

Phytochemical screening, antibacterial and antioxidant assessment of *Leuconotis eugenifolia* leaf extract

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Abstract. Praptiwi, Wulansari D, Fathoni A, Harnoto N, Novita R, Alfridsyah, Agusta A. 2020. Phytochemical screening, antibacterial and antioxidant assessment of *Leuconotis eugenifolia* leaf extract. *Nusantara Bioscience* 12: 79-85. Traditionally, sitahe (*Leuconotis eugenifolia* (Wall. Ex G. Don) A.DC) has been used to maintain stamina by Aceh peoples, Indonesia. The use of *L. eugenifolia* mainly as a spice on a traditional cuisine "ie bu peudah". This research was conducted to determine the phytoconstituents of *L. eugenifolia* leaf extracts, and to investigate their antioxidant potential using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Phytochemical screening was carried out using standard methods. *L. eugenifolia* leaves were extracted successively with n-hexane, dichloromethane, ethyl acetate, methanol, and water. Obtained extracts were tested qualitatively for antioxidant and antibacterial by the TLC-bioautography method, followed by quantitative analysis to determine the MIC and IC₅₀ value using the microdilution method on 96-well microplate. The results showed that it contained alkaloids, flavonoids, steroids, triterpenoids, saponins, phenolics, and tannins. The highest levels of phenolic (259.0486 ± 0.1203 mg GAE/g extract) and flavonoids (84.6575 ± 0.0237 mg QE/g extract) were found in ethyl acetate extract, and dichloromethane, respectively. The antibacterial activity of n-hexane and dichloromethane extracts is moderate against *E. coli*, and weak against *S. aureus*. Ethyl acetate extract, methanol, and water extracts have very strong antioxidant activity. *L. eugenifolia* leaf extracts may be promising new antioxidant sources.

Keywords: Antibacterial, antioxidant, *Leuconotis eugenifolia*, microdilution, TLC-bioautography

INTRODUCTION

Plants have been used in various aspects of human life, including in the health sector. Until now, around 80% of the population still depend on traditional medicinal plants for primary health care. Although there has been a lot of research on medicinal plants, to date the plant is considered a valuable source of chemical compounds for the development of various drugs for various diseases (Angiollela et al. 2018). Traditional medicines in the form of plant extracts have been proven to be clinically effective and relatively less toxic (Awaad 2011).

Chemical ingredients that play a role in bioactivity as a medicinal ingredient include: alkaloids, phenolics, flavonoids, tannins, quinones, saponins, and terpenoids. Alkaloids act as plant protection against pathogens and herbivores and CNS stimulants (Mousavi et al. 2018). Tannins have been used as anti-diarrheal and anti-hemorrhagic (Yogesh and Mala 1998). Phenolic and flavonoids have been known to have many health benefits, can prevent and cure many diseases and are well-known as antioxidants (Tungmunnithum et al. 2018).

The emergence of antibiotic-resistant bacteria has been dramatically increased. Antibiotic-resistant bacteria cause at least 2 million infections and 23,000 deaths per year in the USA (Li and Webster 2017). Therefore, there is a need

for new, effective, affordable for treating microbes infections and to overcome antibiotic-resistant bacteria (Elisha et al. 2017).

Oxidative stress is characterized by an imbalance between the production of reactive oxygen species (ROS) and antioxidant activity, and the increase can lead to several chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular disease (Angilella et al. 2018). ROS in the biological system can damage DNA and oxidation of lipid and protein in the cells (Peng et al. 2014). The antioxidant system in the human body can scavenge ROS and keep the balance between oxidation and antioxidant (Xu et al 2017).

