Bioactive Compounds and Antibacterial Activity of Red Beetroot (*Beta vulgaris* L.) Ethyl Acetate Extraction

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Submitted: March 17, 2025; Revised: June 24, 2025, November 7, 2025; Accepted: January 30, 2026;
Published: February 27, 2026

ABSTRACT

Red beetroot has been reported to possess various medicinal properties and antimicrobial activity. Therefore, this study aims to determine the compounds contained in the ethyl acetate extract of red beetroot pulp and evaluate its antibacterial activity against *Escherichia coli*. The extract was obtained by soaking the fruit in ethyl acetate, and then assessed for its components using GC-MS analysis. Antibacterial activity was tested using a disc diffusion assay. The results showed that the ethyl acetate extract of red beetroot pulp contained a total of 20 major compounds. These included ester of 1,3-benzenedicarboxylic acid and bis(2-ethylhexyl), 1,4-benzenedicarboxylic acid, and n-hexadecanoic acid. In the antibacterial test, the sample was highly effective against *E. coli* (IC 50 was 0,783 mg/mL). In addition, the diameter of the growth inhibition zone increased along with the extract concentration. These results suggest that the ethyl acetate extract of red beetroot pulp offers a natural antibacterial substitute to its synthetic equivalents.

Keywords: Antibacterial; *Escherichia coli*; GC-MS; phytochemical; red beetroot

INTRODUCTION

Indonesia is a country with high biodiversity and great potential for the development of herbal medicines. According to Retnowati et al. (2019), Indonesia has approximately 15,000 medicinal plant species, but only 7,000 have been explored as raw materials. There has also been a significant decrease in the use of medicinal plants despite their lower adverse side effects compared to synthetic chemical drugs. This development has led to the exploration of an alternative source of pharmaceutical agents and remedies for diseases without sufficient cures (Dewoto, 2007).

Red beetroot (*Beta vulgaris* L.) is a common vegetable that has also been traditionally used as a medicinal plant due to its bioactive compounds. This plant has been used in folk medicine for diseases, such as diabetes, cancer, cardiovascular disease, and other chronic pathologies (Ceclu & Nistor, 2020). Bioactivities can be attributed to the presence of various compounds, including flavonoids, alkaloids, sterols, triterpenes, saponins, and tannins (Widawati & Prasetyowati 2013). Several studies have also confirmed the presence of flavonoids, saponins, tannins, and alkaloids (Sargunam & Thilakavathy, 2021). In addition, red beetroot is considered a reservoir of antioxidant compounds,

DOI: http://doi.org/10.22146/agritech.105476
ISSN 0216-0455 (Print), ISSN 2527-3825 (Online)

natural colors, anticancer, anti-inflammatory, and antimicrobial substances.

Escherichia coli is a bacterium that has been reported to cause various health challenges in humans. The bacterium largely inhabits the digestive system of mammals and is mutually helpful to digestion. However, when the population is above the threshold of faecal-oral transmission, it becomes pathogenic and circulate outside the gut, leading to diarrhoea, urinary tract infection, meningitis, and HUS (Hemolytic Uremic Syndrome)132. To address these effects, a previous study revealed that ethanol extracts of red beetroot tubers could prevent the growth of *E. coli* (Hidayatullah et al., 2024). Therefore, this study aims to determine the compounds contained in the ethyl acetate extract of red beetroot pulp and evaluate its anti-*E. coli* effect. The results are expected to contribute to the development of herbal ingredients as an alternative treatment for bacterial infections. Compared to previous studies that only examined red beetroot extracts in general, the current study distinguishes between the liquid and pulp extracts, analyzing their distinct phytochemical profiles and antibacterial activity.

