economic needs, tobacco contains many antibacterial substances and can be used as drugs and antimicrobial health products. One of Indonesia's most common tobacco variants is *Nicotiana tabacum* var Virginia, which includes 63% of tobacco types in this country (Ahsan

* Corresponding Author
E-mail Address: mgozan@che.ui.ac.id

et al. 2014). *Nicotiana tabacum* Var Virginia originated from Virginia (America), brought to Indonesia in 1928, and planted in Bojonegoro for the first time to meet the demand for cigarette tobacco in Indonesia (Pertanian RI 2011). Besides containing high levels of nicotine, pyridine, indole, and d-limonene (Gozan *et al.* 2014; Andhika Priotomo *et al.* 2018). Tobacco can be used as an antimicrobial agent because its leaves contain secondary metabolite compounds, including alkaloids, flavonoids, phenols, saponins, and steroids (Sokunvary Oeung *et al.* 2016; Fauzantoro *et al.* 2017; Fathi *et al.* 2018), which can inhibit the growth of bacteria (Pramono *et al.* 2018).

A common extraction method in extracting antimicrobial compounds of the *Nicotiana tabacum* is Ethanolic Heat Reflux Extraction (EHRE) methods (Fauzantoro *et al.* 2017; Pramono *et al.* 2018; Banožić *et al.* 2020). Some of the studies used the pyrolysis extraction method to extract the antimicrobial compound of the *Nicotiana tabacum* (Ario Putra *et al.* 2019; Fernanda *et al.* 2021). The pyrolysis method involves heating a substance to a high temperature without the oxygen that can cause the disintegration of chemical compounds, this process results in bio-oil compounds (Gozan *et al.* 2014). The advantage of the pyrolisis extraction method is that it can attract active compounds in large amounts, with a high temperature (Andhika Priotomo *et al.* 2018), but the antimicrobial potential from bio oil product still needs to be evaluated. Here we compared the antimicrobial potential of *Nicotiana tabacum* L. var. Virginia extracts produced using the heat reflux and pyrolysis methods from Ponorogo, Indonesia. The comparison with another study is also being discussed.

2. Materials and Methods

2.1. Plant Origin and Sample Collection

Tobacco plants (*Nicotiana tabacum* L. var. Virginia) as raw material for the extracts were obtained from Ponorogo, East Java Province (Indonesia). Fresh leaves were collected and immediately processed. The tobacco leaves used are tobacco leaves that are approximately three months old and are ready to be harvested. The tobacco plant is a subtropical plant but can grow in low pliers wide climate (29–33°C). *Nicotiana tabacum* L. var. Virginia is grown in Ponorogo, Indonesia, at the end of the rainy season, and harvested in the dry season, April-Mei in Ponorogo, East Java, Indonesia (Pertanian RI 2011).

2.2. Preparation of Leaf Extracts Using the Heat-Reflux Extraction Method (Extract)

Several steps are needed to make an extract using the heat reflux method. First, the tobacco leaves are cleaned with water. Second, the tobacco leaves are dried in an oven at 120°C for 2 hours. After the tobacco leaves are dried, they are ground into a dried leaf powder. Up to 50 g of dried leaf powder is then put into an Erlenmeyer glass and dissolved with 150 ml of ethanol solvent. Then, the solution is heated to a temperature of 80°C. This healing process is a vapour, which is put into a condenser until it forms a concentrated tobacco leaf extract (briefly named extract) (Fauzantoro *et al.* 2017).

2.3. Preparation of Leaf Extracts Using the Pyrolysis Method (Bio-oil)

Another extraction method used is the pyrolysis method. First, the tobacco leaves are cleaned and then dried. After that, the tobacco leaves are chopped into smaller pieces, and 250 g of chopped leaves are converted to a powder form. This is done to improve the efficiency of the pyrolysis process. The pyrolysis reaction is carried out inside a closed pyrolysis reactor so that no external gas can enter the reactor. The tobacco leaves are heated to a temperature of 500°C. The heating process produces gas and char. The gas and char are separated using a cyclone separator. Finally, the gas is cooled until it forms a bio-oil (Gozan *et al.* 2014).

2.4. Bacterial Strains and Growth Conditions

The bacteria used in this study were obtained from the American Type Culture Collection (ATCC). They included the following strains: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia Coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853. All bacteria were cultured overnight with agitation at 150 rpm, in flasks containing Mueller-Hinton broth (Merck, Darmstadt, Germany), before being used in the experiment.

2.5. Antibacterial Activity Assessment

The samples used in this research are divided into several groups: 20%, 40%, 60%, 80%, and 100%. The percentage is obtained by diluting the extract with aquadest or bio-oil with propylene glycol. One hundred percent is the initial concentration obtained from direct extraction results. Then 80%, 60%, 40%, and 20% dilutions are obtained by adding a certain amount. The bio-oils and extracts were then analyzed using

the Kirby Bauer antibacterial test (Hudzicki 2009; Balouiri *et al.* 2016). Each experiment used both negative (only with aquadest for EHRE experiment, only with propylene glycol for the pyrolysis extraction experiment) and positive (Ciprofloxacin) control groups. The repetitive amount used for each test was determined using the Federer formula. Based on the Federer analysis, 4 samples are required for each concentration test for the *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa* experiments. Furthermore, to assess the Kirby Bauer antibacterial analysis test's strength, we performed a well diffusion antibacterial analysis test on *Escherichia coli* and *Pseudomonas aeruginosa*.

2.5.1. Disc Diffusion Method

The Kirby-Bauer method was performed using a modification of the disc diffusion method. Bacteria were grown on a solid medium. A 0.5 McFarland standard bacterial suspension was prepared in a sterile saline solution (NaCl 0.85% (w/v); Merck, Darmstadt, Germany) via overnight culturing in a flask containing Mueller-Hinton Broth. Respective bacterial suspensions were seeded on Mueller-Hinton agar (MHA; Merck, Germany) plates, and 6 mm sterile blank discs, which had already been soaked with respective concentrations of extract, were impregnated on top. The prepared plates were incubated at 37°C for 24 hours. The inhibitory zone was identified and recorded carefully (Hudzicki 2009).

