JOBIMB, 2025, Vol 13, No 1, 90-94
https://doi.org/10.54987/jobimb.v13i1.1082

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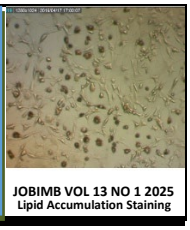

Fractionation-Guided Antibacterial Screening of Selected Medicinal Plant Extracts Against Pathogenic Bacteria

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History

Received: 19th March 2025
Received in revised form: 21st May 2025
Accepted: 10th July 2025

Keywords

Plant extracts
Antibacterial activity
Fractionation
Minimum inhibitory concentration (MIC)
Pathogenic bacteria

Abstract

Fractionation and standardised antibacterial screening are viable concepts employed in separating active compounds in plant extracts that can hinder the growth of tested bacteria. Five plant samples were selected for fractionation, these includes rhizome extract of *Curcuma longa*, seed extract of *Quercus infectoria*, leaf extracts of *Punica granatum, Terminalia catappa* and *Murraya koenigii*. Nine strains of pathogenic bacteria were employed in this research: *Listeria monocytogenes* ATCC 19115, *Staphylococcus aureus* ATCC 700699, *Escherichia coli* O157:H7, *Escherichia coli* ATCC 25922, *Salmonella Paratyphi* ATCC 9150, *Salmonella Typhi* ATCC 14028, *Vibrio alginolyticus* ATCC 17749, *Vibrio paraheamolyticus* ATCC 17802 and *Yersinia enterocolitica* ATCC 23715. The MIC of the extracts was determined using microdilution broth method. The plant extracts were fractionated using paper chromatography. Mixture of butanol, acetic acid and distilled water was prepared in the ratio of 4:1:5. The mixture was used as the mobile phase. Only Fraction 6 of *M. koenigii* exhibited good MIC value of 62.5 µg/mL against *L. monocytogenes* and 31.25 µg/mL against *S. aureus* and *E. coli* O157:H7, but the rest of the fractions showed MIC value ranging from 125-1000 µg/mL. The findings of this study may be useful for developing new natural product-based therapy for pathogenic bacteria.

INTRODUCTION

The investigation of plant bioactive compounds on folk medicinal plants had been strengthened in the 20th century with the intention of discovering novel drugs for treating infections [1; 2].Crude extracts obtained from plants may provide an important information in drug discovery. To achieve this, bioassay fractionation is necessary and important. This method helps in isolating phytochemical principal active components that are naturally contained in plants [3]. Plant extracts are mixture of immense number of secondary metabolites. They differ in their activity and composition which can be influenced by environmental factors and time of plant collection. Moreover, toxic compounds that can counteract the pharmacological activity of the active metabolites can also be contained in plant extracts and must be get rid of. Therefore, standardised separation procedure is paramount. This is necessary for the development of therapeutics that are safe and effective [4].

Due to pathogenic resistant bacteria and side effects of synthetic drugs, great attention has been paid on the bioactive compounds isolated from plants used in traditional medicine. These compounds have vast therapeutic potential in treating infections and at the same time alleviating the side effects associated with synthetic drugs [5]. The advent of disease with no current treatment and the development of resistance to the current antibiotics, makes the search for new antimicrobial from plants a highly significant and important research today.

Techniques used in drug discovery had helped in standardizing herbal medicines. Discovering of drugs from plants involves several fields of knowledge and diverse methods of analysis. The practice usually starts with a botanist, ethnobotanist and plant ecologist (who identifies and collects plants of interest). Pharmacognosy sum up these fields into a separate interdisciplinary science. The process of isolation and identification of bioactive compound can be achieved through

fractionation [6]. Bioassay fractionation can simply be described as a procedure employed in isolating pure bioactive components from plants. This involve employment of some gradual steps (chromatographic fractionation technique) that can help in identifying the biological activity of diverse fractions in an extract; by separating its components based on its physicochemical features using different polarities of solvents [7].

Fractionation is a process that involves separating a mixture into different fractions or components based on their solubility, polarity and molecular weight. This process helps to: 1) remove inactive components and impurities 2) isolate and identify bioactive compounds, and 3) improve bioavailability and extract stability [8]. The plants were selected based on their traditional practice in treating infections as reported by [9]. The study investigated the bioassay fractionation of some selected plants extracts and elucidate the antibacterial activities of the fractions against some selected pathogenic bacteria. The study will hopefully provide science guided evidence on the ethnobotanical use of the plants through investigating their antibacterial potentials.

