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Identification of Antibacterial Compounds in Black Garlic against *Salmonella typhimurium* using HPLC-Fingerprinting and Chemometrics

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ABSTRACT. Black garlic is an aged product derived from fresh garlic and exhibits potential antibacterial properties. This study aims to identify the antibacterial compounds in black garlic against *Salmonella typhimurium* using HPLC fingerprinting and chemometrics. The antibacterial activity of the *n*-hexane fraction, methanol fraction, and water fraction were analyzed using the well diffusion method. Only the water fraction exhibited an inhibitory zone against *S. typhimurium* of 11.96 ± 1.86 mm. The chemical profile of each fraction, analyzed by HPLC-MWD, was correlated with antibacterial activity data using Orthogonal Projection to Latent Structure. A retention interval of 2.50 – 2.99 and 2.00 – 2.49 minutes of the water fraction at a wavelength of 214 nm demonstrated a correlation with antibacterial activity. The peaks identified in HPLC-MWD at a retention interval of 2.50 – 2.99 and 2.00 – 2.49 minutes exhibited a similar pattern to the peaks appearing at a retention time of 1.12 and 1.06 minutes on the UHPLC-Q-Orbitrap-HRMS chromatogram. The resultant peak in the UHPLC-Q-Orbitrap-HRMS analysis of the water fraction indicated that it was attributed to S-allyl cysteine and S-allyl mercaptocysteine.

Keywords: black garlic, metabolomics, S-allyl cysteine, S-allyl mercapto cysteine

INTRODUCTION

Salmonella typhimurium is a Gram-negative bacterial serovar that belongs to the genus *Salmonella*. *S. typhimurium* is considered a pathogenic bacterium capable of causing infections in both humans and animals (Sun et al., 2019). Infections resulting from *S. typhimurium* can lead to disruption in the gastrointestinal tract, potentially resulting in fatal consequences. The source of infection is often traced back to food contaminated with *Salmonella* sp. (Merino et al., 2019). The management of infections caused by *S. typhimurium* typically involves the use of antibiotics. However, it has been observed in some cases that these pathogenic bacteria have resistance to antibiotics (Kirst, 2013). The emergence of antibiotic resistance among pathogenic bacteria underscores the exploration of alternative antibacterial agents derived from natural sources. One such natural ingredient showing potential antibacterial properties is black garlic.

Black garlic is an aged garlic product subjected to heat treatment at 60 – 90 °C with a relative humidity of 70 – 90 % for 30 – 45 days without any additional treatment (Bae et al., 2012). Studies have reported that black garlic exhibits antibacterial potential against bacteria known to cause food product spoilage (Ngan et al., 2017; Jang et al., 2018; Botas et al., 2019; Chang & Jang, 2021; Hue et al., 2022). Black garlic is known to contain organosulfur compound S-allyl cysteine (Wang et al., 2010), polyphenols (Choi et al., 2014), melanoidin (Yuan et al., 2018), and reduction sugars (Martinez-Casas et al., 2017). Despite the extensive examination of the antibacterial potential and compounds found in black garlic, research focus on identifying specific active compounds responsible for its antibacterial properties remains limited. Further scientific investigation is necessary to comprehensively explore this aspect.

The identification of active compounds in a plant can be conducted through a metabolomic approach. Metabolomics is a comprehensive analysis aimed at measuring the metabolites of an organism or biological sample (Idle & Gonzales, 2007). This approach enables the determination of correlations between bioactivity and chemical profiles, facilitating the identification of bioactive components in plants (Yuliana et al., 2011). Metabolomics analysis involves a thorough extraction and fractionation process using solvents with varying polarity levels to achieve a comprehensive dispersion of compounds from each fraction (Yuliana et al., 2011a). The resulting fraction are then used in the analysis of the bioactivity and chemical profile of black garlic. The initial stage in identifying active compounds in plants using a metabolomic approach involves fingerprint profile analysis. Fingerprint profile analysis serves to show the overall chemical compound profile of plant (Liang et al., 2010). High-performance liquid chromatography (HPLC) is one of instrument commonly utilized in the metabolomics approach for identification (Tao et al., 2011). The application of the metabolomics approach with HPLC fingerprint profiles has been successfully used in the identification of antibacterial compounds in extract of *kecombrang* flower (*Etlingera elatior*) (Maser & Yuliana, 2017) and the methanolic extract of *temu tis* (*Curcuma purpurascens*) (Afriyanti et al., 2023). Due to the abundance of data generated from fingerprint profiles, a comprehensive analysis using multivariate data analysis is essential.

Multivariate data analysis is a statistical approach used in a metabolomics, taking into account the vast amount of data acquired. The specific multivariate data analysis technique utilized is orthogonal projection to latent structure (OPLS), which enables the determination of the correlation between the chemical profile and bioactivity of a plant (Yuliana et al., 2011a). The outcomes of OPLS analysis can identify retention intervals based on HPLC profile analysis that are believed to contribute to specific bioactivity. Subsequently, the correlated retention intervals from the active fraction are utilized the identification of active compounds presumed to act as antibacterial agents using ultra-high performance liquid chromatography (UHPLC) Q-Orbitrap high resolution mass spectrometry (HRMS). Therefore, this study aims to identify the antibacterial compounds in black garlic against *S. typhimurium* using HPLC-based metabolomic approach. The results of this study are expected to provide information about the specific compound of black garlic that have potential as antibacterial agents.

