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Research Article

Antibacterial Activity and TLC-Densitometric Analysis of Secondary Metabolites in the Leaves of the Traditional Herb, *Melastoma malabathricum* L.

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Abstract

Indonesia is rich in the biodiversity of medicinal plants used traditionally for healing several ailments. *Melastoma malabathricum* L. is one of the traditional herbs used to treat many diseases. A TLC-densitometric method was developed for determining secondary metabolites such as phenolic compounds and their related compounds, *M. malabathricum* leaves from Riau, Indonesia. This study investigated the secondary metabolites of *M. malabathricum* extract by spraying reagent: $FeCl_3$, *p*-anisaldehyde, and cerium (IV) sulfate and followed by antibacterial assay through broth macro dilution method. Densitometric qualitative analysis of phenolic compounds and their related compounds was employed to examine peaks of the *M. malabathricum* extract through winCATS software. After spraying with particular reagents, the three extracts showed various spots/bands with several colors and exhibited peaks in TLC densitogram profiles. Three extracts were tested for antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The result showed that ethyl acetate extract revealed excellent antibacterial activity against *S. aureus* and *E. coli* with MIC values of 3.125±0.6 mg/mL and 6.25±0.5 mg/mL, respectively. Owing to the presence of a wide variety of secondary metabolites, the leaf extract of *M. malabathricum* is expected to exhibit and help develop as a therapeutic agent.

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INTRODUCTION

Recently, there has been increased interest in natural and herbal remedies. Traditional medicinal plants play a significant role in discovering novel therapeutic compounds[1]. The discovery of antimicrobial and antibiotic agents was one of the major concerns of the twentieth century[2]. Identifying new chemicals with the unique physicochemical properties required for antibiotic or antimicrobial discovery and development is challenging. Natural products still represent the most likely source of materials given advanced invention. Given the relative lack of success in bringing effective synthetic antibiotics to the clinic, the best hope for developing a new generation of anti-infective and antimicrobial drugs is to discover new antimicrobial natural products due to these compounds having their chemical diversity and effectiveness to develop as novel antibiotic agents[3].

Natural products, such as pharmaceutical products, have better tolerability and fewer side effect than chemically synthesized components. The extraction and characterization of bioactive substances from medicinal plants have resulted in the discovery of new drugs with therapeutic value[4]. The wide variety of organic substances that are elaborated and accumulated by plants requires highly sophisticated methods for their separation, purification, identification, and

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quantitative determination[5]. This study aims to show the value of the thin-layer chromatography-densitometry method for the qualitative determination of biologically active constituents in medicinal plants.

Melastoma malabathricum L. is a traditional herb with several biological and pharmacological activities such as antioxidant, antibacterial, anti-inflammatory, antidiabetic, and antiulcer[6]. The majority of the secondary metabolites from *M. malabathricum* leaves are aromatic compounds, especially phenolic compounds that are produced in various amounts. This plant grows in tropical regions, including Asia and the Pacific Islands, Australia, and the South Pacific Ocean. This plant is usually chewed, pounded for traditional uses, and applied to the wound to stop bleeding. Treating diarrhea and dysentery could be applied by boiling the leaves of *M. malabathricum* and then consumed twice a day[7,8].

Thin-layer chromatography (TLC) is an easy-to-use tool for compound identification and separation of herbal extracts. This method is frequently used as a qualitative and quantitative analysis as the low cost of instrumentation and short time. Qualitative analysis can be performed using TLC densitometry. TLC densitometry is a suitable quantitative and qualitative analysis method due to its accurate, precise, and reliable procedure[9]. TLC image analysis using computer software technology has been considered a simple, inexpensive, and convenient quantitation method with good accuracy and precision for chemical compound analysis in medicinal plants[10].

It is a well-known fact that species of medicinal importance contain a wide variety of secondary metabolites, some of which are responsible for biological activity. Based on previous research, many studies have been carried out to explore this plant's biological activity. However, a major cause of the problem relates to the lack of simple, reliable, and economical analytical techniques for the chemical analysis of herbal material. Therefore, the study of the extracts using specific reagents and qualitative analysis used TLC densitometry and detected peaks of secondary metabolites using winCATS followed by an antibacterial activity test was developed. To confirm this view, a TLC densitometry study was undertaken to explore the various secondary metabolites present in the leaves of *M. malabathricum*. Thus, this study aimed to develop and examine TLC densitometry and antibacterial activity of *M. malabathricum* leaves.

