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RESEARCH ARTICLE

Spectroscopic characterization of rosella flower extract (*Hibiscus sabdariffa* L.) and its antibacterial activity against *Enterobacter aerogenes* in suspected typhoid cases

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Typhoid, a serious bacterial disease, has spurred research into natural products like rosella (*Hibiscus sabdariffa* L.) for potential treatments. This study investigates the chemical components found in Rosella extract using spectroscopy approximation, assisted by Fourier transform infrared spectroscopy (FTIR) and Gas chromatography–mass spectrometry (GC–MS). The antibacterial activity of rosella extract on bacteria from stool cultures of suspected typhoid cases continues to be evaluated. The antibacterial experimental employed a post-test-only control group design, using 30 µg Chloramphenicol as a positive control, sterile distilled water as the negative control, rosella extract at concentrations of 25%, 50%, 75%, and 100% as the observed variable. Stool samples from typhoid patients were identified, and *Enterobacter aerogenes* were detected using VITEK®2 testing. Cultivated bacteria from the samples were tested to determine the antibacterial activity of the rosella extract. Phytochemical studies confirmed the presence of tannins, alkaloids, flavonoids, and saponins in the rosella extract. Additionally, the spectroscopic evaluation from FTIR and GC-MS showed the presence of chemical groups, including esters, aldehydes, and aromatics. Further clinical tests demonstrated antibacterial activity at the minimum inhibitory concentration. The results showed an increasing inhibition zone of bacterial growth, correlating with the increase in rosella extract concentration. Although the antibacterial activity of rosella extract was lower compared to commercial Chloramphenicol, this natural product has demonstrated antibacterial activity and shows potential as a candidate for future herbal medicine development.

1. INTRODUCTION

Enterobacter aerogenes classified as a Gram-negative, facultative anaerobic bacterium, has emerged as a significant opportunistic pathogen in healthcare settings. Originally named *Aerobacter aerogenes* and later reclassified as *Klebsiella aerogenes*, this bacterium is known for its ability to cause a wide range of infections, particularly in immunocompromised

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patients (1). The increasing prevalence of *E. aerogenes* in hospital-acquired infections is attributed to its remarkable ability to acquire and disseminate antibiotic-resistance genes, making treatment options increasingly limited (2). *E. aerogenes* has been identified in various clinical settings, including in patients with typhoid. Studies have shown that this bacterium can be a significant pathogen in hospital-acquired infections, often leading to complications in patients with weakened immune systems, such as those suffering from typhoid fever. Molecular techniques, such as restriction endonuclease analysis (REA) and repetitive-element polymerase chain reaction (rep-PCR), have been used to study the epidemiology of infections due to *E. aerogenes*, identifying hospital outbreak-associated strains (3).

Recent studies have explored the potential of natural medicine in combating infections caused by *E. aerogenes*. Research has focused on identifying plant extracts and natural compounds with antimicrobial properties that can inhibit the growth of this bacterium. For instance, certain essential oils and plant-derived compounds have shown promising antibacterial activity against *E. aerogenes* (4).

One study highlighted the effectiveness of essential oils from plants such as oregano, thyme, and tea tree in inhibiting the growth of *E. aerogenes*. These essential oils contain bioactive compounds that exhibit strong antimicrobial properties. Another study investigated the antibacterial activity of various plant extracts, including those from garlic, ginger, and turmeric, against *E. aerogenes* (5). In addition to plant extracts, researchers have also explored the use of natural compounds such as flavonoids, alkaloids, and tannins in combating *E. aerogenes* infections. These compounds, found in various medicinal plants, have demonstrated the ability to disrupt bacterial cell membranes, inhibit enzyme activity, and interfere with bacterial DNA replication, thereby reducing the viability of *E. aerogenes* (5).

Hibiscus sabdariffa, commonly known as rosella, is widely cultivated in Indonesia due to its adaptability to the tropical climate and its economic importance. The plant thrives in regions with full sunlight, well-drained soil, and a pH range of 5.5 to 7 (6). It is known for its high diversity, with various species such as *Hibiscus rosa-sinensis*, kenaf, and waru also present in Indonesia (7). Rosella is valued for its numerous applications, particularly its antibacterial properties. The plant contains various bioactive compounds, including flavonoids, phenolic acids, and anthocyanins, which contribute to its antimicrobial activity. Studies have shown that extracts from *H. sabdariffa* exhibit significant antibacterial effects against pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica* (8). These properties make it a potential natural preservative for food products and a valuable resource for traditional medicine.

In this research, we explore the significant antibacterial activity of the ethanolic extract from rosella against *E. aerogenes* bacteria from stool cultures of typhoid patients. The testing was conducted with various concentrations of rosella extract, and positive control was included using a commercial antibiotic, 30 µg Chloramphenicol, with distilled water as the negative control. Our exploratory process aimed not only to assess the antibacterial activity but also to identify the chemical components of the extract. We performed a thorough phytochemical characterization through qualitative analysis to determine the presence of alkaloids, saponins, flavonoids, and tannins. Further chemical characterization was conducted using sophisticated spectroscopy instruments. Fourier Transformation Infrared (FTIR) and Gas Chromatography Tandem Mass Spectrometer (GC-MS) were the laboratory instruments used for this testing.