EXPERIMENTAL SECTION

Preparation of Black Garlic

Black garlic is derived from fresh garlic sourced from Sembalun village, East Lombok, Indonesia. The fresh garlic undergoes an aging process at a temperature of 70 °C with a relative humidity of 90% for a duration of 30 days (Botas et al., 2019). Subsequently, the black garlic is dried using a freeze dryer (Christ Alpha 2-4 LO Plus, Germany) at a temperature of ± -60 °C and then finely ground using a grinder (Getra IC-10B, Indonesia).

Fractionation of Black Garlic

Fractionation was carried out in successive stages using *n*-hexane, methanol, and water solvents. Fractionation with *n*-hexane and methanol solvents was conducted using the Soxhlet method based on a modified previous study (Rosalina et al., 2023). Black garlic, weighed at 20 g and wrapped in filter paper, was placed into the Soxhlet apparatus lead part. Fractionation was initiated by adding ± 175 mL of *n*-hexane solvent, followed by extraction using the Soxhlet method for ± 2 hours. The black garlic sample was removed, and the solvent mixes with the extract was initially evaporated using Soxhlet system. The remaining extract in the boiling flask was dried using *n*-hexane solvent, was reused, and the same steps were repeated to obtain methanol fraction. Meanwhile, water solvent fractionation was performed through sonication using an ultrasonic bath (Selecta, Indonesia) for 30 minutes at a room temperature, followed by filtration and collection of the filtrate. The resulting fractions had their solvents evaporated using a rotary evaporator (Buchi Heating Bath B-300, Switzerland) at temperature of 40 °C until a concentrated black garlic extract was obtained. The fractionation process was repeated 10 times. All chemical used in this stage were of analytical grade and obtained from the Laboratory of Chemistry, Department of Food Science and Technology, IPB University, Indonesia.

Antibacterial Activity

The antibacterial activity analysis of black garlic fractions was conducted using the agar well diffusion method (Asnani et al., 2017). Each fraction was dissolved in DMSO at a concentration of 150 mg/mL. A single colony of *S.* Typhimurium ATCC 14028 was picked, dissolved in 10 mL NB media, and incubated at 37 °C for 24 hours. A volume of 0.5 mL bacterial suspension was added to sterilized NA media and poured into Petri dishes at ± 25 mL. The media in the Petri dishes were allowed to solidify, and wells with a diameter of 6 mm were created. Each well received 60 µL black garlic fraction, chloramphenicol as a positive control, and DMSO as a negative control. The Petri dishes were then incubated at 37 °C for 24 hours, observed and the diameter of the inhibition zones formed was measured using a digital caliper. The antibacterial activity testing was conducted in triplicate. All chemical used in this stage were of analytical grade and obtained from the SEAFAST Center, Bogor, Indonesia.

HPLC-MWD Analysis

The chemical profile analysis using HPLC was conducted through an optimization process. Each fraction was dissolved and filtered using a 0.22 µm PTFE membrane filter. The instrument utilized was an HPLC-MWD (multiple wavelength detector) system, employing a C_{18} column (150 x 4.6 mm, 5 µm) with a solvent flow rate of 1 mL/minute. The mobile phase consisted of water (A) and acetonitrile (B) with a gradient elution time of 20 minutes, comprising the following composition: 0 – 1 minutes (90% A: 10% B), 1 – 10 minutes (15% A: 85% B), 10 – 18 minutes (10%

A: 90% B), and 18-20 minutes (90% A: 10% B). The wavelength used for detection was 214 nm, and the injection volume was 2.5 µL. The HPLC chromatogram shows the peaks of compounds that have retention time (minutes) and relative area (%). Data on retention time and relative area are tabulated in *excel* form used for multivariate data analysis. All chemical used in this stage were of analytical grade and obtained from the Laboratory of Instrument Analysis, Department of Food Science and Technology, IPB University, Indonesia.

UHPLC-Q-Orbitrap-HRMS Analysis

The fraction exhibiting the highest antibacterial activity was subjected to analysis using UHPLC-Q-Orbitrap-HRMS (Listyorini et al, 2021). The instrument employed for this analysis was the UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS (Thermo Scientific). The column used was C_{18} column (100 x 2.1 mm, 1.5 µm) with a flow rate of 0.2 mL/minutes. The mobile phase consisted of H_2O + 0.1% formic acid (A) and acetonitril + 0.1% formic acid (B), with a gradient elution time of 30 minutes, composed as follows: 0 – 1 minutes (95% A: 5% B), 1 – 25 minutes (95 – 5% A: 5 – 95% B), 25 – 28 minutes (5% A: 95% B), 28 – 33 minutes (95% A: 5% B). The injection volume used was 2.5 µL, mass range 100 – 1500 m/z, employing negative ionization mode. The UHPLC-Q-Orbitrap-HRMS chromatogram was matched with peak pattern data with the HPLC chromatogram. Peaks on the UHPLC-Q-Orbitrap-HRMS chromatogram were identified as peaks correlated with antibacterial activity, then compared to the metabolomics workbench database based on mass spectrum data. All chemical used in this stage were of analytical grade and obtained from the Advanced Research Laboratory of IPB University, Indonesia.

