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ARTICLE

BIOASSAY-GUIDED IN VITRO ANTIBACTERIAL ACTIVITY OF FRACTION FROM *Abelmoschus esculentus* L. FRUIT

[*Aktivitas Antibakteri Fraksi yang Dipandu Bioassay in Vitro dari Buah Abelmoschus esculentus L*]

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ABSTRACT

Research focuses separating antibacterial compounds from *Abelmoschus esculentus* fruit against several pathogenic bacteria including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* using a bioassay-guided method. *A. esculentus* fruit was extracted and its active compounds were separated and tested against several variants of gram negative and gram positive bacteria. The testing began with extracts, followed by fractions, and subfractions. Extracts, fractions and subfractions of *A. esculentus* fruit were obtained all exhibiting antibacterial activity. The ethyl acetate fraction was the most active, yielding the largest inhibition zone diameter for both bacteria. The dominant compound in this fraction was quercetin accounting for 26% of the total. The ethyl acetate fraction was then separated into subfractions then tested for antibacterial and the composition of the compound was identified. One of the subfractions that gave the diameter of the inhibition zone after being separated identification using LC-MS revealed that the dominant compound in the subfraction was flavonoid identified as (3-hydroxy-2,3-dihydroimidazo [1,5-a] pyridin-8(5H)-one-5-β-glucopyranoside 42.46%, followed by quercetin 13.23%, fraxidin 6.34%, scopoletin 1.51% and phenolic compounds such as homovanillic acid 1.67% and 3-(4-hydroxy-3-methoxyphenyl)-n-[2-(4-hydroxyphenyl)ethyl]prop-2-enimidic acid 4.77%. *A. esculentus* fruit has demonstrated significant antibacterial potential, with bioactive compound including flavonoid and phenolic derivate producing a greater inhibitory zone compared to positive control, Ciprofloxacin.

Keywords: Bioassay, Antibacterial, Fraction, *Abelmoschus esculentus*, Fruit

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ABSTRAK

Penelitian berfokus pada pemisahan senyawa antibakteri dari buah Abelmoschus esculentus L. terhadap beberapa bakteri patogen termasuk Staphylococcus aureus, Bacillus subtilis, Eschericia coli dan Pseudomonas aeruginosa menggunakan metode yang dipandu bioassay. Buah A. esculentus diekstraksi dan senyawa aktifnya dipisahkan dan diuji terhadap beberapa varian bakteri gram negatif dan positif. Pengujian dimulai dengan ekstrak, diikuti oleh fraksi dan subfraksi. Ekstrak, fraksi dan subfraksi buah A.esculentus menunjukkan aktivitas antibakteri. Fraksi etil asetat adalah fraksi yang paling aktif, menghasilkan diameter zona hambat terbesar untuk kedua kelompok bakteri. Senyawa dominan dalam fraksi ini adalah quercetin yang mencapai 26% dari total senyawa. Fraksi etil asetat selanjutnya dipisahkan menjadi subfraksi kemudian diuji antibakterinya dan diidentifikasi komposisi senyawanya. Salah satu subfraksi yang memberikan diameter zona hambat setelah dipisahkan dan diidentifikasi menggunakan LC-MS mengungkapkan bahwa senyawa dominan dalam subfraksi adalah flavonoid yang diidentifikasi sebagai (3-hidroksi-2,3-dihidroimidazo[1,5-a] piridin-8-(5H)-satu-5-β-glukopiranosida 42,46%; diikuti oleh quercetin 13,23%; fraxidin 6,34%; skopoletin 1,51% dan senyawa fenolik seperti asam homovanilat 1,67% dan 3-(4-hidroksi-3-metoksifenil)-n-[2-(4-hidroksifenil)etil]prop-2-asam enamat 4,77%. Buah A.esculentus telah menunjukkan potensi antibakteri yang signifikan, dengan senyawa bioaktif termasuk flavonoid dan turunan fenolik menghasilkan zona hambat yang lebih besar dibanding konrol positif Ciprofloxacin.

Kata kunci: *Bioassay, antibakteri, Abelmoschus esculentus, buah*

INTRODUCTION

As one of the main causes of death globally, infectious illnesses remain a significant public health concern (Bloom and Cadarette, 2019). According to literature sources, 17 million fatalities globally are attributed to these illnesses each year (Asrani *et al.*, 2019). The rise of bacterial diseases resistant to all antimicrobial medications is making it more difficult to treat microbial infections (Abushaheen *et al.,* 2020). Finding novel, alternative antibacterial components with enhanced action is critical. In many impoverished nations, traditional herbal medicine served as a vital source of antibiotics (Seukep *et al*., 2023). Medicinal plants have long been considered a valuable natural source for innovative treatments (Jamshidi-Kia *et al*., 2017). Natural medicinal herbs have been utilized all over the world to treat a variety of illnesses and have shown positive therapeutic effects (Jamal, 2023).