Sitahe (*Leuconotis eugenifolia*) has been used to strengthen stamina by the people of Aceh, Indonesia. This plant species distributed in Sumatra, Kalimantan, and Peninsular Malaysia and secondary metabolites vary depending on the place of growth (Tang et al. 2019). To the best of our knowledge, publications on *L. eugenifolia* collected from Indonesia are still very limited. Abe and Yamauchi (1993) have reported that *L. eugenifolia* from Sumatra, Indonesia without specifying a more specific location contains 6 types of indole alkaloids ie. yohimbine, β-yohimbine, leukonolam, 21-O-leuconolam, diazaspilukonolam, and rhazinalin-N6-oxide. These secondary metabolites did not found in plant samples

collected from the Peninsular area (Gih et al. 1989; Gan 2009a, b; Deguchi et al. 2010; Tang et al. 2019). Variations in secondary metabolites could produce different biological activities of the plant extract. Therefore, this study aims to determine the phytochemical compounds, antibacterial, and antioxidant activity of sitahe (*Leuconotis eugenifolia*) collected from Aceh, Indonesia.

MATERIALS AND METHODS

Collection and preparation of plant material

Leuconotis eugenifolia leaves were collected from Aceh. The plant was identified at the Herbarium Bogoriense (BO), Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Bogor, Indonesia. The leaves were washed and air-dried followed by grinding the sample.

Preparation of extracts: maceration

Leuconotis eugenifolia leaves powder was macerated for 3 days and stirred occasionally. Ethanol 70% was used as an extractant using a ratio of 1: 10 of *L. eugenifolia* leaves to ethanol 70%. The extraction process is done 3 times. The filtrate was collected and then concentrated with a rotary evaporator to get concentrated extract. The concentrated extract was partitioned using the different polarity of solvents, i.e., *n*-hexane, dichloromethane, ethyl acetate, methanol, and water.

Phytochemical screening

Phytochemical content of *L. eugenifolia* leaf extract was analyzed qualitatively according to the standard method as follows:

Test for alkaloid. 1.5 mL of extract was added with 2 mL of HCl 2%. To this mixture was added with Mayer reagent and the formation of a white precipitate indicates the presence of alkaloid. At the addition of Dragendorff, the presence of alkaloids was indicated by the presence of orange or orange-red precipitate.

Test for glycoside. 1 mL of extract was dissolved in ethanol followed by heat- evaporation. Then, it dissolved in 1 mL of anhydrous acetic acid and added 5 drops of concentrated sulfuric acid. Blue or green color indicates the presence of glycosides.

Test for steroids and terpenoids. The extract was dissolved in 0.5 mL chloroform, add 0.5 mL anhydrous acetic acid. This mixture was added with 2 mL of concentrated sulfuric acid through the tube wall. Green color indicating the presence of sterols, while brownish ring indicating the presence of triterpenes.

Test for saponin. 1.5 mL of extract in the test tube was shaken. The presence of saponin indicated by foam formation as high as 1 cm that persistent for 15 minutes.

Test for polyphenol and tannin. 1-1.5 mL of extract was added with 1 mL of 10% FeCl₃ solution. The presence of dark blue, black-blue, or greenish-black color indicated the presence of polyphenol and tannin.

Test for flavonoid. 1.5 mL of extract added with magnesium powder, then heated followed by the addition

of concentrated HCl. Positive results indicated by the presence of red color in the extract solution.

Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) was carried out by Folin Ciocalteu's method. Two hundred μ L extract (1000 μ g/mL) and standard gallic acid at the concentration range of 6.25- 200 μ g/mL added 0.2 mL of 50% Folin-Ciocalteu solution and vortex for 1 minute. The mixture was added 4 mL of 2% sodium carbonate (Na₂CO₃) solution then incubated under dark at room temperature for 30 minutes. The absorbance of the extract was read at a wavelength of 750 nm using a UV-Vis spectrophotometer and performed in triplicates. The results are expressed as mg gallic acid/g extract (Ismail et al. 2012).

Determination of Total Flavonoid Content (TPC)

0.5 mL extract (1000 μ g mL) and standard quercetin at the concentration range of 3.125-100 μ g mL each added with 1.5 ethanol pa, 0.1 mL 10% AlCl₃, 0.1 mL CH₃COONa.3H₂ 1M and 2.8 mL aquabidest. The mixture was incubated at room temperature for 30 minutes under dark conditions. The absorbance of the extract solution is read at a wavelength of 417 nm with a UV-Vis spectrophotometer (UVmini-1240, Shimadzu) and performed in triplicates. The results are expressed as mg quercetin/g extract (Pourmorad et al. 2006).