Statistical Analysis

The antibacterial activity data of black garlic were subjected to a complete randomized design analysis. Differences indicated by the variance analysis were further investigated using Duncan's Multiple Range Test (DMRT) at a significance level of 5%, employing SPSS software (IBM SPSS Statistics, 25, USA) version 25.0. Chemical profile data were correlated with antibacterial activity data to identify active compounds using multivariate data analysis, specifically OPLS, in SIMCA software version 13.0 (Sartorius Stedim Biotech GmbH, Gottingen, Germany). Several OPLS outputs utilized for data interpretation included score plot, S-plot, Y-related coefficient plot, and VIP plot. Retention time intervals correlating with antibacterial activity were determined using OPLS. Subsequently, fractions with dominant retention times were analyzed using UHPLC-Q-Orbitrap-HRMS. UHPLC-Q-Orbitrap-HRMS chromatograms were aligned with peak pattern data from HPLC-MWD chromatograms. Peak in the UHPLC-Q-Orbitrap-HRMS chromatogram, identified as correlating with antibacterial activity, were then cross-referenced with the metabolomic workbench database based on mass spectrum data.

RESULTS AND DISCUSSION

Antibacterial Activity of Black Garlic

The antibacterial activity of black garlic fractions against *S. typhimurium* was expressed in terms of the diameter of inhibition zone (DIZ) (**Figure 1**). The water fraction exhibited the highest antibacterial activity (11.96 ± 1.86 mm), while the *n*-hexane and methanol fractions did not show any DIZ (0 ± 0 mm) against *S. typhimurium*. These results indicate that the water fraction is the active fraction as it formed an inhibition zone, whereas the *n*-hexane and methanol fractions are considered inactive fractions. Chloramphenicol, utilized as the positive control, demonstrated a significantly high DIZ (36.37 ± 0,73 mm), while DMSO, employed as the negative control, did not yield any inhibition zone (0 ± 0 mm).

The analysis of antibacterial activity in the black garlic fraction showed that only the water fraction formed the inhibitory zone against *S. typhimurium*, while the *n*-hexane and methanol fraction do not show an inhibition zone. These findings suggest that the compounds responsible for the antibacterial activity in black garlic predominantly originate from polar constituents present in the water fraction. *S. typhimurium* is a Gram-negative bacterium characterized by hydrophilic outer membrane that contains lipopolysaccharide molecules, serving as a protective barrier against antibacterial agents (Sharma et al., 2013). Nevertheless, the observed capability of the water fraction from black garlic to inhibit *S. typhimurium* suggest that antibacterial compounds may penetrate bacterial cells through general bacterial porins. This penetration is believed to impact bacterial enzymes, potential leading to cellular lysis and subsequent cell death (Patra et al., 2015). Black garlic reportedly contains alkaloid compounds, flavonoids, and organosulfur compounds believed to contribute to its bioactivity, particularly its antibacterial properties (Wang et al., 2010; Choi et al., 2014).

Correlation of Chemical Profile and Antibacterial Activity of Black Garlic based on Multivariate Data Analysis.

Results of multivariate data analysis based on principal components analysis (PCA)

The PCA score plot demonstrates good grouping and separation among fractions based on the polarity of each solvent (**Figure 2**). The *n*-hexane fractions, characterized as the most non-polar, are clustered on the left side of the plot, whereas the semi-polar methanol fraction and the polar water fraction are clustered on the right side. These findings indicate that all fractions have been distinctly grouped and separated according to their respective levels of polarity. PCA serves as an initial analysis aimed at

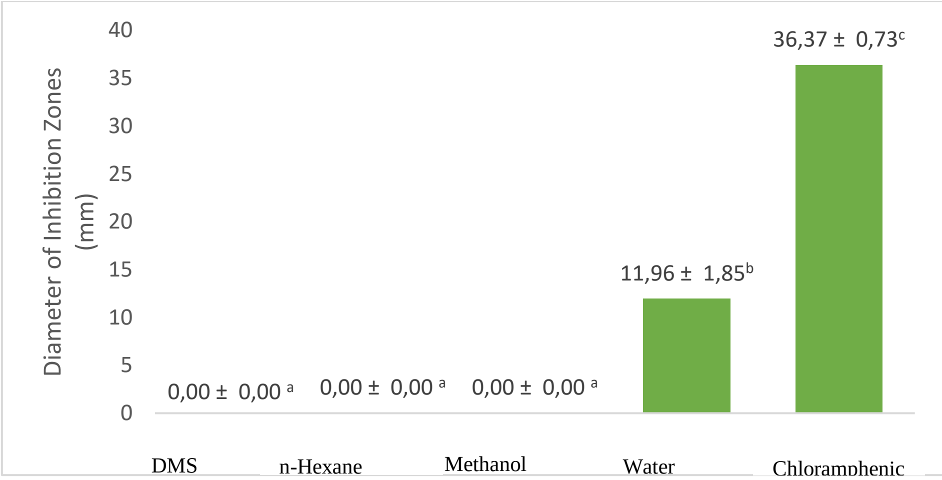

Figure 1. The antibacterial activity of the black garlic fraction at a concentration of 150 mg/mL against *S. typhimurium*. Note: The values are expressed as mean ± standard deviation (SD). Superscript differences indicate statistically significant variations based on the DMRT at $p < 0.05$