Phytochemicals in medicinal plants are abundant and have been utilized for their antibacterial, antifungal, and antiviral properties (Behl *et al.,* 2021). New antimicrobial drugs were developed as a result of data from plants that showed the presence of antibacterial components (Álvarez-Martínez *et al.,* 2021). Nearly 80% of people worldwide depend on traditional plants or herbs for their primary medical care (Khan and Ahmad, 2019). Fortunately, a number of plant products, either as separated compounds or as raw materials, have been investigated for possible antimicrobial and antibacterial therapy in Indonesia. This is because there are very few plants and isolated bioactive chemicals with potential antimicrobial qualities. One plants that has been extensively research its antibacterial activity is *A. esculentus* L. has been found to yielded a variety of chemicals compounds, including flavonoids (Yang *et al.,* 2022), quinones phenolic (Winata *et al.,* 2024; Zhu *et al.,* 2020), anthocyanin pigments (Yora *et al.,* 2018) and saponin (Veshalini *et al.,* 2022). Numerous biological characteristics, including antidiabetic, antibacterial, antioxidant, and anti-inflammatory action, have been identified for it (Younis *et al.,* 2024). The carotene, chlorophyll, and phenolic chemicals present in *A. esculentus* are responsible for these characteristics. Conditions like depression, ulcers, sore throats, lung inflammations, and gastrointestinal irritations have all been treated with it (Hafeez *et al.,* 2020). The pods and seeds of *A. esculentus* are abundant in phenolic compounds, such as hydroxycinnamic derivatives, catechin oligomers, and derivatives of quercetin and flavonol. These substances provide *A. esculentus* with its antioxidant and antibacterial activity (Huerta *et al.,* 2024).

Based on many studies, it is know that the flavonoid of *A. esculentus* is quercetin (Islam, 2019). Recent findings also revealed that 80% methanol is most effective solvent, showing enhance many pharmacology activities potential in *A. esculentus* leaf (Rachida, 2019). Antibacterial activity was screened using disk diffusion method against *Staphylococcus aureus*, *Bacillus subtilus, Escherichia coli*, and *Pseudomonas aerigunosa*. *A.seculentus* can be applied to the pharmaceutical sector

and antimicrobial bioactive functional foods (Ong *et al.,* 2021). However, *E. coli* and *S. aureus* strains show the best antimicrobial activities with a MIC of 3.75 mg/mL for crude ethanol extract and ethanol fraction of leaves and seeds of *A.esculentus* (Petropoulos *et al.,* 2017). Testing of antibacterial activity with different solvents, namely petroleuem ether, acetone, ethanol and water, obtained acetone extract results gave the largest inhibitory zone diameter results against gram positive bacteria *S. aureus*, *B. subtilis*, and gram negative *E. coli* and *Klebsiella pneumoniae* (Diouf *et al.,* 2023). As a result, neither phytochemical research on this plant nor its application in traditional medicine have been previously reported. Therefore, using the *bioassay-guided* separation approach, we sought to separate antibacterial compounds from *A. esculentus* to target bacterial pathogens, *S. aureus*, *B. subtilis E. coli,* and *P. aeruginosa*.

MATERIALS AND METHODS

Fresh fruit of *A. esculentus* were collected from Toroh, Purwodadi, Jawa Tengah, in December 2023. The fruit was pulverized into powders using a grinder after being dried in a dryer cabinet at 50-60°C for 24 hours. A sieve No. 30/40 was subsequently used to filter the resultant particles.

Microbial strain the *Stapylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 39320), *Eeschericia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 9027) were obtained from the Diponegoro University, Semarang, Indonesia.

Chemical materials used methanol, n-hexane, chloroform, ethyl acetate, n-butanol, DMSO, NaCl, silica gel for column, silica gel for preparative, silica gel GF 254, gelatin, amyl alcohol, $FeCl_3$, Mayer, ammonia, Dragendroff reagent, ciprofloxacin, Eosin Methylene Blue Agar*,* Nutrient Agar*,* Nutrient Broth, DMSO (Dimethyl sulfoxidea), Mannitol Salt Agar, *Pseudomonas Cetrimide Agar,* Muller Hinton Agar.

Preparation of extracts

Dried fruit powder (200 g) was macerated with methanol (1 L) for 3 days or for 72 hours, with the solvent being replaced daily. The extracts were then separated, and the solvents were evaporated under reduced pressure at 50°C (Astutiningsih, 2021).

Fractination Extracts

The methanol extract from the fruit (25 g) was added to 100 mL of aquadest, then fractionated with 100 mL n-hexane separated by n-hexane fraction and residue again fractionation stopped when the obtained n-hexane fraction was clear. The same thing is also done with the next solvents, namely ethyl acetate and n-butanol. The insoluble part of the solvents n-hexane, ethyl acetate and n-butanol are reffered to as residual fractions or water fractions. Following the separation of the fractions, the solvents were evaporated at 50°C with reduced pressure.

