

In vitro antibacterial activity and phytochemical profiling of Indonesian *Anacardium occidentale* L. leaf extract and fractions

[Actividad antibacteriana *in vitro* y perfil fitoquímico del extracto de hoja y fracciones de *Anacardium occidentale* L. de Indonesia]

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Abstract

Context: Uncontrolled practice of antibiotics leads to bacterial resistance. Therefore, investigating the possible utilization of several medicinal plant extracts is necessary. Traditionally, numerous medicinal plants have been widely utilized to treat some human diseases including *Anacardium occidentale*.

Aims: To investigate the antibacterial activity of the ethanolic extract and fractions from *A. occidentale* as well as characterize the phytochemical profile of *A. occidentale* leaves associated with antibacterial capacity.

Methods: The antibacterial activity was determined using minimum inhibitory concentration and time kill assay, which confirmed by streak agar plate. Phytochemical profiling of *A. occidentale* leaves extract and fractions were evaluated by their total phenolic contents, total flavonoid contents, terpenoid contents, and metabolite analysis by UHPLC-HR-ESI-MS.

Results: Among the extract and all fractions, the ethyl acetate fraction had high killing efficiency against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, which was confirmed by its MIC value of 0.5, 0.5, 0.5, and 0.25 mg/mL, respectively. Corresponding to its antibacterial capacity, the ethyl acetate fraction showed the highest of phenolic (508.89 mg GAE/g dw) and flavonoid (184 mg QE/g dw) contents. Based on HRMS investigation, the extract and fractions contain amino acids, phenolic acids, alkaloids, flavonoids, fatty acid amides, fatty acids, and organic acids as classes of molecules. Moreover, avicularin/quercetin-3-O-arabinofuranoside, myricitrin, and gallic acid were identified as major compounds from the active fraction.

Conclusions: The ethyl acetate fraction showed the most remarkable antibacterial activity. There was a significant correlation between the antibacterial activity of active fraction and its phenolic and flavonoid contents.

Keywords: *Anacardium occidentale*; antibacterial; antibiotics; phytochemical.

Resumen

Contexto: La práctica incontrolada de antibióticos conduce a la resistencia bacteriana. Por lo tanto, es necesario investigar la posible utilización de varios extractos de plantas medicinales. Tradicionalmente, numerosas plantas medicinales se han utilizado ampliamente para tratar algunas enfermedades humanas, entre ellas *Anacardium occidentale*.

Objetivos: Investigar la actividad antibacteriana del extracto etanólico y las fracciones de *A. occidentale*, así como caracterizar el perfil fitoquímico de las hojas de *A. occidentale* asociado a la capacidad antibacteriana.

Métodos: La actividad antibacteriana se determinó utilizando la concentración inhibitoria mínima y el ensayo de tiempo de muerte, que se confirmó mediante una placa de agar. El perfil fitoquímico del extracto y las fracciones de las hojas de *A. occidentale* se evaluó mediante el contenido total de fenoles, flavonoides y terpenoides, y el análisis de metabolitos por UHPLC-HR-ESI-MS.

Resultados: Entre el extracto y todas las fracciones, la fracción de acetato de etilo tuvo una alta eficacia letal contra *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* y *Pseudomonas aeruginosa*, lo que se confirmó por su valor MIC de 0,5, 0,5, 0,5 y 0,25 mg/mL, respectivamente. En correspondencia con su capacidad antibacteriana, la fracción de acetato de etilo mostró los mayores contenidos fenólicos (508,89 mg GAE/g extracto seco) y flavonoides (184 mg QE/g extracto seco). Según la investigación HRMS, el extracto y las fracciones contienen aminoácidos, ácidos fenólicos, alcaloides, flavonoides, amidas de ácidos grasos, ácidos grasos y ácidos orgánicos como clases de moléculas. Además, se identificaron avicularina/quercetina-3-O-arabinofuranosido, miricitrina y ácido gálico como compuestos principales de la fracción activa.

Conclusiones: La fracción de acetato de etilo mostró la actividad antibacteriana más notable. Se observó una correlación significativa entre la actividad antibacteriana de la fracción activa y su contenido en fenoles y flavonoides.

Palabras Clave: *Anacardium occidentale*; antibacteriano; antibióticos; fitoquímica.

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INTRODUCTION

Infectious diseases are a big problem faced by countries. These diseases originated from pathogenic microorganisms such as parasites, viruses, fungi, and bacteria, which are one of the main causes of death worldwide (Dirga et al., 2021). Uncontrolled practice of antibiotics leads to bacterial resistance due to no inhibition in the growth of bacteria by systematic practicality of antibiotics with less or minimal inhibitory doses. Therefore, it requires serious handling to reduce the impact of antibiotic resistance by investigating the possible utilization of several medicinal plant extracts. Numerous medicinal plants have traditionally been widely used to treat some human diseases (Khan et al., 2013).

Anacardium occidentale L. (Anacardiaceae), commonly called the cashew tree, is commonly found in tropical regions. Different parts of this tree have many uses and have been utilised as folk medicine in many countries (Palombo and Semple, 2001). Cashew nuts are very popular in the food industry for snack manufacturing. The fruit is used as medicine, food, flavour, and beverages. The leaves of *A. occidentale* have been used traditionally to treat some diseases. Particularly, in Indonesia, the young leaves are often consumed as a vegetable in daily diet.

According to several studies, *A. occidentale* leaf extracts displayed antibacterial, antifungal, anti-inflammatory, antioxidant, and hypoglycemic properties. A study on ethanolic and petroleum ether extracts of *A. occidentale* showed that they had antimicrobial activity against pathogens such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, *Aspergillus niger*, *Klebsiella pneumonia*, *Micrococcus luteus*, *Streptococcus mutans*, *Shigella sonnei*, and *Salmonella typhoid fever* (Onasanwo et al., 2012). Meanwhile, *n*-hexane extract from the leaves and stems of *A. occidentale* could inhibit *Staphylococcus aureus* using agar diffusion. Lately, many phytochemicals have been reported contained in this plant including phenols, flavonoids, steroids, terpenoids, essential oils, anacardic acid, tannol, saponins, tannins, alkaloids, flavones, flavonols, xanthenes, chalcones, catechins, and glycosides (Anand et al., 2015; Michodjehoun-Mestres et al., 2009; Onasanwo et al., 2012; Santos et al., 2013). However, the phytoconstituents characterization of *A. occidentale* leaves associated with antibacterial capacity remains less investigated. In our present study, we comprehensively investigated the antibacterial activity of the extract and its fractions from *A. occidentale* against both Gram-positive and Gram-negative bacteria using successive methods. In this way, we specifi-

cally analysed the killing curve of bacteria for 24 h using microdilution method in order to determine the optimum concentration and time of extract or fractions to kill the bacteria. An agar streak plate assessment was visually conducted to confirm the time-kill assay. Moreover, the phytochemical composition of the extract and fractions were investigated using LC-HRMS. This investigation provides a complete metabolite profile of *A. occidentale* leaves, which most likely has antibacterial property. To date, there remains a lack of information about the phytochemical profiling and the comprehensive antibacterial activity of the fractions from *A. occidentale*.

MATERIAL AND METHODS

Chemicals

Solvents and reagents for analytical purposes, such as methanol, AlCl_3 , KCH_3COO , Na_2CO_3 , Folin Ciocalteu, HClO_4 , acetic acid (glacial), and HCl , were purchased from Merck, Germany. Quercetin, gallic acid, ursolic acid, and vanillin standard reagents were obtained from Sigma-Aldrich, United States. Nutrient Broth (Merck, Germany), Mueller-Hinton Agar, and Mueller-Hinton Broth (Himedia, India) were used as media for bacterial culture and antibacterial assay. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) were used for detection of antibacterial activity, which was initially diluted in isopropyl alcohol (Merck, Germany) and acidified by HCl (Merck, Germany). All samples were diluted in dimethyl sulfoxide (DMSO) (Merck, Germany), while ampicillin (Sigma Aldrich, USA) was used as a positive control.

Plant material

The *A. occidentale* leaves were harvested from the Yogyakarta area, Indonesia (7°55'53.4"S 110°33'35.0"E, 209 m above sea level). They were collected as the mature leaves at the upper part of the plant. The leaves were identified and deposited (voucher specimen 1860046) at Botanical Characterization Laboratories, The National Research and Innovation Agency, Indonesia.

Extraction and fractionation

Three hundred grams of *A. occidentale* ground leaves were macerated with ethanol fifth fold proportion (w/v) using an ultrasonic bath (Elmasonic S10H, Elma, Germany, 37 kHz, 90 W) for 1 h at room temperature. The extraction was repeated three times. The filtrates resulting from sonication were filtered and evaporated with a rotary evaporator (Buchi, Switzerland). The collected extract (9.63 g) was dis-

solved using distilled water (300 mL). The dissolved extract was then partitioned by adding 300 mL of n-hexane, chloroform, and ethyl acetate in each stage using a separating funnel. The mixture was shaken and allowed to stand to form two layers. This partitioning was repeated until each supernatant was clear. All obtained fractions were concentrated using a rotary evaporator and lyophilized.

Total phenolic content (TPC)

The samples and standard gallic acid solution were prepared at 1 mg/mL concentration in a methanol solution. The standard solution was then diluted at 20, 40, 60, 80, 100, 125, and 150 µg/mL concentrations. The samples or standard solutions were added with 3.75 mL of distilled water and 2.5 mL of Folin Ciocalteu reagent in the dark test tube. The mixture was homogenized and incubated for 8 min. After incubation, the mixture was added with 0.75 mL of 20% Na₂CO₃ solution. The mixture was incubated for 2 h, and the absorbance was observed at 765 nm (Cary 60 UV-Vis Spectrophotometer, Agilent, USA). The total phenolic content was defined as mg GAE/g of dry weight (Nisa et al., 2019).

Total flavonoid content (TFC)

The samples and standard quercetin solution were prepared at 1 mg/mL concentration in a methanol solution. The standard solution was then diluted at 25, 50, 100, 200, 400, and 800 µg/mL concentrations. Each extract, fraction, and standard solution was added into a 96-well microplate, followed by adding 60 µL methanol, 10% aluminium chloride, and 10 µL of 1 M potassium acetate. Finally, 120 µL of distilled water was added and then properly homogenized. After incubation for 30 min, the absorbance was observed at a wavelength of 415 nm (Multiskan™ FC microplate reader, Thermo Scientific, USA). The total flavonoid content was defined as mg QE/g of dry weight (Nisa et al., 2022).

Total terpenoid content

The extract, fractions, and standard ursolic acid solution were prepared at 1 mg/mL concentration in a methanol solution. The standard solution was then diluted at 100, 200, 300, 400, 500, and 600 µg/mL concentrations. A 0.1 mL of each sample and standard solution was added with 0.2 mL of 5% vanillin and 1 mL of perchloric acid. The mixture was then incubated for 45 min at 60°C. After incubation, the samples were let to stand at room temperature for 15 min. Finally, 2.5 mL of acetic acid was added and well homogenized. The absorbance was measured using UV-Vis at a wavelength of 548 nm (Cary 60 UV-Vis Spectrophotometer, Agilent, USA).