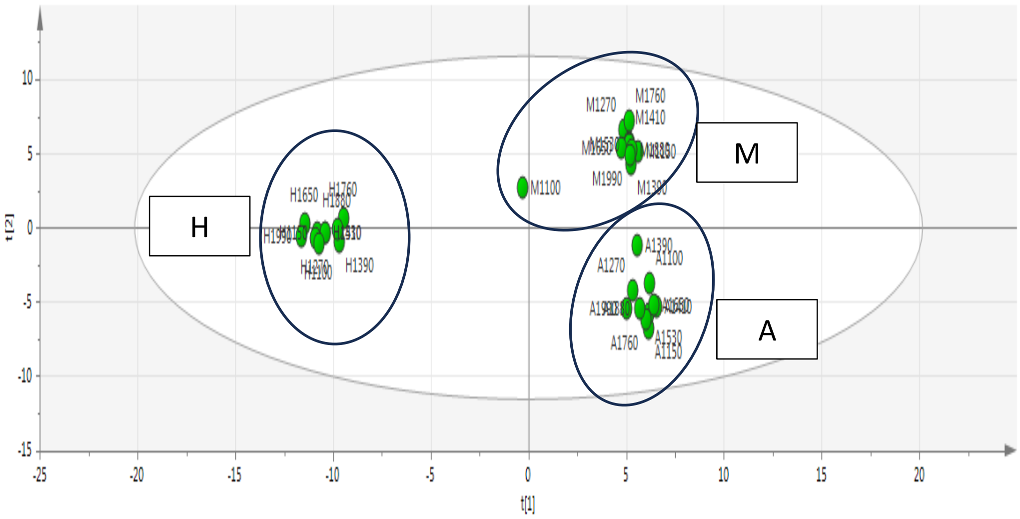

Figure 2. Score plot (H: *n*-hexane fraction, M: methanol fraction, A: water fraction)

observing the sample grouping based on the solvent's polarity level. The PCA model achieved an R^2X value of 0.91 and a Q^2 of 0.84. The model has a value of ≥ 0.5 so it is included in the category of good in representing data, so that it can be used for further analysis, particularly with OPLS (Worley & Powers, 2016).

***Results of multivariate data analysis based on orthogonal projection to least square Analysis* (OPLS)**

The objective of OPLS analysis is to correlate two types of data matrices, specifically metabolite chemical profile data and bioactivity data (Yuliana et al., 2011a). In this study, the metabolite chemical profile data represents the retention intervals in the HPLC-MWD chromatogram, while the bioactivity data represents the antibacterial activity of black garlic. The OPLS score plot (**Figure 3a**) shows a grouping of fractions based on antibacterial activity, where the water fraction (A) is identified as the active fraction, while the *n*-hexane fraction (H) and methanol fraction (M) are categorized as inactive fractions. The water fraction (A), identified as the most active, is positioned on the right side of the plot and is denoted by a blue circle. Conversely, the *n*-hexane (H) and methanol (M) fractions are on the left side of the plot and are marked with a red circle indicating they are inactive fractions. The S-plot (**Figure 3b**) showed a dominant retention interval in the active and inactive fractions, indicating positive, uncorrelated, and negative correlations with antibacterial activity.

Y-related coefficient plots showed retention intervals that exhibited correlation with the antibacterial activity of black garlic (**Figure 4a**). Notably, the retention interval of 2.50 - 2.99 and 2.00 – 2.49 minutes had the highest Y-related coefficient value, which was 1.53 and 1.24, indicating the strongest correlation with antibacterial activity. VIP plots show the effect of the retention intervals on sample clustering based on