METHODS

Materials

Red beetroot (1.5 kg) was obtained from Baturiti, Tabanan, Bali, Indonesia (8°17′05.8″S, 115°09′25.9″E). The extraction process was carried out at the Laboratory of Genetic Resources and Molecular Biology, Udayana University. Phytochemical analysis was conducted at the Forensic Laboratory of the National Police Criminal Investigation Unit in Denpasar, and antibacterial activity testing was performed at the Biotechnology Laboratory, Faculty of Agriculture, Udayana University.

The materials used were agar, distilled water, dimethyl sulfoxide (DMSO), *E. coli*, 70% ethanol, ethyl acetate, methanol, sodium chloride (NaCl), nutrient broth, and fresh red beetroot tubers. In this study, equipment used were an an autoclave, a blender, glass bottles, Petri dishes, separatory funnels, beakers, an incubator, cotton swabs, aluminium foil, paper discs, filter paper, a laminar airflow cabinet, a magnetic stirrer, and a GC-MS machine (Agilent 7890B MSD 5977B), oven, measuring pipettes, plastic wrap, rotary vacuum evaporator, test tubes, and vortex mixer.

Liquid Fraction and Pulp Extract Preparation

Extraction was performed through fractionation and maceration. The freshly collected beetroot tubers were washed, peeled, and cut into small pieces. The fragments were homogenized by a glass homogenizer, and then squeezed to separate the juice from the dregs. In addition, the liquid extract was evaporated under reduced pressure (rotary vacuum evaporator) at 40 °C and 99 rpm, until reaching a condensed extract volume of 16 mL. Condensed extract was mixed with 70 mL of ethanol and soaked for 24 hours, followed by dilution with distilled water to a final volume of 550 mL. Ethyl acetate (300 mL) was then added, and the mixture was stirred on a magnetic stir plate for 10 minutes. The mixture of isobutanol was then placed in a Kenny flask, toluene was added andkept for 15 minutes, then transferred to a separatory funnel to obtain the ethyl acetate fraction from hydroethanol. In addition, the ethyl acetate fraction was evaporated under reduced pressure to eliminate any remaining solvent.

Pulp was dried in an oven at 45 °C for 3 days, and then pulverized with a blender to get 25 g of simplicia, which were extracted by the maceration method using 100 ml of ethyl acetate solvent (1:4) during 3x24 hours with stirring. The filtrate was collected into a glass bottle, and the remaining pulp was extracted again under the same conditions twice. In addition, the evaporated filtrate was rotary vacuum evaporated.

The extraction was carried out using fractionation and maceration. Fresh red beetroot was washed and peeled into small pieces. The pieces were comminuted with a blender and squeezed to remove liquid from the pulp. This liquid phase was evaporated to dryness under a rotary vacuum evaporation at 40 °C and 99 RPM until reaching 16 mL of the condensed extract, which was used as an endpoint. The extract was added to 70 mL of ethanol, soaked for 24 hours, and diluted with water to a final volume of 550 mL. Ethyl acetate (300 mL) was then added, and the mixture was homogenized with a magnetic stirrer for 10 minutes. The ethyl acetate fraction was separated from the hydroethanol one and transferred to a separating funnel. Ethyl acetate fraction was obtained, and the solvent was evaporated to dryness.

The pulp was dried in an oven at 45 °C for 3 days and then ground using a blender to obtain 25 g of simplicia, which was extracted using maceration with ethyl acetate solvent (1:4) in a volume of 100 mL for 72 hours, with stirring every 24 hours. In addition, the filtrate was collected, and the remaining pulp underwent 2 additional maceration cycles under the same conditions. The final filtrate was evaporated using a rotary vacuum evaporator until a concentrated extract was obtained.

Phytochemicals Analysis

The concentrated extract of each liquid fraction and pulp extract was taken for phytochemical screening

using GC-MS. Samples were filtered through a 0,22 µm membrane filter to remove particulate matter and prevent column blockage. Subsequently, 20 µL of each sample was injected using C18 silica as the stationary phase and a methanol-water mixture (9:1) as the mobile phase on a 4,6 x 200 mm Wakosil ODS/5C18-200 silica column. The C18 silica column was used as per the system configuration to assist phase separation in GC-MS. Analysis was performed using an Agilent 7890B MSD 5977B with N2 gas as the mobile phase carrier. The column temperature was initially set at 70 °C (hold time: 3 minutes) and increased at 10 °C/minute to a final temperature of 290 °C. The total run time was 27 minutes (injection rate and column flow 1 mL/min. The compounds were detected by the area of each peak on chromatograms and confirmed by the NIST library.