2. MATERIALS AND METHODS

The rosella flower samples were obtained from Semendo, Muara Enim, South Sumatera Province, from private plant cultivation. The sample was identified at the Biology Laboratory facility at Muhammadiyah University, Palembang, Indonesia. According to the result of determination, the sample was classified as *Hibiscus sabdariffa* L. The determination statement is recorded in the legal document with reference number 146/Lab.Vio.FKIP/IV/2024.

In this research, an in vitro experimental test with a post-test-only control group design was applied to identify the correlation between ethanol extract from rosella and antibacterial activity. The research was carried out from October 2023 to January 2024 at the Biomedical Laboratory of the Faculty of Medicine, Muhammadiyah University of Palembang; the Integrated Testing Laboratory of the Faculty of Mathematics and Natural Sciences, Sriwijaya University; the Internal Medicine Clinic of the Muhammadiyah Hospital, Palembang; and the Laboratory of the Ministry of Health of the Republic of Indonesia, Directorate General of Health Services, Palembang City Health Laboratory Centre.

2.1. Extraction Process

Rosella flowers (*Hibiscus sabdariffa* L.), sourced from private plant cultivation in Semendo, Muara Enim, South Sumatera Province, were processed by air drying for 72 hours. The dried flowers were then ground into powder using a commercial grinding machine. The grinding process was conducted to homogenize the sample and enhance the extraction efficiency by increasing the surface area. Afterward, the extraction process was carried out using the maceration method with 2000 gr of rosella powder, which was soaked in absolute ethanol (Cat. No. 1070174000, Merck, Darmstadt, Germany) for 24 hours. The extract was then filtered and separated from the remaining residue using Whatman filter paper, and evaporated using a vacuum rotary evaporator until a thick extract was obtained. The final extract was stored in a closed vial in a refrigerator at a temperature of 5°C. The rosella sample and processed extract are depicted in Figure 1.

Figure 1. *Hibiscus sabdariffa* L. (A) Sample, (B) Ethanolic extract after processing

2.2. Phytochemical Screening of The Ethanol Extract

2.2.1. Test for Flavonoid

The flavonoid test was performed by reacting the testing samples with 0.1 grams of magnesium and 10 drops of hydrochloride acid. A positive result, indicating the presence of flavonoid, is identified by the formation of a dark red color in the testing sample (9,10).

2.2.2. Test for Alkaloids

The alkaloid identification test was performed using qualitative analysis. The reaction between the Wagner Reagent (Cat. No. 902600, CDH, New Delhi, India), which contains iodine, potassium iodide, water, and the alkaloid compounds results in the formation of a brown precipitate, indicating the presence of alkaloid compounds (11).

2.2.3. Test for Saponins

The saponin test was performed by adding several drops of sodium carbonate (Na_2CO_3) 0.1 M (Cat. No. 230952, Sigma Aldrich, Saint Louis Missouri, USA) to the testing sample. The formation of stable white foam indicates a positive result for the presence of saponin compounds. The chemical reaction in this test involves saponins having hydrophobic and hydrophilic active sites, reducing the testing sample's surface tension (9,10).

2.2.4. Test for Tannins

The presence of tannin compounds was identified using qualitative analysis, where a certain amount of ethanol extract was reacted with lead (ii) acetate 0.01 N (Cat. No. 316512, Sigma Aldrich, Saint Louis Missouri, USA). A positive result is indicated by forming a brown precipitate (9-11).

2.3. Gas Chromatography and Mass Spectrometer (GC-MS) and Attenuated Total Reflectance - Fourier Transformation Infrared (ATR-FTIR) Extract Characterization

Extract characterization was carried out by testing compound profiles using chemical instrumentation. This testing was performed at the Integrated Testing Laboratory, Faculty of Mathematics and Natural Sciences Sriwijaya University, with Fourier Transformation Infrared (FTIR) spectroscopy from Thermoscientific Nicolet™ iS50 FTIR USA, equipped with an Attenuated Total Reflectance (ATR) measurement module. Chemical identification was performed using a Gas Chromatography Mass Spectrometer (GC-MS) from Thermoscientific TSQ 9000 Triple Quadrupole GC-MS USA, equipped with the NIST library as a reference. The results of the extraction of rosella in ethanol solvent were injected into the GC-MS instrument with a proportional volume of 1 µL. The injection results produce a chromatogram showing the separation of chemical constituents and a mass spectrum, providing information on the compounds resulting from the separation. The mass spectrum was compared with the database in the NIST standard library (12).