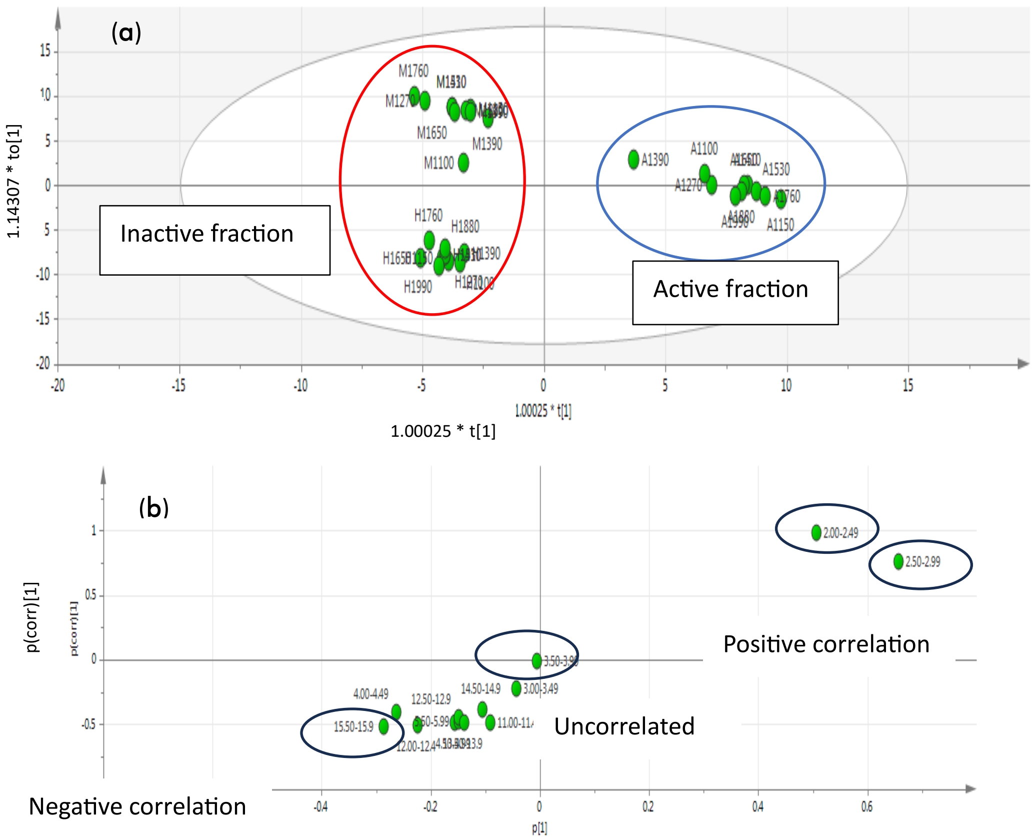

Figure 3. (a) Score plot OPLS and (b) S-plot OPLS.

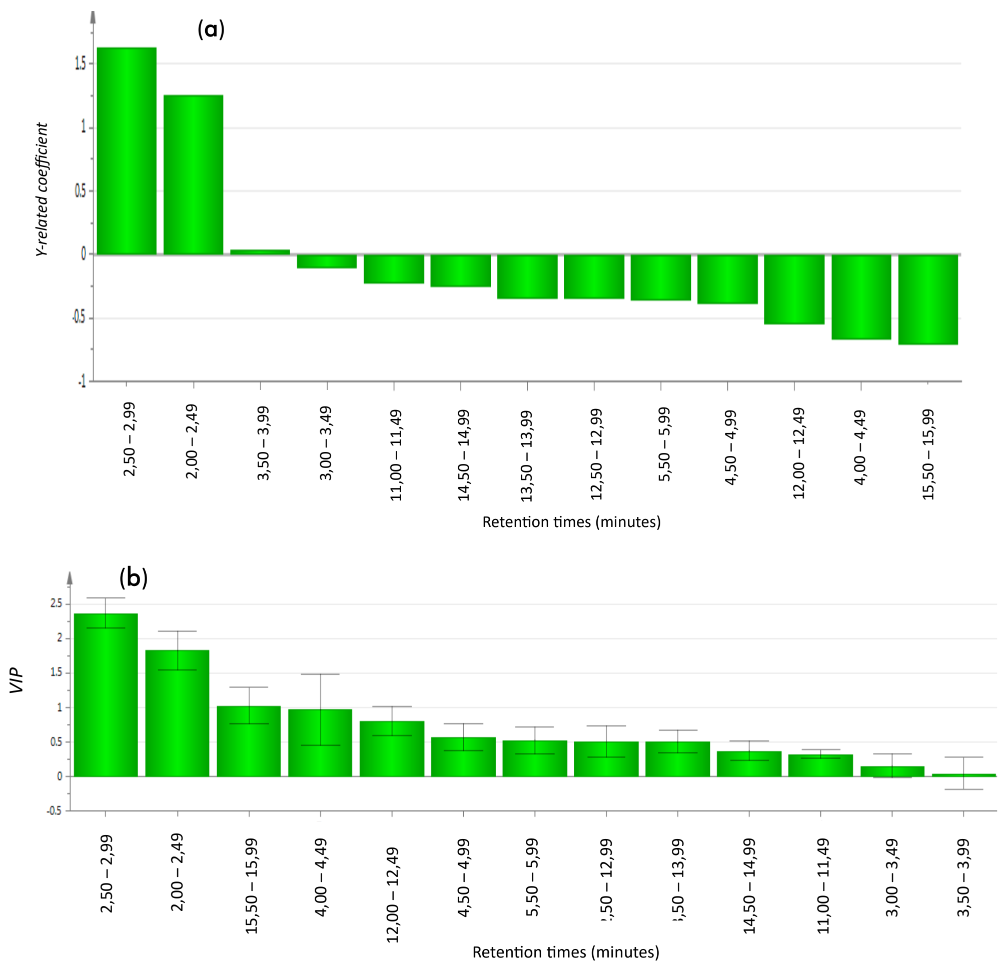

Figure 4. (a) Y-related coefficient plot and (b) VIP plot.

antibacterial activity (**Figure 4b**) (Rosalina et al., 2023). Specifically, VIP plots with values exceeding 1 and error bars not touching the X axis were utilized (Worley & Powers., 2016). The retention interval of 2.50 – 2.99 and 2.00 – 2.49 minutes, which demonstrated the highest Y-related coefficient value, also has the highest VIP value of 2.23 and 1.76. Consequently, based on the OPLS analysis, it can be concluded that the retention interval of 2.50 – 2.99 and 2.00 – 2.49 minutes exhibits the most significant correlation with antibacterial activity.