2.5.2. Well Diffusion Method

Bacteria were grown on a solid medium. 0.5 McFarland standard bacterial suspensions (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853) were prepared in sterile saline solution (NaCl 0.85% (w/v); Merck, Darmstadt, Germany) via overnight culturing in a flask containing Mueller-Hinton Broth. The well diffusion method involved making a 6 mm diameter hole in the MHA media containing the respective bacteria. The extract was then placed inside the hole carefully, and the prepared agars were incubated at 37°C for approximately 24 hours. The inhibitory zone was identified and recorded carefully (Balouiri *et al.* 2016).

2.6. Qualitative Phytochemistry

Phytochemical testing was carried out on extract and bio-oil. Phytochemical testing aims to determine the qualitative content of secondary metabolites contained in a sample.

2.6.1. Alkaloid Test

The extract or bio-oil was added 1 ml of 2N hydrochloric acid and 9 ml of water, heated in a water bath for 2 minutes, cooled, and filtered. Then, 3 ml of the filtrate was transferred to the watch glass, and two drops of Dragendorf reagent were added. If brown sediment occurred, the extract or bio-oil contained alkaloids. If a white or yellow clot residue is dissolved in methanol with the Mayer reagent, there is a positive for alkaloid substances.

2.6.2. Flavonoid Test

1 ml of the extract or bio-oil is evaporated, and the rest is dissolved in 1-2 ml of ethanol (95%) P. Then, 500 mg of zinc powder and 2 ml of 2N hydrochloric acid are added, the solution is allowed to stand for 1 minute, and then ten drops of concentrated hydrochloric acid are added. If the solution turned red in 2-5 minutes, it meant it contained flavonoids.

2.6.3. Tanin Test

Simplicia extract was added 50 ml of aquadest, boiled for 5 minutes, and then cooled. Then, 5 ml of the filtrate was transferred to a test tube, and $FeCl_3$ reagent was dropped in. If there was a greenish-black colour, that indicated the presence of tannin compounds.

2.6.4. Saponin Test

Extract or bio-oil was put into a test tube, 10 ml of hot water was added, and the mixture was cooled and then shaken vigorously for 10 seconds to form a stable white foam as high as 1-10 cm for not less than 10 minutes. If, with the addition of 1 drop of 2N hydrochloric acid, the foam did not disappear, that indicated that the extract or bio-oil contained saponins.

2.6.5. Steroid/Triterpenoid Test

The extract or bio-oil was added to 20 ml ether and macerated for 2 hours. Then, three drops of the filtrate were transferred to watch glass and dripped with a Lieberman-Burchard reagent (glacial acetic acid-concentrated sulfuric acid). If it turned red, that indicated the presence of steroid compounds, green indicated triterpenoid compounds.

2.7. Quantitative Phytochemistry

Quantitative Phytochemical testing was carried out on extract and bio-oil to measure the total alkaloids and polyphenols in the extract and bio-oil.

2.7.1. Total Flavonoid

A total of 10.0 mg of quercetin was put into a 10 ml volumetric flask and dissolved with ethanol until the limit mark to obtain a 1,000 µg/ml concentration. The standard solution of quercetin with a concentration of 1,000 µg/ml is used for the manufacture of series (3 µg/ml, 5 µg/ml, 7 µg/ml, 9 µg/ml, 11 µg/ml). Then from each concentration, 3 ml of ethanol, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1M of Na acetate were added and distilled water to a volume of 10 ml. Then the standard solution at which concentration has been determined is left to stand for operating time, then the absorbance is read at a wavelength of 434.5 nm on a spectrophotometer.

2.7.2. Total Polyphenol

A total of 300 µl of a gallic acid solution with a 50 µg/ml concentration added with 1.5 ml of Folin-Ciocalteau reagent (1:10), then shaken and let stand for 3 minutes. The solution was added1 with 1.2 ml of 7.5% Na_2CO_3 solution, shaken until homogeneous, and allowed to stand at room temperature in 0-90 minute. The absorbance was measured at a wavelength of 600-850 nm on a spectrophotometer.

2.8. Data Analysis

This experiment was performed in duplicate with a minimum of four repeats. Data were analyzed using a one-way parametric test with the statistical program SPSS 21.0 (Statistical Package for the Social Sciences). A parametric test can be done if the data variance is homogeneous and normal. An independent T-test was performed to equalize the bacterial tests with different concentrations and analyze the differences between the two extraction methods and two antibacterial activity assessments. The significance level for the difference was set at $p<0.05$.

3. Results

We have previously performed EHRE extraction methods (resulting extract) with ethanol solvent for *Nicotiana tabacum* Var Virginia leaves from Indonesia. This study performed the pyrolysis extraction method (resulting bio-oil) with propylene glycol solvent. The yields produced using the EHRE method are lower (23wt%) than those produced using pyrolysis extraction methods (47.6wt%). Higher nicotine yields of up to 31.1% were obtained using pyrolysis extraction compared to the EHRE method (6.3%). The EHRE method's extraction procedure was done in 6 hours with 1 atm and 150 rpm centrifuge speed (heated at a temperature of 80°C). In the pyrolysis method, the extraction is done in 50 minutes with 1 atm and heated at 500°C.

3.1. Qualitative Phytochemistry, Quantitative Phytochemistry, and Yield Extract Efficiency

Qualitative phytochemical tests are performed to determine the active compounds in tobacco leaf extracts, which can be useful as antibacterial agents. Based on the phytochemical test results of the tobacco leaf extracts examined in this study, it is confirmed that the active antimicrobial compounds in tobacco leaf extracts are alkaloids, flavonoids, saponin, tannin, phenolic, glicoside, and triterpenoid (Table 1). The number of qualitative total alkaloids is greater in bio-oil, but total polyphenol was still higher in the tobacco extract (Table 2).