LC-HRMS analysis

Untargeted metabolomics evaluation of extract and each fraction were performed using a liquid chromatography (Thermo Scientific™ Vanquish™ UHPLC Binary Pump), high-resolution mass spectrometry (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High-Resolution Mass Spectrometer) and an autosampler device. The samples were subjected into a column (Accucore™ Phenyl-Hexyl 100 mm × 2.1 mm ID × 2.6 µm) and eluted gradiently with water (0.1% formic acid) and methanol (0.1 % formic acid) as mobile phases for 25 min in a 0.30 mL/min flow rate. The data were detected using an Orbitrap mass spectrometer. Full MS/dd-MS² acquisition mode was used to collect untargeted metabolites in positive and negative ionization polarities. The detected metabolites were scanned in the range of 66.7-1000 m/z using 70,000 for full MS and 17,500 for dd-MS² resolutions. Data analysis was processed on Xcalibur software 4.4 version (Thermo Scientific, Bremen, Germany).

Bacterial strains

The antibacterial activity was tested using four pathogenic bacterial strains, namely *Staphylococcus aureus* FNCC 0047, *Bacillus subtilis* FNCC 0059, *Escherichia coli* FNCC 0091, and *Pseudomonas aeruginosa* FNCC 0070 bacteria. All the tested bacteria were provided from the Food and Nutrition Culture Collection (FNCC), Universitas Gadjah Mada, Yogyakarta, Indonesia.

Preparation of inoculums

Approximately 300 µL bacterial strains were subjected to 3 mL Nutrient Broth agar media and subcultured overnight at 37°C. The Optical density (OD) was measured using a microplate reader (Multiskan™ FC microplate reader, Thermo Scientific, USA) at 600 nm and calculated according to McFarland Standard 0.5 method (1.5×10^8 CFU/mL). The inoculum suspension was further diluted to obtain a viable cell count of approximately 10^7 CFU/mL standard bacterial turbidity.

Antibacterial evaluations

The antibacterial activity of *A. occidentale* was evaluated using minimum inhibitory concentrations (MIC) (Nisa et al., 2022) and minimum bactericidal concentrations (MBC) determination and time-kill assay by microdilution method (Galgano et al., 2022). In a 96-well microplate, 20 mg/mL stock solution of extract and fractions in DMSO were diluted at various concentrations (0.125, 0.25, 0.5, 1, 2 mg/mL) in a total volume of 100 µL Mueller Hinton Broth (MHB) medium. Then, 100 µL of bacterial colonies were added

into extract, fractions, and positive and negative controls. Ampicillin was served as a positive control, and DMSO was used as a negative control. All tests were performed in triplicate. The samples were incubated at 37°C for 24 h. The time kill assay was evaluated by monitoring the OD using a microplate reader (Multiskan™ FC microplate reader, Thermo Scientific, USA) ($\lambda = 600$ nm) with 2 h as intervals time of observation. The OD data of each antibacterial concentration was calculated as ΔOD (interval incubation time – initial incubation time). The ΔOD was plotted as bacterial killing times curves, which were processed using the GraphPad Prism 9.5.1 (May et al., 2000). After 24 h of incubation for MBC determination, one loop of the mixtures treatment in each level of concentration was inoculated on Mueller Hinton Agar (MHA) media and further incubated for 24 h. Finally, 15 μ L MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide] was added into the samples, followed by incubation for 1 h at 37°C. The colour (purple-yellow) alteration was observed visually in each well group. The lowest concentration, which indicated the absence of bacterial growth (yellow colour), was considered the MIC value. The MBC value was expressed as the lowest concentration without bacterial growth on the agar plate.

Statistical analysis

The obtained phytochemical data were expressed as the mean $\pm$ SD (standard derivation) of triplicate experiments and examined by one-way analysis of variance (ANOVA) and Duncan's post-hoc test. The significance of data was statistically defined to be $p < 0.01$.

RESULTS AND DISCUSSION

Extraction

Comparing the chemical composition of ethanol extract and all fractions, the ethyl acetate fraction was observed to have more strongly positive results in

secondary metabolite contents. Table 1 showed that the ethyl acetate fraction of *A. occidentale* had the highest value of phenolic content (508.89 ± 5.0 mg GAE/g dw) followed by aqueous fraction (314.64 ± 3.7 mg GAE/g dw) and the crude ethanol extract (249.25 ± 1.6 mg GAE/g dw) ($p < 0.01$). Same as phenolic contents, ethyl acetate fraction also exhibited the highest flavonoid contents (184 ± 3.6 mg QE/g dw) followed by an aqueous fraction as well ($p < 0.01$). While in the terpenoid contents evaluation, the ethanol extract and all fractions displayed a similar value ($p < 0.01$).

These results show that the phenolic compounds in *A. occidentale* are mostly semipolar compounds that are easy to dissolve in semipolar solvents such as ethyl acetate. According to Chaves et al. (2020), ethyl acetate solvent extraction can bind flavonoids with low polarity, such as isoflavones, flavanones, methyl flavones, and flavonols. In contrast, all solvents seem to have a similar capacity to attract nonpolar compounds based on their terpenoid contents. Terpenoids are one of the largest groups of secondary metabolites derived from natural sources. They are considered volatile, colourless, and fragrant liquids. Terpenoids are incompatible with water but easily soluble in organic solvents, such as petroleum ether, chloroform, or ethanol (Fan et al., 2023).

Determination of minimum inhibitory concentration (MIC)

Antibacterial activity of *A. occidentale* was investigated by MIC and MBC determinations and time-kill assay confirmed by streak agar plate evaluation.

The MIC of the *A. occidentale* ethanol extract and fractions were performed by microplate dilution method to evaluate their bacteriostatic properties. The MIC is determined as the lowest concentration of the *A. occidentale* extract or fractions, in which no microbial growth was visually observed after incubation for 24 h, as presented in Table 2.

Table 1. The yields, phenolic, flavonoid, terpenoid contents of extract and fractions.

Samples	Yields (g)	Phenolic content (mg GAE/g dw)	Flavonoid content (mg QE/g dw)	Terpenoid content (mg UAE/g dw)
Ethanol extract	9.63	249.25 ± 1.6^c	52.89 ± 4.1^c	673.11 ± 0.8^a
<i>n</i> -hexane fraction	0.56	58.89 ± 0.6^e	84.04 ± 2.3^{bc}	177.36 ± 5.2^a
Chloroform fraction	0.64	90.80 ± 0.2^d	79.11 ± 1.5^{bc}	164.94 ± 7.8^a
Ethyl acetate fraction	0.49	508.89 ± 5.0^a	184.00 ± 3.6^a	159.72 ± 5.9^a
Aqueous fraction	4.89	314.64 ± 3.7^b	118.00 ± 14.6^b	244.00 ± 2.1^a

The same column marked with a different letter shows that the values are significantly different by Duncan's post-hoc test ($p < 0.01$).

Table 2. Minimum inhibitory concentration of extract and fractions.

Samples	MIC value (mg/mL)			
	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
Ethanol extract	≤1.0	≤1.0	≤0.25	≤0.5
<i>n</i> -hexane fraction	≤1.0	≤1.0	≤1.0	≤1.0
Chloroform fraction	≤2.0	≤1.0	≤1.0	≤2.0
Ethyl acetate fraction	≤0.5	≤0.5	≤0.5	≤0.25
Aqueous fraction	≤2.0	≤1.0	≤0.5	≤0.5
Ampicillin	≤0.001	≤0.001	≤0.001	≤0.001

A. occidentale extract could inhibit the bacteria started from a concentration of 0.25 mg/mL to 1.0 mg/mL with selective to *S. aureus* and *P. aeruginosa* yet less effective against *E. coli* and *B. subtilis*. The results are in accordance with those reported by Anand et al. (2015), Kaewpiboon et al. (2012), and Santos et al. (2013). The ethyl acetate fraction suppressed the bacterial growth from 0.25 mg/mL to 0.5 mg/mL. Among other fractions, the ethyl acetate fraction was the most effective against all tested bacteria, followed by the aqueous fraction. *S. aureus* and *P. aeruginosa* exhibited the highest susceptibility to *A. occidentale* aqueous fraction. *E. coli*, as a Gram-negative bacteria, was apparently more resistant to the extract and most of the fractions. However, it was sensitive only to ethyl acetate fraction. Gram-negative bacteria resist because they possess a wall structure consisting of many layers of peptidoglycan and diverge in chemical conformation. Hence, it is quite complicated than Gram-positives even though the thicker types only present a single form of macro molecule (Gonçalves and Gobbo, 2012). However, in contrast, Gram-negative bacteria *P. aeruginosa* has sensitiveness rather than the extract and fractions except for the chloroform fraction. The investigation by those three assays of extract and fractions suggested that *A. occidentale* can be used as an antibiotic agent to treat such pathogenic bacteria causing several diseases. *E. coli* and *P. aeruginosa* are the two common sources for producing toxins that cause human gastroenteritis diseases. While *S. aureus* is the organism that contributed to pneumonia, skin infections, oral cavity, and bacteremia.

Time-kill assay evaluation

The minimum bactericidal concentration (MBC) and time effectiveness to kill the four tested pathogenic bacteria are presented in Fig. 1.