The accuracy of the OPLS model can be evaluated by the values of R^2Y and Q^2Y. The R^2Y value signifies the extent to which the Y variables can be explained by the model and serves as a measure of model fitness, while the Q^2Y values is an indicator of cross-validation, quantifying how well the predicted results align with the actual data (Worley & Powers, 2016). In the OPLS analysis based on antibacterial activity, the R^2Y and Q^2Y values are 0.90 and 0.88, respectively. These values indicate that the generated OPLS model include into the category of being good at representing data, as it possesses R^2Y and $Q^2Y \geq 0,5$ (Worley & Powers, 2016). Furthermore, the validation of the OPLS model can be ascertained through CV-ANOVA. The CV-ANOVA validation outcome is determined by the obtained p-value, which in this case is 3.67×10^{-11}. The resulting p-value is smaller than the acceptable maximum threshold ($p < 0.05$), leading to the conclusion that the OPLS model exhibits sufficient validity (Errikson et al., 2008).

Antibacterial Compounds of Black Garlic

The OPLS results indicate that the peak potentially contributing to antibacterial activity in black garlic corresponds to a retention interval of 2.50 – 2.99 and 2.00 – 2.49 minutes, predominantly present in the water fraction. To confirm the compound identify, chromatogram patterns of the water fraction were compared between HPLC-MWD and UHPLC-Q-Orbitrap-HRMS analyses (**Figure 5**). Peaks observed in the water fraction at a retention time of 2.60 and 2.34 minutes on the HPLC-MWD chromatogram align with the retention interval of 2.50 – 2.99 and 2.00 – 2.49 minutes. Furthermore, this peak exhibits a consistent pattern with the peak at a retention time of 1.12 and 1.06 minutes on the UHPLC-Q-Orbitrap-HRMS chromatogram.

Compounds detected at a retention time of 1.12 and 1.06 minutes were matched against the Metabolomic Workbench database using mass spectral data. Subsequent to the database matching, the compound was identified as S-allyl cysteine (SAC), characterized by molecular ions at 161 m/z and fragment ions (m/z) at 71, 85, 95, 113, 145, and 161, and S-allyl mercaptocyteine (SAMC), characterized by molecular ions at 193 m/z and fragment ions (m/z) at 85, 87, 105, 111, and 191 (**Figure 6**).