The ATR-FTIR analysis was conducted using the Thermo-Scientific Nicolet FTIR spectrophotometer equipped with an ATR module. The rosella ethanol extract was placed on top of the ATR FTIR Zinc Selenium (ZnSe) crystal. The analysis was conducted in the wave-number range of 4000 - 450 cm^{-1}. The spectrum obtained from the FTIR spectrophotometer was interpreted using the literature-specific signal database. These signals were used to identify the presence of functional groups in the active compounds of the rosella ethanol extract (13,14).

2.4. Source of Test Organisms (Stool Sample Culture)

The research samples were obtained from stool samples of patients suspected of typhoid fever with positive Widal test results. A total of 5 gr of stool samples were placed in a sterile cooler box, which had been pre-cooled to maintain the temperature and condition of the samples. The samples were then transported to the laboratory for further analysis.

2.5. Antimicrobial Sensitivity Testing

2.5.1. Agar Well Diffusion Method

Bacterial cultivation was performed by culturing and identifying bacteria. Distilled water was sterilized using an Ultraviolet C system equipped with an ultrapure water purification system from Merck Millipore. The agar medium was prepared by dissolving 15 grams of agar powder, 5 grams of peptones, 3 grams of yeast extract, 1 gram of glucose, and 5 grams of sodium chloride in 1 L of sterilized water. The mixture was then heated until the agar was completely dissolved. The agar was sterilized using an autoclave at 121°C for 15 minutes. After the sterilization process, the medium was allowed to cool to about 45-50°C, then poured into petri dishes or test tubes. The medium was left to solidify at room temperature. Once the agar medium was ready to be used, the stool sample was dissolved in sterilized water and 20 µL of the dissolved sample was inoculated in a straight line on the surface of the agar medium. The plate was then incubated at 35(±2) ^{o}C for 18 to 24 hours.

2.5.2. Determination of Minimum Inhibitory Concentration (MIC)

Determining of the minimum inhibitory concentration (MIC) of antimicrobial agents against *Enterobacter aerogenes* adheres to rigorous and standardized protocols to ensure scientific accuracy and reproducibility. Due to its established compatibility with antimicrobial susceptibility testing, the experiment commences with preparing a growth-supportive medium Mueller-Hinton Broth (Cat. No CM0405B, Thermo Fischer Scientific, Hempshire, UK). Subsequently, the bacterial inoculum is prepared by adjusting a fresh culture of *E. aerogenes*, corresponding to approximately 2 ×10^8 CFU/mL, ensuring a homogenous bacterial density for reliable testing.

The antimicrobial agent under investigation is formulated as a stock solution and subjected to serial two-fold dilutions across a defined concentration gradient. The testing is performed using the broth microdilution technique, wherein aliquots of 100 µL from each dilution are dispensed into designated wells of a sterile microtiter plate. Each well is inoculated with 100 µL of bacterial suspension, standardized to a final concentration of 5 ×10^5 CFU/mL in the test system. To ensure optimal conditions for growth and activity, the inoculated microtiter plates are incubated aerobically at 35 ± 2°C for 16–20 hours, unless specific testing conditions demand modifications.

Adequate controls are integrated to validate the experimental outcomes. Positive growth controls include wells containing the bacterial inoculum without the antimicrobial agent, confirming the viability of *E. aerogenes.* Negative controls consist of uninoculated wells containing the medium alone, verifying the absence of contamination and ensuring sterility. Additionally, a standard reference antibiotic may be included as a comparator to ascertain the consistency of MIC values the positive control group, which was given 30 µg of Chloramphenicol; the negative control group, which was given sterilized water. The observed variables in this experiment were *H. sabdariffa* ethanol extract with four different concentrations: 25%, 50%, 75%, and 100% (v/v).

The inhibitory effect on bacterial proliferation was observed by calculating the colony area of the bacteria in the petri dish. The results were used to determine the inhibitory rate of growth of the test bacteria (13). Post-incubation, MIC determination is based on the visual inspection of wells to identify the lowest concentration of the antimicrobial agent that inhibits visible bacterial growth. This meticulous methodology provides a robust framework for assessing the inhibitory efficacy of antimicrobial agents against *E. aerogenes*, offering valuable insights into its potential applications in therapeutic or clinical microbiology. The inhibitory dimension obtained from the experiment will be plotted into the linear regression to observe the influence of *H. sabdariffa* extract in order to inhibit the *E. aerogenes* activity.

2.6. Antibacterial Identification Using VITEK® 2

Antibacterial characterization was conducted using the VITEK® 2 instrument. This testing was performed at the external laboratory located at the Ministry of Health of the Republic of Indonesia, Directorate General of Health Services, Palembang City Health Laboratory Centre. The Kirby Bauer diffusion disk method was used in the form of VITEK ® 2: Healthcare. MIC testing followed the Global Clinical Laboratory Standard Institute (CLSI) 2023 MIC Interpretation Guideline and the Natural Resistance modification on the Advanced Expert System (AES) software, which validates testing online.