Detection of antibacterial activity by TLC-Direct bioautography

TLC-direct bioautography for antibacterial activity was carried out by the dot-blot method and developed TLC-plates aseptically. The antibacterial activity of extracts was carried out against *Escherichia coli* Ina-CC B5 and *Staphylococcus aureus* B4. **Dot-blot method:** Ten μ L of extract (10 μ g/mL) was transferred onto silica TLC-plate (Merck, silica gel 60 F254) and air-dried. Chloramphenicol was used as a positive control, while the solvent used as the negative control. **Developed-TLC plates:** The extracts were developed with eluent system as follows: hexane extract was developed with eluent system of hexane: ethyl acetate (3: 1), dichloromethane and ethyl acetate extracts were developed with dichloromethane: methanol (10: 1), while methanol and water extracts were developed with chloroform: methanol: water (6: 4: 1). After finish transferring and developing the extracts, TLC-plate were dipped in the bacterial suspension, and then incubated for 18-24 hours at 37°C under humid condition by adding sterile wet-cotton. After incubation, plates were sprayed with iodinitrotetrazolium (4 mg/mL) aqueous solution. Growth inhibition was indicated by white zones against the purple background.

Detection of antioxidant activity by TLC-direct bioautography

TLC-direct bioautography for antioxidant activity was carried out by the dot-blot method and developed TLC-plates. The antibacterial activity of extracts was carried out against *E. coli* and *S. aureus*. **Dot-blot method:** Ten μ L of extract (10 μ g/mL) was transferred onto silica TLC-plate

(Merck, silica gel 60 F254) and air-dried. *Developed-TLC plates*: The extracts were developed with eluent system as follows: hexane extract was developed with eluent system of hexane: ethyl acetate (3: 1), dichloromethane and ethyl acetate extracts were developed with dichloromethane: methanol (10: 1), while methanol and water extracts were developed with chloroform: methanol: water (6: 4: 1). The plates were sprayed with 0.2% DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in methanol. The yellow color against purple background indicated active antioxidant extract or compounds.

Determination of Minimum Inhibitory Concentration (MIC)

Determination of Minimum Inhibitory Concentration (MIC) was performed by broth microdilution in 96-well microplate against *E. coli* and *S. aureus*. The extract concentrations for determining MIC value were in the range of 2-256 µg/mL. Positive control chloramphenicol ranges from 2 to 32 µg/mL.

The first well in row A was introduced with 100 µL of Mueller Hinton Broth (MHB), 10 µL of extract (1024 µg/mL), and 90% sterile aqua dest. The following row was introduced with 100 µL of MHB (row B-H). Twofold dilution was carried out as follows: one hundred µL was removed from row A and transferred to the next column in the same row and mixed properly. It was repeated to the last row. In the last row, 100 µL was discarded. After finish dilution, bacterial suspension (10⁵ CFU/mL) was added into each well. The microplate was incubated at 37°C for 24 hours. After incubation, each well of microplate was added with 10 µL of INT (4 mg/mL) and incubated for a few minutes. The inhibition of bacterial growth can be observed by changes in the color of the wells. The lowest concentration of well with no color change was determined as MIC value.

Determination of IC₅₀ for antioxidant activity

IC₅₀ for antioxidant of the extract was carried out by two-fold microdilution in the 96-well microplate. Catechin was used as a positive control. The first row (A) was introduced with 195 µL methanol and 5 µL extract (1024 µg/mL), mixed properly. Each well on the next row was introduced with 100 µL methanol. One hundred µL of the 1st row was transferred into the 2nd at the same column and mixed properly. It was repeated up to the last row, and 100 µL was discarded from the last row. After finish dilution, 100 µL DPPH (61.5 mg/50 mL) was added into each well. The microplate was incubated at room temperature for 90 min under dark conditions. After incubation, the absorbance was recorded at 517 nm wavelength with a microplate reader (Varioskan Flash). Percent inhibition concentration (IC) was calculated as follows:

$$IC (\%) = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100\%$$

Where; A control is the absorbance of DPPH without samples, A sample is the absorbance of DPPH along with different concentrations of extracts. The IC₅₀ values of