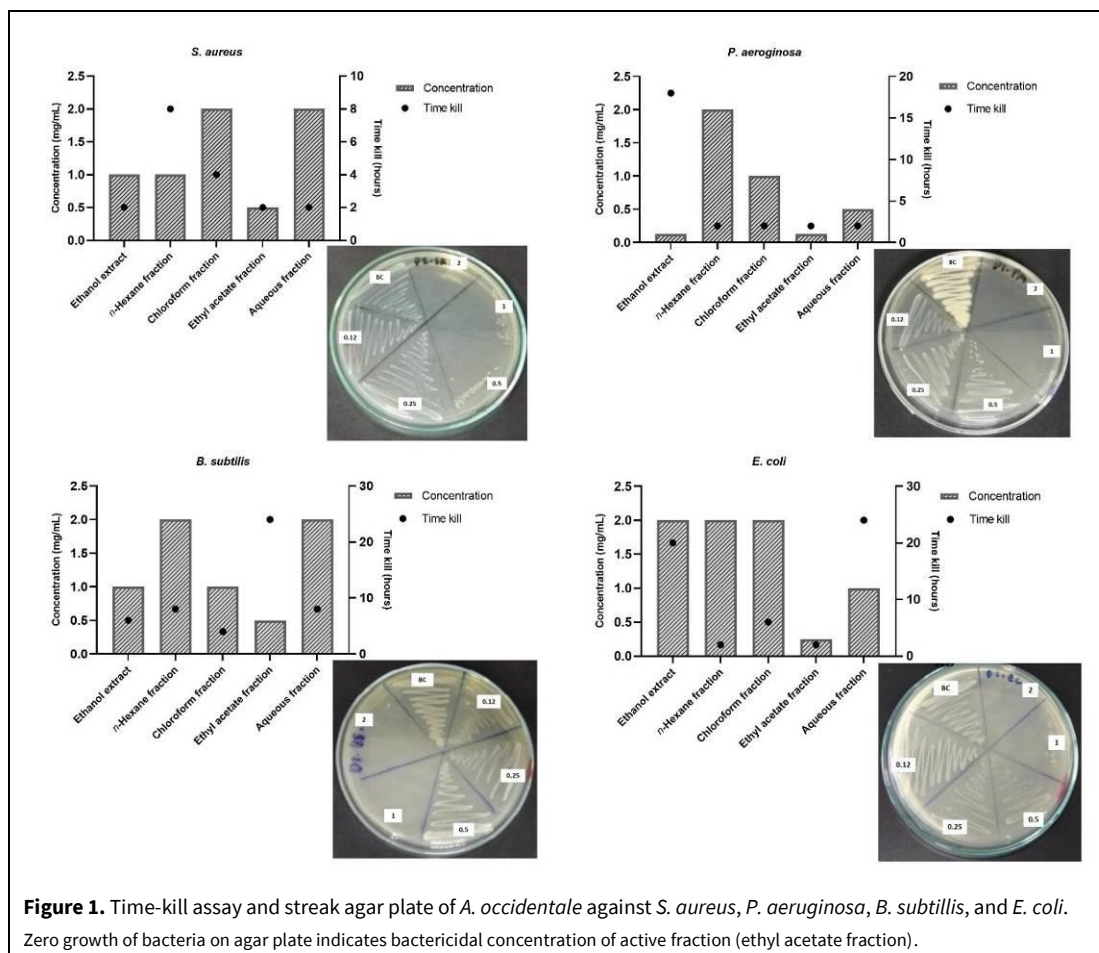
The Fig. 1 displayed that the ethanol extract could kill *E. coli* in 18 h at a concentration of 2 mg/mL while at the same concentration, ethanol extract, *n*-hexane fraction, and chloroform fraction were able to kill *E. coli* in the 20 h, 2 h, and 6 h, respectively. Moreover,

aqueous fraction could kill *E. coli* in the lower concentration (1 mg/mL), but needs a longer time (24 h). Compared with extract as well as other fractions, ethyl acetate fraction showed a very strong ability to kill *E. coli* until the lowest tested concentrations (0.25 mg/mL) in two hours of observation.

Antibacterial evaluation toward *B. subtilis* exhibited that at a concentration of 1 mg/mL, either ethanol extract or chloroform fraction were able to kill in 6 h and 4 h, respectively. The *n*-hexane fraction required a minimum concentration of 2 mg/mL to kill *B. subtilis* in 8 h, similar to the aqueous fraction. While at a low concentration (0.5 mg/mL), the ethyl acetate fraction could kill the bacteria longer (24 h). As an antimicrobial agent, less concentration is taken to minimize the use of higher doses in drug administrations to prevent their toxicity effects (Sun et al., 2019). Toward *B. subtilis*, the ethyl acetate fraction presented a very strong capacity to kill bacteria at all ranges of tested concentrations.

From Fig. 1, either extract or fractions afforded to kill microbial growth of *P. aeruginosa*. The ethanol extract and ethyl acetate fraction were observed to possess bactericidal activity to *P. aeruginosa* at low concentrations (0.125 mg/mL) in 18 h and 2 h, respectively. Moreover, the aqueous fraction exhibited good bactericidal activity at a high concentration (0.5 mg/mL) in 2 h of growth observation. In contrast, *n*-hexane (2 mg/mL) and chloroform fractions (1 mg/mL) were able to kill bacteria at this concentration in 2 h. Within all fractions, ethyl acetate fraction has the highest bactericidal activity against *P. aeruginosa*.

The ethanol extract and all fractions displayed good antibacterial activity by killing *S. aureus* microbial growth. The ethanol extract and *n*-hexane fraction could kill the bacteria at a minimum 1 mg/mL concentration in 2 h and 8 h, respectively. While the chloroform and aqueous fraction required a 2 mg/mL concentration to kill bacteria in 4 h and 2 h, respectively. The ethyl acetate fraction showed a very strong



ability to kill *S. aureus* even in 2 h at 0.5 mg/mL of concentrations.

According to those results, the ethyl acetate fraction was the most active against all tested bacteria. Flavonoids and phenolic compounds might attribute it as the main constituents in ethyl acetate fraction. Several studies explained that ethyl acetate fraction derived from plant extract contains high flavonoid and phenolic content with antimicrobial activity. As reported in *Vernonia amygdalina* Delile leaves, its ethyl acetate fraction had improved the microbial activity of tetracycline against *P. aeruginosa* and MRSA (Satria et al., 2023). Flavonoids have good antimicrobial activity and have recently been paid much attention (Tungmunthum et al., 2018). Several flavonoid compounds with antibacterial capacity, such as apigenin, quercetin, and luteolin, have been isolated (Basile et al., 2000; Rauha et al., 2000; Torrenegra et al., 1989).

Alkaloid and phenolic compounds, which are abundantly obtained in plant extract, work as antimicrobial components by disrupting the function of the cytoplasmic membrane, which may cause cell death or hinder essential enzymes for the biosynthesis of amino acids (Burt, 2004). At low concentrations, it can

damage the cytoplasmic membrane, causing leakage of important metabolites that inactivate the microbial enzyme system, while the cytoplasmic membrane and precipitate cell proteins are destroyed by increasing its concentrations (Suliani et al., 2016).

Streak agar plate

The lowest MIC value of *A. occidentale* extract and fractions was confirmed by the Minimum Bactericidal Concentration (MBC) evaluation using a streak agar plate to show potentially bactericidal capacity against the tested pathogenic bacteria (Fig. 1). After incubation for 24 h, the lowest concentration from an antimicrobial agent, which works out to kill bacteria up to 99.9% expresses as an MBC value. By streaking agar plate method, MBC is recognized by the appearance of scratches on the plate that has been streaked and incubated for 24 h (Balouiri et al., 2016).

Fig. 1 displays that the ethyl acetate fraction of *A. occidentale* has a good bactericidal capacity against *E. coli*, *P. aeruginosa*, and *B. subtilis* bacteria at a concentration of 1 mg/mL and, at lower concentration (0.5 mg/mL), was more effective against *S. aureus* bacteria. The MIC determination assay of *A. occidentale* complied with the time-kill assay, which demonstrat-

ed that ethyl acetate fraction was the most active antimicrobial against all tested pathogenic bacteria. See also Figs. S1-S3.

LC-HRMS compounds profiling

The untargeted LC-HRMS chromatogram, as displayed in Fig. 2, was examined. The following analytical parameters comprised the metabolite profile analysis: the conformation of candidate molecules to the fragmentation pattern in mzCloud was used to select them, and their molecular mass deviation did not exceed 2 ppm. High abundance compounds in the aqueous, chloroform, and hexane fractions appeared between 13 and 25 min (Fig. 2).

As shown in Table 3, the fraction from ethanolic extracts of *A. occidentale* was analysed with an LC-HRMS system to predict the metabolite variation to determine the fractionation impact. In general, the leaf extract of *A. occidentale* and its fractions contain amino acids, phenolic acids, alkaloids, flavonoids, fatty acid amides, fatty acids, and organic acids as classes of molecules. *A. occidentale* is an exceptionally flavonoid-rich herb. The flavonoids were profused between 3.75 and 17.7 min of retention time (Table 3 and Fig. 2). Except for the stearamide compound, all fractions contained fatty acid amide compounds. Moreover, excluding for L-phenylalanine, trans-3-indoleacrylic acid, catechin gallate, quercetin-3 β -D-glucosides, luteolin, palmitic acid, myristamide, and

stearamide, the ethyl acetate fraction contains all putative compounds in the extract (n = 31). Therefore, only the fractions of chloroform and *n*-hexane contain fatty acids, specifically palmitic acid.

Taking a retention time range of 5 to 25 min, the ethyl acetate fraction contained a large variety and abundance of compounds from semipolar to nonpolar regions. Flavonoids are identified as the main contents in ethyl acetate fraction, with avicularin/quercetin-3-O-arabinofuranoside and myricitrin recognized as major compounds. Due to its structure, which includes both a polar group (-COOR) and a nonpolar group (-CH), ethyl acetate is classified as a semipolar compound. It should, therefore, attract either polar or nonpolar compounds. Like flavonoid aglycones, a semipolar polyphenol could be dissolved in cell walls by an ethyl acetate solvent. The solvent ethyl acetate can bind flavonoids with low polarity, including isoflavones, flavanones, methyl flavones, and flavonols, during extraction (Chaves et al., 2020). Flavonoid compounds are present in most of the evergreen vegetation. Most flavonoid compounds in plants exist as sugar-bound glycosides. The hydroxyl group in the flavonoid molecule (aglycone) bonds to the carbonyl group of sugar (glycone) to form flavonoid glycosides (Kumar and Pandey, 2013). Moreover, the phenolic compound, such as gallic acid, was also abundantly contained in ethyl acetate fraction. See also Table S1.

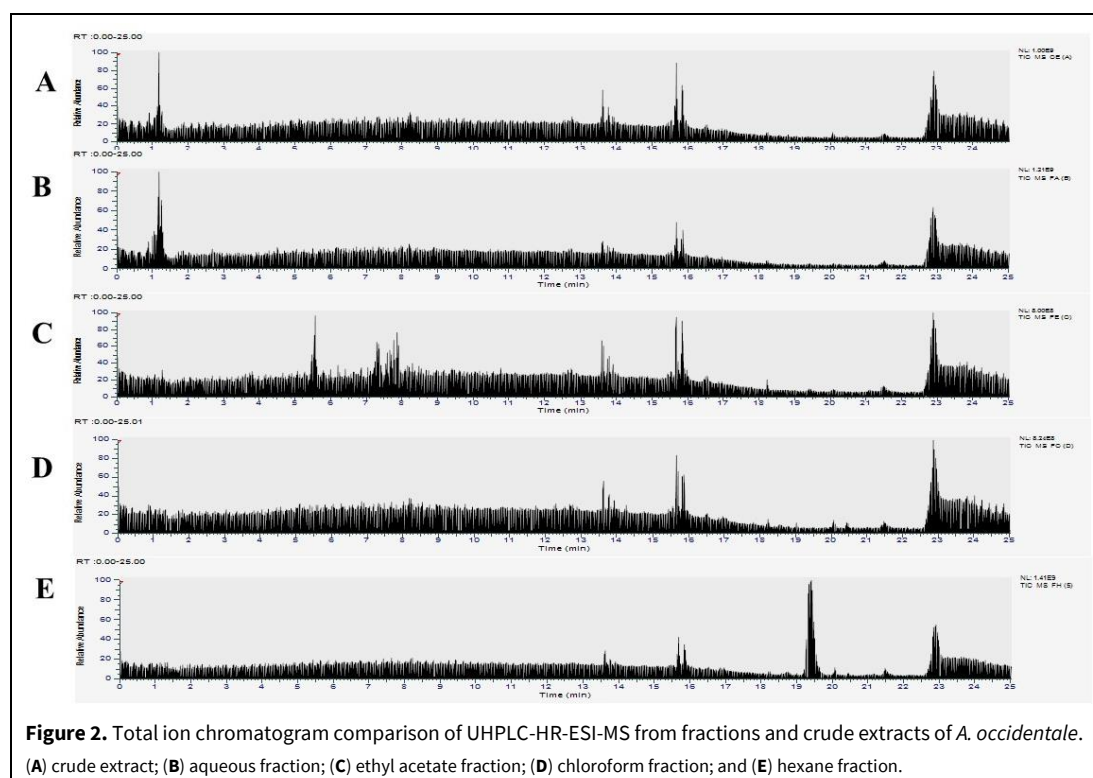


Table 3. Putative compound identified from *A. occidentale*.