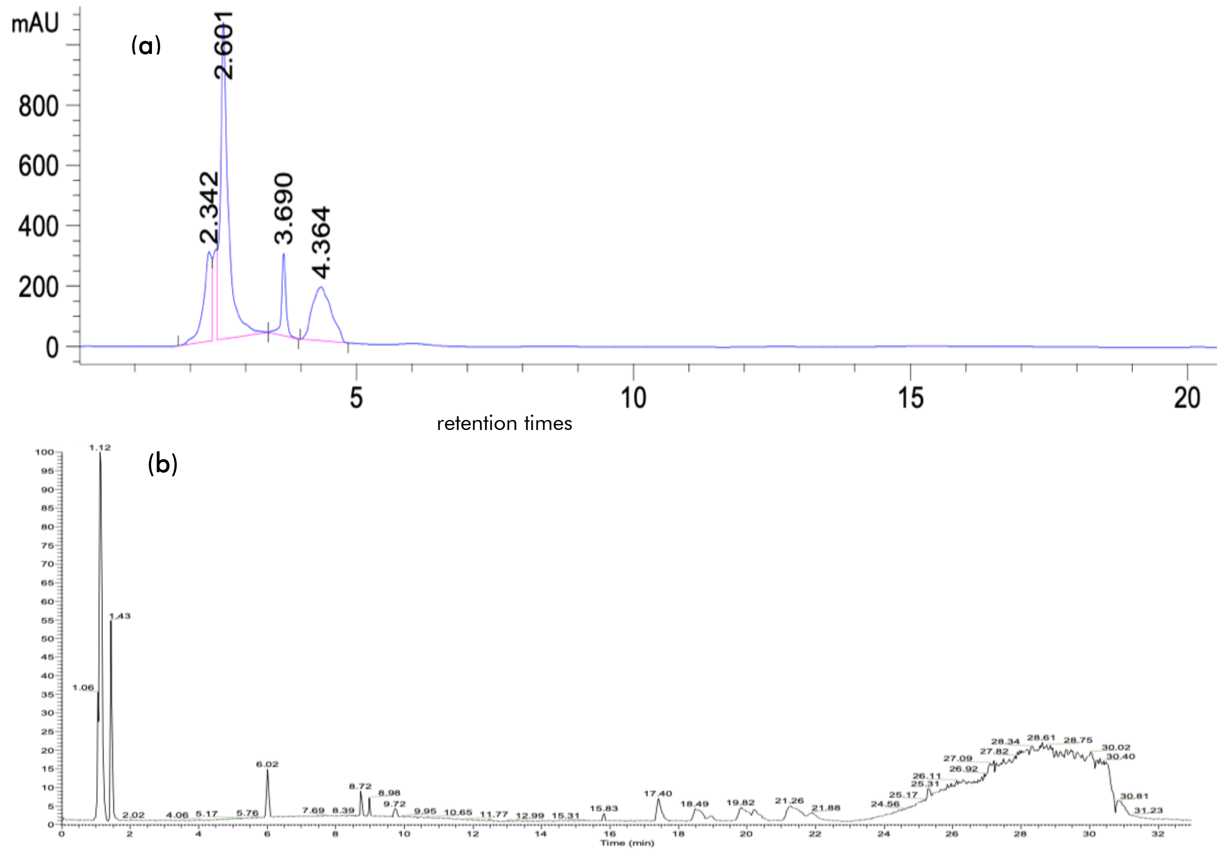

Figure 5. Chromatogram of (a) HPLC-MWD and (b) UHPLC-Q-Orbitrap-HRMS from water fraction of black garlic.

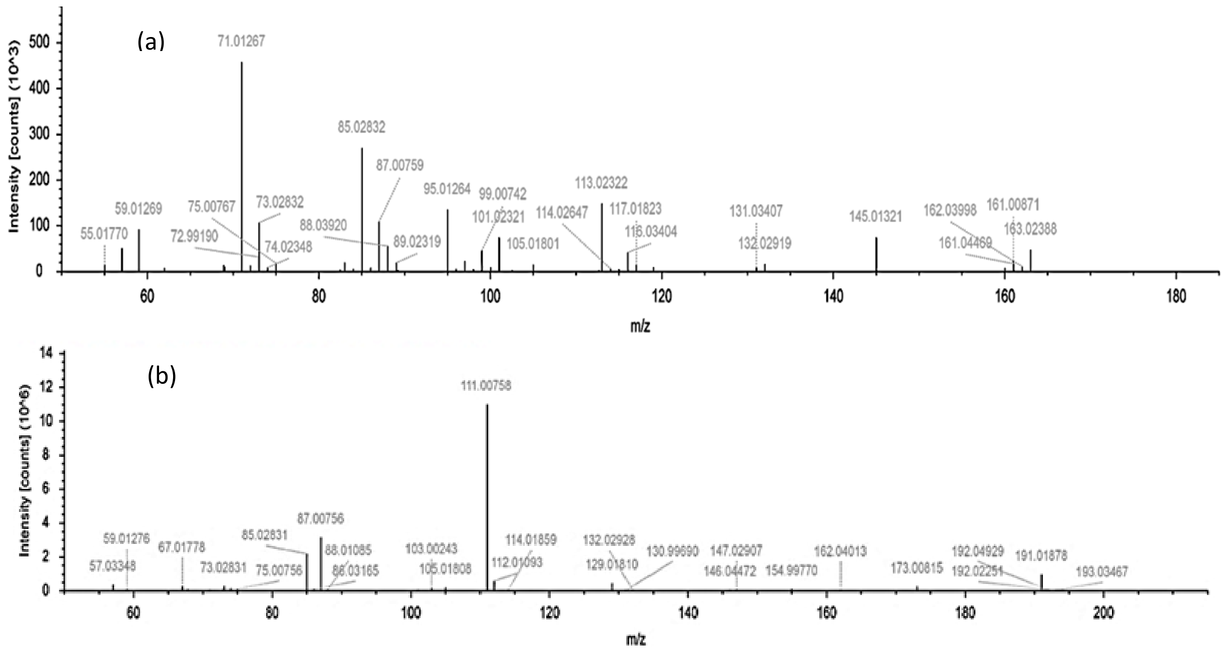

Figure 6. Fragmentation patterns (m/z) in the [M-H]$^-$ ionization mode of (a) SAC and (b) SAMC