MATERIALS AND METHODS

Materials

All solvents: *n*-hexane, ethyl acetate, ethanol, formic acid, methanol, and chloroform were of analytical grade supplied from Merck (Merck, Darmstadt, Germany). $FeCl_3$, *p*-anisaldehyde, and cerium (IV) sulfate reagents were used for the phytochemical assay. Extracts were monitored by TLC silica gel 60 F_{254} nm plates (Merck). Amoxicillin was used as a positive control, and dimethyl sulfoxide was used for the antibacterial test. All chemical solvents used were analytical grade. The fresh mature leaves of *M. malabathricum* (**Figure 1**) were collected from the Kuantan Singingi District, Riau, Sumatera Island, Indonesia. Plant specimens were authenticated at the Department of Pharmaceutical Biology, Faculty of Pharmacy Universitas Gadjah Mada, by a botanist (Dr. Djoko Santoso). Voucher specimens (29560/M/03/02) were deposited at the Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The authentic sample was dried in a hot air oven at 40°C, homogenized to a fine powder, and stored for further analysis.

Methods

Preparation extracts of M. malabathricum leaves

One hundred grams of the leaf powder of *M. malabathricum* were soxhlet with *n*-hexane. The extract was filtered, and the solvent was evaporated by a rotary evaporator. The residues were macerated by ethyl acetate and ethanol, and the solvent was evaporated in a rotary vacuum evaporator to obtain a viscous semi-solid mass. The yield of the extract was calculated and recorded. This semi-dry crude *n*-hexane, ethyl acetate, and ethanol extract were subjected to TLC densitometry analysis and continued to sprayer with specific reagents.

Figure 1. *Melastoma malabathricum* leaves.

Thin-layer chromatography

TLC experiments were carried out in 10 x 20 cm TLC silica 60 plates with the mobile phases containing chloroform 100% for *n*-hexane extract, chloroform : methanol : ethyl acetate : formic acid (50 : 15 : 30 : 5 v/v/v/v) for ethyl acetate extract, *n*-hexane : ethyl acetate (8 : 2 v/v) in a saturated chamber for 15 minutes. After development, the plates were dried in a stream of warm air. Next, the plates were scanned at a wavelength of 245 nm and used for phytochemical screening by spraying several reagents.

Phytochemical screening of M. malabathricum extract

The extracts were spotted manually using a capillary tube on pre-coated silica gel GF to separate different phytochemical compounds in *n*-hexane, ethyl acetate, and ethanol extract of *M. malabathricum* leaves TLC plates (1.5 x 5 cm, 3 mm thickness). The spotted plates were developed in different solvent systems to detect a suitable mobile phase. After separating the phytochemical constituents, reagents, including 5% ferric chloride, *p*-anisaldehyde, and cerium (IV) sulfate, were sprayed to identify the respective compounds. The colors of the spots were noted, and R_f values were calculated.

TLC densitometry method

Ten microliters of the sample were loaded as 5 mm band length separately on pre-coated silica gel GF 60 using a Hamilton syringe with the help of a Linomat 5 applicator attached to a Camag system, which was programmed through winCATS software. After applying spots, the chromatogram was developed in a twin-through glass chamber (20 x 10 cm) pre-saturated respective mobile phase. The air-dried plates were kept in a photo documentation chamber (CAMAG Reprostar 3), and images were captured at visible light and UV 254 nm. The chromatograms were scanned by a densitometer at 254 nm. The peak numbers with their height, area, and R_f values of fingerprint data were recorded by winCATS (1.3.4 version) software.