DPPH were obtained from linear calibration between the percentage of inhibition and sample concentration. The antioxidant activity index (AAI) acquired by the formula below:

$$AAI = \frac{\text{The final concentration of DPPH}}{IC_{50}}$$

Statistical analysis

Statistical analysis of variance of total phenolic contents (TPC) and total flavonoid contents (TFC) values was performed by Duncan's Multiple- Range Tests using SPSS 16.0. The experiment was performed in triplicate and expressed as mean ± SD. Values in each column with the different letters are significantly different (P<0.05).

RESULTS AND DISCUSSION

Phytochemical content of sitahe leaf extract

Phytochemical screening of *L. eugenifolia* leaf extracts in various organic solvents showed the presence of alkaloid, flavonoid, steroid, triterpenoid, saponin, phenolic, and tannin, although some chemical compounds were absent in some extracts (Table 1). It showed that some of the chemical compounds are absent, i.e. triterpenoid was absent in *n*-hexane, dichloromethane, and ethyl acetate extracts.

Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The total phenolic content (TPC) and total flavonoid content (TFC) were presented in Table 2. Total phenolic contents were ranging from 4.9931 to 259.0486 mg GAE/g extract, while TFC were ranging from 9.1691 to 84.6575 mg QE/g extract. The results showed that the highest TPC was in the ethyl acetate extract (259.0486 ± 0.1203 mg GAE/g extract), while the highest TFC was in the dichloromethane extract (84.6575 ± 0.0237 mg QE/g extract). Total phenolic contents and total flavonoids contents of *L. eugenifolia* leaf were solvent dependent

Table 1. Phytoconstituents of *Leuconotis eugenifolia* leaf extracts in various solvents

Chemical compounds	Extract				
	<i>n</i> -Hexane	Dichloro methane	Ethyl acetate	Methanol	Water
Alkaloid	+	+	+	+	+
Flavonoid	-	+	+	+	+
Steroid	+	+	+	-	+
Triterpenoid	-	-	-	+	-
Saponin	-	-	-	-	+
Phenolic	+	+	+	+	+
Tannin	-	-	+	+	+
Glycoside	-	-	-	-	-