Although the polyphenol compound was found higher in the EHRE methods, on the contrary, nicotine yields showed the opposite around. The higher nicotine yields were obtained from pyrolysis extraction until 31.1% of yields (Table 3). The yields produced from

Table 1. Qualitative phytochemical test results for *Nicotiana tabacum* L. var Virginia leaf extracted using the heat reflux method (extract) and the pyrolysis method (bio-oil)

Compound	Qualitative results	
	Heat reflux extraction (extract)	Pyrolysis extraction (bio-oil)
Alkaloid	+	+
Saponin	+	+
Tanin	+	+
Phenolic	+	+
Flavonoid	+	+
Triterpenoid	+	+
Glicoside	+	+
Steroid	-	-

Table 2. Quantitive phytochemical test results for *Nicotiana tabacum* L. var Virginia leaf extracted using the heat reflux method (extract) and the pyrolysis method (bio-oil)

	Qualitative results	
	Heat reflux extraction (Extract)	Pyrolysis extraction (Bio-oil)
Total flavonoid	2,125.60 ppm	2,579.36 ppm
Total polyphenol	73,273.73 ppm	15,800.00 ppm

EHRE methods (23%) are less than those from pyrolysis extraction (47.6%).

3.2. Antimicrobial Activity

3.2.1. Antibacterial Potential of Extracts Produced using Pyrolysis and Heat Reflux Methods against *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli,* and *Pseudomonas aeruginosa*

The first analysis compared the antibacterial agents' effectiveness using different extraction methods, namely heat reflux (EHRE) and pyrolysis extraction, on several bacterial tests. The study results showed that the pyrolysis extraction method produced higher antibacterial potential than the heat reflux method (Figure 1). A significant statistically difference was only seen in *Enterococcus faecalis*, but not in other bacteria. Even so, pyrolysis is still better in inhibiting other bacteria even though it does not show significance in statistical tests.

The data also showed a significant difference in extracts' effectiveness ranging from 40 to 100% against *Enterococcus faecalis* compared to their effectiveness

Table 3. Comparison of Ethanolic Heat Reflux Extraction (EHRE) and pyrolysis extraction yield

Methods	Solvent	Operation conditions	Yields (wt%)	Nicotine yields (wt%)	Reference
Ethanolic Heat Reflux Extraction (EHRE)	Ethanol	Ethanol bp, 1 atm, 6 h, 150 rpm, heated at a temperature of 80°C	23.0	6.3	This research
Pyrolysis extraction	Propylene glycol	Propylene glycol, 1 atm, 50 minutes, heated at a temperature of 500°C	47.6	31.1	This research

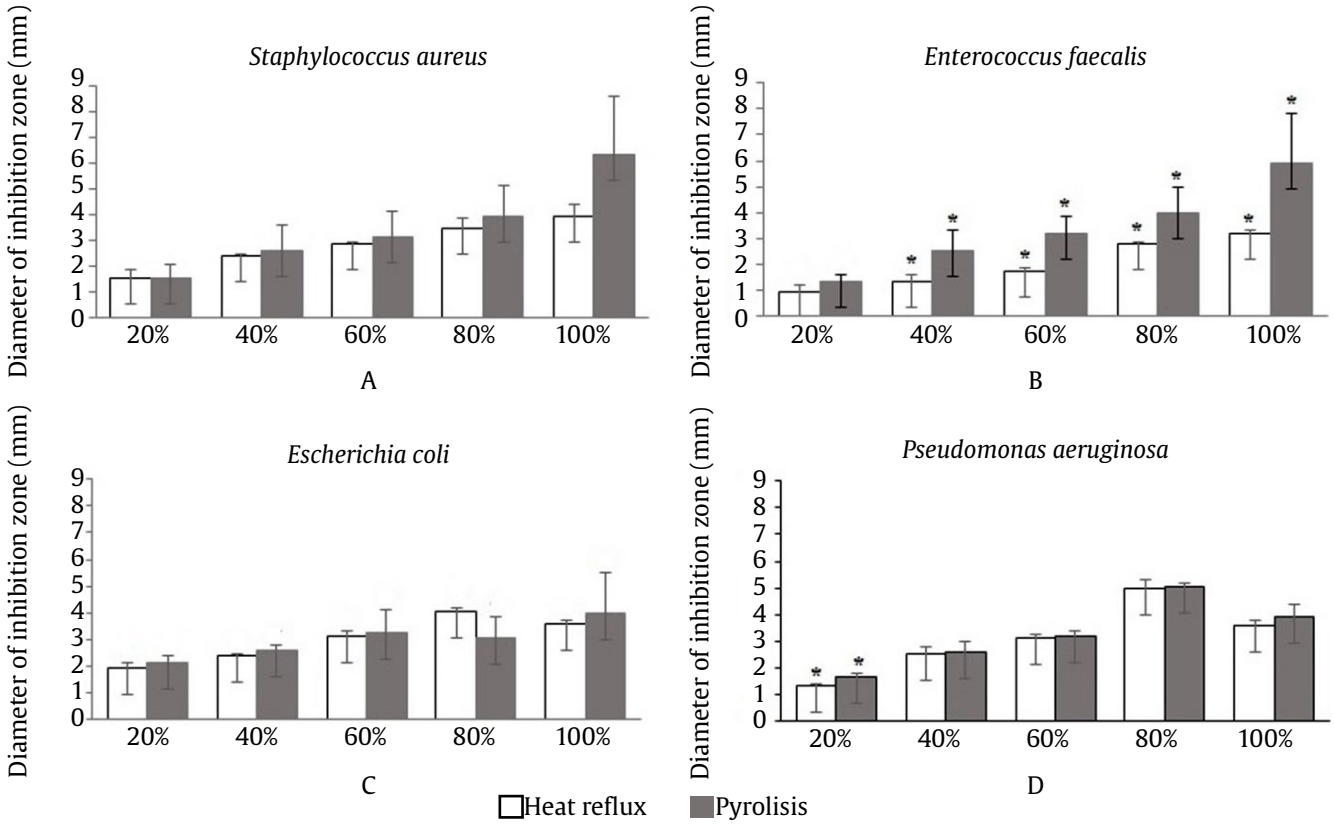

Figure 1. Comparison of the antibacterial potential of extracts produced using pyrolysis and heat reflux extraction (mm). (A) *Staphylococcus aureus*, (B) *Enterococcus faecalis*, (C) *Escherichia coli*, (D) *Pseudomonas aeruginosa*. *The mean difference is significant at 0.05

against the other three test bacteria (Figure 1). This study showed that *Enterococcus faecalis* is more susceptible to active antimicrobial substances in tobacco extracts and bio-oils. *Enterococcus faecalis* belongs to the susceptible bacteria in analyzing the potential antimicrobial differences between extractions methods, especially in EHRE and pyrolysis extraction methods.