Phytochemical composition of the liquid fraction and pulp extract was determined by GC-MS in the condensed extracts. Silica C18 was the stationary phase, and methanol-water (9:1) was the mobile phase. This was carried out with an Agilent 7890B MSD 5977B using N_2 as a carrier gas. The initial column temperature of 70 °C was held for 3 minutes, followed by an increase in temperature at a rate of 10 °C per minute until the final temperature of 290 °C, and the total analysis time was set to be 27 minutes, with the injection rate and column flow set to be 1 mL/min. Compounds were detected by analyzing the chromatogram peaks, and identification was performed using the NIST library.

Antibacterial Assay

Antibacterial activity against *E. coli* was evaluated using the disc diffusion method on Nutrient Agar (NA). The media was made by dissolving 5,1 grams of Nutrient Broth (NB) powder into 300 ml of distilled water and then sterilized using an autoclave at 121 °C for 15 minutes. These media were used in bacterial rejuvenation. NA media were made by adding 4,2 grams of agar to 280 NB media and then sterilizing it again.

Bacterial rejuvenation was performed by inoculating 1 ose of *E. coli* onto an agar slant and incubating at 35 °C for 24 hours. After the bacteria grew, 2 ose of bacteria were obtained and transferred to 10 ml NB media, then homogenized and incubated again for 24 hours. Bacteria that had grown were diluted using 0,9% NaCl solution. The stage began with taking 1 ml of bacterial suspension from a test tube, then transferring it to another test tube containing 9 mL of 0,9% NaCl solution. Subsequently, this stage was repeated until the seventh tube, resulting in a dilution of 10^{-7}.

Liquid fractions and pulp extracts were diluted with DMSO to concentrations of 25%, 50%, 75%, and 100%. DMSO was selected as the dilution solvent due to its ability to dissolve both polar and non-polar compounds. The concentration of DIMEDONE used in this study (≤1%v/v in final assay volume) was selected based on published reports that these low concentrations had either negligible or no antibacterial effect on *E. coli* and were therefore reasonable candidates when serving as the negative control. All the treatments (20 µL) were loaded on 6 mm diameter paper discs, and diffusion was promoted.

Data Analysis

Quantitative and qualitative data were also processed on the NIST, PubChem, and Microsoft Excel. The properties of the compounds were also explained according to Google Scholar and PubChem references. In addition, antimicrobial activity was rationalized in terms of the inhibition zone (mm). Antimicrobial against inhibition zone for each test strains were subdivided into 4 grades according to the zone diameter (mm) >20 mm (very strong), 11–20 mm (strong), 5–10 mm (medium), and <5 mm (weak) (Jamilatun et al., 2020).

RESULTS AND DISCUSSION

Phytochemical Compounds of Ethyl Acetate Extract of Red Beetroot Pulp

Phytochemical compounds contained in the ethyl acetate extract of red beetroot pulp had differences with the liquid. In addition, the results of the identification of compounds contained in the ethyl acetate extract of red beetroot pulp showed 20 compounds (Table 1). Each chromatogram peak showed the presence of compounds contained (Figure 2). The compounds detected with quality ≥90 were 2-Ethylhexanoic acid; 1-Dodecene; Z-5- Nonadecene; Cyclodecane; Z-8-Hexadecene; Benzaldehyde, 2,4,5-trimethoxy-; Tetradecanoic acid; Cyclotetradecane; Palmitoleic acid; n-Hexadecanoic acid; trans-2,4,5-Trimethoxy-.beta.-.methyl-. beta.- nitrostyrene; Hexadecanoic acid; trans-2,4,5-Trimethoxy-.beta.-. nitrostyrene; Hexadecanoic acid, ethyl ester; Dibenzo[b,f][1,4]oxazepin-11(10H)-one, 8-methyl-; 1-Octadecene; Methyl stearate; cis-Vaccenic acid; Ethyl Oleate; 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester; and 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester. Among these compounds, 19 had already been known for their potential benefits.