No.	RT (min)	Putative compound	Class	Formula	Annot. Delta Mass [ppm]	Calc. MW	MS/MS fragmentation	Area*				
								A	B	C	D	E
1	1.251	L-Valine	Amino acid	C ₅ H ₁₁ NO ₂	0.75	117.07907	55.05383; 68.95245; 72.08123; 91.05432; 118.03487	1.2E+08	2.1E+08	1.2E+07		
2	1.515	Shikimic acid	Phenolic acid	C ₇ H ₁₀ O ₅	-5.96	174.05179	71.01268; 81.03339; 93.03343; 111.04402; 137.02338; 143.03384 (negative mode)	7.4E+07		3.0E+07		
3	2.311	Gallic acid	Phenolic acid	C ₇ H ₆ O ₅	-6.43	170.02043	89.07113; 109.02844; 125.02316; 127.03877; 153.01797; 171.02852	5.9E+07	3.9E+07	6.4E+08	1.0E+07	
4	2.723	L-Phenylalanine	Amino acid	C ₉ H ₁₁ NO ₂	-0.11	165.07896	79.05464; 93.07007; 103.05441; 107.04921; 120.08077; 131.04909	1.9E+08	1.7E+08			
5	3.750	Gallocatechin	Flavonoid	C ₁₅ H ₁₄ O ₇	-1.55	306.07348	139.03871; 181.04915; 247.06029; 307.08029	7.4E+08	1.7E+07	4.2E+08		
6	4.542	trans-3-Indoleacrylic acid	Alkaloids	C ₁₁ H ₉ NO ₂	-1.25	187.06309	91.05447; 118.06522; 146.05992	1.5E+08	1.2E+08		1.7E+07	

Table 3. Putative compound identified from *A. occidentale* (continued...)

No.	RT (min)	Putative compound	Class	Formula	Annot. Delta Mass [ppm]	Calc. MW	MS/MS fragmentation	Area*				
								A	B	C	D	E
7	5.484	Catechin	Flavonoid	C ₁₅ H ₁₄ O ₆	-1.46	290.07861	97.02832; 109.02833; 123.04400; 203.08059; 245.08139; 289.07147 (negative mode)	2.1E+08	6.5E+06	2.9E+08		
8	6.231	Epigallocatechin gallate	Flavonoid	C ₂₂ H ₁₈ O ₁₁	-1.14	458.08439	69.03343; 125.02329; 169.01326; 161.02342; 269.04605 (negative mode)	1.7E+07		6.6E+07		
9	6.757	Myricetin 3-O-β-D-galactopyranoside	Flavonoid	C ₂₁ H ₂₀ O ₁₃	-0.14	480.08984	153.01804; 245.04384; 319.04398; 385.05838	3.8E+07	2.9E+07	1.6E+08		
10	7.251	Catechin gallate	Flavonoid	C ₂₂ H ₁₈ O ₁₀	-1.41	442.08937	151.03882; 123.04403; 139.03877; 273.07544	1.1E+07				
11	7.305	Quercetin-3β-D-glucosides	Flavonoid	C ₂₁ H ₂₀ O ₁₂	-1.10	486.07687	71.50153; 96.21252; 185.04170; 255.39217; 325.03085	6.9E+07	2.5E+07			
12	7.314	Myricitrin	Flavonoid	C ₂₁ H ₂₀ O ₁₂	-1.27	464.09548	71.04960; 129.05457; 147.06473; 245.04453; 319.04410	1.4E+08	1.7E+08	2.4E+09	1.4E+08	

Table 3. Putative compound identified from *A. occidentale* (continued...)

No.	RT (min)	Putative compound	Class	Formula	Annot. Delta Mass [ppm]	Calc. MW	MS/MS fragmentation	Area*				
								A	B	C	D	E
13	7.322	(2r)-2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydro-4h-chromen-4-one	Flavonoid	C ₁₅ H ₁₀ O ₇	-2.35	302.04194	109.02831; 121.02949; 274.04694; 303.04922	9.4E+07	3.5E+07	4.4E+08	2.2E+07	
14	7.367	Desmanthin-1	Flavonoid	C ₂₈ H ₂₄ O ₁₆	-2.01	616.10520	97.02862; 153.01805; 297.05994; 303.04922	2.7E+07		1.9E+08		
15	7.731	Avicularin / quercetin-3-O-arabinofuranoside	Flavonoid	C ₂₀ H ₁₈ O ₁₁	-1.53	434.08491	57.03408; 73.02885; 85.02875; 165.01791; 303.04938	2.1E+08		1.3E+09	5.7E+07	
16	8.142	Juglalin / kaempferol 3-O-arabinoside	Flavonoid	C ₂₀ H ₁₈ O ₁₀	-1.05	418.08956	107.01297; 135.00787; 255.02926; 284.03247; 417.08252	4.5E+07		8.9E+07		
17	8.288	Azelaic acid	Organic acids	C ₁₉ H ₁₆ O ₄	-4.31	188.10486	57.03348; 97.06476; 125.09613; 187.09682	7.5E+06		7.5E+06		
18	8.362	Myricetin	Flavonoid	C ₁₅ H ₁₀ O ₈	-0.82	318.03731	68.99761; 109.02860; 137.02338; 255.02853	8.5E+06		8.5E+06		
19	9.141	5,7-Dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]chromen-4-one	Flavonoid	C ₂₂ H ₂₂ O ₁₂	-0.55	478.11086	61.02888; 91.03920; 271.05984; 317.06458	9.3E+06		9.2E+07		

Table 3. Putative compound identified from *A. occidentale* (continued...)

No.	RT (min)	Putative compound	Class	Formula	Annot. Delta Mass [ppm]	Calc. MW	MS/MS fragmentation	Area*				
								A	B	C	D	E
20	9.490	Quercetin	Flavonoid	C ₁₅ H ₁₀ O ₇	-1.12	302.04265	65.00217; 107.01275; 151.00266; 245.04509; 301.04503 (negative mode)	3.4E+07		7.2E+07	7.1E+06	
21	10.508	Luteolin	Flavonoid	C ₁₅ H ₁₀ O ₆	-0.77	286.04752	63.02294; 107.01269; 143.04926; 285.04016 (negative mode)	9.6E+06	2.6E+07			
22	11.376	Palmitic acid	Fatty acid	C ₁₆ H ₃₂ O ₂	-1.01	273.26663	70.06553; 106.08653; 230.24518; 274.27383	6.6E+06			5.7E+06	5.4E+06
23	16.514	5,5',7,7'-Tetrahydroxy-2,2'-bis(4-hydroxyphenyl)-4H,4'H-6,6'-bichromene-4,4'-dione	Flavonoid	C ₃₀ H ₁₈ O ₁₀	-1.29	538.08930	68.99787; 121.02843; 163.03906; 275.01895; 403.04379; 539.09680	2.9E+08		2.6E+08	2.6E+08	1.2E+08
24	17.770	Myristamide	Flavonoid	C ₁₄ H ₂₉ NO	-2.14	227.22443	71.0851; 254.2473; 272.2585	2.9E+09			2.2E+09	
25	18.235	(4E)-5-Methyl-4-[(5-methyl-3-oxo-2-phenyl-1H-pyrazol-4-yl)methylidene]-2-phenylpyrazol-3-one	NA	C ₂₁ H ₁₈ N ₄ O ₂	-2.27	358.14216	93.05757; 118.06523; 268.10818; 266.09219; 359.14948	3.1E+07	8.9E+07	1.9E+08	1.3E+08	

Table 3. Putative compound identified from *A. occidentale* (continued...)

No.	RT (min)	Putative compound	Class	Formula	Annot. Delta Mass [ppm]	Calc. MW	MS/MS fragmentation	Area*				
								A	B	C	D	E
26	19.406	bis(2-Ethylhexyl)phthalate	Organic compound	C ₂₄ H ₃₈ O ₄	-3.51	390.27701	57.07042; 71.08594; 149.02312; 279.15747	2.9E+07	9.2E+07	1.3E+08	4.0E+07	1.3E+10
27	19.839	Hexadecanamide	Fatty acid amide	C ₁₆ H ₃₃ NO	-1.36	255.25587	57.07046; 74.06046; 116.10697; 148.67090. 258.26331	2.8E+07	5.5E+06	5.7E+06	3.4E+07	3.0E+07
28	20.078	Oleamide	Fatty acid amide	C ₁₈ H ₃₅ NO	-1.92	281.27133	69.07031; 83.08582; 95.08567; 149.13232; 163.14767; 247.24138; 282.27863	2.4E+08	9.8E+07	1.7E+08	2.6E+08	4.7E+08
29	20.142	Ostruthin	Terpenoid	C ₁₉ H ₂₂ O ₃	-0.12	298.15980	57.45317; 89.44093; 183.01117; 239.07391; 297.15244	1.4E+07	1.3E+07	1.7E+07	2.1E+07	1.3E+07
30	21.498	Erucamide	Fatty acid amide	C ₂₂ H ₄₃ NO	-3.04	337.33344	69.07029; 83.08581; 97.10133; 109.10123; 149.13225; 226.21590; 303.20365; 338.34058	4.5E+08	6.0E+08	5.5E+08	4.7E+08	7.4E+08
31	22.660	Strearamide	Fatty acid amide	C ₁₈ H ₃₇ NO	-1.12	283.28720	57.07042; 74.06034; 116.10705; 284.29446	2.2E+07			2.4E+07	3.8E+07

*(A) crude extract; (B) aqueous fraction; (C) ethyl acetate fraction; (D) chloroform fraction; and (E) *n*-hexane fraction.