Antimicrobial Test

An antibacetrial test agains was performed on these fractions, *Staphylococcus aureus* and *Bacillus subtilis*, *Eschericia coli* and *Pseudomonas aeruginosa.* The bacteria in media were incubated at 37°C for 24 hours. A few bacterial colonies were mixed with sterile Ringer's solution (0.85% NaCl) to create inocula, and the turbidity was measured and compared to the standard 0,5 McFarland solution solution, or 10^8 CFU/mL. 20 mL of media were transferred into sterilized petri dishes and allowed to solidity: Mannitol Salt Agar for *S. aureus*, Eosin Methylene Blue agar for *E. coli*, Muller Hinton Agar for *B. subtilis*, Pseudomonas Cetrimide Agar for *P. aeruginosa*. Using the agar well diffusion technique, six sterile cylinder cups were arranged within the Petri dishes, forming wells in each medium. Five microliters of each medium, initiallty in liquid form and heated to a temperature of 40-50°C, were mixed with cultures of *S. aureus*, *E. coli*, and *B. Subtilis, P. aeruginosa* at concentration of 1.0 x 108 CFU/mL. The insides of the cylinder cups were left empty as the microbial containing medium was poured into the Petri dish over the initial layer (Indriyanti *et al*., 2022; 2023). Utilizing sterile tweezers, gently take out the cylinder cup to form a well after the bacterial suspension

(second layer) media has set. Introduce the positive and negative control solutions into the wells along with 50 µL of each concentration from the test solution. Incubate at 37°C for a period of 24 hours. Following the incubation period, measure and assess the clear zone that appears at each concentration using a capillary (Wulandari *et al.,* 2022). The efficacy of fractions against microorganisms was evaluated at concentrations of 5%, 10% and 15%. For the antibacterial testing, regular Ciprofloxacin was utilized as the positive control and DMSO as the negative control (Panyo *et al.,* 2016). Ciprofloxacin 20 ppm is the reference standard for testing against all microbial.

Identification Antimicrobial Compounds

The fraction showing the greatest inhibitory activity among all bacteria was chemically identified using TLC and LC-MS. TLC was performed on all fractions, namely n-hexane fraction, chloroform fraction, ethyl acetate fraction, n-butanol fraction and water fraction with the following chromatography system (Table 1).

Table 1. Chromatographic Conditions and Spray Reagents for Compound Identification (*Kondisi kromatografi dan reagen semprot untuk identifikasi senyawa*).

Sample (*Sampel*)	Stationary Phase (*Fase Diam)*	Mobile Phase (*(Fase Gerak)*	Spotting Sighting (*Penampak bercak)*
Alkaloid	Silica Gel GF 254	Ethyl acetate:Methanol:water (100:13.5:10) (Harbone, 1987)	Dragendroff
Saponin	Silica Gel GF 254	Chloroform:Methanol:Water (64:50:10) (Hanani, 2015)	Anisaldehyde-H_2SO_4
Steroid	Silica Gel GF 254	n-Hexane:Ethyl acetate (5:5) (Hanani, 2015)	Lieberman Burchard
Flavonoid	Silica Gel GF 254	n-Butanol: Acetate Acid: Water (4:5:1) (Harbone, 1987)	Amoniac vapour
Phenolic	Silica Gel GF 254	Methanol: Water (6:4) (Hanani, 2015)	$FeCl_3$ 5%

TLC is also performed on subfractions to determine whether the active compounds are in accodance with the results of the identification of compounds group from the LC-MS in the most active fraction.

Furthermore, the most active fraction was separated again using the column chromatography method using a chromatography system for the dominant compounds that were successfully identified and separated based on the results of chemical reactions, TLC and LC-MS. Column chromatography was performed for 5 g fraction of 15-gram silica gel and eluted with step gradient elution. The gradient column chromatography used 100% n-hexane eluent, ethyl acetate and 100% methanol (Nalini *et al.,* 2017).

The subfractions were then subjected to an antimicrobial assay, using the same test method and bacteria type as in the antibacterial test conducted on the fraction. The subfraction that provides thew greatest inhibitory power among all bacteria was chemically identified using TLC. Furthermore, the most active subfractions were separated again using preparative thin-layer chromatography method using a chromatographic system for the dominant compounds that were successfully identified and separated based on the results of chemical reaction, TLC and LC-MS. The fractions or subfraction that were successfully separated were subsequently tested for antibacterial using the same system, method and bacteria type as the extract and identified by the TLC and LC-MS.

RESULTS

The study began with the extraction of 1 kg of dry *A. esculentus* fruit powder using methanol 1 liter per day for 3 x 24 hours and obtained an extract yield of 25.5%. A fairly high extract yield indicates the success of the extraction process and the bioactive compounds are optimally extracted (Jha and Sit, 2022). The extraction results indicated that an effective extraction process is characterized by high extract yields. The extract was then subjected to phytochemical screening tests to determine the secondary metabolites contained in the extract; the results were as Table 2.

Table 2. Phytochemical Profile Methanol Extract of *A. esculentus* Fruit (*Profil Fitokimia dari Ekstrak Metanol Buah A. esculentus*).

Chemical Content (*Kandungan Kimia*)	**Screening Result** (*Hasil Skrining*)	**TLC Result** (*Hasil KLT*)
Flavonoid	+ orange color on amyl alcohol	Rf 0.87 yellow stain
Tanin	+ blackish green + precipitate with gelatin	Rf 0.43 blackish green stain
Saponin	+ stable foam formed	Rf 0.86 white stain
Alkaloid	+precipitate with bourchadat reageb + precipate with mayer reagen	Rf 0.13 brownish-orange stains
Triterpenoid/Steroid	+ green color	Rf 0.42 purple stain

Next, antibacterial activity testing was carried out on the extract. The antibacterial activity of *A. esculentus* using methanol extract against bacterial pathogens *S. aureus*, *B. subtilis E. coli* and *P. aeruginosa* were studied. The result obtained from antibacterial activity methanol extract of *A. esculentus* was presented in Figure 1.