When it comes to the well diffusion test, *Nicotiana tabacum* var Virginia's showed the highest antibacterial activity in bio-oil produced using the pyrolysis extraction method compared to the EHRE method, which is tested against *Escherichia coli* (Table 3). This is because of the pyrolysis extraction method that we have done has reached 47.6wt%, which is very much higher than the EHRE method, which is only 23wt%. An increase in extraction yield could be accompanied by an increase in *Nicotiana tabacum* var Virginia's antimicrobial compound and enhancement of the inhibition diameter. This is also supported by the GC-MS from our bio-oils results which show that dominant compounds in the bio-oils from the pyrolysis process were organic acids and pyridine (besides nicotine), which the antibacterial potential are well known. In contrast, the heat reflux extract was dominated by γ-sitosterol, where the antimicrobial potential is still unclear.

When comparing the extraction methods for the *Nicotiana tabacum* in this study with other previous extraction studies from the same plant species (*Nicotiana tabacum* sp.), it seems that the maceration method produces more significant inhibition than the pyrolysis method in this study (Table 4). The factor that influences these results must be studied further.

When comparing the antibacterial test, N*icotiana tabacum* antimicrobial test against *Escherichia coli* with well diffusion in this study, with other previous studies against other bacteria, showed that *E. coli* has a higher susceptibility to the antibacterial compound in *Nicotiana tabacum*, compared to the different bacteria that have been examined (Table 4). It can reach up to 26.66 mm in inhibition diameter. This might be due to the initial concentration used for the experiment is quite high (3 g/ml) compared to the concentration used in the pyrolysis method (0.33 g/ml) in this study. Higher concentrations for increasing the diameter of inhibition in the pyrolysis method could be an alternative for further research. This is also seen in the antimicrobial test of other plant species against *E. coli*, where *E. coli* also show high susceptibility to active substances from other plant extracts as well.

3.2.2. Comparison of Disc Diffusion Antibacterial Test to the Well Diffusion Antibacterial Test against *Escherichia coli* and *Pseudomonas aeruginosa*

A second analysis was carried out to compare the differences in antibacterial activity for bio-oils used with different test methods, namely the disc diffusion test method and the well diffusion test method when deployed against the two test bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The analysis results indicate that extracts implemented using the well diffusion test method create greater inhibition diameters with a significant difference (Figure 2).

Our study confirms previous studies' findings, which showed that the well diffusion method is more effective than the disc diffusion method. Is also confirm from previous studies against *Escherichia coli* using different plant extracts, except *Adenium obesum, Syzygium aromaticum,* and *Capparis spinosa* plant extracts produced using the maceration extraction method. In testing using *Pseudomonas aeruginosa*, it also showed a similar trend, except for *Adenium obesum* and *Syzygium aromaticum* plant extracts.

Table 4. Antibacterial activity of *Nicotiana tabacum* leaf and other plants (expressed in diameter of inhibition zone (mm))

Plant	Bacteria	Type of antibacterial test	Solvent	Concentration (w/v)	Extraction method	Inhibition diameter (mm)	Reference
N. tabacum Var Virginia	*S. aureus*	DD	Ethanol	0.20 g/ml	HR	3.95±0.45	TS
	E. faecalis					3.20±0.10	TS
	E. coli					3.54±0.16	TS
	P. aeruginosa					3.60±0.15	TS
	S. aureus	DD	PG	0.33 g/ml	Pyrolisis	6.35±2.24	TS
	E. faecalis					5.90±1.96	TS
	E. coli					3.97±1.49	TS
	P. aeruginosa					3.92±0.46	TS
	E. coli	WD	PG	0.33 g/ml	Pyrolisis	11.65±1.99	TS
	P. aeruginosa					8.72±0.26	
N. tabacum L	*S. aureus*	DD	Ethanol	0.10 g/ml	Maceration	7.13±0.25	(Ameya *et al.* 2018)
	P. aeruginosa					6.16±0.47	
	K. pneumonia					4.59±0.387	
	S. aureus (CI)					5.57±0.54	
	S. typhi (CI)					6.16±0.47	
N. tabacum L	*B. substilis*	DD	Ethanol	0.25 g/ml	Maceration	13	(Akinpelu and Obuotor 2000)
	C. pyogenes					15	
	P. aeruginosa					11	
	S. marcescent					11	
	S. dysenteriae					15	
	S. aureus					14	
N. tabacum L	*B. cereus*	DD	EA	3.00 g/ml	Maceration	22.33	(Bakht and Shafi 2012)
			Butanol			15.33	
	S. aureus		EA			15.33	
			Acetone			12.33	
			Butanol			20.00	
	E. carotavora		EA			22.33	
			Acetone			9.66	
			Butanol			15.66	
	E. coli		EA			26.66	
			Butanol			20.00	
			Water			10.66	
	A.tumefaciens		EA			17.00	
			Butanol			15.66	
	P. aeruginosa		Ethanol			10.33	
			EA			24.66	
			Butanol			18.33	
			Water			12.33	
	S. typhi		Ethanol			14.33	
			EA			18.33	
			Acetone			15.33	
			Butanol			16.66	
N. tabacum L	*S. pyogenes*	DD	Methanol	0.2 g/ml	Maceration	13.00	(Ck *et al.* 2019)
			Water			10.00	
N. tabacum L	*S. aureus*	DD	Methanol		Maceration	3.10	(Gebremedhin Romha *et al.* 2018)
	E. coli		Methanol			3.38	
	P. aeruginosa		Methanol			2.53	

HR: heat reflux, DD: disc diffusion, WD: well diffusion, EA: ethyl acetate, PG: polyethylene glycol, TS: this study