MATERIALS AND METHODS

Plant materials

Plant samples that comprise of rhizome extract of *Curcuma longa* (SFS/IB/10), seed extract of *Quercus infectoria* (SFS/IB/40), leaf extracts of *Punica granatum* (SFS/IB/39), *Terminalia catappa* (SFS/IB/43) and *Murraya koenigii* (SFS/IB/29) were identified by Professor Shaida Fariza Sulaiman [School of Pharmaceutical Sciences, University Sains Malaysia (USM), Penang]. Vouchered specimens were deposited in school of biological science, USM. The samples were collected from various places within Penang, Malaysia during rainy season between June to July 2015. The concentration of 40 mg/mL of each extract was subjected to six two-fold serial dilution and different concentrations were obtained; 40 mg/mL, 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL respectively.

Bacterial strains

Nine species of pathogenic bacteria were selected in this research; these comprised of seven Gram-negative bacteria and two Gram-positive bacteria. The bacteria were obtained from Laboratory of Food Safety and Quality (Butterworth, Penang, Malaysia). The bacteria include: *Listeria monocytogenes* ATCC 19115, *Staphylococcus aureus* ATCC 700699, *Escherichia coli* O157:H7, *Escherichia coli* ATCC 25922, *Salmonella Paratyphi* ATCC 9150, *Salmonella Typhi* ATCC 14028, *Vibrio alginolyticus* ATCC 17749, *Vibrio paraheamolyticus* ATCC 17802 and *Yersinia enterocolitica* ATCC 23715. Screening plant extracts against both Gram-negative and Gram-positive bacteria allows for the identification of broad-spectrum antibacterial agents, which is crucial given that both types of bacteria can cause human infections, thereby ensuring the extract's potential therapeutic value.

Preparation of nutrient agar

Preparation was made according to the manufacturer's instruction. Briefly, a microbalance was employed to weigh fourteen grams (14 g) of the agar. This was then mixed in 500 mL of distilled water. The mixture was then subjected to heat for complete dissolution. The dissolved mixture was autoclaved at the temperature of 121ºC for 15 minutes. The sterilized nutrient agar was allowed to cool and then transferred into petri dishes for complete solidification.

Preparation of nutrient broth

Nutrient broth (Oxford, UK) was used as a stock media for preparing the inoculums for the antibacterial screening. Thirteen grams (13 g) of the nutrient broth was weighed and dissolved in 1000 mL. The mixture was stirred until the broth became well dissolved. The mixture was boiled and allowed to cool. Twenty empty universal bottles were filled with 10 mL of the solution and the remaining were filled with 15 mL per bottle, respectively. The universal bottles containing nutrient broth were autoclaved at the temperature of 121oC for 15 minutes. Finally, the nutrient broths were kept in a sterilized condition for the antibacterial screening.

Preparation of nutrient agar and broth against vibrio species

Nutrient agar and broth (Oxford, UK) were used as stock media against vibrio species. 1.5 % of sodium chloride (NaCl) is required in 100 ml of distilled water [10]. To obtain one litre of the mixture, nutrient agar and broth were separately dissolved in each of one litre of distilled water containing 15 grams of NaCl. The mixtures were subjected to heat for complete dissolution. The dissolved mixtures were autoclaved at the temperature of 121ºC for 15 minutes. The agar media was allowed to cool and then transferred into petri dishes for complete solidification, while the broth media was kept in a sterilized condition prior to the antimicrobial screening.

Preparation of inoculum

One loop full of each test bacteria from the stock culture were streaked onto the surface of nutrient agar media. All plates were incubated for 24 hours at 37 °C. The pure colonies of the bacteria were subsequently taken by a needle loop from the agar plate and were grown separately in 10 mL of sterile nutrient broth. The inoculated broth was then incubated at 37 °C for 20 hours. Next, the bacteria in inoculated broth were pipetted into new sterile universal bottles containing saline solution to prepare the bacterial suspension. The density of the bacterial suspension in the saline solution was adjusted until the turbidity matched with 0.5 McFarland turbidity standards (1.5 x 10^8 CFU/mL Colony Forming Units) (Lalitha, 2004). The bacterial suspension's turbidity was standardized to 0.5 McFarland units by visual inspection. Then, the solution was diluted according to 1:100 ratio by pipetting 150 µL of the saline solution containing inoculums into 15 mL sterile broth which gives the final density of inoculums approximately 1.5 x 10^6 CFU/mL.