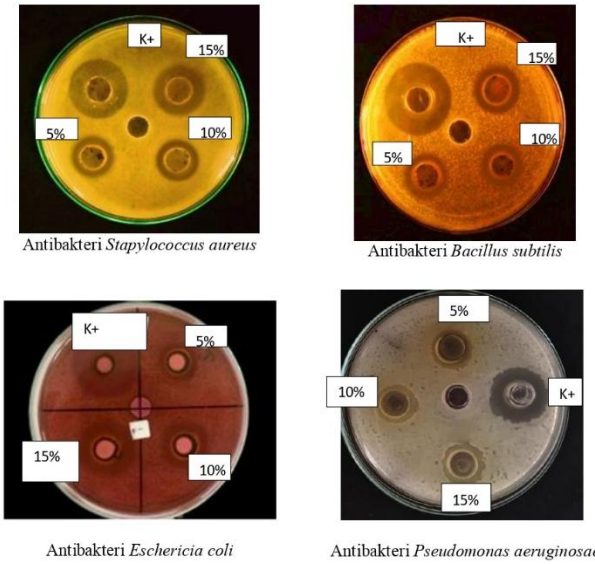

Figure 1. Antibacterial Test from Methanol Extract *A. esculentus* Fruit (*Uji Antibakteri dari Ekstrak Metanol Buah A. esculentus*).

Samples tested for antibacterial activity were conducted at concentration of 5%, 10% and 15%, and concentration selected based on the result of previous orientation. The results of the activity test are presented in the following Table 3 which is the results of measuring the diameter of the inhibition zone.

Table 3. Average Diameter of Inhibition Zone of *A. esculentus* Fruit Extract (*Rata-rata Diameter Zona Hambat Ekstrak Buah A. esculentus*).

Type Bakteri *(Jenis Bakteri)*	**Concentration** (*Konsentrasi)*	**Average Diameter of Inhibition Zone** *(Rata-rata Diameter Zona Hambat)* **(cm)**
S. aureus	5%	6.67±0.132
	10%	9.44±0.1432
	15%	10.17±0.198
	Ciproloxacin 20 ppm	11.32±0.129
	DMSO	0
B. subtilis	5%	5.04±0.176
	10%	7.21±0.143
	15%	8.94±0.121
	Ciproloxacin 20 ppm	10.32±0.193
	DMSO	0
E. coli	5%	6.674±0.1322
	10%	9.44±0.1432
	15%	10.174±0.1983
	Ciproloxacin 20 ppm	11.32±0.1288
	DMSO	0
P. aeruginosa	5%	6.674±0.1322
	10%	9.44±0.1432
	15%	10.174±0.1983
	Ciproloxacin 20 ppm	6.40 ± 0.5090
	DMSO	0

Furthermore, methanol extracts were fractionated using liquid-liquid method, obtained in Table 4.

Table 4. *A. esculentus* Fruit Fraction Yield Data (*Data Rendemen Fraksi Buah A. esculentus*).

No	**Sample** (*Sample*)	**Yield** (*Rendemen)* **(%)**
1	n-Hexane Fraction (FH)	26.73%
2	Chloroform Fraction (FC)	12.68%
3	Ethyl acetate Fraction (FE)	11.45%
4	n-Butanol Fraction (FN)	22.57%
5	Water Fraction (FA)	26.57%

The results of TLC identification of all fractions can be seen in the following Figure 2.

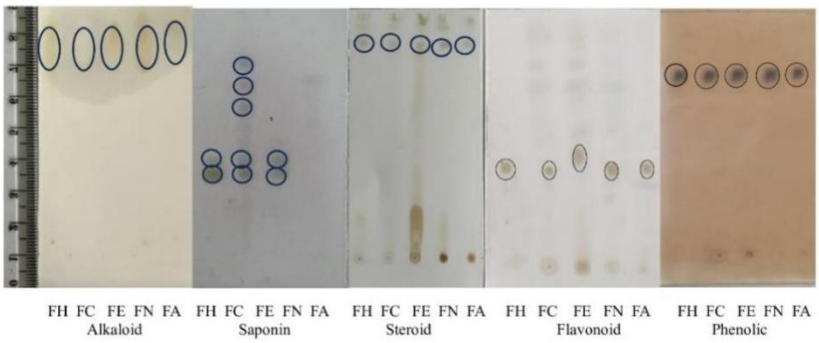

Figure 2. Identification TLC Results from Fraction of *A. esculentus* Fruit (*Hasil Identifikasi KLT dari Fraksi Buah A. esculentus*). Description: FH = n-Hexane Fraction, FC = Chloroform Fraction, FE = Ethyl acetate Fraction, FN = n-Butanol Fraction and FA = Water Fraction*(Keterangan: FH = Fraksi n-Hexana, FC = Fraksi Kloroform, FE = Fraksi Etil asetat, FN = Fraksi n-butanol, dan FA = Fraksi Air*).

The antibacterial activity results of the fraction against bacteria are shown in Table 5.

Table 5. Antibacterial Test from Fractions of *A. esculentus* Fruit (*Uji Antibakteri dari Fraksi Buah dari Fraksi Buah A.esculentus*).