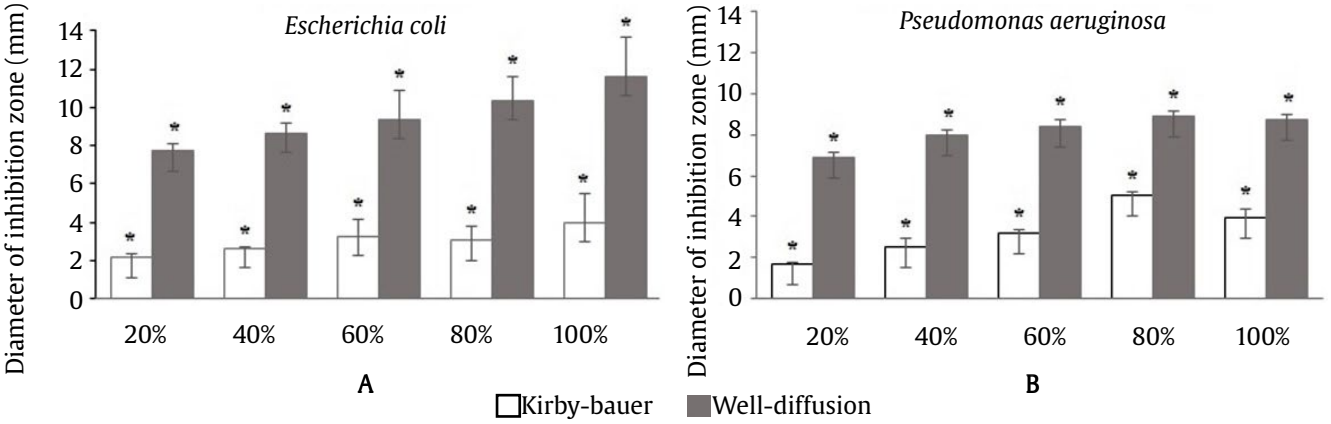

Figure 2. The average diameter of the Kirby-Bauer and well diffusion methods (expressed in diameter of inhibition zone (mm)) produced by (A) *Escherichia coli* (B) *Pseudomonas aeruginosa*. *The mean difference is significant at 0.05

4. Discussion

4.1. Pyrolisis Extraction Degraded Some Tobacco Active Compounds but Still Have Functional Bacterial Growth Inhibition

Qualitative total alkaloids are greater in bio-oil, but total polyphenol was still higher in the tobacco extract (Table 2). This study also shows that polyphenol compounds were less in bio-oil than tobacco extract (Table 2). This can be caused by the polyphenols themselves, which are easily degraded at high temperatures done in pyrolisis, compared to alkaloids (Hanuka Katz *et al.* 2020). Our study showed that the extraction of pyrolysis degraded some active tobacco compounds with antimicrobial potential. However, pyrolysis still showed higher antimicrobial activity than EHRE. The pyrolysis extraction can isolate more other antimicrobial components (such as nicotine) than EHRE (Table 3). However, also some components are degraded due to the use of hot temperatures. Previous research from Cardoso *et al.* 2017 on tobacco extraction using the pyrolysis method showed that the phenolic compounds found in the bio-oil were a less significant number than other compounds, such as nicotine, furfural, and acetic acid (Cardoso and Ataíde 2013).

4.2. Organic Acids and Pyridine (besides Nicotine) as Bio-oils Dominant Compounds (in Pyrolysis Extract) Showed Higher Antimicrobial Properties

Our study results showed that the pyrolysis extraction method produced higher antibacterial potential than the heat reflux method (Figure 1). This could be due to the higher number of alkaloids in pyrolysis. It may also be caused by differences in the dominance of the active substance produced by the heat reflux method and the pyrolysis in our study. Through the pyrolysis process, Bio-oil produces products with more varied compounds in higher concentrations and contain many active compounds. Our GC-MS analysis study showed that the bio-oils dominant compounds from the pyrolysis process were organic acids and pyridine, besides nicotine. Organic acid and pyridine have antimicrobial properties (Gozan *et al.* 2014). Unlike the tobacco extract produced by heat reflux, pyridine is not the dominant substance found other than nicotine, but rather γ-sitosterol (Andjani *et al.* 2019). The pyridine ring is a compound with a spectrum of biological activity the antimicrobial potential. Pyridine has antimicrobial potential against several bacteria (Desai *et al.* 2017; Radwan *et al.* 2020). Some plants that contain predominant γ-sitosterol have antimicrobial potential (Zhang *et al.* 2011; Silveira *et al.* 2017). The possibility from our results can be concluded indirectly that pyridine has more antimicrobial properties than gamma sitosterol. But how far gamma sitosterol can be antimicrobial compared to pyridine remains proven further.

Organic acids can inhibit bacterial growth by increasing the entry of transition metals into the bacteria. A strong synergy was found between low concentrations of non-toxic metals and organic acids, with an inhibitory effect on bacterial growth (Zhitnitsky *et al.* 2017). Previous studies have also shown that organic acids have an inhibitory effect on Gram+and Gram-bacteria (Kovanda *et al.* 2019).

In addition, the dominant antimicrobial action of pyrolysis can also be caused by the relatively higher number of alkaloids. The antibacterial mechanism of flavonoid compounds causes damage to the bacterial cell walls and disrupts cell metabolic processes (Cushnie and Lamb 2011; Khameneh *et al.* 2019).

4.3. *Enterococcus faecalis* is more susceptible to Active Antimicrobial Substances compared to other Bacteria for Both EHRE (extract) and Pyrolysis (Bio-oil) Extraction

Comparing the bacteria used in our test shows that *Enterococcus faecalis* are more susceptible to active microbial substances from *Nicotiana tabacum* var Virginia than the other three test bacteria (Figure 1) in both tobacco extracts and bio-oils. Some bacterial species are more susceptible to antimicrobial compounds, and some are innately resistant. This is due to each bacterial pathogen's unique profile characteristics and antibacterial compound mechanisms (Khameneh *et al.* 2019). This was also shown in previous studies (Chandrappa *et al.* 2015; Noushad *et al.* 2018).