2.7. Statistical Analysis

Statistical analysis was performed using Microsoft Excel 365, version 16.0. A correlation test was performed by generating a linear function between the concentration of ethanolic extract of rosella and the diameters of the inhibitory zones. The correlation coefficient was calculated from the independent and measured variables (15).

3. RESULTS AND DISCUSSION

3.1. Phytochemical Screening Result

The Phytochemical screening methodology was used to identify four main compound groups in the testing sample, including for flavonoids, alkaloids, tannins, and saponins. These four compound groups were identified using qualitative testing methods, as described in part 2.2. The result showed the presence of these four compound groups in rosella. A summary of these results is shown in Figure 2.

Previous research on the qualitative phytochemical identification of chemical constituents in *H. sabdariffa L* has been commonly conducted. These compounds are known for their diverse pharmacological activities, including antimicrobial, anti-inflammatory, and analgesic effects. The presence of alkaloids in *H. sabdariffa* was confirmed through qualitative and quantitative phytochemical analyses. For instance, a study conducted by Okereke et al. (16) revealed that the dried calyces of *H. sabdariffa* contain approximately 2.14% alkaloids. Recent studies also confirmed the presence of alkaloids in significant quantities, highlighting their potential antioxidant and antihypertensive activities (17).

Rosella, is also known to be a rich source of flavonoids, which are recognized for their antioxidant properties. Studies have shown that the calyx of *H. sabdariffa* contains significant amounts of flavonoids, contributing to its health benefits. For instance, previous studies found that the acetone extract of *H. sabdariffa* calyx had the highest flavonoid content, measuring 53.6 mg QE/g DW (18). Another study also reported that the calyx extracts of *H. sabdariffa*, fertilized with chicken manure, contained 322 mg EC3G/g MS of flavonoids (19). These findings highlight the potential of *H. sabdariffa* as a natural source of antioxidants, which can benefit for human health.

Tannins were also present in this testing sample. This chemical group consists of polyphenolic compounds known for their astringent properties and potential health benefits. Research has shown that the calyces of *H. sabdariffa* contain significant amounts of tannins. The previous studies revealed that the dried calyces of *H. sabdariffa* contain approximately 17.0% tannins (16). Another study by Adamu and Ngwu (20) confirmed the presence of tannins in the methanolic leaf extracts of *H. sabdariffa*. These findings highlight the potential of *H. sabdariffa* as a natural source of tannins, which can be helpful in various medicinal and nutritional applications.

Saponins are secondary metabolites synthesized by many plant species, including *H. sabdariffa*. These compounds are known for their surfactant properties, which allow them to form stable soap-like foam when shaken in an aqueous solution. The presence of saponins in *H. sabdariffa* has been confirmed through various phytochemical screenings. For instance, a recent study revealed the probable presence of saponins in the leaves of *H. sabdariffa* Linn. using cold maceration extraction methods (21). Another study highlighted the antimicrobial and antioxidative properties of the phytochemical compounds extracted from *H. sabdariffa* L., including saponins. These findings suggest that saponins in *H. sabdariffa* contribute to its medicinal properties, such as anti-cancerous, analgesic, anti-inflammatory, antihypertensive, antitumor, and antiviral activities (22).

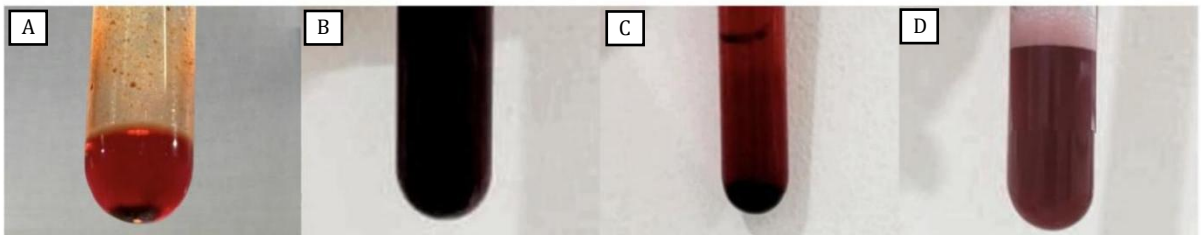

Figure 2. Phytochemical test result. (A) positive test result for alkaloids test; (B) positive test result for flavonoids test; (C) positive test result for tannin test; and (D) positive test result for saponin test.

3.2. Attenuated Total Reflection Fourier Transformation Infrared (ATR-FTIR) Measurement Results

Spectroscopy characterization aims to identify the chemical constituents present in the testing sample. The ATR-FTIR technique provides information on the molecular vibration of various functional groups, which can be used to predict the chemical structure of the compounds in the sample. The ATR-FTIR spectroscopy results, along with the molecular vibration data, are tabulated in Table 1.