Bacteria (*Bacteria*)	**Average of Diameter zone** (*Rata-rata Diameter Zona Hambat)* **(mm) ± SD**						
	DMSO	**Ciprofloxacin**	**F. n-hexane**	**F. chloroform**	**F. Ethyl acetate**	**F.n-butanol**	**F. water** (*F. air*)
S. aureus	0	11.32 ± 0.1288	2.85 ± 0.1304	6.34 ± 0.1625	10.03 ± 0.1077	1.41 ± 0.1562	3.29 ± 0.0735
B. subtilis	0	10.31 ± 0.2586	2.82 ± 0.1054	5.87 ± 0.0806	9.53 ± 0.2033	1.26 ± 0.1405	3.07 ± 0.0911
E.coli	0	7.54 ± 0.1765	1.24 ± 0.2145	4.66 ± 0.1399	8.072 ± 0.14005	0.85 ± 0.05636	2.67 ± 0.2096
P.aeruginosa	0	6.40 ± 0.5090	3.08 ± 0.6554	2.52 ± 0.6554	8.28 ± 0.6295	3.31 ± 0.2223	2.52 ± 0.3932

The ethyl acetate fractions as the most active fraction is then identified using the LC-MS instrument the results are obtained as shown in Table 6.

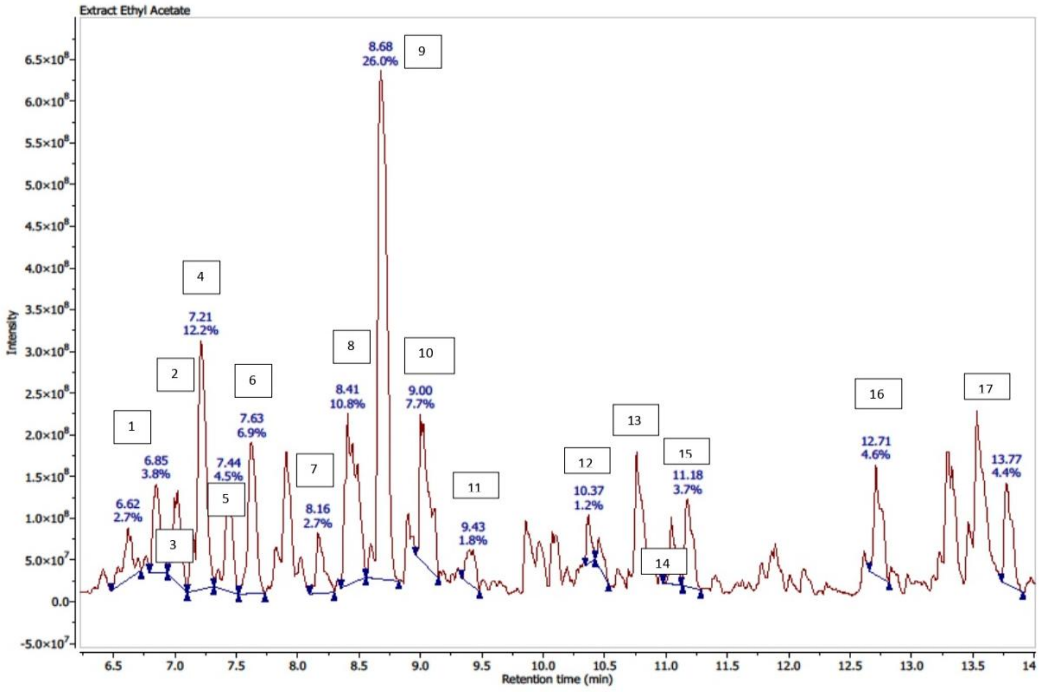

Figure 3. LC-MS Chromatogram ethyl acetate fraction of *A. esculentus* fruit *(Kromatogram LC-MS dari fraksi etil asetat*).

Table 6. Results of chromatogram LC-MS from ethyl acetate fraction of *A. esculentus* (*Hasil Kromatografi LC-MS dari fraksi etil asetat A. esculentus*)