Antimicrobial assay

The MICs for the test solutions were determined by broth macrodilution per CLSI 2012 standard protocols[11]. The examined bacteria were Gram-positive strains, *Staphylococcus aureus* (ATCC 25923), and Gram-negative strains, *Escherichia coli* (ATCC 25922). The cultures were then incubated and serially diluted to reach the density of 2×10^4 cells per mL. Two milliliters of MHA broth were dispensed in tubes, and 100 µL of cell culture was inoculated. Then, 100 µL of different concentrations of extract (50, 25, 12.5, 6.25, 3.125 mg/mL) was added to each tube. Amoxicillin (10 µg/mL) was used as a positive control for bacteria, and disc without plant extract was used as a negative control. Growth control was run in parallel with every

experiment. All the experimental tubes were incubated in anaerobic jars for 48 hours. After completion of the incubation period, the density of the tubes was compared to the standard solution Mc Farland (the inoculum and McFarland standard must be in the same sized tubes).

Statistical analysis

For statistical data analysis, multiple comparisons were performed using one-way analysis of variance (ANOVA). Statistical significance was accepted as a level of $p < 0.05$. The statistical analysis was carried out using SPSS 17 (SPSS Inc., Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Phytochemical profiling

The chromatographic condition for analysis of the secondary metabolite of *M. malabathricum* leaves was examined using silica gel GF_{254}. The selected mobile phase demonstrated the best separation of *n*-hexane, ethyl acetate, and ethanol extract. *Melastoma malabathricum* belongs to the family *Melastomataceae*. The medicinal uses of this species are manifold. Traditional healers prescribed the tubers of this species for wound healing, stopping the bleeding, stomachache, toothache, cholera, and dysentery[6,7,12].

Conventional chromatography fingerprinting techniques are typically used in authenticity and identification, mostly analyzed for qualitative analysis to identify similarity among extract samples[13]. Qualitative similarity analysis could be identified the type and shape of peaks of each extract of the samples. The capability of TLC-densitometric analysis in quality control of plant extract samples has been verified using a densitometer combined with winCATS software[14].

The current study was taken up to screen *M. malabathricum* extract for secondary metabolites and develop fingerprints using TLC densitometry techniques. The n-hexane, ethyl acetate, and ethanol extract of leaves were subjected to TLC in which different mobile phases were tried to separate bioactive compounds like alkaloids, flavonoids, glycosides, terpenoids, and saponins. The dried chromatographic plates were subjected to non-targeted chemical derivatizations with $FeCl_3$, *p*-anisaldehyde, and cerium (IV) sulfate (**Figure 2**)