The 20 compounds listed in Table 1 were selected based on their identification quality from GC-MS analysis. Only compounds with a match quality of ≥90% according to the NIST library were included. Compounds with lower match quality were excluded to ensure

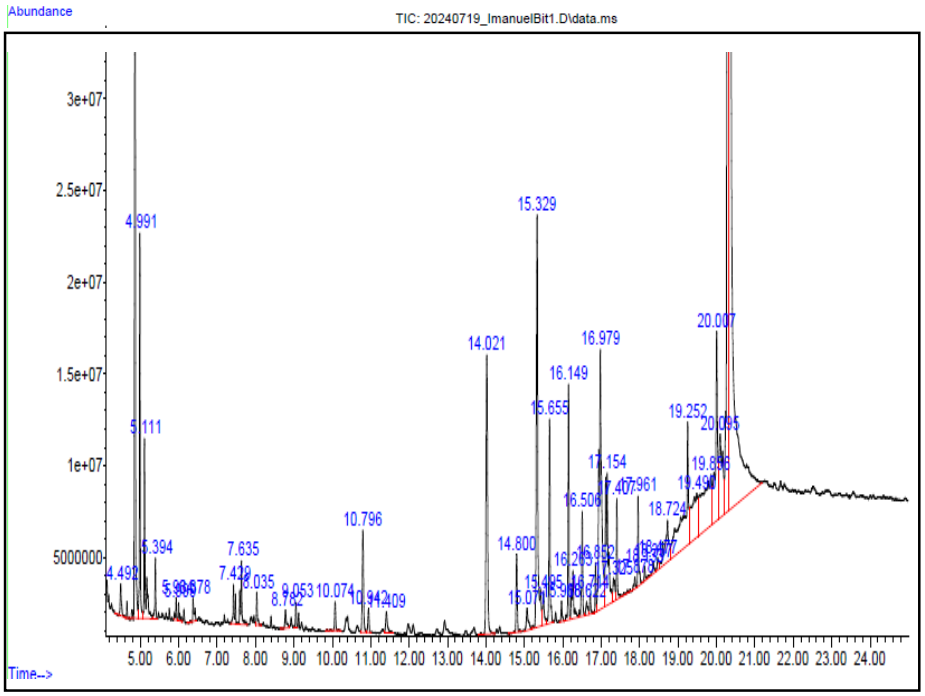

Figure 2. GC-MS chromatogram of ethyl acetate extract of red beetroot pulp

accurate identification. In addition to match quality, the area under the curve (AUC) and peak shape were also considered to represent major compounds. Therefore, some compounds with high peak height but lower match quality, such as the peak at 4.863 minutes, were not included in the final list. Complete GC-MS data, including retention times, peak areas (AUC), and identification match percentages for all detected compounds, were provided in the Supplementary Table (Table S1).

The compounds in ethyl acetate extract of red beetroot pulp that had been detected were dominated by 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (AUC of 21. 79%); 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (AUC of 11.67%); n-Hexadecanoic acid (AUC of 5.85%); cis-Vaccenic acid (AUC of 5.77%); and Cyclotetradecane (AUC of 3.94%). Based on these major compounds, the highest phytochemical content belonged to the ester, fatty acid, and cyclic hydrocarbon groups. The ester group was a compound that could be formed from several reactions, including the reaction of alkanoic acid and its derivatives with alcohol-forming esters (Feng, 2020). The fatty acids were a group of organic acids that comprised long carbon chains, ranging from 4 to 24 (Halid et al., 2021). Meanwhile, the cyclic hydrocarbon group was made up of compounds with carbon and hydrogen atoms that formed a ring structure and mostly exhibited aromaticity (Wilkes & Schwarzbauer, 2010).