CONCLUSION

Ethyl acetate fraction showed remarkable antimicrobial activity against *E. coli*, *B. subtilis*, *P. aeruginosa*, and *S. aureus* bacteria strains. The results were in accordance with its phytochemical profile, which possessed the highest phenolic and flavonoid contents. The antibacterial capacity of the active fraction might be attributed mainly to the presence of those flavonoid and phenolic compounds, as confirmed in LC-HRMS profiling.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Nisa K	Hasanah AU	Damayanti E	Frediansyah A	Anwar M	Khumaira A	Anindita NS
Concepts or ideas	x		x				
Design	x	x	x				
Definition of intellectual content	x	x					
Literature search		x					
Experimental studies	x	x	x				
Data acquisition	x	x	x	x	x		
Data analysis	x	x	x	x	x		
Statistical analysis		x	x				
Manuscript preparation	x	x				x	x
Manuscript editing	x		x	x			
Manuscript review	x	x	x	x	x	x	x

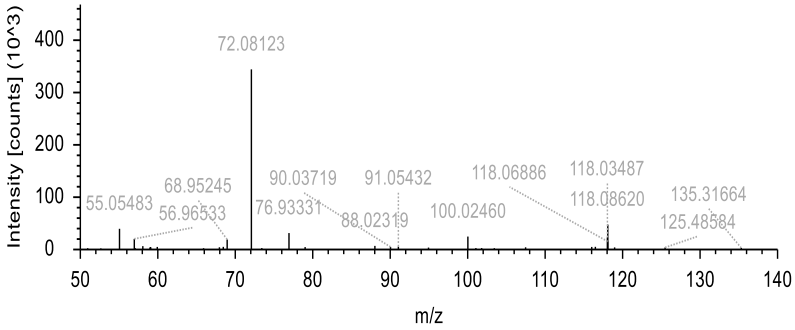
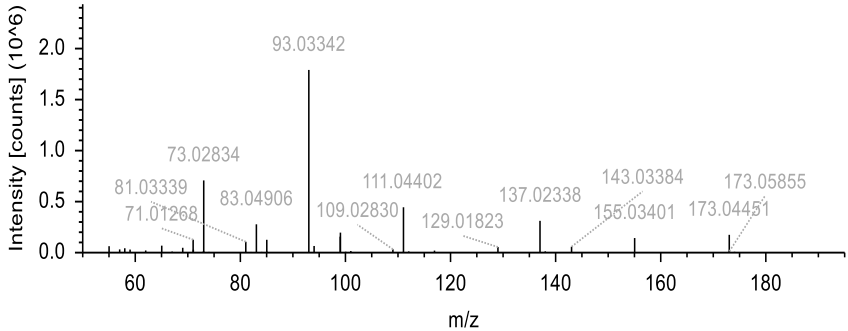
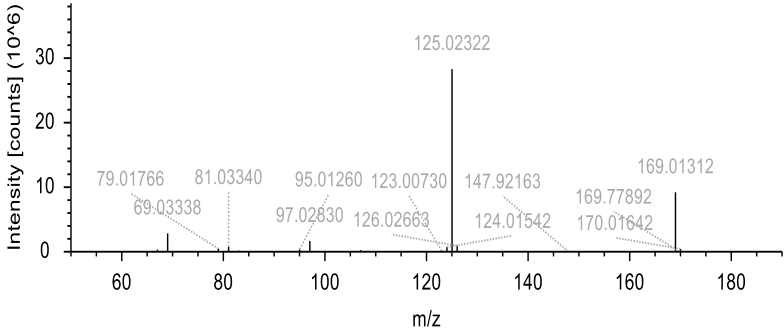
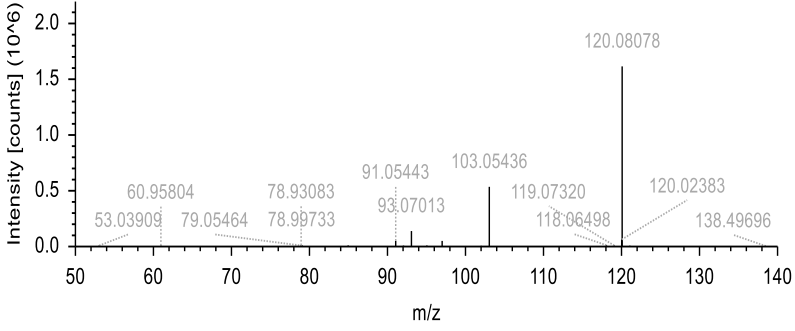
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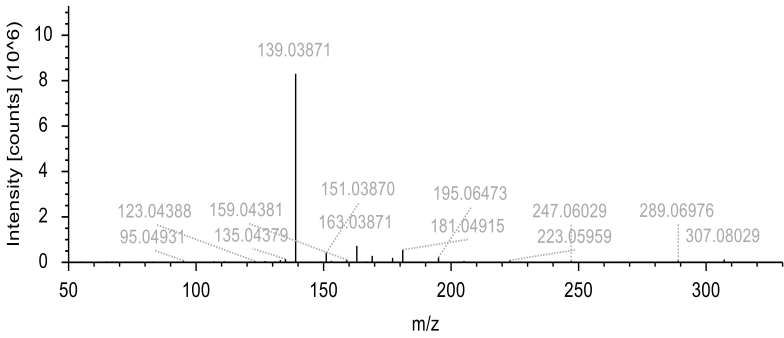
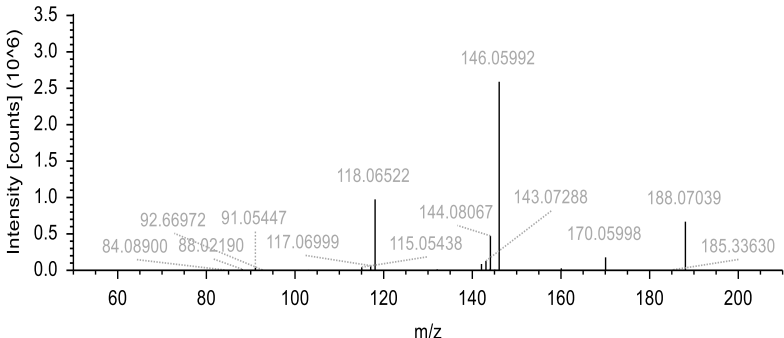
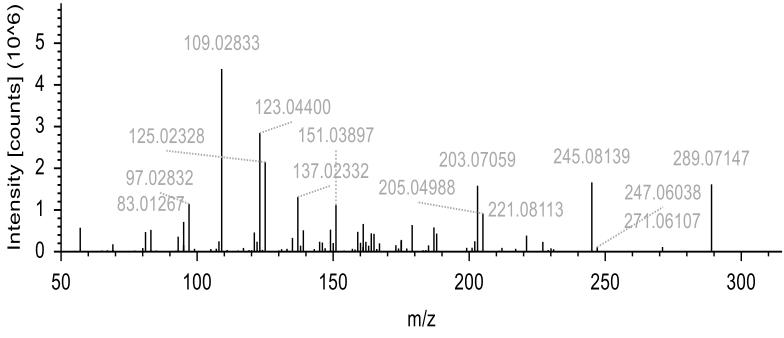
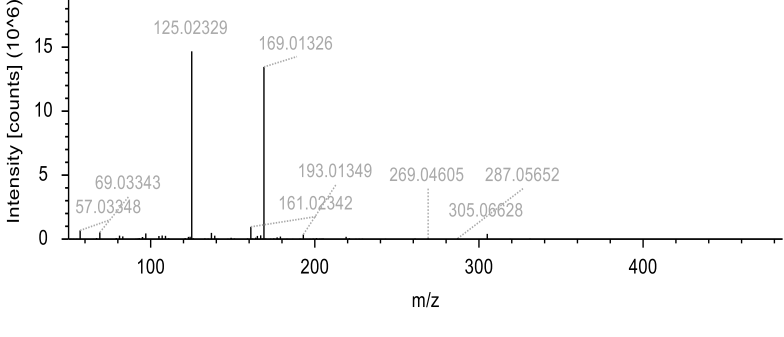
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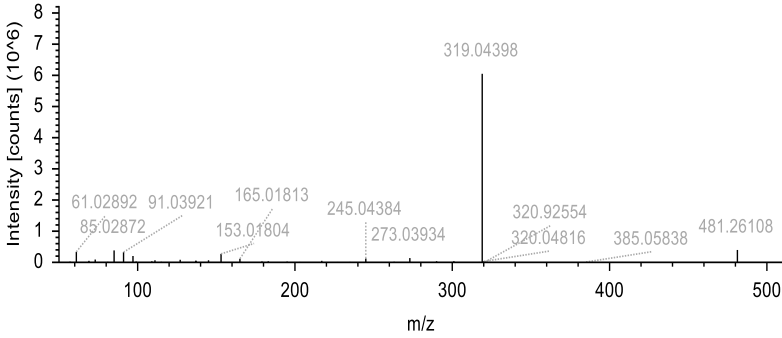
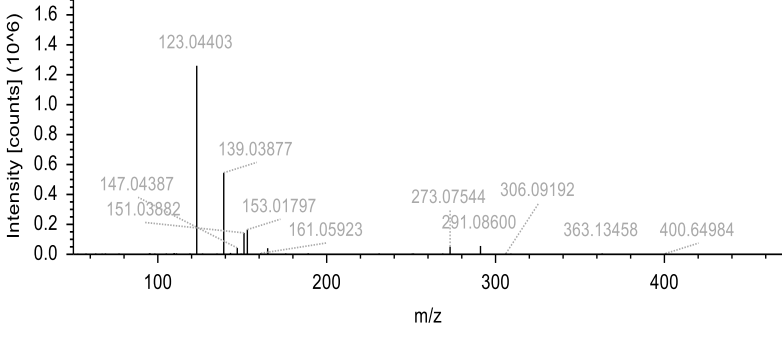
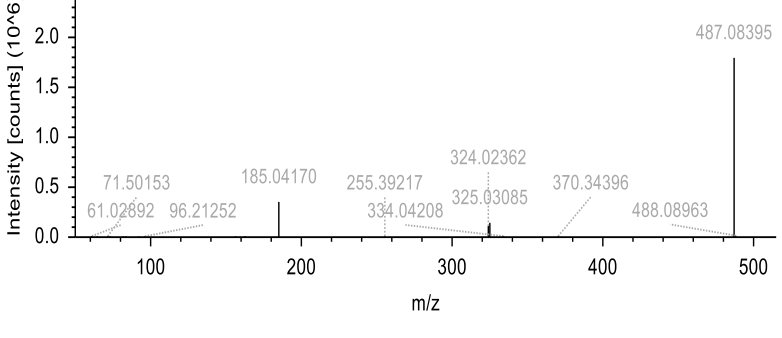
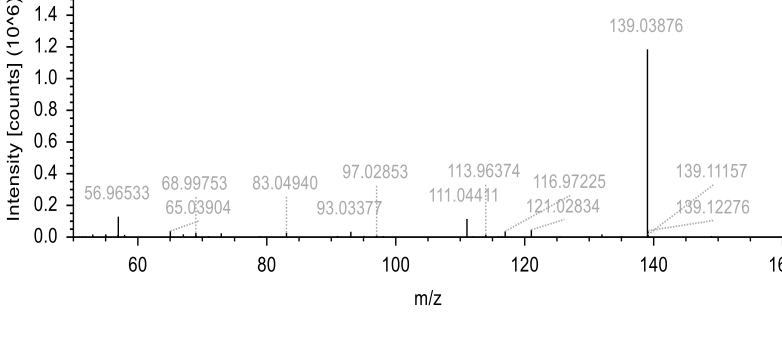
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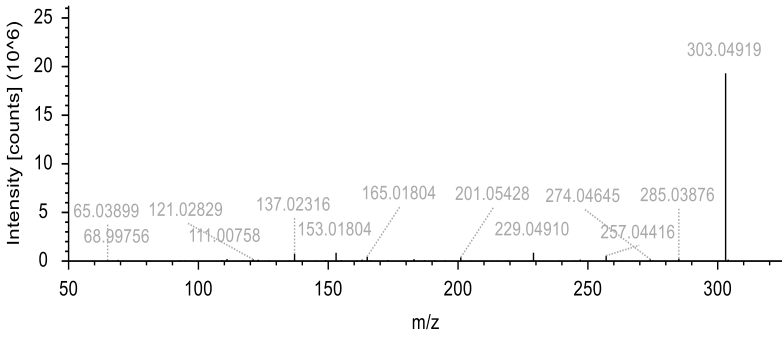
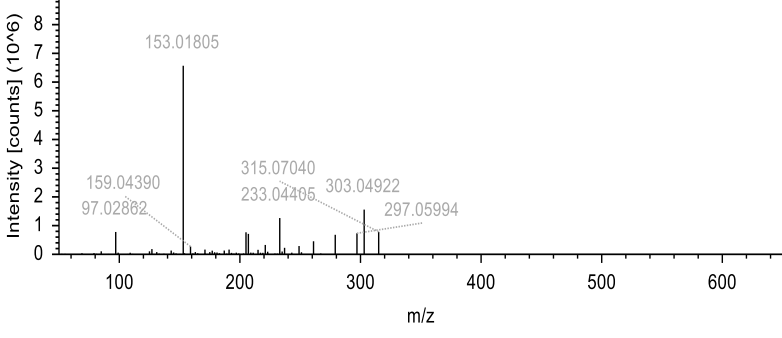
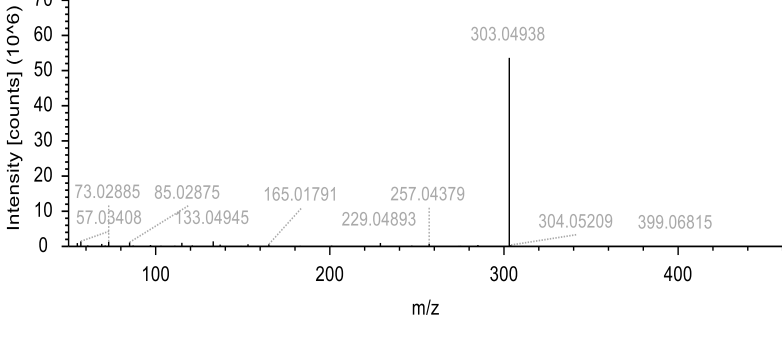
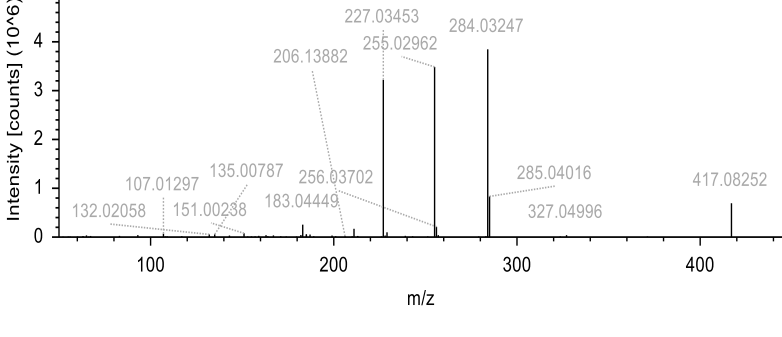
Supplementary data