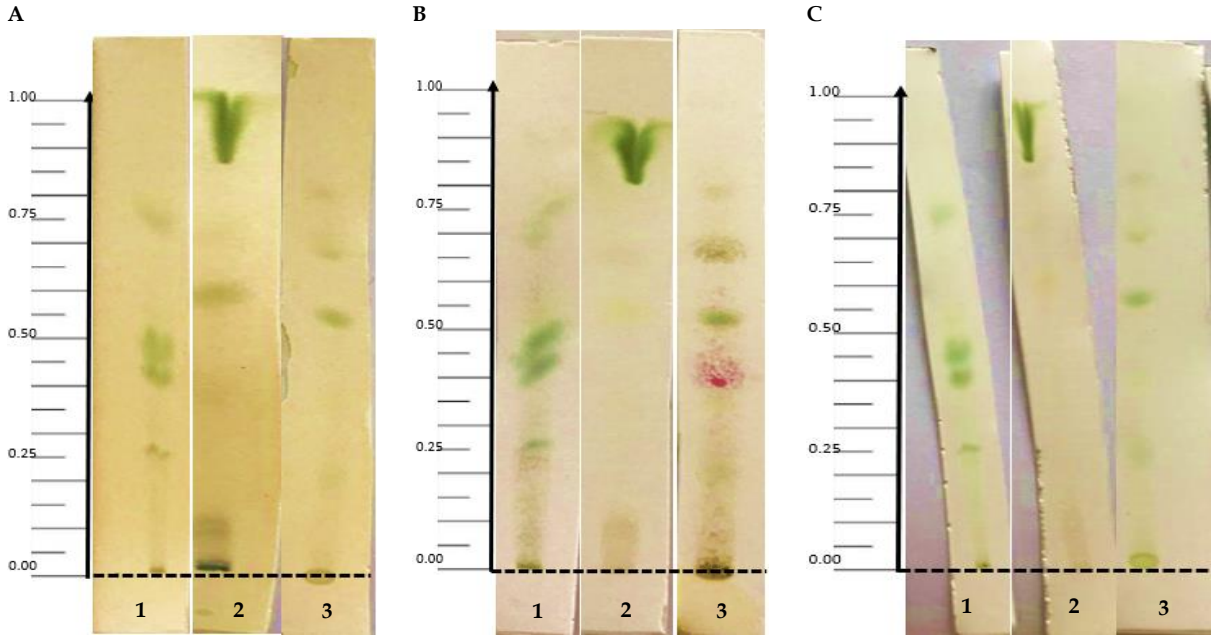

Figure 2. TLC plates of *n*-hexane (**1**), ethyl acetate (**2**), and ethanol (**3**) extract after spraying reagent of $FeCl_3$ (**A**), *p*-anisaldehyde (**B**), and cerium (IV) sulfate (**C**).

The study revealed the development of several colored bands of compounds in three different solvent systems of *n*-hexane, ethyl acetate, and ethanol extract. For *n*-hexane extract, it showed various colors of each sprayer, such as the blackish green of $FeCl_3$, grey of *p*-anisaldehyde, and green of cerium (IV) sulfate. In addition, a pink-colored spot of ethyl acetate was identified from *p*-anisaldehyde and cerium (IV) sulfate spraying reagents in the range R_f of 0.60. Next, the ethanol extract showed pinkish-violet-colored spots at the R_f of 0.40. The secondary metabolites were distinguished based on color, and R_f values were recorded. The study revealed that relatively high polarity solvents like chloroform : methanol : ethyl acetate : formic acid (50 : 15 : 30 : 5 v/v/v/v) and *n*-hexane : ethyl acetate (8 : 2 v/v) were more suitable as a mobile phase for the separation of bioactive compounds in the leaves of *M. malabathricum*. Planar layer chromatography enhances further usefulness, including minimal sample preparation, as crude extracts can be applied directly to plates (without losing components) and enable parallel profiling of sample extracts in different assays[15,16].

After derivatization with *p*-anisaldehyde, all samples revealed additional bands in the upper part of chromatograms. Spraying of *p*-anisaldehyde reagents can be used to detect phenols, terpenes, and steroids. If compounds contain phenols, terpenes, and steroids, it turns violet, blue, grey, and green bands[17]. After derivatization, the pinkish violet color after the *p*-anisaldehyde sprayer reagent of ethyl acetate extract confirmed the presence of phenol compounds. The plates were sprayed with $FeCl_3$ reagents that identified flavonoid and polyphenol compounds detection and will turn to brown, grey, and black colors, indicating flavonoid and polyphenols content in extracts[18]. Cerium (IV) sulfate identified organic compounds. Several reagents are more informative because they reveal the specific colors of several compounds, including phenols, monoterpenes, triterpenes, steroids, and other organic compounds[15]. Based on the chromatogram in **Figure 1**, TLC profiles showed some spots with R_f values that can be seen in **Table I**.

Table I. TLC screening of phytochemicals in *n*-hexane, ethyl acetate, and ethanol extract of the leaves of *M. malabathricum*.

Extract	Mobil phase	Spraying reagent	Color of the spots/bands	R_f
n-hexane	Chloroform 100%	$FeCl_3$	Blackish-green	0.25; 0.43; 0.48; 0.75
		p-anisaldehyde	Grey	0.25; 0.42; 0.48; 0.70; 0.75
		Cerium (IV) sulphate	Green	0.25; 0.41; 0.45; 0.75
Ethyl acetate	Chloroform : methanol : ethyl acetate : formic acid (50 : 15 : 30 : 5 v/v/v/v)	$FeCl_3$	Blackish-green	0.05; 0.10; 0.20; 0.40; 0.60; 0.85; 0.90
		p-anisaldehyde	Grey/pink	0.05; 0.10; 0.60; 0.65; 0.85; 0.90
		Cerium (IV) sulphate	Grey/pink	0.05; 0.10; 0.60; 0.65; 0.85; 0.90
Ethanol	*n*-hexane : ethyl acetate (8 : 2 v/v)	$FeCl_3$	Blackish-green	0.20; 0.55; 0.70; 0.80
		p-anisaldehyde	Pinkish-violet	0.40; 0.53; 0.65; 0.75
		Cerium (IV) sulphate	Green	0.25; 0.60; 0.70; 0.80