Based on the FTIR spectrum and predictions of active compounds in rosella extract, vibrations at a wave number value of 3401.90 cm^{-1} were observed, which corresponds to the stretching vibrations of the hydroxy functional group (-OH). The presence of this functional group typically indicates alcohol or phenolic compounds. The vibration observed at 2929.90 cm^{-1} suggests the presence of alkane compounds due to the stretching vibration of the -CH(CH_2) functional group. This hypothesis is further supported by the presence of vibrations at 1629.38 cm^{-1}, indicating alkane compounds with a cis structure -C=C stretching vibration.

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Table 1. Molecular vibration of rosella flower extract sample using ATR-FTIR

Wave-number (cm^{-1})	Functional Group	Compound Group Prediction
522	C-H	Aromatics Groups
778.29	-N-H *bending out of plane*	Amine Groups
832.22	=CH_2 *wagging*	Alkene Groups
1064.79	C-O *stretching*	Carboxylic Groups
1103.11	C-O-C *bending*	Nucleic Acid Groups
1225.30	-PO^{2-}	Phospholipid Groups
1321.70	-C-H(CH_3) *bending symmetric*	Lipid Group
1408.05	=C-H (cis) *bending rocking*	Fatty acid and amino acid Groups
1629.38	-C=C- (cis) *stretching*	Alkane Compounds
1741.31	-C=O (ester) *stretching*	Aldehyde Compounds
1791.15	-C=O *stretching*	Carboxylate group
2929.90	-CH(CH_2) *stretching*	Alkane compounds
3401.90	-OH *stretching*	Alcohol, Phenol

FTIR analysis also revealed the presence of carboxylic acid compounds, characterized by stretching vibrations of the -C=O and C-O functional groups at wave numbers of 1791.15 and 1064.79 cm^{-1}. Amino acids were identified in the rosella extract, with peaks appearing at 1408.05, 1103.11, and 778.29 cm^{-1}, which correspond to molecular vibrations typical of nucleic acids, such as the =C-H (cis) functional group, C-O-C bending vibrations, and the -N-H functional group. Additionally, lipid or fat compounds were identified with vibrations at wave numbers 1321 and 1225 cm^{-1}, which indicate symmetric bending vibrations of the -C-H(CH_3) functional group and the PO^{2-} group. These compound groups provide a basis for predicting the presence of target compounds in the rosella extract obtained using the maceration method with ethanol solvent. Further confirmation of the extracted target compounds was carried out using a gas chromatography instrument equipped with a mass spectrometer detector (Figure 3).

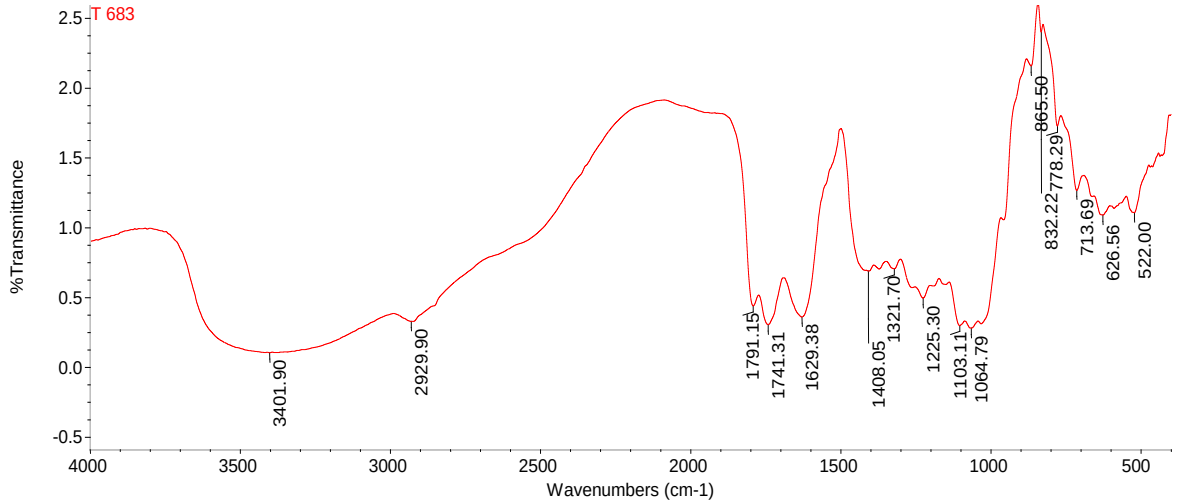

Figure 3. ATR-FTIR spectrum from rosella flower extraction.

The results obtained were also compared with those of previous studies. Previously, studies were discovered that *H. sabdariffa* extract exhibited characteristic peaks corresponding to various functional groups. For example, the broad absorption band around 3300 cm^{-1} indicates of O-H stretching vibrations associated with phenolic compounds and water content in the extract. The peaks observed at approximately 2920 cm^{-1} and 2850 cm^{-1} correspond to C-H stretching vibrations, suggesting the presence of aliphatic hydrocarbons. The absorption bands around 1600 cm^{-1} and 1500 cm^{-1} are attributed to C=C stretching vibrations, which are characteristic of aromatic compounds. Furthermore, the presence of peaks around 1200 cm^{-1} and 1000 cm^{-1} indicates C-O stretching vibrations, which are commonly found in polysaccharides and flavonoids (14). Based on this comparison, the results are consistent with those of previous studies.