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibition concentration values of all the extracts were determined using micro-dilution technique as described by [11], which was modified to be performed in sterile 96 wells microplate (Nunc). Briefly, 5 µL of the extracts were mixed with 195 µL of inoculums, which gives a total amount of 200 µL of the mixture per well. To obtain the negative control, 195 µL of the inoculums was filled along with 5 µL of 99.9% dimethyl sulfoxide (DMSO). Tetracycline was employed as positive control. The experiment was performed in triplicate.

The 96 wells microplate containing the solutions was covered and sealed with a paraffin film and then incubated for 20-22 hours at 37 °C. To evaluate growth inhibition, 40 µL of p-iodonitrotetrazolium chloride (INT) (Sigma Aldrich) solution was added into each well. The results were then observed and recorded after 30 minutes re-incubation (37 °C) period. Appearance of yellow to pinkish-red color is an indication of bacterial growth. The minimum inhibitory concentration was defined as the lowest concentration of the extracts with zero visible growth of bacteria.

Paper chromatography

The procedure was adopted as described by [12] Whatman No. 3MM (23 × 57 cm) chromatographic filter papers were cut at equal half size, with one side been folded. The selected extracts; rhizome extract of *Curcuma longa*, seed extract of *Quercus infectoria*, leaf extracts of *Punica granatum*, *Terminalia catappa* and *Murraya koenigii*, were dissolved in 80% methanol solvent and then applied on the folded side of the chromatographic paper. The applications of the extracts were done repeatedly using micro haematocrit tubes until the extracts became dense on the chromatographic paper.

The mixture of butanol, acetic acid and distilled water (BAW) was prepared in the ratio of 4: 1: 5. The mixture was poured into a separation funnel and then shook vigorously in a fume cupboard. This was then allowed to settle, two layers were formed, the upper layer and the lower layer. The lower layer was discarded, and the upper layer was collected and was used as the mobile phase. The papers containing the extracts were hanged in a tank glass inside a drain glass supported with two rod glasses per each drain. The BAW was poured in the drained glass containing the chromatographic papers and covered with a glass slide. The solvent fronts were allowed to develop for 15 hours. The solvent phases were marked for each paper, and the papers were hanged and allowed to dry in the fume cupboard.

The dried chromatographic papers were subjected to ultraviolet light, and different colours of layers were observed, each layer was marked and numbered. The retention factor (Rf) for each fraction was obtained by measuring the distance covered by an individual fraction divide by the distance covered by the solvent in centimeter (cm), and the percentage value for each fraction of the extracts was calculated according to [13].

R_f value (%) = (Distance travelled by fraction / Distance travelled by solvent) × 100

Each separate fraction or layer observed using ultraviolet light was cut out using scissors; this was then cut into smaller pieces and collected in a beaker assign for each fraction. The plant fractions were dissolved in 80% methanol solvent and then filtered using Whatman filter paper No. 1. The filtrates were dried in an oven. The process was repeated until all extracts were obtained. The fractions were refrigerated at −4 ºC until further use.

RESULT

Fractionation using paper chromatography

Mixture of butanol, acetic acid and distilled water was prepared in the ratio of 4:1:5. The mixture was used as the mobile phase for the fractionation of the selected plant samples. **Table 1** showed the fractionation results of the selected plant samples using paper chromatography. Five plant samples were selected for fractionation, this includes; rhizome extract of *Curcuma longa*, seed extract of *Quercus infectoria*, leaf extracts of *Punica granatum, Terminalia catappa* and *Murraya koenigii*. The rhizome extract of *Curcuma longa* and the seed extract of *Quercus infectoria* cannot be separated when viewed using visible light, long-wave and short-wave ultra-violet illumination. Thus, only three separated extracts were selected for antibacterial screening.