Note: +: present, -: absent

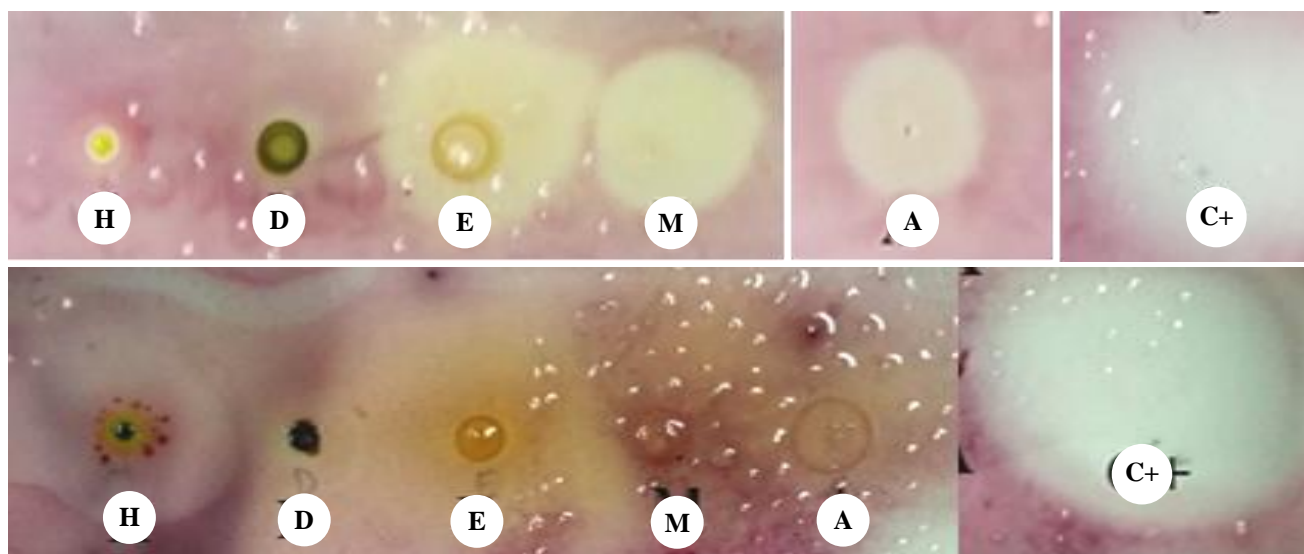


Figure 1. Dot-Blot Test for antibacterial activity of *Leuconotis eugenifolia* leaf extract against *Staphylococcus aureus* (top) and *Escherichia coli* (bottom), extract of: H: *n*-Hexane; D: Dichloromethane; E: Ethyl Acetate; M: Methanol; A: Water, and C+: Chloramphenicol

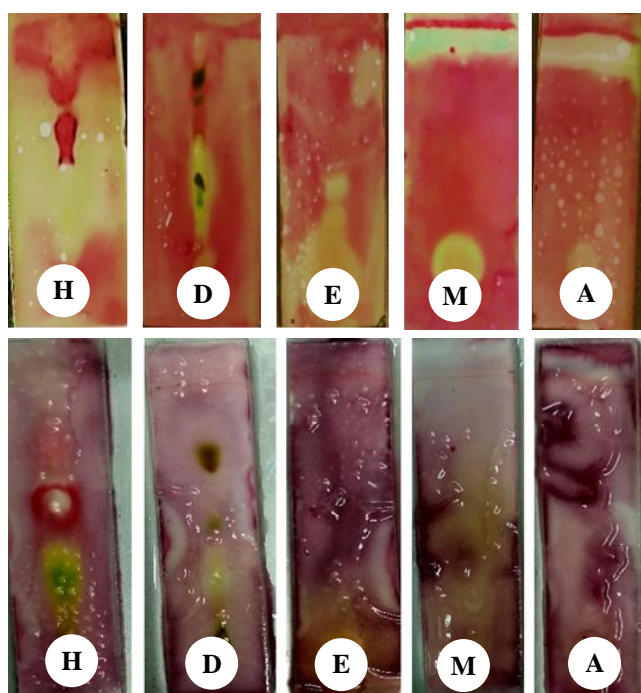


Figure. 2. TLC-Bioautogram for antibacterial activity of *Leuconotis eugenifolia* leaf extract against *Staphylococcus aureus* (top) and *Escherichia coli* (bottom). H: *n*-Hexane; D: Dichloromethane; E: Ethyl Acetate; M: Methanol; and A: Water

TLC-Bioautography for antibacterial assay

Antibacterial activity of *L. eugenifolia* leaf extracts was performed by the Dot-Blot method (Figure 1) and Developed Plates (Figure 2). Dot-Blot method for antibacterial activity against *S. aureus* showed that ethyl acetate, methanol, and water extracts have wider yellowish-white spots compared to hexane and dichloromethane extract.

Table 2. Total phenolic content (TPC) and Total flavonoid content (TFC) of *Leuconotis eugenifolia* leaf extract

Extract	TPC (mg GAE/g extract)	TFC (mg QE/g extract)
<i>n</i> -Hexane	4.9931 ^e ± 0.0120	9.1691 ^e ± 0.0079
Dichloromethane	88.5903 ^d ± 0.0241	84.6575^a ± 0.0237
Ethyl acetate	259.0486^a ± 0.1203	62.9817 ^b ± 0.0079
Methanol	133.2153 ^c ± 0.0241	30.7808 ^d ± 0.0362
Water	164.2083 ^b ± 0.0361	37.0228 ^c ± 0.0174

Note: Each value was represented by mean±SD (n=3). Values in the same column with different letters are significantly different (P<0.05)

The developed plates of antibacterial activity of *L. eugenifolia* leaf extract against *S. aureus* and *E. coli* showed white bands that indicated the presence of bioactive chemical compounds in the extracts.

Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentration of extracts was carried out by microdilution in 96-well microplate. The results showed that ethyl acetate extract has moderate antibacterial activity against *S. aureus*, while hexane and dichloromethane extracts have moderate antibacterial activity against *E. coli*. The MIC of the extracts against *E. coli* and *S. aureus* ranged 256->256 µg/mL.

Antioxidant activity of si Tahe leaf extracts

TLC-bioautography for antioxidant either by Dot-Blot or Developed Plates showed the formation of yellowish spots or bands indicated antioxidant activity. The developed plates (Figure 4) showed several yellow spots indicated the chemical compounds possess antioxidant activity.

Table 3. Minimum inhibitory concentration (MIC) of *Leuconotis eugenifolia* leaf extract against *Staphylococcus aureus* and *Escherichia coli*

Extract	MIC (µg/mL)			
	<i>S. aureus</i>	Category	<i>E. coli</i>	Category
<i>n</i> -Hexane	ND	-	256	Moderate
Dichloromethane	ND	-	256	Moderate
Ethyl acetate	256	Moderate	>256	Weak
Methanol	>256	Weak	>256	Weak
Water	>256	Weak	>256	Weak
Chloramphenicol	16	Strong/good	16	Strong/good

Note: ND = Not determined



Figure 3. Dot-blot test for DPPH free radical scavenging (antioxidant) activity of *Leuconotis eugenifolia* leaf extract. H: *n*-hexane, D: dichloromethane, E: ethyl acetate, M: methanol, A: water, C+: catechin

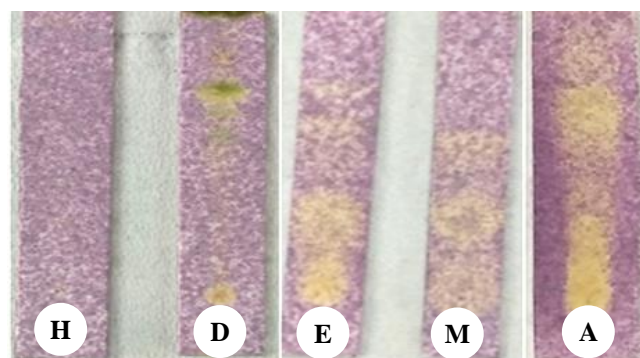


Figure 4. TLC-bioautogram for antioxidant activity of *Leuconotis eugenifolia* leaf extracts. H: *n*-hexane, D: dichloromethane, E: ethyl acetate, M: methanol, A: water

Table 4. The IC₅₀ value for antioxidant activity and antioxidant activity index (AAI) of *Leuconotis eugenifolia* leaf extracts

Extract	IC ₅₀ (µg/mL)	AAI	Category
Hexane	NT	NT	-
Dichloromethane	NT	NT	-
Ethyl acetate	11.50	2.67	Very strong
Methanol	5.25	5.86	Very strong
Water	11.44	2.69	Very strong
Catechin	2.36	13.02	Very strong

Note: ND: not determined. Category of antioxidant activity of the extracts based AAI value (weak < 0.05 < moderate < 1 < strong < 2 < very strong) (Scherer and Godoy 2009)

Determination of IC₅₀ for antioxidant activity by DPPH-method

Microdilution analysis for antioxidant activity in 96-well microplate showed that ethyl acetate, methanol, and water extracts possess very strong antioxidant activity on DPPH- free radical scavenging method.

Discussion

The medicinal plants are widely used in the primary health-care system. It is due to the phytoconstituents of the plant as the basis for traditional herbal medicine (Govindarajan et al. 2005). Plants are considered natural sources of new compounds of medical and biotechnological interest since they synthesize a large variety of bioactive compounds (Farias et al. 2013).