4.4. Well Diffusion Test Method has a more Efficient Diffusion Process compared to the Disc Diffusion Method on Bio-oils against *Escherichia coli* and *Pseudomonas aeruginosa*

A second analysis of bio-oils on different test methods (disc diffusion test method and the well diffusion test method) against the two test bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) indicate that the well diffusion test method has greater inhibition diameters with a significant difference (Figure 2). This may be due to the more efficient diffusion process of the well method. This result is confirmed by another study on antimicrobial tests (Valgas *et al.* 2007; Mansour Saleh Saif *et al.* 2017) and suggests that the well diffusion test method is more effective than the disc diffusion test method (Figure 2). In another study on a different plant extract, the well diffusion test method produced a greater inhibitory zone diameter than the disc diffusion test method (Valgas *et al.* 2007; Mansour Saleh Saif *et al.* 2017). This may be because the tobacco leaf extract's osmolarity is higher in the well diffusion test method than in the disc diffusion test method. The well diffusion test method involves making a hole in the Muller Hinton Agar (MHA), filled with tobacco leaf extract. The tobacco leaf extract diffusion into the medium will be complete and homogeneous (Balouiri *et al.* 2016). In contrast to the well diffusion test method, in the disc diffusion test method, the tobacco leaf extract is not directly poured into the MHA; instead, the disc paper is first soaked in each component of each tobacco leaf extract before being placed on top of the MHA (Balouiri *et al.* 2016). In a well diffusion test against *Escherichia coli* from different plant extracts besides *Nicotiana tabacum* (Table 1), the disc diffusion test method produced a smaller inhibitory zone diameter when compared to the well diffusion test method because the greater absorption of the antimicrobial compound to the disc paper in the disc diffusion method made the process of antimicrobial compound diffusion to the media less than optimal (Valgas *et al.* 2007; Mansour Saleh Saif *et al.* 2017).

Moreover, another antibacterial test against *Escherichia coli* using different plant extracts showed the same trend, except the tests that used *Adenium obesum, Syzygium aromaticum*, and *Capparis spinosa* plant extracts using the maceration extraction method (Mansour Saleh Saif *et al.* 2017). Thus, it is most likely that the findings in our study on the well diffusion test method are more effective than the disc diffusion test method against *Escherichia coli* are comparable to the findings of other antibacterial test experiments (Elisha *et al.* 2017; Mansour Saleh Saif *et al.* 2017; Valgas *et al.* 2007).

Disc diffusion and well diffusion antibacterial testing with *Nicotiana tabacum* have been conducted against *Pseudomonas aeruginosa*, as shown in Table 1. Our study produced results in line with previous studies, which indicated that the well diffusion test method is more effective than the disc diffusion test method. Moreover, another antibacterial test against *Pseudomonas aeruginosa* using other plant extracts showed the same trend, except for where *Adenium obesum* and *Syzygium aromaticum* plant extracts derived using the maceration extraction method were used (Mansour Saleh Saif *et al.* 2017). Thus, it is most likely that the findings of this study that the well diffusion test method is more effective than the disc diffusion test method are comparable to the findings of other antibacterial test experiments against *Pseudomonas aeruginosa*. This could be due to the different antibacterial compounds that reside in plants. Some of these antibacterial compounds might be more effectively captured in the disc diffusion and inhibit bacteria growth more effectively. In contrast, other antibacterial compounds might work better in well diffusion test methods since they are abundant in liquid form in the well (Balouiri *et al.* 2016).

In conclusions, this study's antibiotic testing comparison indicates that bio-oils produced using the pyrolysis method are better than the extracts produced using the heat reflux method for extracting the antibacterial compounds from *Nicotiana tabacum* var. Virginia from Ponorogo, Indonesia. This is proven by our result, where the pyrolysis method produces a greater diameter of inhibition compared to the EHRE method, against *Staphylococcus aureus* (6.35 mm), *Enterococcus faecalis* (5.90 mm), *Escherichia coli* (3.97 mm), and *Pseudomonas aeruginosa* (5.025 mm). This is related to antimicrobial compounds inside in the bio-oils, where alkaloids are higher in

bio-oils. However, fewer polyphenols are found in bio-oils, but there are still antimicrobial compounds predominantly found in bio-oils (pyridine) besides nicotine.

Moreover, our study showed that antibacterial using the well diffusion test method was more effective for delivering the antimicrobial compound than agar diffusion, indicated by the higher inhibition diameters produced against *Escherichia coli*. This research proves that bio-oil in the well diffusion test method showed the greatest antibacterial effectiveness. It created the biggest inhibition zone, with a maximum of 11.65 mm and 8.90 mm for *E. coli* and *P. aeruginosa* (Table 3). Nevertheless, the initial concentration used in the experiments, the test method and susceptibility of bacteria against the active antimicrobial compound were the factor that should be considered to influence the inhibition diameter enhancement on antibacterial testing.

Funding

The works were financially supported by the Universitas Indonesia PUTI Q1 2020 (Contract Nr.NKB-1415/UN2.RST/HKP.05.00/2020).

Conflicts of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Acknowledgments

We would like to thank Mrs. Titi Sudjiati (research assistant in our microbiology laboratory) for her support in this study.

References

Ahsan, A., Wiyono, N., Kiting, A., 2014. Bunga Rampai-Fakta Tembakau dan Permasalahan di Indonesia, fifth ed. Tobacco Control Support Center- IAKMI, Jakarta.

Akinpelu, D.A., Obuotor, E.M., 2000. Antibacterial activity of *Nicotiana tabacum* leaves. *Fitoterapia.* 71, 199–200. https://doi.org/10.1016/S0367-326X(99)00148-3

Ameya, G., Manilal, A., Merdekios, B., 2018. *In vitro* antibacterial activity and phytochemical analysis of *Nicotiana tabacum* L. extracted in different organic solvents. *Open Microbiol. J.* 11, 352–359. https://doi.org/10.2174/1874285801711010352

Andhika Priotomo, R., Fauzantoro, A., Gozan, M., 2018. Fractionation and characterization of semi-polar and polar compounds from leaf extract *Nicotiana tabaccum* L. reflux ethanol extraction results. *In: AIP Conference Proceedings*. Bali: American Institute of Physics Inc, pp. 1-9. https://doi.org/ 10.1063/1.5023969.