Six fractions were obtained from *Murraya koenigii* with different retention factors that are 18%, 40%, 54%, 68%, 80% and 90% respectively. The fractions revealed different colors of layers when viewed using visible light, long-wave and short-wave ultra-violet light. The colors of the first two layers from the top using long-wave ultra-violet light were yellow then followed by dull brown, dark grey, blue and green. For the visible light, the color of the first layer from the top was grey then followed by brown, dull brown, light yellow, another light yellow and white. For *Punica granatum,* seven fractions were obtained with the following retention factors; 28%, 46%, 54%, 66%, 74%, 80% and 90%. The fractions showed various colors when viewed under visible light, long-wave and short-wave ultra-violet light. From the top, the first fraction was mixed, and then followed by dark grey, yellow, dark grey, yellow, blue and red. The visible light revealed the following color starting from the top; dark brown, brown, orange, dark grey, pale orange, dark grey and yellow. The fractions of *Terminalia catappa* showed different retention factors, which are; 3%, 22%, 45%, 52%, 64%, 84% and 94%, respectively. When viewed under long-wave ultra-violet illumination, the first two fractions appeared to be dark yellow, the next three fractions were light yellow, and then followed by yellow and pale yellow. The color of the fractions from the top using visible light revealed the following colors; yellow, dark yellow, light yellow, yellow, white and another white.

Table 1. Fractionation results of selected plant extracts using paper chromatography by using Butanol: Acetic acid: Water (B:A:W) as the mobile phase.

		UV Illumination			
Sample	Fraction	Long-wave	Short-wave	Visible light	R_f value (%)
Murraya koenigii	1	Yellow	Yellow	Grey	18
	2	Yellow	Yellow	Brown	40
	3	Dull brown	Dull brown	Dull brown	54
	4	Dark	Dark grey	Light yellow	68
	5	Blue	Blue	Light yellow	80
	6	Green	Green	white	90
Punica granatum	1	Mix	Brown	Dark brown	28
	2	Dark grey	Dark grey	Brown	46
	3	Yellow	Light yellow	Orange	54
	4	Dark grey	Dark grey	Dark grey	66
	5	Yellow	Yellow	Pale orange	74
	6	Blue	Light blue	Dark grey	80
	7	Red	Red light	Yellow	90
Terminalia catappa	1	Dark yellow	Dark grey	Yellow	3
	2	Dark yellow	Dark yellow	Dark yellow	22
	3	Light yellow	Dark yellow	Light yellow	45
	4	Light yellow	Light yellow	Yellow	52
	5	Light yellow	Light yellow	Yellow	64
	6	Yellow	Light yellow	White	84
	7	Pale yellow	Yellow	White	90

Antibacterial activity of the fractions of selected methanol extracts

Twenty fractions of the three extracts were subjected to antibacterial screening: *Murraya koenigii* (six Fractions), seven Fractions *Terminalia catappa* and *Punica granatum* respectively. The MIC results obtained are shown in **Table 2**. Tetracycline (antibiotic) was used as the positive control. All concentrations displayed were the final concentrations. No MIC value was obtained from Fraction 1, 2 and 3 of *M. koenigii* against the tested bacteria, except for *S. aureus*, which recorded MIC value of 1000 µg/mL. Fractions 4 and 5 of *M. koenigii* did not show any MIC value against the entire tested bacteria**.** Fraction 6 was found to inhibit *L. monocytogenes* with MIC value of 62.5 µg/mL, *S. aureus* and *E. coli* O157:H7 with MIC value of 31.25 µg/mL. The same fraction (Fraction 6) recorded MIC value of 1000 µg/mL against *S. Paratyphi* and *Y. enterocolitica*, and MIC value of 250 µg/mL against *V. paraheamolyticus*.

Table 2. Minimum inhibitory concentration (MIC) (µg/mL) of shortlisted extracts and their individual fractions.
Minimum inhibitory concentration (µg/mL).