3.3. Characterization of Gas Chromatography Tandem Mass Spectrometer (GC-MS)

The GC-MS test results showed several candidate compounds with potential antibacterial activity, as indicated by the chromatogram peaks. The GC-MS data interpretation was conducted by aligning the mass spectrum fragmentation patterns with those in the library identification. The reference spectra, known for their reliability, were obtained from the library package installed in GC-MS software. All identified chemical compounds are listed in Table 2. The spectrum comparisons were performed with the NIST library as the reference, using the deconvolution spectrum extraction algorithm as the standard procedure for the mass spectrum identification process.

Table 2. Identified chemical compound detected in the GC-MS analysis results

Retention Time	Identified Compound	Area Abundance
5.62	3-Pentenoic Acid, 3- Ethyl-, Methyl Ester	21.91%
11.66	Butanedioic Acid, 3-Hydroxy-2,2-Dimethyl-, Dimethyl Ester	24.44%
15.19	Hexadecanoic Acid, Methyl Ester	3.34%
15.40	Benzenepropanoic Acid, 3,5-Bis (1,1-Dimethylethyl)-4-Hydroxy-, Methyl Ester	0.60%
15.85	Hexadecanoic Acid, Ethyl Ester	1.00%
17.47	Octadecanoic Acid	1.17
27.52	Gamma-Sitosterol	0.53%

The chromatogram peak with the highest abundance was found at a retention time of 5.62 minutes, with an abundance of 21.91%. The mass spectrum determination revealed prominent peaks at m/z 82, 55, 142, and 110, which were identical to the compounds 3-Pentenoic Acid, 3- Ethyl-, and Methyl Ester. Another major compound identified appeared at a retention time of 11.66 minutes, with an abundance of 24.44%. The mass spectrum determination for this compound showed identical peaks for Butanedioic Acid, 3-Hydroxy-2,2-Dimethyl-, Dimethyl Ester. Additionally, another compound with a notable abundance was found at a retention time of 15.19 minutes, with an abundance of 3.34%. The molecular fragmentation at m/z 74, 87, and 143 was identical to Hexadecanoic Acid, Methyl Ester.

Another group of ester compounds was found at a retention time of 15.85 minutes, identified as Hexadecanoic Acid, Ethyl Ester, with an abundance of 1.00%. The carboxylic acid compound Octadecanoic Acid and the cyclic chain hydrocarbon compound Gamma-Sitosterol were also detected, with an abundance of 0.53%. Other components with the highest chemical structure similarity to the database reference were observed at a retention time of 15.40 minutes, with an abundance of 0.60% and a matching score of 94.67%. These compounds were identified as Benzenepropanoic Acid, 3,5-Bis (1,1-Dimethylethyl)-4-Hydroxy-, Methyl Ester. The results of this identification are consistent with those reported in previous studies from the extraction of rosella using methanol solvent (12). The chromatogram resulting from the separation of the analyzed compounds is shown in Figure 4.

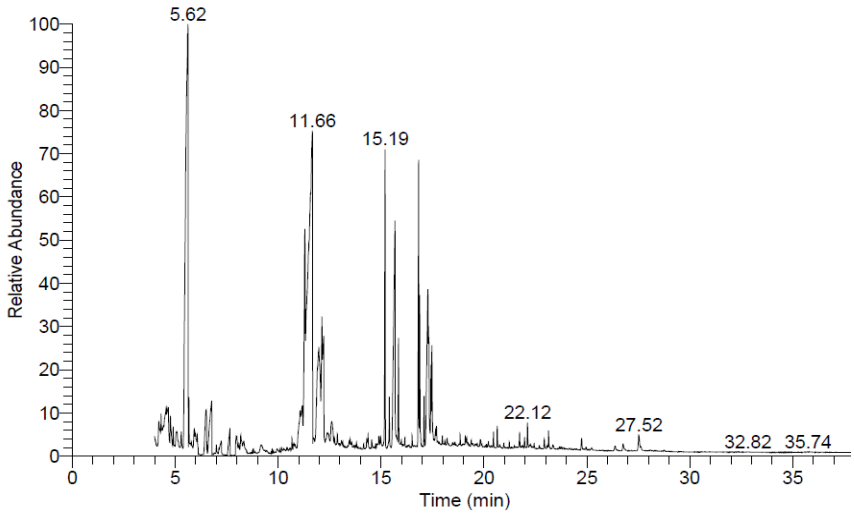

Figure 4. Chromatogram of the results of separating rosella extract compounds from Ethanol extract.