Different compounds were observed out of four bands in the n-hexane extract after spraying of $FeCl_3$, five bands after spraying of *p*-anisaldehyde, and six bands after spraying of cerium (IV) sulfate. The R_f values determined in the *n*-hexane extract were in the range of 0.25-0.75. Ethyl acetate extract showed several bands containing seven bands after spraying $FeCl_3$ and six bands after spraying *p*-anisaldehyde and cerium (IV) sulfate. The R_f values determined in the ethyl acetate extract were in the range of 0.25-0.90. After spraying $FeCl_3$, *p*-anisaldehyde, and cerium (IV) sulfate, the ethanol extract showed several bands containing four bands. The R_f values determined in the ethanol extract were in the range of 0.20-0.80.

TLC densitometry profile

Identifying secondary metabolites in plants helps detect potential bioactive substances responsible for biological and pharmacological activities[19]. The TLC profile was generated in a solvent system of different polarities to ascertain the total number of chemical moieties, which will also help in designing the method of isolation and characterization of bioactive compounds[20] (**Figure 3**). The TLC profile of the *n*-hexane extract of the *M. malabathricum* leaves is presented in **Table II**. Nine compounds were separated at the R_f range of 0.05 – 0.91. The highest peak was 19740.6 AU, and the lowest was 587.5 AU, observed at R_f 0.27 and 0.79, respectively. Other significant peaks were detected in R_f of 0.05, 0.14, 0.21, and 0.52, with areas 12926.6, 4056.2, 10228.1, and 9570.6 AU, respectively.