	Gram-positive				Gram-negative				
Samples	*L. monocytogenes*	*S. aureus*	*E. coli 0157:H7*	*E. coli*	*S. paratyphi*	*S. typhi*	*V. alginolyticus*	*V. parahaemolyticus*	*Y. enterocolitica*
Murraya koenigii	-	1000	-	-	-	-	-	-	-
1	-	1000	-	-	-	-	-	-	-
2	-	1000	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-
5	62.5	31.25	31.25	-	1000	-	-	250	1000
6									
Punica granatum	-	-	-	-	1000	-	-	-	-
1	1000	-	1000	-	125	-	-	-	500
2	1000	1000	-	1000	500	1000	-	-	-
3	1000	1000	1000	1000	500	-	-	-	-
4	-	-	-	1000	1000	-	-	-	500
5	-	-	1000	1000	1000	1000	-	-	-
6	-	-	1000	-	-	-	-	-	-
				Terminalia catappa					
1	1000	-	1000	-	1000	1000	-	-	-
2	-	-	-	-	500	-	-	-	-
3	1000	-	-	1000	250	1000	-	-	-
4	1000	1000	1000	-	1000	-	-	-	-
5	-	-	1000	-	1000	-	-	-	500
6	-	-	1000	1000	250	1000	500	-	500
7	-	-	1000	-	1000	1000	-	-	-

Note: *L. monocytogenes: Listeria monocytogenes* ATCC 19115; *S. aureus: Staphylococcus aureus* ATCC 700699; *E. coli* 0157:H7: *Escherichia coli* 0157:H7; *E. coli: Escherichia coli* ATCC25922; *S. Paratyphi: Salmonella Paratyphi* ATCC 9150; *S. Typhi: Salmonella Typhi* ATCC 14028; *V. alginolyticus: Vibrio alginolyticus* ATCC 17749; *V. parahaemolyticus: Vibrio parahaemolyticus* ATCC 17749; *Y. enterocolitica: Yersinia enterocolitica* ATCC 23715

Only *S. Paratyphi* was found to be inhibited by Fraction 1 of *Punica granatum* with MIC value of 1000 µg/mL. For Fraction 2 of the same extract (*P. granatum*), *L. monocytogenes* and *E. coli* 0157:H were found to be inhibited with MIC value of 1000 µg/mL, while *S. Paratyphi* was inhibited with MIC value of 125 µg/mL, which was the least value that was obtained from all the fractions of *P. granatum*. The same fraction (Fraction 2 of *P. granatum*) recorded MIC value of 500 µg/mL against *Y. enterocolitica***.**

Listeria monocytogenes, *S. aureus, E. coli* and *S. Typhi* were inhibited by Fraction 3 of *P. granatum* with MIC value of 1000 µg/mL, while *S. Paratyphi* was inhibited by the same fraction with MIC value of 500 µg/mL. Fraction 4 of *P. granatum* was potent against *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *E. coli* with MIC value of 1000 µg/mL, and also effective against *S. Paratyphi* but with MIC value of 500 µg/mL. Fraction 5 of *P. granatum* was able to inhibit *Y. enterocolitica* with MIC of 500 µg/mL, while *E. coli,* and *S. Paratyphi* were inhibited by the same fraction with MIC value of 1000 µg/mL. Furthermore, Fraction 6 of *P. granatum* successfully inhibited the growth of *E. coli* 0157:H, *E. coli, S. Paratyphi* and *S. Typhi* with MIC value of 1000 µg/mL. Only *E. coli* O157:H7 was found to be inhibited by fraction 7 with MIC value of 1000 µg/mL

Fraction 1 of *Terminalia catappa* recorded MIC value of 1000 µg/mL against *L. monocytogenes, E. coli* O157:H7, *S. Paratyphi* and *S. Typhi*. No inhibition of the whole tested bacteria was observed by Fraction 2 of *T. catappa* except for *S. Paratyphi* with recordable MIC value of 500 µg/mL. Least value (250 µg/mL) was exhibited with Fraction 3 against *S. Paratyphi*, but in the case of *L. monocytogenes*, *E. coli*, and *S. Typhi* the MIC value of 1000 µg/mL was observed. Fraction 4 of *T. catappa* gave MIC value of 1000 µg/mL against *L. monocytogenes***,** *S. aureus, E. coli* O157:H7 and *S. Paratyphi*. However, fraction 5 of the same plant extract (*T. catappa*) was found to exhibit the MIC value of 1000 µg/mL against *E. coli* O157:H7 and *S. Paratyphi*, and MIC value of 500 µg/mL against *Y. enterocolitica*.
For fraction 6, the inhibition was observed against *E. coli* O157:H7, *E. coli* and *S. Typhi* with MIC value of 1000 µg/mL, and for *V. alginolyticus* and *Y. enterocolitica,* the MIC value was 500 µg/mL. The least value (250 µg/mL) recorded for fraction 6 of *T. catappa* was established against *S. Paratyphi.* Only *E. coli* O157:H7, *S. Paratyphi* and *S. Typhi* were inhibited with MIC value of 1000 µg/mL for fraction 7 of *T. catappa*.