Andjani, H.N., Sentosa, Y., Yati, K., Jufri, M., Fauzantoro, A., Gozan, M., 2019. Determination of LC_{50} value of *Nicotiana tabacum* L. extracts against *Gryllus bimaculatus* imago and *Galleria mellonella* larvae. *In: AIP Conference Proceedings*. Padang: American Institute of Physics Inc, pp. 1-7. https://doi.org/10.1063/1.5139361

Ario Putra, D.A., Pramono, A., Anisah, A., Fauzantoro, A., Gozan, M., 2019. The effect of tobacco leaves pyrolysis extract (*Nicotiana tabacum* L.var. Virginia) against the formation of biofilm by *Staphylococcus aureus*: an *in vitro* study. *IOP Conf. Ser. Mater. Sci. Eng.* 508, 012147. https://doi.org/10.1088/1757-899X/508/1/012147

Bakht, J., Shafi, M., 2012. Antimicrobial activity of *Nicotiana tabacum* using different solvent extracts. *Pak. J. Bot.* 44, 459-643.

Balouiri, M., Sadiki, M., Ibnsouda, S.K., 2016. Methods for *in vitro* evaluating antimicrobial activity: a review. *J. Pharm. Anal.* 6, 71-79. https://doi.org/10.1016/j.jpha.2015.11.005

Banožić, M., Babić, J., Jokić, S., 2020. Recent advances in extraction of bioactive compounds from tobacco industrial waste-a review. *Ind. Crops Prod.* 144, 112009. https://doi.org/10.1016/j.indcrop.2019.112009

Cardoso, C.R., Ataíde, C.H., 2013. Analytical pyrolysis of tobacco residue: effect of temperature and inorganic additives. *J. Anal. Appl. Pyrolysis,* 99, 49–57. https://doi.org/10.1016/j.jaap.2012.10.029

Chandrappa, P.M., Dupper, A., Tripathi, P., Arroju, R., Sharma, P., Sulochana, K., 2015. Antimicrobial activity of herbal medicines (tulsi extract, neem extract) and chlorhexidine against *Enterococcus faecalis* in Endodontics: an *in vitro* study. *J. Int. Soc. Prev. Community Dent.* 5, 89-92.

Cheesman, M.J., Ilanko, A., Blonk, B., Cock, I.E., 2017. Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? *Pharmacogn. Rev.* 11, 57-72.

Ck, A., Mi, N., Cc, O., Io, N., Fc, I., 2019. Antimicrobial activities of extracts of tobacco leaf (*Nicotiana tabacum*) and Its Grounded Snuff (Utaba) on *Candida albicans* and *Streptococcus pyogenes*. *J.Trop Dis.* 7, 1000300. https://doi.org/10.4172/2329-891X.1000300

Cushnie, T.P.T., Lamb, A.J., 2011. Recent advances in understanding the antibacterial properties of flavonoids. *Int. J. Antimicrob. Agents.* 38, 77-107. https://doi.org/10.1016/j.ijantimicag.2011.02.014

Desai, N.C., Patel, B.Y., Dave, B.P., 2017. Synthesis and antimicrobial activity of novel quinoline derivatives bearing pyrazoline and pyridine analogues. *Med. Chem. Res.* 26, 109–119. https://doi.org/10.1007/s00044-016-1732-6

Elisha, I.L., Botha, F.S., McGaw, L.J., Eloff, J.N., 2017. The antibacterial activity of extracts of nine plant species with good activity against *Escherichia coli* against five other bacteria and cytotoxicity of extracts. *BMC Complement. Altern. Med.* 17, 133. https://doi.org/10.1186/s12906-017-1645-z

Fathi, R.M., Fauzantoro, A., Rahman, S.F., Gozan, M., 2018. Column chromatography isolation of nicotine from tobacco leaf extract (*Nicotiana tabaccum* L.). *In: AIP Conference Proceedings.* Bali: American Institute of Physics Inc., pp. 1–9. https://doi.org/10.1063/1.5023958

Fauzantoro, A., Muharam, Y., Gozan, M., 2017. Improvement of nicotine yield by ethanolic heat reflux extraction of *Nicotiana tabacum* var. Virginia origin of Ponorogo. *Int. J. Appl. Eng. Res.* 12, 13891–13897.

Fernanda, S.A., Amru, B.A.,Rahmani, H.A., Gozan, M., Irsyad, N.S., Bahar, M., Puspita, O.S., Zulfa, F. Pramono, A., 2021. Antibacterial Potential of *Nicotiana tabacum* L. var Virginia Pyrolysis Extract Against *Staphylococcus aureus,Enterococcus faecalis, Escherichia coli*, and *Pseudomonas aeruginosa. IOP Conf. Ser.: Earth Environ. Sci.* 755 012013. https://doi.org/10.1088/1755-1315/755/1/012013

Gebremedhin Romha, Birhanu Admasu, Tsegaye Hiwot Gebrekida, Hailelule Aleme, Gebreyohans Gebru, 2018. Antibacterial activities of five medicinal plants in ethiopia against some human and animal pathogens. Evidence-Based complement. *Altern. Med.* 2018, 1–10. https://doi.org/10.1155/2018/2950758

Gentile, B., Grottola, A., Orlando, G., Serpini, G.F., Venturelli, C., Meschiari, M., Anselmo, A., Fillo, S., Fortunato, A., Lista, F., Pecorari, M., Mussini, C., 2020. A retrospective whole-genome sequencing analysis of carbapenem and colistin-resistant *Klebsiella pneumoniae* nosocomial strains isolated during an MDR surveillance program. *Antibiotics.* 9, 246. https://doi.org/10.3390/antibiotics9050246

Gozan, M., Yasman, Wulan, P.P.D.K., Dawitri, E., 2014. Tobacco leaves pyrolysis for repellent active compound production. *Int. J. Appl. Eng. Res.* 9, 9739–9750.