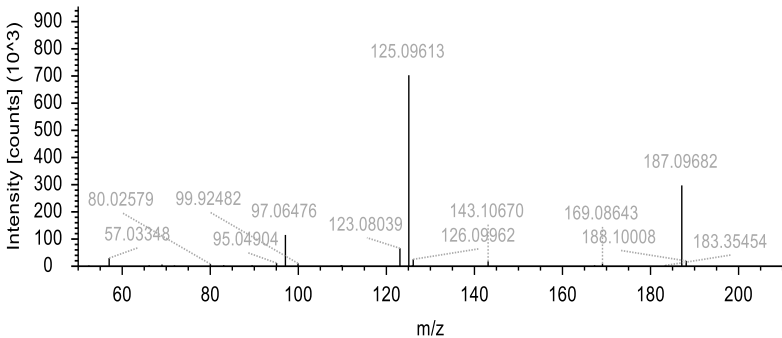
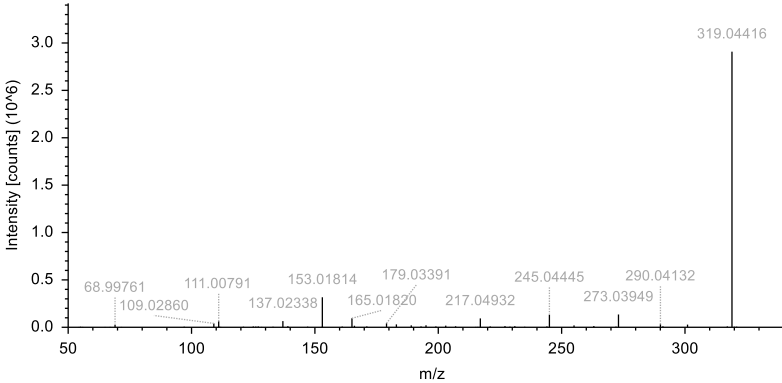
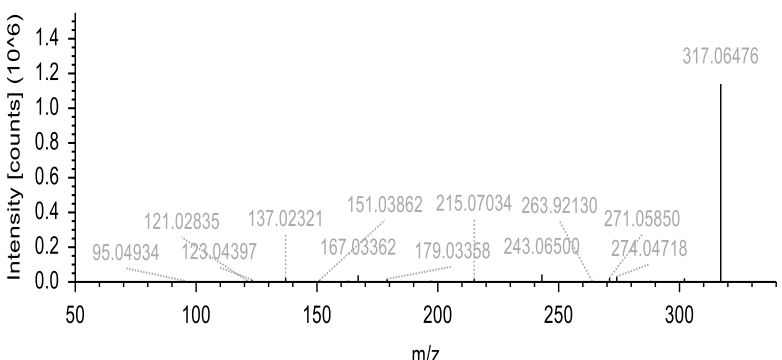
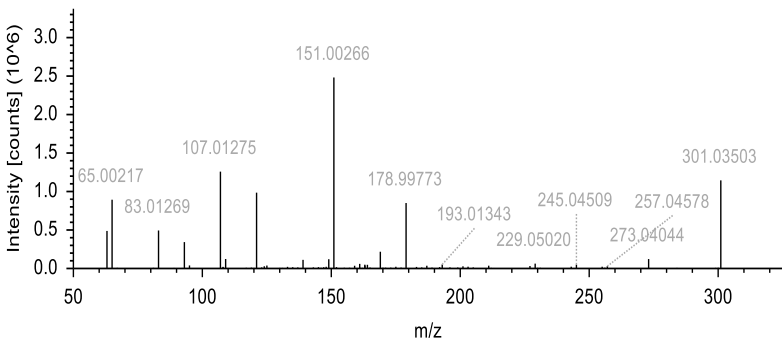
Table S1. Putative compound chromatograms.

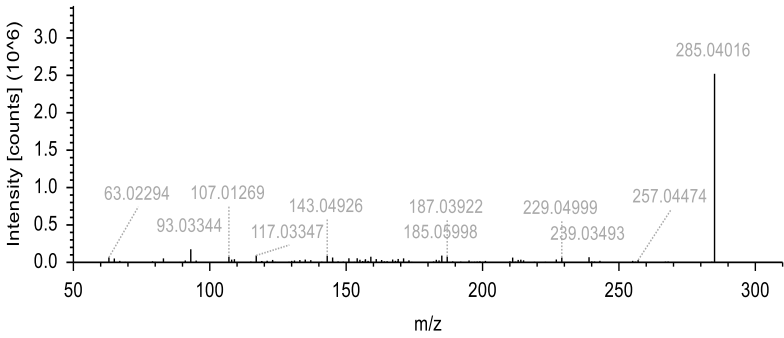
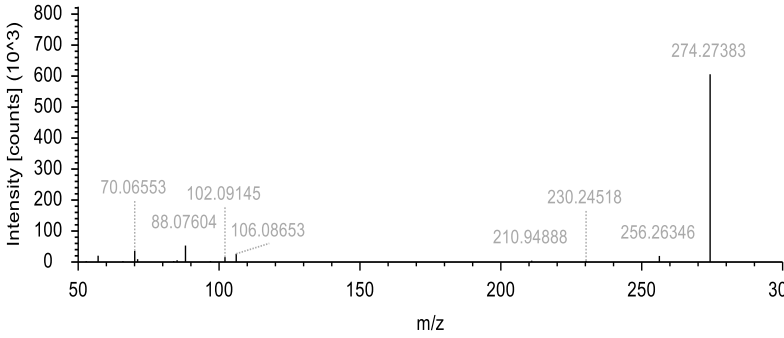
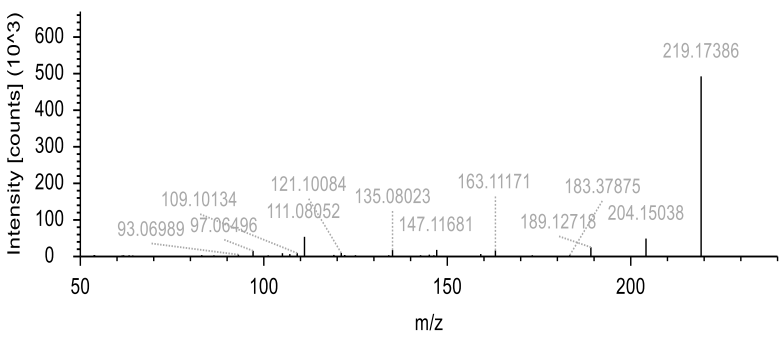
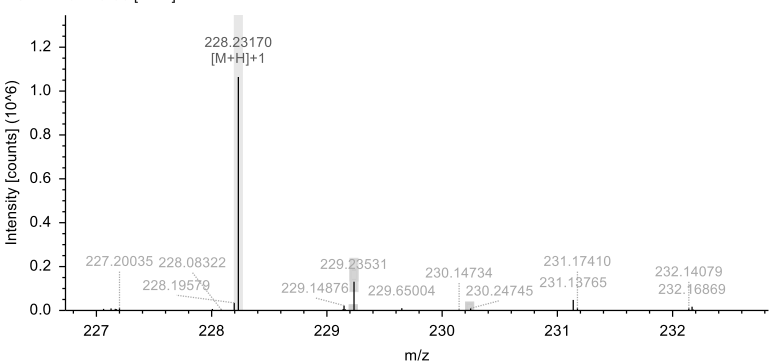
Putative compound	Fragmentation pattern
L-Valine	CE (A) (F1) #434, RT=1.490 min, MS2, FTMS (+), (HCD, DDA, 118.0864@(18;30;50), +1) 
Shikimic acid	CE (A) (F1) #439, RT=1.508 min, MS2, FTMS (-), (HCD, DDA, 173.0447@(30;50;70), -1) 
Gallic acid	FE (C) (F6) #654, RT=2.305 min, MS2, FTMS (-), (HCD, DDA, 169.0132@(30;50;70), -1) 
L-Phenylalanine	CE (A) (F1) #767, RT=2.656 min, MS2, FTMS (+), (HCD, DDA, 120.0808@(18;30;50), +1) 