3.4. Correlation Test of Antibacterial Activity Against Identified Chemical Compounds

The identified chemical compounds and their chemical properties were compared with previous research. Historically, phenolic compounds play an important role in the antibacterial activity against several bacterial test media. Previous studies also reveal that the phenolic compounds contained in rosella were proven to inhibit bacterial growth in test samples (7).

In general, the action mechanism of phenolic compounds in inhibiting bacterial growth involves damaging the bacteria cell membrane, which results in extracellular damage and the destruction of the bacterial cell wall structure (23). The results of compound identification using GC-MS showed that Benzenepropanoic Acid, 3,5-bis(1,1-Dimethylethyl)-4-Hydroxy-, Methyl Ester is a phenolic compound contributing to antibacterial activity. Acidic compounds, such as Hexadecanoic Acid and Octadecanoic Acid, exhibit antibacterial properties, although not much research has explored these compounds, primarily from rosella. Another study is also reported that these compounds were effectively inhibited the growth of *E. coli* bacteria from neem oil samples (24). Regarding antibacterial activity, a chemical compound with antioxidant properties, Gamma Sitosterol, was also identified. This compound is widely used in the pharmaceutical industry for its antibacterial, antioxidant properties and use in diabetes therapy. Recently, studies were reported that Gamma Sitosterol has anti-hyperglycemia activity, which increases insulin secretion in response to glucose (25). However, the toxicity and appropriate dosage for consumption should still be considered.

3.5. Antibacterial Testing using VITEK® 2 and Commercial Antibiotics Microbiology Inhibitory Concentration (MIC) Test

Based on a series of laboratory tests, identifying microbes from suspected typhoid patients with positive Widal results, who were infected with *Salmonella typhi,* revealed other microorganisms in the stool samples, namely *Enterobacter aerogenes*. The testing was carried out at the Laboratory of the Ministry of Health of the Republic of Indonesia, Directorate General of Health Services, Palembang City Health Laboratory Centre, using the Kirby Bauer diffusion disk method with VITEK ® 2: Healthcare. MIC testing was conducted according to the Global Clinical Laboratory Standard Institute (CLSI), 2023: MIC Interpretation Guideline and natural resistance modification was made using the Advanced Expert System (AES) software, which validates testing online. The results confirmed the identification of *Enterobacter aerogenes* bacteria with a quantification of acceptance interval of 95%.

The negative control results from the series experiment showed no bacterial presence, indicating no bacterial contamination during the testing process. Further identification was confirmed by MIC testing using several commercial antibiotics. It was found that the tested bacteria were resistant to several antibiotics, including Ampicillin (MIC = 8 µg/mL), Ampicillin/Sulbactam (MIC <= 2 µg/mL), Piperacillin/Tazobactam (MIC <= 4 µg/mL), and Cefazolin (MIC >= 64 µg/mL). Other antibiotics were also tested to observe the interaction of *Enterobacter aerogenes* with different antibiotics. The bacteria were found to be sensitive to several antibiotics, including Ceftazidime (MIC <= 1 µg/mL), Ceftriaxone (MIC <= 1 µg/mL), Cefepime (MIC <= 1 µg/mL), Aztreonam (MIC <= 1 µg/mL), Ertapenem (MIC <= 0.5 µg/mL), Meropenem (MIC <= 0.25 µg/mL), Amikacin (MIC <= 2 µg/mL), Gentamicin (MIC <= 1 µg/mL), Ciprofloxacin (MIC <= 0.25 µg/mL), Tigecycline (MIC = 1 µg/mL), and Trimethoprim/Sulfamethoxyazole (MIC <= 20 µg /mL). In the test samples, it was also found that *E. aerogenes* bacteria exhibited intermediate inhibition against the antibiotic Nitrofurantoin, with an MIC value of 64 µg/mL.

3.6. Microbiology Testing Inhibitory Concentration (MIC) of Rosella Flower Extract

FTIR spectroscopy and GC-MS tests were carried out on rosella extracts. MIC testing was performed to determine the microbiological inhibitory concentration of rosella ethanol extract. Systematically, it was observed that the extract inhibited the growth of microorganisms found in the stool of patients suspected of having typhoid. This was indicated by the diameter of the inhibition zone resulting from bacterial isolation on the test medium in the petri dish. An increase in the concentration of rosella extract led to a corresponding increase in the rate of inhibition of bacterial growth. As a positive control, 30 µg/mL Chloramphenicol was used.