Phytoconstituents of *L. eugenifolia* leaf extracts extracted in various solvents with different polarity (Table 1) showed that different polarity of solvent extracted different chemical compounds. The chemical compound was extracted optimally in the solvent of the same polarity. Therefore, the solvents are responsible for extracting the chemical compounds of the plants (Siddurhaju and Becker 2003). Phenolic, alkaloid, and tannin present in all extract, however, they may have different molecular structures. Alkaloids are reported to have analgesic, anti-inflammatory, and adaptogenic activities which help to alleviate pains developed resistance against disease and endurance against stress (Manhas and Dahiya 2017). Phenolic and flavonoids have several biological activities with health benefits such as antiatherosclerosis, and cardiovascular protection (Batta 2016). Saponins and tannins have anti-inflammatory effects (Shah and Hossain 2014). Tannin also can bind proline-rich protein (Ahmed et al 2014). Therefore, the phytochemical constituents of *L. eugenifolia* leaf extract may have biological activities.

The leaf extracts of *L. eugenifolia* were tested for antibacterial activity against *S. aureus* and *E. coli* by Direct TLC-bioautography method due to its fast, inexpensive, and simple and easy. TLC-bioautography enables rapid detection for antimicrobial activity of the extract and allows the localization of antimicrobial activity directly on the chromatographic plate (Navarro et al. 1998). The TLC bioautographic method combines chromatographic separation and determination of in situ activities that facilitate localization and target-directed isolation of active constituents in the mixture (Shahverdi et al. 2007).

In the TLC-bioautography test, antibacterial activity was demonstrated by the appearance of a white area with a purple-red background on the chromatogram. Living microorganisms can reduce INT to a purple-red color (Begue and Klein 1972). Based on the white area formation, it showed that ethyl acetate, methanol, and water extracts inhibit the growth of *S. aureus*, while *E. coli* was inhibited by ethyl acetate. The eluted plate showed several spots that indicated the active components of the extract. Although in the TLC-Bioautography results showed there were yellowish-white several spots or white bands on *L. eugenifolia* leaf extracts indicated the capability of extracts to inhibit the growth of *S. aureus* or

E. coli, however the MIC value of extracts categorized as weak to moderate antibacterial activity.

Antioxidant activity of *L. eugenifolia* leaf extracts was carried out by the TLC-bioautography assay with DPPH reagent. DPPH is a stable free radical that is mostly used technique to evaluate antioxidant activity due to its quick, easy and simple test (Dudonne et al. 2009; Ali et al. 2013). Antioxidative compounds change the purple color of DPPH to yellow because the antioxidant compounds scavenge the DPPH free radicals (Ali et al. 2013). The dot-blot method and eluted plate showed that ethyl acetate, methanol, and water extracts possess antioxidant activity.

Ethyl acetate, methanol, and water extracts have very strong antioxidant activity. There was a correlation between total phenolic compounds (TPC) with IC₅₀ of the extract. Phenolic compounds are important chemical components in plants because they are redox which is responsible for antioxidant activity (Soobrattee et al. 2005). Phenolics can donor electrons and their hydroxyl group contribute to the antioxidant activity (Bendary et al. 2013). The results showed that different solvent results in different antioxidant activity. It is suggested that different solvents with different polarity extracted different chemical compounds.

The content of phenolic and flavonoid compounds in *L. eugenifolia* leaves is quite high. Compounds from this group are generally well known to have a positive effect on blood circulation in the body, including improving blood flow in the brain (Rees et al. 2018). Therefore, it is suspected that the high content of flavonoid and phenolic groups in *L. eugenifolia* leaves play an important role in restoring stamina by consuming "*ie bu peudah*" in the people of Aceh. The yohimbine alkaloid, known as aphrodisiacs (Ostojic 2006), may also play an important role in strengthening stamina, but in-depth studies are still needed.

To conclude, the results of the study showed that *L. eugenifolia* leaves have a high content of flavonoids and phenolics, and several other classes of compounds such as alkaloids. *L. eugenifolia* leaf extract showed good potential as an antioxidant, but moderate and weak antibacterial activity against *E. coli* Ina-CC B5 and *S. aureus* Ina-CC B4. Isolation of active constituents is ongoing.

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