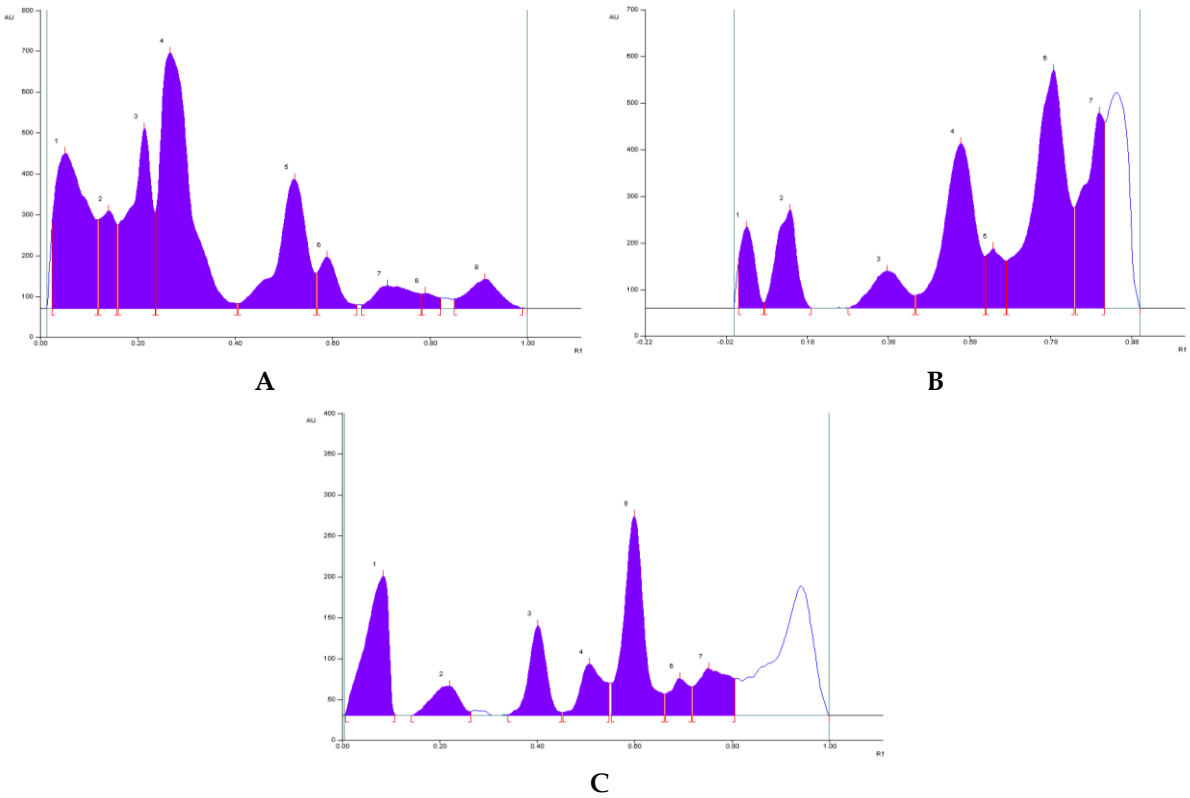

Figure 3. TLC densitometry profiles of *M. malabathricum* extracts. (**A**) *n*-hexane extract, (**B**) ethyl acetate extract, and (**C**) ethanol extract.

Various compounds can be observed in the ethyl acetate extract on TLC densitogram profiles (**Table III**). The highest peak area was identified at R_f of 0.79, with an area of 21066.9 AU. The lowest peak was in the R_f of 0.38, with an area of 3308.6 AU. Other peaks were observed at R_f of 0.03, 0.14, 0.56, 0.64, and 0.90 with areas 3145.5, 5356.3, 13645.4, 13645.4, 2644.3, and 10751.0, respectively. Ethanol extract revealed seven peaks in the R_f range of 0.08 to 0.0.75 (**Table IV**). A reliable solvent system to observe the above separation is *n*-hexane : ethyl acetate (8 : 2 v/v). The highest peak area, 5215.2 AU, and the lowest peak area, 917.2 AU, were observed at R_f of 0.60 and 0.69, respectively.

Table II. TLC densitometry profiles of n-hexane extract of the leaves of *M. malabathricum*.

Peak	R_f	Height (mm)	Area (AU)	Area (%)
1	0.05	379.8	12926.6	20.10
2	0.14	238.4	4056.2	6.31
3	0.21	440.2	10228.1	15.91
4	0.27	624.8	19740.6	30.70
5	0.52	317.1	9570.6	14.89
6	0.59	126.2	2494.4	3.88
7	0.71	55.8	2273.9	3.54
8	0.79	37.4	587.5	0.91
9	0.91	72.1	2417.5	3.76

Table III. TLC densitometry profiles of ethyl acetate extract of the leaves of *M. malabathricum*.

Peak	R_f	Height (mm)	Area (AU)	Area (%)
1	0.03	174.3	3145.5	5.25
2	0.14	209.8	5356.3	8.94
3	0.38	79.5	3308.6	5.52
4	0.56	352.8	13645.4	22.77
5	0.64	127.9	2644.3	4.41
6	0.79	510.5	21066.9	35.16
7	0.90	419.3	10751.0	17.94

Table IV. TLC densitometry profiles of ethanol extract of the leaves of *M. malabathricum*.

Peak	R_f	Height (mm)	Area (AU)	Area (%)
1	0.08	170.4	4105.7	24.40
2	0.22	35.9	1067.3	6.34
3	0.40	109.8	2011.1	11.95
4	0.51	62.8	1551.2	9.22
5	0.60	244.2	5215.8	31.00
6	0.69	45.4	917.2	5.45
7	0.75	57.0	1958.2	11.64

Phenolic compounds can be observed in the ethyl acetate and ethanol extract because of the results of the phytochemical spraying reagents. The two extracts showed positive in spraying of $FeCl_3$ reagents. TLC-densitogram profiles revealed that the extracts contained positively phenolic compounds having the largest area on the densitometry qualitative analysis in the R_f 0.60 for the ethanol and 0.79 for the ethyl acetate extract.