DISCUSSION

Antibacterial activities of the fractionated plant extracts

Three out of five samples were selected for fractionation, this includes; leaf extracts of *Punica granatum, Terminalia catappa* and *Murraya koenigii*. The remaining two (rhizome extract of *Curcuma longa* and the seed extract of *Quercus infectoria*) were ignored since their fractions cannot be separated when viewed using visible light, long-wave and short-wave ultra-violet illumination. The pH, concentration and the nature of the mobile phase might affect the separation of the extracts, since variations in the mobile phase composition have been reported to affect separation of mixtures [14].

Out of the fractions obtained from the leaf of *M. koenigii*, Fraction 6 was found to have the least value of 31.25 µg/mL against *S. aureus* (ATCC 700699) and clinical strain of *E. coli* O157:H7. The antibacterial activity of the fraction might be as a result of the activity of the active compounds of the leaf fractions against the strains of the bacteria. A study conducted by [15] revealed a good antibacterial activity of the leaf fraction of *M. koenigii* against clinical isolate of *S. aureus* with MIC value of 62.5 µg/mL. No record was found on the antibacterial activity of the leaf fraction of the plant against *E. coli* O157:H7. Fraction 2 of *P. granatum* exhibited a good antibacterial activity with the least value of 125 µg/mL against *S. Paratyphi* (ATCC 9150). The activity was better when compared with the MIC value (500

µg/mL) of the plant extract (*P. granatum*) in the initial screening (Table 3) against the tested bacteria. The disparity might be as a result of an antagonistic or combine behavior of the phytochemical constituents of the plant extract [16]. The fractionation of the fruit extract of *P. granatum* using methanolic extract was investigated by [17] the zone of inhibition (11.2 mm and 14.3 mm) at the concentration of 0.5 mg/mL was determined against clinical isolates of *S. Paratyphi* A and *S. Paratyphi* B, using agar well diffusion method.

Fraction 3 and 6 of *T. catappa* were found to inhibit the growth of *S. Paratyphi* (ATCC 9150) with low MIC value of 250 µg/mL. The finding of this study is in conformity with the result obtained by [18], revealing a moderate antibacterial activity of the leaf Fraction of the plant (*T. catappa*) against *S. Paratyphi* with 25 mm zone of inhibition (the concentration was not recorded). The possible reasons for the antibacterial activity of the fractions might be presence of bioactive compounds, disruption of bacterial cell membrane, inhibition of bacterial enzymes, interference with bacterial DNA or protein synthesis, synergistic effects, and antioxidant activity of the fractions. Fractions that show no activity or very week MIC might not contain sufficient amounts of the bioactive compounds responsible for the desired activity, or the fractionation process might lead to degradation, loss, or degradation of bioactive compounds. Another possible reason might be inadequate separation or purification of bioactive compounds, synergistic effect lost, solvent or method incompatibility, and inactivity of the specific fraction. Tetracycline is a broad-spectrum antibiotic that inhibits bacterial protein synthesis. Its mechanism of action involves, binding to the bacterial ribosome, inhibition of aminoacyl-tRNA binding, inhibition of protein synthesis and bacteriostatic effect.

CONCLUSION

The result of the present study revealed different Minimum Inhibitory Concentration (MIC) of the 3 plant extracts against the tested bacteria. The fractionated extracts did not show good antibacterial activity, since only Fraction 6 of *M. koenigii* exhibited good MIC value of 62.5 µg/mL against *L. monocytogenes* and 31.25 µg/mL against *S. aureus* and *E. coli* O157:H7, but the rest of the fractions showed MIC value ranging from 125-1000 µg/mL. Further research will focus on the detection of the bioactive compounds using Gas Chromatography Mass Spectrometry (GCMS).

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