No	RT (min)	Parent Ion (m/z)	Molecular Weight (*Berat Molekul*)	Molecular Formula (*Rumus Molekul*)	Compound Name (*Nama Senyawa*)	Compound Composition (*Komposisi Senyawa*) (%)	Classification (*Klasifikasi*)
1	6.62	280.0650	279.25	$C_{13}H_{13}NO_6$	2-[3-(4-Hydroxyphenyl)prop-2-enoylamino]butanedioic acid (Stark and Hofmann., 2005)	2.7	Fatty Acid
2	6.85	210.1159	209.24	$C_{11}H_{15}NO_3$	2-(dimethylamino)-3-(4-hydroxyphenyl)propanoic acid (Bernard *et al.,* 1981)	3.8	Fatty Acid
3	7.02	310.0854	309.27	$C_{14}H_{15}NO_7$	3-hydroxy-2,3-dihydroimidazo [1,5-a] pyridin-8(5H)-one-5-β-glucopyranosid (Kehinde *et al.,* 2022)	3.9	Flavonoid
4	7.21	209.1009	208.21	$C_{11}H_{12}O_4$	Sinapyl aldehide (Farah & Samuelsson, 1992)	12.2	Aldehid Derivate
5	7.44	300.1819	299.36	$C_{15}H_{25}NO_5$	Isolycopsamine (Ravi & Lakshmanan, 1990)	4.5	Alkaloid
6	7.63	227.1908	226.36	$C_{14}H_{26}O_2$	(1e)-dodec-1-en-1-yl acetate (Nee *et al.,* 1986)	6.9	Terpenoid
7	8.16	197.1157	196.29	$C_{12}H_{20}O_2$	Geranyl acetate (Venskutonis *et al.,* 1997)	2.7	Terpenoid
8	8.41	465.1160	464.38	$C_{21}H_{20}O_{12}$	Hyperoside (Bandyukova and Ligai, 1997)	10.8	Flavonoid
9	**8.68**	**303. 200**	**303.14**	**$C_{21}H_{20}O_{12}$**	**Quercetin-3-O-glucoside** (Felipe *et al.,* 2014)]	**26.0**	**Flavonoid**
10	9.00	336. 279	335.4817	$C_{20}H_{33}NO_3$	3,7-dimethoxy-2-methyl-5-octyl-5,6,7,8-tetrahydro-1h-quinolin-4-one (Nkunya *et al.,* 2004)	7.7	Alkaloid
11	9.43	625.166	624.5452	$C_{28}H_{32}O_{16}$	Sexangularetin 3-glucoside 7-rhamnoside (Elliger, 1984)	1.8	Flavonoid
12	10.37	245.1257	244.2863	$C_{15}H_{16}O_3$	Heritol (Miles *et al.,* 1987)	1.2	Terpenoid
13	10.46	246.1574	255.3968	$C_{15}H_{29}NO_2$	1-[6-(2-hydroxybutyl)-1-methyl-3,6-dihydro-2h-pyridin-2-yl]pentan-2-ol (Ahmad and Nasir, 1987)	0.9	Alkaloid
14	11.04	194.0892	193.2	$C_{15}H_{25}NO_3$	Cichorine (Komissaranko and Kovalev, 1992)	2.1	Phytotoxin
15	11.18	283.1943	282.2913	$C_{17}H_{14}O_4$	2-(4-Hydroxyphenethyl)-6-hydroxychromone (Konishi *et al.,* 2002)	3.7	Flavonoid
16	12.71	253.1385	252.2205	$C_{12}H_{12}O_6$	8-hydroxy-5,6,7-trimethoxychromen-2-one (Yun *et al.,* 2001)	4.6	Flavonoid
17	13.77	250.1466	249.3923	$C_{16}H_{27}NO$	Microcosamine A (Still *et al.,* 2013)	4.4	Phytotoxin

The subfraction of the chromatography results of the column were obtained 3 subfractions. . The results of the identification of the three subfractions (Subfractiona A, Subfraction B and subfraction C) can be seen in Figure 4.

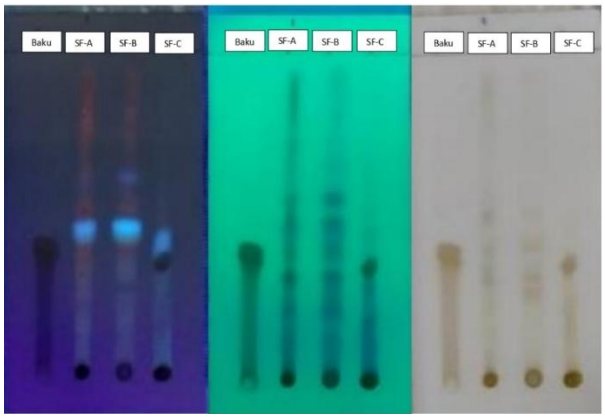

Figure 4. Results of Identification TLC from Subfractions of *A. esculentus* Fruit; Stationary Phase: silica GF 254 and Mobile Phase: Chloroform:Ethyl acetate:n-Butanol:Formic acid (5:2:2:1). (*Hasil Identifikasi KLT dari Subfraksi Buah A.esculentus; Fase Diam: Silica Gel 254; Fase Gerak; Kloroform:: Etil Asetat: n-butanol: asam format (5:2:2:1*).

The three subfraction obtained were tested for antibacterial using the same type of bacteria, the results were obtained as shown in Table 7.

Table 7. Antibacterial Test from Subfraction of *A. esculentus* Fruit (*Uji Antibakteri dari Subfraksi Buah A. esculentus*).

Bacteria (*Bakteri)*	**Average of Diameter zone** (*Rata-rata Diameter Zona Hambat)* **(mm) ± SD**				
	DMSO	**Ciprofloxacin**	**Subfraction A**	**Subfraction B**	**Subfraction C**
S. aureus	0	11.32 ± 0.1288	3.97 ± 0.1030	7.80 ± 0.3955	16.95 ± 0.0941
B. subtilis	0	10.31 ± 0.2586	3.88 ± 0.068	6.79 ± 0.1129	13.95 ± 0.2059
E. coli	0	7.54 ± 0.1765	2.20 ± 0.1690	5.13 ± 0.1806	9.51 ± 0.3591
P. aeruginosa	0	6.40 ± 0.5090	4.04 ± 0.1667	2.85 ± 0.0916	9.16 ± 0.1283

Subfraction C was then identified using LC-MS. The identification results were also carried out using LC-MS and the chromatogram results can be seen in Figure 5.