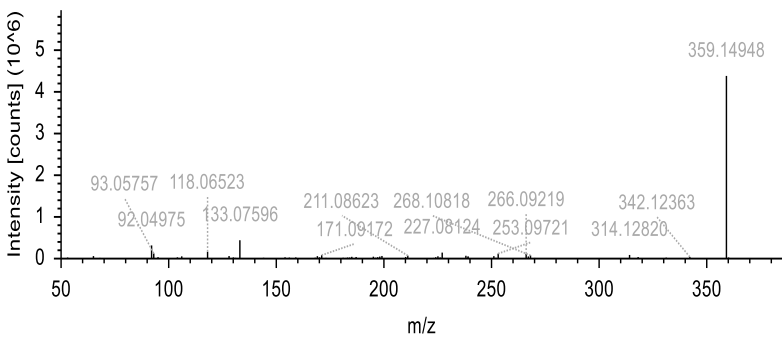
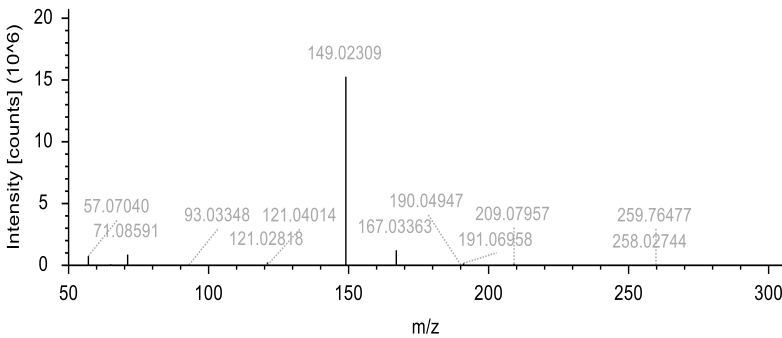
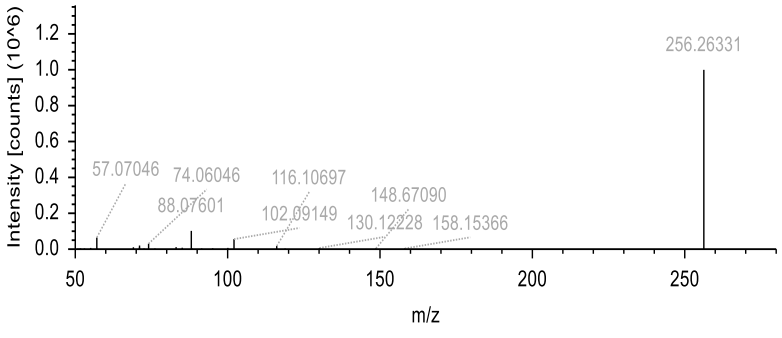
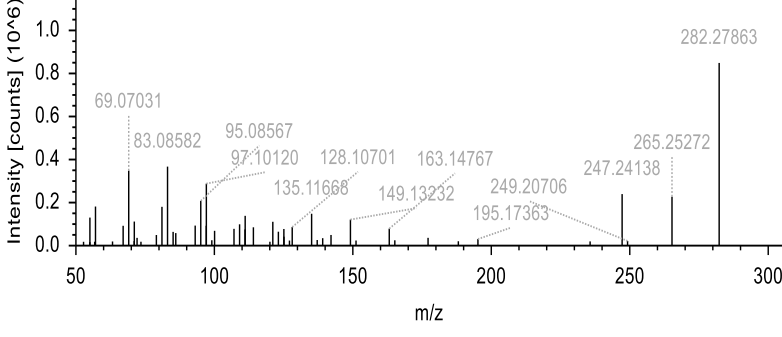
Gallocatechin	FE (C) (F6) #1042, RT=3.667 min, MS2, FTMS (+), (HCD, DDA, 307.0807@(18;30;50), +1) 
trans-3-Indoleacrylic acid	CE (A) (F1) #1284, RT=4.507 min, MS2, FTMS (+), (HCD, DDA, 188.0704@(18;30;50), +1) 
Catechin	FE (C) (F6) #1545, RT=5.441 min, MS2, FTMS (-), (HCD, DDA, 289.0714@(30;50;70), -1) 
Epigallocatechin gallate	FE (C) (F6) #1790, RT=6.235 min, MS2, FTMS (-), (HCD, DDA, 457.0773@(30;50;70), -1) 

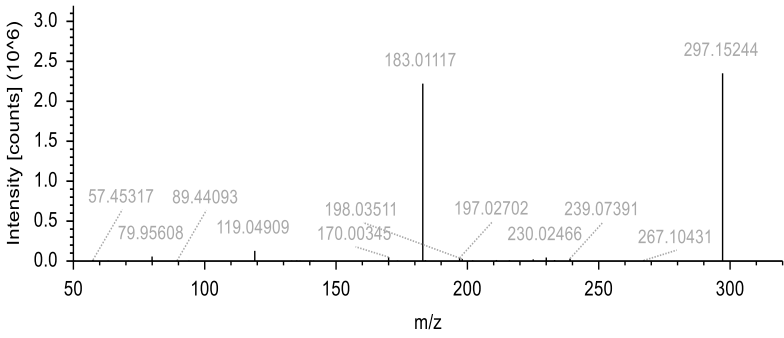
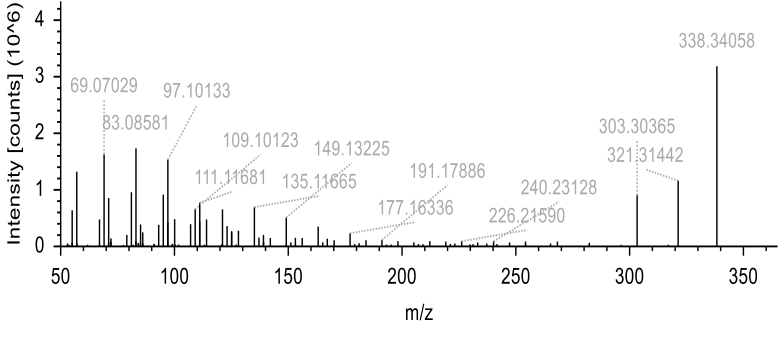
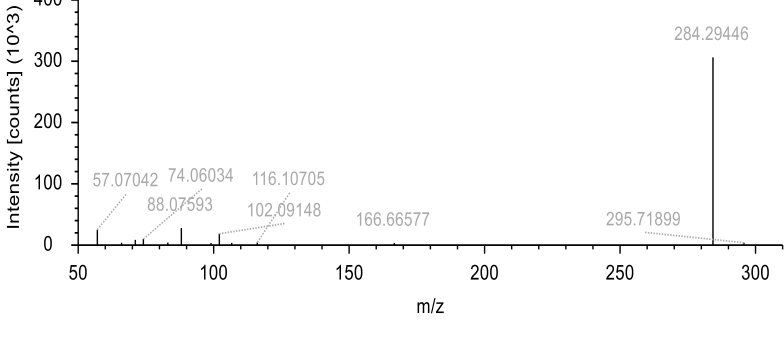
Myricetin 3-O-β-D-galactopyranoside	FE (C) (F6) #1932, RT=6.706 min, MS2, FTMS (+), (HCD, DDA, 481.0972@(18;30;50), +1) 
Catechin gallate	CE (A) (F1) #2074, RT=7.264 min, MS2, FTMS (+), (HCD, DDA, 443.0966@(18;30;50), +1) 
Quercetin-3β-D-glucosides	CE (A) (F1) #2086, RT=7.300 min, MS2, FTMS (+), (HCD, DDA, 487.0840@(18;30;50), +1) 
Myricitrin	FE (C) (F6) #1060, RT=3.732 min, MS2, FTMS (+), (HCD, DDA, 139.0387@(18;30;50), +1) 

(2r)-2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydro-4h-chromen-4-one	FE (C) (F6) #2106, RT=7.270 min, MS2, FTMS (+), (HCD, DDA, 303.0491@(18;30;50), +1) 
Desmanthin-1	FE (C) (F6) #2130, RT=7.342 min, MS2, FTMS (+), (HCD, DDA, 617.1119@(18;30;50), +1) 
Avicularin / quercetin-3-O-arabinofuranoside	FE (C) (F6) #2270, RT=7.771 min, MS2, FTMS (+), (HCD, DDA, 435.0916@(18;30;50), +1) 
Juglalin / kaempferol 3-O-arabinoside	FE (C) (F6) #2374, RT=8.083 min, MS2, FTMS (-), (HCD, DDA, 417.0824@(30;50;70), -1) 

Azelaic acid	FE (C) (F6) #2440, RT=8.295 min, MS2, FTMS (-), (HCD, DDA, 187.0968@(30;50;70), -1)  Intensity [counts] (10³) m/z
Myricetin	FE (C) (F6) #2073, RT=7.167 min, MS2, FTMS (+), (HCD, DDA, 319.0446@(18;30;50), +1)  Intensity [counts] (10%) m/z
5,7-Dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one	FE (C) (F6) #2904, RT=9.887 min, MS2, FTMS (+), (HCD, DDA, 317.0652@(18;30;50), +1)  Intensity [counts] (10⁶) m/z
Quercetin	FE (C) (F6) #2780, RT=9.466 min, MS2, FTMS (-), (HCD, DDA, 301.0352@(30;50;70), -1)  Intensity [counts] (10⁶) m/z

Luteolin	FE (C) (F6) #3082, RT=10.501 min, MS2, FTMS (-), (HCD, DDA, 285.0402@(30;50;70), -1) 
Palmitic acid	CE (A) (F1) #3282, RT=11.387 min, MS2, FTMS (+), (HCD, DDA, 274.2740@(18;30;50), +1) 
5,5',7,7'-Tetrahydroxy-2,2'-bis(4-hydroxyphenyl)-4H,4'H-6,6'-bichromene-4,4'-dione	FH (5) (F8) #4674, RT=16.484 min, MS2, FTMS (+), (HCD, DDA, 219.1742@(18;30;50), +1) 
Myristamide	FC (D) (F7) #5018, RT=17.806 min, MS1, FTMS (+) C14 H29 N O as [M+H]⁺1 