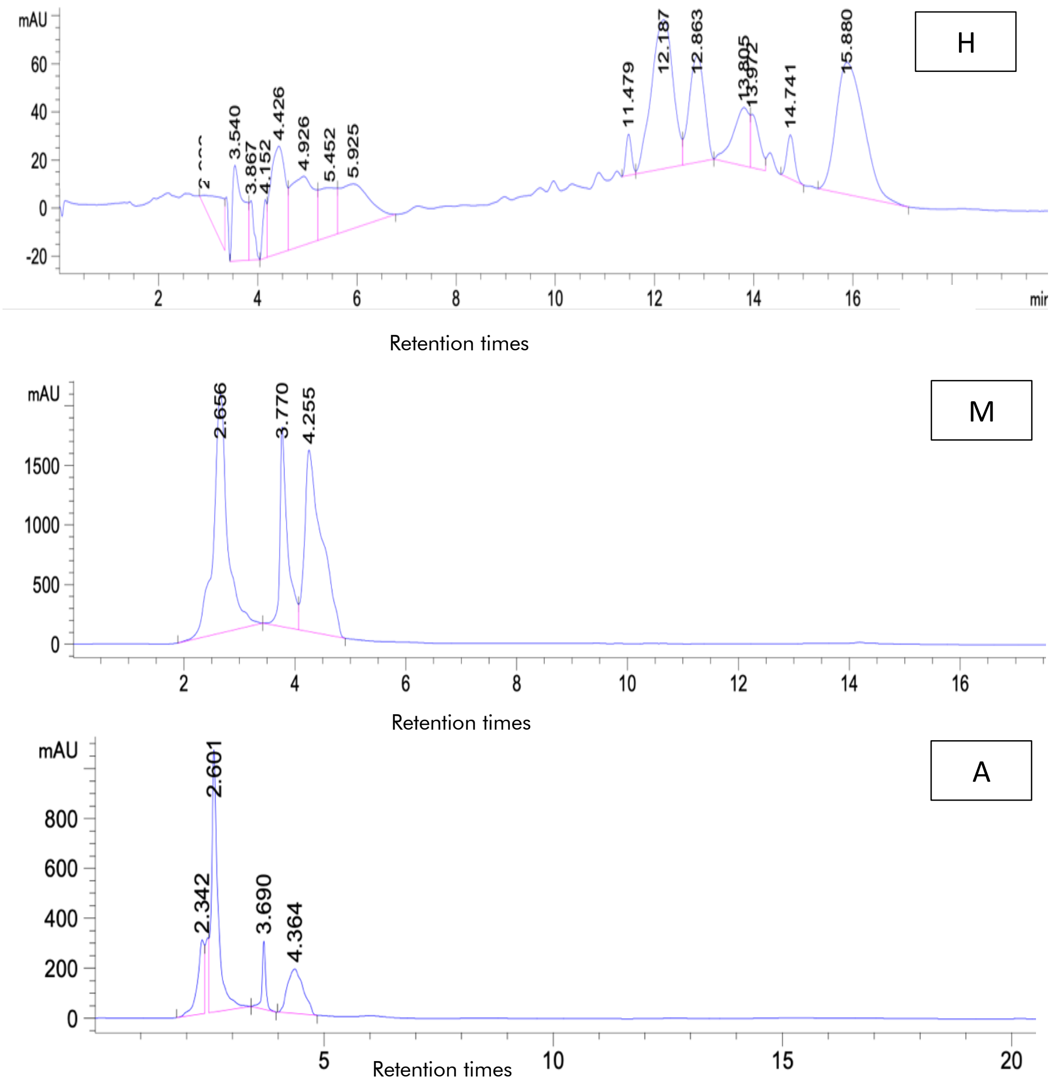

Figure 7. HPLC-MWD chromatogram of *n*-hexane fraction (H), methanol fraction (M), and water fraction (A)

SAC is an organosulfur compound formed due to the activity of γ-glutamyl-transpeptidase enzyme which converts γ-glutamyl-S-allyl-L-cysteine (GSAC) in fresh garlic into SAC in black garlic. Organosulfur compounds in both fresh garlic (allicin) and black garlic (SAC) are often associated with their bioactivity, including antibacterial properties. Organosulfur compounds, with SAC, being the dominant compound, are prevalent in black garlic. SAC compounds are formed during the high-temperature treatment applied in the aged garlic process. The concentration of SAC compounds in black garlic is known to significantly increase compared to fresh garlic (Sasaki et al., 2007). While research on SAC compounds antibacterial properties is still limited, it has been reported that black garlic aged for 30 – 35 days has better antibacterial activity than fresh garlic against *E. coli, P. aeruginosa, S. aureus, L. monocytogenes* (Botas et al., 2019; Chang & Jang, 2021). This enhanced antibacterial activity is attributed to the heightened concentration of SAC compounds following the aging process, suggesting a potential role for SAC compounds in the antibacterial efficacy of black garlic.

In addition to the SAC compound, during the aging process, another compound called SAMC is formed. SAMC is a derivative of the allicin compound. The potential of SAMC in the bioactivity of both fresh garlic and black garlic has been extensively studied, such as its role as an antioxidant (Pedraza-Chaverri et al., 2004), anticancer (Yi et al., 2019), and antibacterial (Fujisawa et al., 2009). Prior study showed that allicin-derived compounds, including SAMC, have antibacterial potential (Miron et al., 2000). Similar results were also demonstrated that SAMC exhibits antibacterial properties against *S. aureus* and *E.coli* (Fujisawa et al., 2009).

For comparison, HPLC-MWD chromatograms of each fraction are also provided (**Figure 7**). The retention interval of 2.50 - 2.99 minutes was not observed in the *n*-hexane fraction, but was detected in both the methanol fraction and the water fraction. Notably, the retention interval of 2.50 - 2.99 minutes is prevalent in the water fractions, consistent with the findings of antibacterial activity analysis, which indicates that the water fraction exhibits the highest antibacterial activity. Meanwhile, the retention interval of 2.00 – 2.49 minutes only detected in the water fraction. Both 2.50 – 2.99 and 2.00 – 2.49 minutes are the dominant peaks in water fraction, so it can be predicted that they contribute to antibacterial activity of black garlic.

CONCLUSIONS

The results of the antibacterial activity analysis showed that the water fraction of black garlic had the highest antibacterial activity against *S. typhimurium*. The results of the OPLS analysis demonstrated that the retention interval most correlated with the antibacterial activity of black garlic was 2.50 – 2.99 and 2.00 – 2.49 minutes, which can be attributed to SAC and SAMC. A suggestion for further research is to re-evaluate the antibacterial activity using standard SAC and SAMC. Additionally, there is a need for further investigation into the antibacterial mechanisms of black garlic.

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