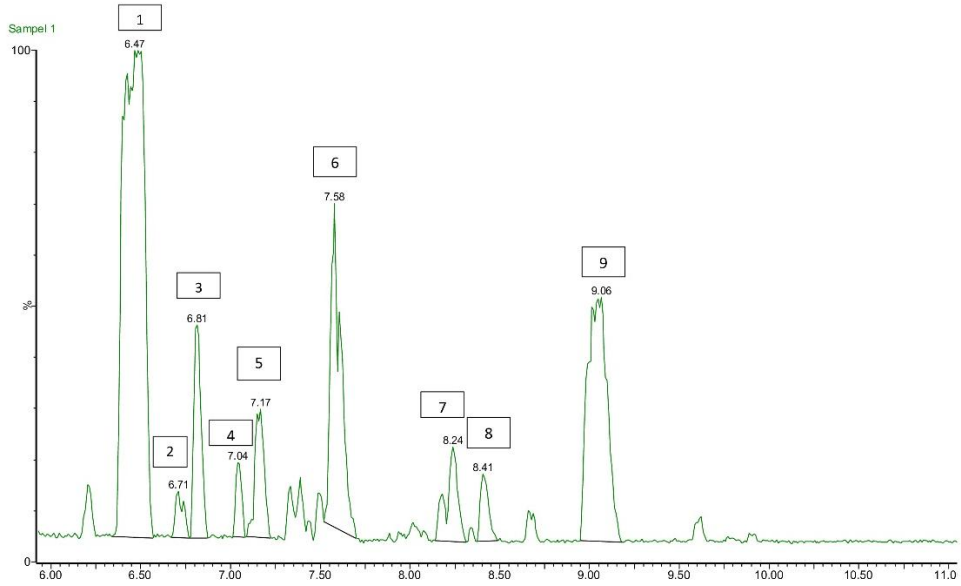

Figure 5. Chromatogram of Subfractions of *A. esculentus* Fruit with LC-MS (*Kromatogram dari Subfraksi Buah A. esculentus dengan LC-MS*).

Table 8. Composition of the compounds from Subfractions of *A. esculentus* Fruit with LC-MS (Komposisi kimia *dari Subfraksi Buah A.esculentus dengan LC-MS*).

No	RT (min)	Parent Ion (m/z)	Molecular Weight (*Berat Molekul*)	Molecular Formula (*Rumus Molekul*)	Compound Name (*Nama Senyawa*)	Compound Composition (*Komposisi Senyawa*) (%)	Classification (*Klasifikasi*)
1	6.47	246.0879	245.23	$C_{12}H_{11}N_3O_3$	3-hydroxy-2,3-dihydroimidazo [1,5-a] pyridin-8(5H)-one-5-β-glucopyranosid (Kehinde *et al.,* 2022)	42.46	Flavonoid
2	6.71	193.0501	192.17	$C_{10}H_8O_4$	Scopoletin (Ahmed *et al.,* 2017)	1.51	Flavonoid
3	6.81	223.0606	222.37	$C_{11}H_{10}O_5$	Fraxidin (Kasmi *et al.,* 2021)	6.34	Phenolic
4	7.04	197.0814	196.20	$C_{10}H_{12}O_4$	Homovanillinic acid (Aguilar-Camacho *et al.,* 2024)	1.67	Phenolic acid
5	7.17	314.1392	313.35	$C_{18}H_{19}NO_4$	3-(4-hydroxy-3-methoxyphenyl)-n-[2-(4-hydroxyphenyl) ethyl]prop-2-enimidic acid (Højmark , 2020)	4.77	Phenolic acid
6	7.58	303.0505	302.24	$C_{15}H_{10}O_7$	Quercetin (Felipe *et al.*, 2014)	13.23	Flavonoid
7	8.24	245.1542	244.33	$C_{16}H_{20}O_2$	2-hydroxy-7-methoxycadalene (Stipanovic *et al.,* 1981)	4.33	Terpenoid
8	8.41	227.0188	228.00	$C_{15}H_{16}O_2$	2-methoxy-4-(2-phenylethyl)phenol (Schwab *et al.,* 1990)	2.14	Phenol
9	9.06	286.1443	283.34	$C_{17}H_{19}NO_3$	*N-E*-Coumaroyl tyramine (Hu, 2024)	19.18	Phenol Amida