(4E)-5-Methyl-4-[(5-methyl-3-oxo-2-phenyl-1H-pyrazol-4-yl) methylidene]-2-phenylpyrazol-3-one	FE (C) (F6) #5203, RT=18.199 min, MS2, FTMS (+), (HCD, DDA, 359.1498@(18;30;50), +1) 
bis(2-Ethylhexyl)phthalate	FH (5) (F8) #5429, RT=19.431 min, MS2, FTMS (+), (HCD, DDA, 279.1584@(18;30;50), +1) 
Hexadecanamide	FC (D) (F7) #5509, RT=19.801 min, MS2, FTMS (+), (HCD, DDA, 256.2631@(18;30;50), +1) 
Oleamide	FH (5) (F8) #5615, RT=20.153 min, MS2, FTMS (+), (HCD, DDA, 282.2783@(18;30;50), +1) 

Ostruthin	FC (D) (F7) #5581, RT=20.104 min, MS2, FTMS (-), (HCD, DDA, 297.1524@(30;50;70), -1)  <table><caption>Peak Data for Ostruthin</caption><thead><tr><th>m/z</th><th>Relative Intensity (10⁶)</th></tr></thead><tbody><tr><td>57.45317</td><td>~0.5</td></tr><tr><td>79.95608</td><td>~0.2</td></tr><tr><td>89.44093</td><td>~0.5</td></tr><tr><td>119.04909</td><td>~0.2</td></tr><tr><td>170.00345</td><td>~0.2</td></tr><tr><td>183.01117</td><td>~2.2</td></tr><tr><td>197.02702</td><td>~0.5</td></tr><tr><td>198.03511</td><td>~0.5</td></tr><tr><td>230.02466</td><td>~0.2</td></tr><tr><td>239.07391</td><td>~0.5</td></tr><tr><td>267.10431</td><td>~0.2</td></tr><tr><td>297.15244</td><td>~2.5</td></tr></tbody></table>	m/z	Relative Intensity (10 ⁶)	57.45317	~0.5	79.95608	~0.2	89.44093	~0.5	119.04909	~0.2	170.00345	~0.2	183.01117	~2.2	197.02702	~0.5	198.03511	~0.5	230.02466	~0.2	239.07391	~0.5	267.10431	~0.2	297.15244	~2.5						
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239.07391	~0.5																																
267.10431	~0.2																																
297.15244	~2.5																																
Erucamide	FH (5) (F8) #5936, RT=21.453 min, MS2, FTMS (+), (HCD, DDA, 338.3407@(18;30;50), +1)  <table><caption>Peak Data for Erucamide</caption><thead><tr><th>m/z</th><th>Relative Intensity (10⁶)</th></tr></thead><tbody><tr><td>69.07029</td><td>~1.5</td></tr><tr><td>83.08581</td><td>~1.0</td></tr><tr><td>97.10133</td><td>~1.5</td></tr><tr><td>109.10123</td><td>~1.0</td></tr><tr><td>111.11681</td><td>~0.5</td></tr><tr><td>135.11665</td><td>~0.5</td></tr><tr><td>149.13225</td><td>~1.0</td></tr><tr><td>170.00345</td><td>~0.2</td></tr><tr><td>177.16336</td><td>~0.5</td></tr><tr><td>191.17886</td><td>~1.0</td></tr><tr><td>226.21590</td><td>~0.2</td></tr><tr><td>240.23128</td><td>~0.5</td></tr><tr><td>303.30365</td><td>~1.5</td></tr><tr><td>321.31442</td><td>~1.0</td></tr><tr><td>338.34058</td><td>~3.5</td></tr></tbody></table>	m/z	Relative Intensity (10 ⁶)	69.07029	~1.5	83.08581	~1.0	97.10133	~1.5	109.10123	~1.0	111.11681	~0.5	135.11665	~0.5	149.13225	~1.0	170.00345	~0.2	177.16336	~0.5	191.17886	~1.0	226.21590	~0.2	240.23128	~0.5	303.30365	~1.5	321.31442	~1.0	338.34058	~3.5
m/z	Relative Intensity (10 ⁶)																																
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Strearamide	FH (5) (F8) #6211, RT=22.586 min, MS2, FTMS (+), (HCD, DDA, 284.2944@(18;30;50), +1)  <table><caption>Peak Data for Strearamide</caption><thead><tr><th>m/z</th><th>Relative Intensity (10³)</th></tr></thead><tbody><tr><td>57.07042</td><td>~50</td></tr><tr><td>74.06034</td><td>~50</td></tr><tr><td>88.07593</td><td>~20</td></tr><tr><td>102.09148</td><td>~20</td></tr><tr><td>116.10705</td><td>~50</td></tr><tr><td>166.66577</td><td>~20</td></tr><tr><td>284.29446</td><td>~300</td></tr><tr><td>295.71899</td><td>~20</td></tr></tbody></table>	m/z	Relative Intensity (10 ³)	57.07042	~50	74.06034	~50	88.07593	~20	102.09148	~20	116.10705	~50	166.66577	~20	284.29446	~300	295.71899	~20														
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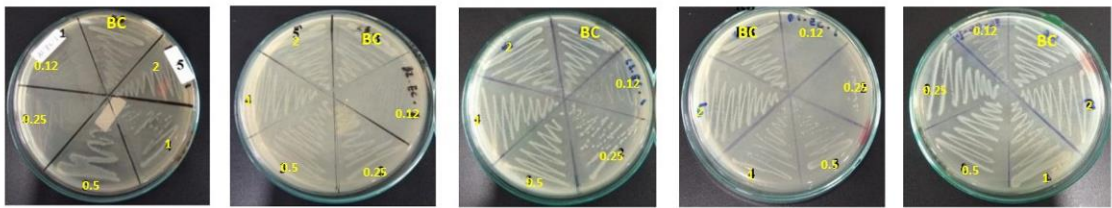


Figure S1-1. Streak Agar Plate of ethanol extract (a), n-hexane fraction (b), chloroform fraction (c), ethyl acetate fraction (d), water fraction (e) against *E. coli*

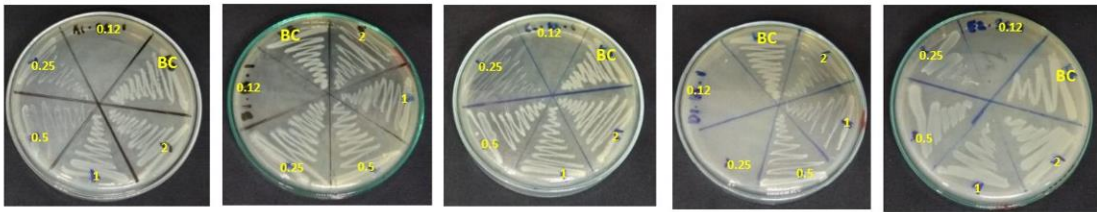


Figure S1-2. Streak Agar Plate of ethanol extract (a), n-hexane fraction (b), chloroform fraction (c), ethyl acetate fraction (d), water fraction (e) against *B. subtilis*

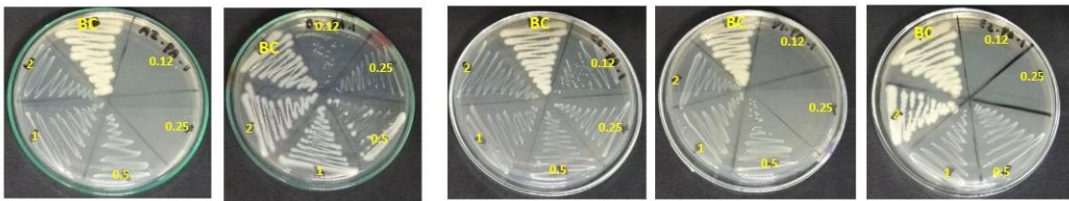


Figure S2-1. Streak Agar Plate of ethanol extract (a), n-hexane fraction (b), chloroform fraction (c), ethyl acetate fraction (d), water fraction (e) against *P. aeruginosa*

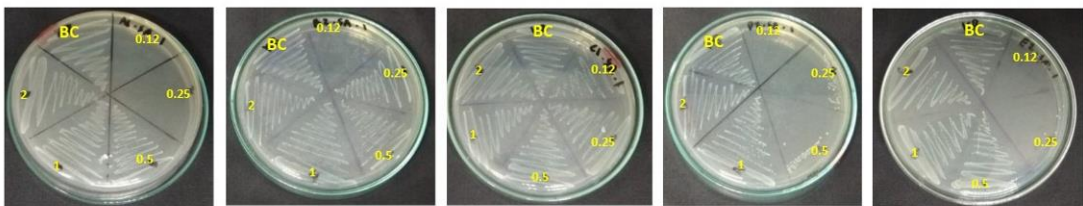


Figure S2-2. Streak Agar Plate of ethanol extract (a), n-hexane fraction (b), chloroform fraction (c), ethyl acetate fraction (d), water fraction (e) against *S. aureus*

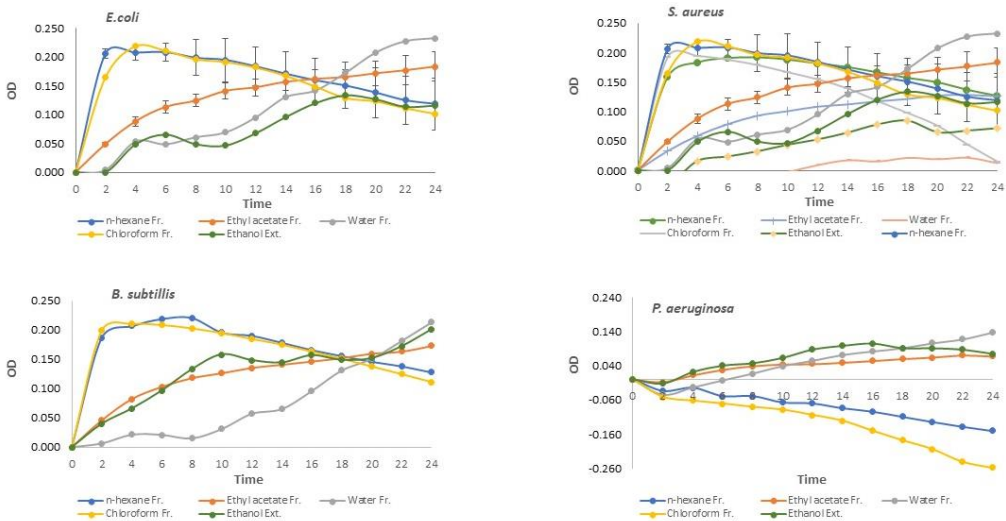


Figure S3. Bacterial growth observation for 24 h incubation