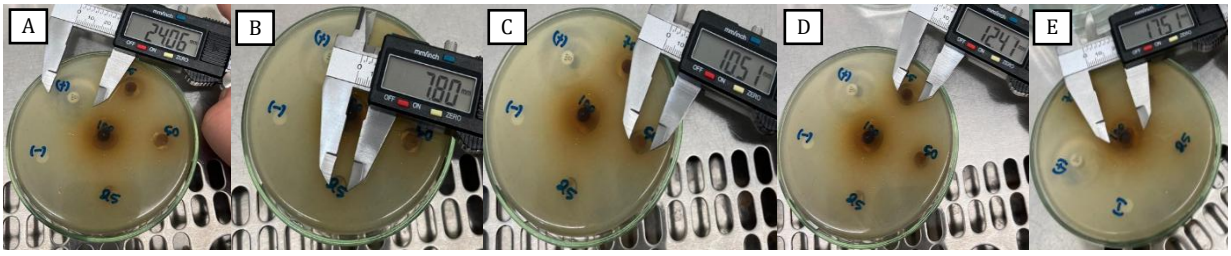

Figure 5. The inhibition zone diameter from microbiological growth inhibition rate tests with various concentrations of rosella flower extract. (A) Commercial chloramphenicol, (B) Rosella Extract 25%, (C) Rosella Extract 50%, (D) Rosella Extract 75%, (E) Rosella Extract 100%,

The correlation between the dosage of rosella extract and bacterial inhibition was determined by applying a linear equation. The measured variable in this test was the diameter of the microbial inhibition zone in response to changes in the concentration of rosella extract, expressed as volume percent concentration per volume. The inhibitory rate for

bacterial development was observed at 0.125 mm/% volume of rosella extract. These results confirm that rosella extract can inhibit the growth of microorganisms in stool samples from patients suspected of having typhoid.

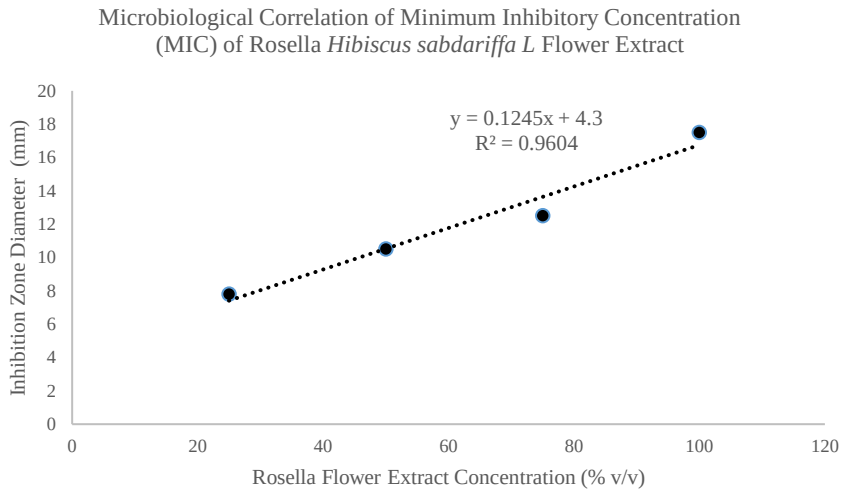

Figure 6. Evaluation of the linear correlation between bacterial inhibition zone diameter and rosella flower extract at various concentrations against *Enterobacter aerogenes* bacteria from cultivated stool samples of positive typhoid patients

Experimentally, it can be stated that the inhibitory effect of rosella extract on the antibacterial activity of the stool of patients suspected of typhoid, containing the microorganism *E. aerogenes*, produced positive results. These findings are consistent with the research were reported antibacterial activity from ethyl acetate and ethanol extracts of *H. sabdariffa* L. (26). Additionally, this natural product has numerous other applications, such as in the treatment of hypertension, antioxidant activity, antimicrobial growth and various other health benefits (27-30). This suggests that rosella extract has potential as a natural medicinal product for future use.

4. CONCLUSIONS

The rosella extract contains various chemical groups, including esters, aldehydes, and aromatics. This was confirmed by the FTIR peak spectrum positions, indicating these chemical groups' presence. Additionally, GC-MS identification results, including chromatograms and mass spectra, reflected the presence of compounds such as 3-Pentenoic Acid, 3-Ethyl, Methyl Ester; Butanedioic Acid, 3-Hydroxy-2,2-Dimethyl-, Dimethyl Ester; Hexadecanoic Acid, Methyl Ester; Benzenepropanoic Acid, 3,5-Bis(1,1-Dimethylethyl)-4-Hydroxy-, Methyl Ester; Hexadecanoic Acid, Ethyl Ester; Octadecanoic Acid, and Gamma-Sitosterol. Among these, Benzenepropanoic Acid, 3,5-Bis(1,1-Dimethylethyl)-4-Hydroxy-, Methyl Ester was identified as the most active contributor to antibacterial activity. Further exploration of the other chemical components is needed to understand their mechanisms, supported by microbiological research. The antibacterial evaluation of rosella extract against *E. aerogenes* demonstrated a correlation between increasing bacterial inhibition rates and higher extract concentrations. Although the inhibition rates of rosella extract were lower than those of commercial antibiotics, this natural product shows promise as a safe and effective herbal medicine for future development.

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Conflict of interest: All the author member from this publication declares there is no any conflict of interest from any entity and department during this scientific research conducted.

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