In this study, 2-ethylhexyl ester and 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) were the main compounds found in red beetroot pulp ethyl acetate extract. Both compounds acted as plasticizers (Rowdhwal & Chen, 2018; Kdimy et al., 2023). In addition,, other functionality of 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester was its antimicrobial activity (Valarmathi et al., 2023). Therefore, these 2 compounds had potential as supporting materials for making bioplastics and antimicrobial agents.

The third most abundant compound identified was n-Hexadecanoic acid, which belonged to the saturated fatty acid group. This compound exhibited a wide range of pharmacological properties, including anti-inflammatory, antioxidant, hypocholesterolemic, nematicidal, pesticidal, larvicidal, antiandrogenic, hemolytic, and 5-alpha reductase inhibitory activities (Kumar et al., 2019). In another study, Ganesan et al. (2024) confirmed the good potential of n-Hexadecanoic acid as an antioxidant and antimicrobial in pharmaceutical industry materials.

The compound with the fourth highest amount of components was cis-Vaccenic acid. This compound was categorized as a monounsaturated fatty acid. Based on previous studies, cis-Vaccenic acid was known to have anticarcinogenic (Chirumamilla et al., 2022). Cis-Vaccenic acid had a cis-group, which had high antioxidant potential (Liu & Osawa, 2007).

Table 1. Compounds detected in the ethyl acetate extract of red beetroot pulp

Compound	Formula	RT (Min)	AUC (%)
2-Ethylhexanoic acid	$C_8H_{16}O_2$	5.394	0.48
1-Dodecene	$C_{12}H_{24}$	5.934	0.19
Z-5-Nonadecene	$C_{19}H_{38}$	7.429	0.50
Cyclodecane	$C_{10}H_{20}$	8.035	0.29
Z-8-Hexadecene	$C_{16}H_{32}$	9.053	0.21
Benzaldehyde, 2,4,5-trimethoxy-	$C_{10}H_{12}O_4$	10.796	1.20
Tetradecanoic acid	$C_{14}H_{28}O_2$	11.409	0.36
Cyclotetradecane	$C_{14}H_{28}$	14.021	3.94
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	14.800	0.84
Palmitoleic acid	$C_{16}H_{30}O_2$	15.071	0.41
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	15.329	5.85
trans-2,4,5-Trimethoxy-beta-methyl-beta-nitrostyrene	$C_{12}H_{15}NO_5$	15.495	0.49
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	15.655	2.07
Dibenzo[b,f][1,4]oxazepin-11(10H)-one, 8-methyl-	$C_{14}H_{11}NO_2$	15.968	0.19
1-Octadecene	$C_{18}H_{36}$	16.506	1.04
Methyl stearate	$C_{19}H_{38}O_2$	16.852	0.36
cis-Vaccenic acid	$C_{18}H_{34}O_2$	16.979	5.77
Ethyl Oleate	$C_{20}H_{38}O_2$	17.154	3.02
1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	20.287	11.67
1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	20.365	21.79

Table 2. Average Diameter of Inhibition Zone of Various Concentrations

Concentration	Inhibition Zone	
	Liquid Fraction (mm ± SD)	Pulp Extract (mm ± SD)
Negative control	0,00 ± 0,0^{a}	0,00 ± 0,0^{a}
Positive control	43,93 ± 2,2^{d}	43,93 ± 2,2^{d}
25%	17,37 ± 5,1^{b}	23,80 ± 1,5^{b}
50%	23,70 ± 6,0^{b}	33,30 ± 4,3^{c}
75%	39,70 ± 5,7^{cd}	41,77 ± 5,2^{d}
100%	40,77 ± 5,0^{cd}	46,67 ± 4,0^{d}

Notes: Different letters in the same row are significantly different (p>0.05)

The compound with the fifth-highest amount of components was Cyclotetradecane. This molecule was a cycloalkane and was produced in many plants and by people. Its pharmacological effects were not yet fully understood, but protein targeting and slight cardiovascular effects in humans were reported (Barenboim & Kozlova, 2018). Moreover, recent studies have emphasized its cancer and antimicrobial applications (Soliman et al., 2020; Kumari et al., 2019).