DISCUSSION

Antibacterial activity is closely linked to presence of compounds that either kill bacteria or inhibit their growth. The antibacterial activity of the extracts against various bacterial strains is presented in Figure 1. The results showed that *A. esculentus* fruit extract has significant antibacterial activity, particularly at higher concentrations. Methanol extract of *A. esculentus* showed better inhibition of Gram-positive bacteria such as *Staphylococcus aures* and *Bacillus subtillis* compared to gram negative bacteria like *Pseudomonas aeruginosa* and *Eschericia coli*. Gram positive bacteria tend to be more sensitive to antibacterial agents than Gram-negative bacteria, which explains the larger inhibition zone observed with methanol extract *A. esculentus* against gram positive bacteria. The difference is attributed to the structural variation in cell wall: Gram-positive bacteria have thick peptidoglycan layer, teikoic acid, and a smaller amount of lipids (Ganiswarna, 1995), which are more easily inhibited by methanol extract of *A. esculentus*. In contrast, Gram negative bacteria have a more complex outer membrane composed of protiens, phospholipids, lipopolysaccahrides and periplasmic space (Giordano *et al.,* 2020), which contributes to a smaller inhibition zone. The results of the antibacterial activity test showed that the inhibitory effect increased with higher concentration, similar to the positive control, ciprofloxacin. The *A. esculentus* fruit extract demonstrated antibacterial activity, which led to further bioassay-guided fractionation of the extract based using 5 solvents with varying polarities. Each fraction was then tested for antibacterial activity with the same type of bacteria in the extract test by using only one concentration that provided the greatest inhibitor power, namely a concentration of 15%.

During the fractionation stage, the n-hexane fraction yielded a relatively high percentage of 26.73% suggesting the presence of a significant number of nonpolar compounds, such as lipids, sterol and hydrocarbons. The chloroform and the ethyl acetate fractions were able to dissolve more semipolar. Two types of semipolar solvents with different polarity indices were employed, with the

expectation that they would dissolve more semipolar compounds than a single solvent. Meanwhile, two solvents, n-butanol and water were used to dissolve more polar compounds.

The results of phytochemical and TLC screening tests showed that the *A. esculentus* fraction contained flavonoid, phenolic, saponins, alkaloid and triterpenoids (Astutiningsih & Anggraeny, 2023; Febrianti *et al.,* 2021). TLC analysis was conducted on all fractions that had been separated from the extract (Figure 2). The results showed that the compound in the n-hexane, choloroform and ethyl acetate fractions were similar to those in the original, while the n-butanol and water fractions did not contain saponins. This is likely due to the nonpolar nature of hydrocarbon, which cannot be dissolved in polar solvents. Although fractionation with solvent of different polarities aims to separate compounds based on their polarity, some compounds with similar chemical properties remain difficult to separate perfectly.

The antibacterial activity results of the fraction against bacteria are shown in Table 5. The results indicated moderate antibacterial activity (Pan *et al*., 2009) with the ethyl acetate fraction showing highest activity (larger zone of inhibition), followed by the chloroform, n-hexane, n-butanol and water fractions. However, for *P. aeruginosa* bacteria, the n-butanol fraction exhibited better activity than the n-hexane and the water fraction. The ethyl acetate fraction provided the greatest antibacterial resistance to all types of bacteria it was separated and identified for the type of active compound content. To determine the type of dominant active compound contained in the ethyl acetate fraction, identification was carried out using LC-MS and the result showed that the dominant compound in the ethyl acetate fraction was quercetin compound with a percentage of 26% and it could be separated from 17 other compounds. The ethyl acetate fractions as the most active fraction is then identified using the LC-MS instrument, the results are obtained as shown in Figure 3 and Table 6.

The ethyl acetate fraction was further fractionated using gravity column chromatography with a stationary phase of 60 GF 254 silica gel and phase mobile from n-hexane to ethyl acetate and methanol. Separation was performed to obtain subfractions based on similar stain or spot patterns identified by TLC. The separation results were obtained 52 fractions combined into 3 subfractions identified compounds with a Rf 0.33; 0.42 and 0.56. The three subfractions obtained were then tested for antibacterial activity. The results were obtained that the C subfraction provided the largest inhibitory zone diameter for the same type of bacteria used in the antibacterial testing of extracts and fractions. The results of the subfraction antibacterial test showed a much larger diameter than the extract or fraction. They exceeded the diameter of the inhibition zone given the positive control of Ciprofloxacin. The results of the identification of the three subfractions can be seen in Figure 5.

The results of subfraction C using LC-MS revealed that the dominant compound in the subfraction was flavonoid consisting of 3-hydroxy-2,3-dihydroimidazo[1,5-a] pyridin-8(5H)-one-5-β-glucopyranoside at 42.46%, quercetin at 13.23%, fraxidin at 6.34%, scopoletin at 1.51%. The phenolic compounds in the subfraction included homovanillic acid at 1.67% and 3-(4-hydroxy-3-methoxyphenyl)-n-[2-(4-hydroxyphenyl) ethyl] prop-2-enimidic acid at 4.77%. Other compounds found in the subfraction included phenol amide at 19.18% and terpenoids at 4.33%. The single dominant compound in the subfraction can be separated by chromatography. The okra fruit subfraction has considerable antibacterial activity, most likely because of the several active chemicals in the subfraction working in concert.

CONCLUSION

A. esculentus fruit has antibacterial potential with bioactive compound are flavonoid and phenolic derivate compounds that can provide a greater inhibitory zone than positive control of Ciprofloxacin.

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AUTHOR CONTRIBUTIONS

CA: conceptualization, software, formal analysis, investigation, resources, data curation, writing – original draft, writing – review & editing, visualization, supervision, project administration, funding acquisition. MM: methodology, validation, formal analysis, investigation, resources, data curation, writing – review & editing, visualization. NAR: methodology, validation, writing – original draft, writing – review & editing, visualization, supervision. All authors have read and agreed to the published version of the manuscript.

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