Hanuka Katz, I., Eran Nagar, E., Okun, Z., Shpigelman, A., 2020. The link between polyphenol structure, antioxidant capacity and shelf-life stability in the presence of fructose and ascorbic acid. *Molecules.* 25, 225. https://doi.org/10.3390/molecules25010225

Hudzicki, J., 2009. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. American Society for Microbiology. Available at: https://asm.org/Protocols/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Pro. [Date accessed: 5 September 2021]

Iseppi, R., Di Cerbo, A., Aloisi, P., Manelli, M., Pellesi, V., Provenzano, C., Camellini, S., Messi, P., Sabia, C., 2020. *In vitro* activity of essential oils against planktonic and biofilm cells of extended-spectrum β-lactamase (ESBL)/carbapenemase-producing gram-negative bacteria involved in human nosocomial infections. *Antibiotics.* 9, 272. https://doi.org/10.3390/antibiotics9050272

Jean, S.S., Chang, Y.C., Lin, W.C., Lee, W.S., Hsueh, P.R., Hsu, C.W., 2020. Epidemiology, treatment, and prevention of nosocomial bacterial pneumonia. *J. Clin. Med.* 9, 275. https://doi.org/10.3390/jcm9010275

Khameneh, B., Iranshahy, M., Soheili, V., Fazly Bazzaz, B.S., 2019. Review on plant antimicrobials: a mechanistic viewpoint. *Antimicrob. Resist. Infect. Control.* 8, 118. https://doi.org/10.1186/s13756-019-0559-6

Kolar, M., Cermak, P., Hobzova, L., Bogdanova, K., Neradova, K., Mlynarcik, P., Bostik, P., 2020. Antibiotic resistance in nosocomial bacteria isolated from infected wounds of hospitalized patients in the czech republic. *Antibiotics.* 9, 342. https://doi.org/10.3390/antibiotics9060342

Kovanda, L., Zhang, W., Wei, X., Luo, J., Wu, X., Atwill, E.R., Vaessen, S., Li, X., Liu, Y., 2019. *In vitro* antimicrobial activities of organic acids and their derivatives on several species of gram-negative and gram-positive bacteria. *Molecules.* 24, 3770. https://doi.org/10.3390/molecules24203770

Kudo, M., Nomura, T., Yomoda, S., Tanimoto, K., Tomita, H., 2014. Nosocomial infection caused by vancomycin-susceptible multidrug-resistant *Enterococcus faecalis* over a long period in a university hospital in Japan. *Microbiol. Immunol.* 58, 607–614. https://doi.org/10.1111/1348-0421.12190

Mansour Saleh Saif, M., Ali Al-Fakih, A., Abdu Hassan, M.M., Mohammed Mansour Saleh Saif, C., 2017. Antibacterial activity of selected plant (aqueous and methanolic) extracts against some pathogenic bacteria. *J. Pharmacogn. Phytochem.* 6, 1929–1935.

Noushad, M.C., Balan, B., Basheer, S., Usman, S. Bin, Askar, M.K.M., 2018. Antimicrobial efficacy of different natural extracts against persistent root canal pathogens: an *in vitro* study. *Contemp. Clin. Dent.* 9, 177–181. https://doi.org/10.4103/ccd.ccd_754_17

Pertanian RI, K., 2011. Tembakau Virginia. In Balitbangtan Kementrian Pertanian. Balai Penelitian Tanaman Tembakau dan Serat, Malang.

Pramono, A., Fauzantoro, A., Hidayati, I.R., Hygea, A., Puspita, O.S., Muktamiroh, H., Simanjuntak, K., Gozan, M., 2018. *In vitro* assay of ethanolic heat reflux extract of *Nicotiana tabacum* L . var Virginia against nosocomial bacteria pathogen *in vitro* assay of ethanolic heat reflux extract of *Nicotiana tabacum* L. var Virginia against nosocomial bacterial pathogen. *J. Phys. Conf. Ser.* 970, 012021. https://doi.org/10.1088/1742-6596/970/1/012021

Radwan, M.A.A., Alshubramy, M.A., Abdel-Motaal, M., Hemdan, B.A., El-Kady, D.S., 2020. Synthesis, molecular docking and antimicrobial activity of new fused pyrimidine and pyridine derivatives. *Bioorg. Chem.* 96, 103516. https://doi.org/10.1016/j.bioorg.2019.103516

Silveira, D., Maia, V., Camargo Aranha, B., Chaves, F.C., Padilha Da Silva, W., 2017. Antibacterial activity of *Butia odorata* Barb. Rodr. Extracts antibacterial foodborne pathogens synthetic preservatives chemical characterization phytosterols. *Trends Phytochem. Res.* 1, 169-174.

Sokunvary Oeung, Nov, V., Ung, H., Roum, K., Yin, V., Keo, S., Sin Chea, 2016. Phytochemical analysis of different extracts of leaves of *Nicotiana tabacum* L. of Cambodia. *Asian J. Pharmacogn.* 1, 18–26.

Suleyman, G., Alangaden, G., Bardossy, A.C., 2018. The role of environmental contamination in the transmission of nosocomial pathogens and healthcare-associated infections. *Curr. Infect. Dis. Rep.* 20, 1–11. https://doi.org/10.1007/s11908-018-0620-2

Valgas, C., De Souza, S.M., Smânia, E.F.A., Smânia, A., 2007. Screening methods to determine antibacterial activity of natural products. *Brazilian J. Microbiol.* 38, 369–380. https://doi.org/10.1590/S1517-83822007000200034

Zhang, W., Abdel-Rahman, F.H., Saleh, M.A., 2011. Natural resistance of rose petals to microbial attack. *J. Environ. Sci. Health, Part B.* 46, 381–393. https://doi.org/10.1080/03601234.2011.572502

Zhitnitsky, D., Rose, J., Lewinson, O., 2017. The highly synergistic, broad-spectrum, antibacterial activity of organic acids and transition metals. *Sci. Rep.* 7, 1–13. https://doi.org/10.1038/srep44554