Antibacterial activity

The broth macrodilution method is one of the most basic antimicrobial susceptibility assay methods. The procedure involves preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium dispensed in tubes containing a minimum volume of 2 mL (microdilution). Then, each tube is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland standards. After well-mixing, the inoculated tubes are incubated under suitable conditions depending on the test microorganisms[21]. The MICs are the lowest concentration of antimicrobial agent that completely inhibits the organism's growth in tubes as detected by the unaided eye. Inoculum size, the type of growth medium, the incubation time, and the inoculum preparation method can affect MIC values. The MIC is defined as the lowest concentration of antibiotic at which the organism has no visible growth. The growth of one or two colonies or a fine film of growth should be disregarded. The result of the broth microdilution method of *M. malabathricum* extract is represented in **Table V**.

Table V. Broth macrodilution method of antibacterial activity of extracts against *S. aureus* and *E. coli*.

Microorganisms	MIC (mg/mL)		
	n-hexane extract	Ethyl acetate extract	Ethanol extract
Staphylococcus aureus	50±0.4	3.125±0.6	12.5±0.6
Escherichia coli	50±0.2	6.25±0.5	25±0.7

Antibacterial activity of ethyl acetate extract was found to be the highest compared to ethanol and *n*-hexane extract, with MIC values of 3.125±0.6 mg/mL and 6.25±0.5 mg/mL for *S. aureus* and *E. coli*, respectively. This finding revealed that secondary metabolites in the ethyl acetate extract were the potential to inhibit the growth of the pathogen bacteria. A previous study revealed that ethyl acetate extract of the *M. malabathricum* leaves had excellent activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA) with MIC 1 mg/mL (0.1% b/v) and antioxidant activity with IC_{50} of 43.30 µg/mL[6]. Another antibacterial study about leaves of *M. malabathricum* showed that ethanol extract exhibited the highest antibacterial activity against *S. aureus* and MRSA with a diameter of inhibition zone 14.0±0.8 mm and 12.5±0.4 mm, respectively[22]. Besides, flavonoid compounds enhance the ability of LDL to bind the receptors and reduce oxidative stress by inhibiting cellular oxygenase and activating cellular antioxidants[23].

Among the tremendous variety of secondary metabolites from the leaves of *M. malabathricum* are polyphenolic compounds and other related compounds such as alkaloids, terpenoids, steroids, and saponins. Polyphenolic compounds such as flavonoids, glycosides, tannins, and phenolic acids are considered a significant contribution to the antioxidant activity of *M. malabathricum* leaves[8,24]. Bioactive of the leaves of *M. malabathricum* is due to the presence of phenolic compounds, including quercetin, quercitrin, rutin, kaempferol, kaempferol-3-O-(2",6"-di-O-p-trans-coumaroyl)-β-D-glucoside, naringin, malabathrin A, B, C, and D, nobotanin B, D, G, and H, casuarictin, strictinin, pterocarinin C, pedunculagin, epicatechin, epicatechin gallate, and patriscabatrin[7,8,25,26]. The chemical substances of *M. malabathricum* leaves can be found in **Figure 4**.