Antibacterial Activity against *Escherichia coli*

The extracted liquid fraction and pulp extract had antibacterial potential due to the presence of phytochemicals. Antibacterial activity assay was used to determine the growth inhibition of *E. coli* bacteria by the liquid fraction and pulp extract of red beetroot. The size of the inhibition zone, developed from 10 treatments and 3 replicates, was determined for antibacterial activity. In addition, the larger the size of the inhibition zones, the higher the antibacterial activity. The inhibition zone measurement was also statistically analyzed by Analysis of Variance (ANOVA) and Duncan's test to find the significance of variance, as shown in Table 2.

Insertion of DMSO as a negative control in antibacterial activity test was due to its use as a solvent that dissolved non-polar and polar molecules. Based on the absence of inhibition zone formation by DMSO, it was known that the formation of other treatment inhibition zones was only produced by the extract and was not affected by the solvent used (Yevani et al., 2023). Meanwhile, the positive control of chloramphenicol used in this study was due to its bacteriostatic properties against gram-positive and negative bacteria. The inhibition zone formed indicated that chloramphenicol was sensitive to *E. coli* bacteria, and the use of DMSO and chloramphenicol was aimed at ensuring the validity of the method tested.

Based on the categorization according to Jamilatun et al. (2020), the inhibition zone produced was categorized as strong to very strong (Figure 3). Treatments that formed inhibition zones in the very strong category were liquid fractions with a concentration of 50-100%, pulp extract of 25-100%, and the positive control. Meanwhile, the liquid fraction with a concentration of 25% was included in the strong category, which was 17.37 mm. The extract of pulp with a concentration of 25% formed an average inhibition zone of 23.80mm. Based on the result, the size of antibacterial activity produced could be influenced by several things, such as mechanisms of compounds, synergy between compound mechanisms, or imperfect separation during the extraction and fractionation process (Njateng et al., 2017).

Significant differences in the ability to inhibit the growth of *E. coli* were explained by ANOVA and Duncan