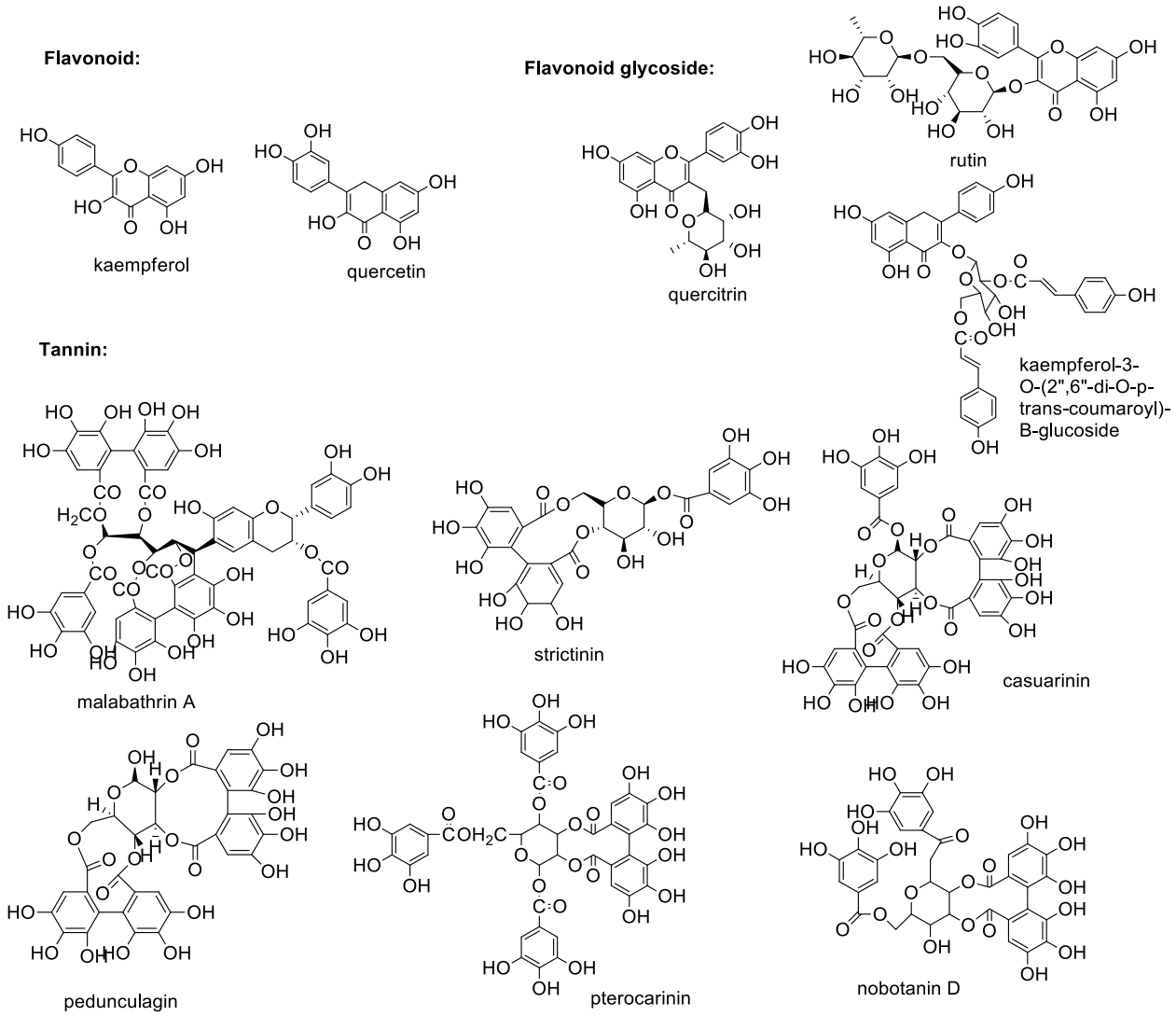

Figure 4. Molecule structures of secondary metabolites of the *M. malabathricum* leaves[8].

Flavonoids are bioactive substances in many medicinal plants that play a prominent role in biological activity[27]. Based on the previous report, *M. malabathricum* has biological activity such as antibacterial[22], antioxidant[6], wound healing[28], antidiarrheal[29], anti-diabetic[30], and anti-inflammatory activity[31] regarding its phenolic compounds and other related compounds. The bioactive compounds of *M. malabathricum* were influenced by the geographical origins of where this plant was grown[8,24]. The developed TLC densitometry analysis will help the manufacturer with quality control and standardization of herbal formulation, including fingerprinting analysis in differentiation and distinct species from the adulterant[32].

CONCLUSION

Melastoma malabathricum extract has considerable amounts of secondary metabolites, including a phenolic compound such as flavonoid, flavonoid glycosides, tannin, and other related compound such as terpenoids and saponins, some of which could be developed as biological and pharmacological agents in future. The TLC densitometry profiles have identified several spots responsible for *M. malabathricum* extracts inhibiting pathogen bacteria, *S. aureus*, and *E. coli*. The fingerprints developed in this study could be helpful as an assessment of quality control and standardization of herbal formulations containing *M. malabathricum*. Hence, this discovery could lead to future experiments regarding the invention of new lead compounds from this plant.

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AUTHORS' CONTRIBUTION

Dian Mayasari: conducted the research and wrote the original manuscript; **Yosi Bayu Murti**: designed the experiment and helped analyze the data; **Sylvia Utami Tunjung Pratiwi** and **Sudarsono**: contributed to reviewing and editing the article.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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