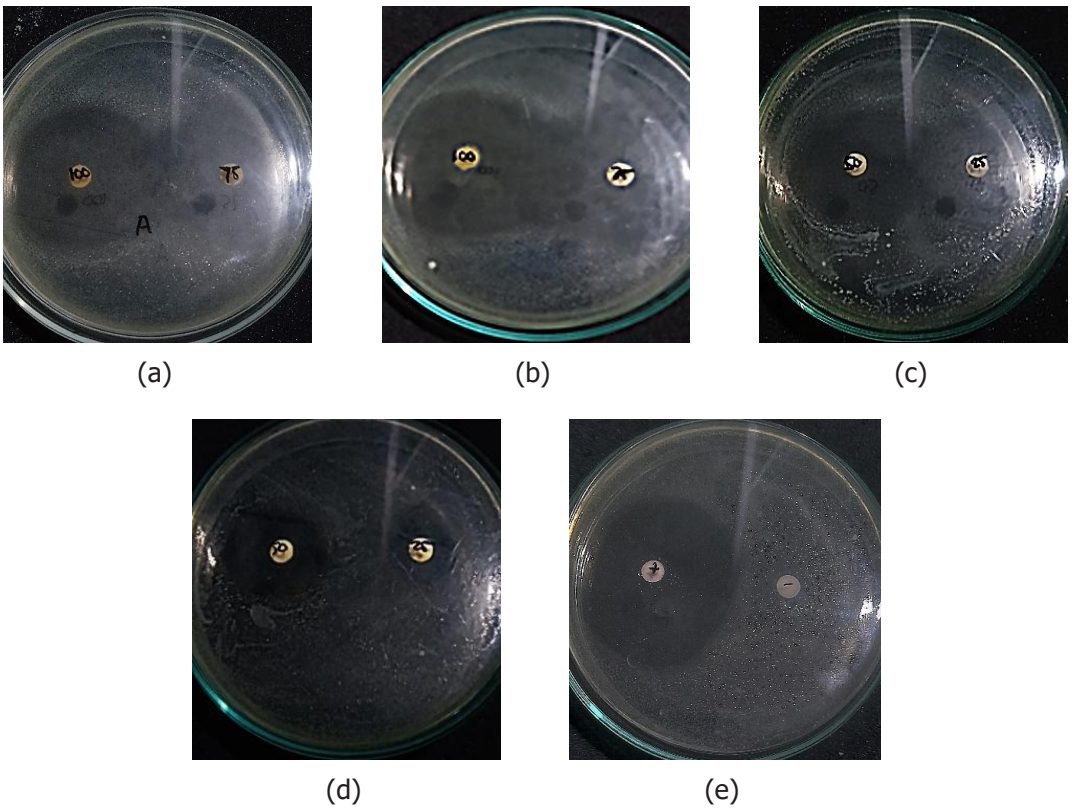

Figure 3. Inhibition zones of liquid fractions and pulp extracts against *E. coli*

Note: A. Pulp extract 100% and 75%, B. liquid fraction 100% and 75%, C. pulp extract 50% and 25%, D. liquid fraction 50% and 25%, E. chloramphenicol and DMSO

tests. ANOVA results showed a highly significant difference ($p < 0.05$). The 100% pulp extract exhibited the highest inhibition zone, averaging 46.67 mm, which was not different from the positive control, 75% pulp extract, and 75-100% liquid fraction. However, the 75 to 100% pulp extract and positive control were significantly different from the 25-50% liquid fraction, 25-50% pulp extract, and negative control. This suggested that the pulp extract from a high concentration value was as effective as the positive control. The results revealed that a decrease in dregs extract and liquid fraction concentration by 25 to 50% significantly reduced inhibitory activity. One of the possible explanations for the low antibacterial activity of the 25% liquid compared to that of the 25% pulp could be related to only a major compound being detected in the liquid extract. Meanwhile, 20 principal compounds in pulp extract synergistically contributed to the antibacterial efficacy of olive leaf. The inferior activity presented by the 25% liquid fraction in comparison to the 25% pulp extract was related to as little as a single compound found in the liquid fraction. A total of 20 main compounds detected in pulp extract interacted together, and could be related to the synergistic effect of pulp antibacterial activity.

For the liquid extract with a predominant component, decamethyltetrasiloxane was detected, which also exhibited significant antibacterial properties. This suggested that the material had considerable antimicrobial potential, and it was consistent with the previous results. In the future, additional studies must be conducted to investigate its antibacterial mechanism. The discrepancy in compound detection between liquid and pulp extraction could be due to varying solubility and matrix characterizations. Some compounds were more easily extracted or retained during the extraction, depending on the extract type and polarity.

CONCLUSION

In conclusion, the results of this study indicate that the ethyl acetate extract of red beetroot pulp contains 20 major bioactive compounds, including acid groups, ester groups, and cyclic hydrocarbons. These bioactive compounds confer strong antibacterial activity on *E. coli*. With the concentration of extract used as a guide, the diameter of the inhibition zone accordingly increases. These results confirm that ethyl acetate extract from red beetroot pulp has the potential to become a source of natural antibacterial compounds. Further study is needed for isolating the most influential active principles and obtaining a more comprehensive understanding of the mechanisms of their action.

CONFLICT OF INTEREST

The author declares no conflict of interest with any party.

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