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Optimization of Extraction Process and Effect of Time on Chemical Composition and Antimicrobial Activity of *Allium sativum* (Garlic) and *Allium schoenoprasum* (Chives) Volatile Fractions

Vo Thi Thu Hang[1,2], Nguyen Thi Van Anh[1]

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ABSTRACT

Background: This study optimized the hydrodistillation kinetics of *Allium sativum* (garlic) and *Allium schoenoprasum* (chives) bulbs from North and Central Vietnam and evaluated their bioactivity.

Methods: Extraction parameters were investigated by varying pretreatment methods, material-to-water ratios and distillation time. The chemical compositions of the obtained volatile fractions were analyzed *via* GC-MS, while biological activities were assessed through antioxidant assays and antibacterial testing against *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 12943 using inhibition zone diameters and MIC values.

Result: Ultrasound-assisted pretreatment yielded the best results ($p \leq 0.05$) at a 1:3 material-to-water ratio over 3 hours (1.00% for garlic; 1.34% for chives). Chemically, garlic volatile fraction was stable with diallyl disulfide as the major component, whereas chives volatile fraction showed a transient accumulation of allyl propyl sulfide at 3 hours. Biologically, garlic volatile fraction displayed high antioxidant capacity comparable to vitamin C and potent antibacterial activity against *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 12943 (Inhibition zone: 21 mm; MIC: 12.5 µg/mL). In contrast, chives volatile fraction showed significantly lower efficacy (Inhibition zone: 9.0 mm; MIC: 250 µg/mL).

Key words: *Allium sativum*, *Allium schoenoprasum*, Antimicrobial, Antioxidant, Hydrodistillation, Volatile fraction.

INTRODUCTION

The genus *Allium* (Amaryllidaceae family), comprising 600-700 species, includes some of the most ancient and widely consumed cultivated plants in the world, most notably garlic (*Allium sativum*) and chives (*Allium schoenoprasum*) (Calimpang *et al.,* 2024). Beyond their culinary ubiquity as flavoring agents, these species are renowned for their broad spectrum of biological activities, including antimicrobial, antioxidant, anti-inflammatory and anticancer properties (Danquah *et al*., 2022; Iwar *et al*., 2024). These bioactive effects are primarily attributed to their rich content of organosulfur compounds (Dai *et al*., 2025). Unlike typical essential oils which are predominantly terpene-based, the volatile fractions of *Allium* species are characterized by a complex matrix of sulfur-containing constituents such as diallyl disulfide (DADS), trisulfides and vinyldithiins. Crucially, these compounds are not inherently present in intact tissues but are formed enzymatically and thermally from non-volatile precursors like alliin upon tissue rupture and extraction processing (Edris and Fadel, 2002).

Conventional extraction is often hindered by prolonged processing and thermal degradation of labile sulfur constituents (Stratakos and Koidis, 2016). To mitigate this, green intensification via ultrasound and microwave pretreatments is increasingly adopted. Mechanistically, ultrasound employs acoustic cavitation for cell disruption, whereas microwaves utilize ionic conduction and dipole rotation to induce rapid heating (Mandal *et al*., 2007; Tiwari, 2015; Patil *et al*., 2018). However, the comparative efficacy of these interventions on *Allium* tissues during hydrodistillation requires further investigation.

Furthermore, most extraction optimization studies prioritize yield maximization, often overlooking the temporal evolution of chemical constituents. While recent literature has evaluated the chemical profiles of *Allium* volatile fractions, these studies predominantly rely on end-point analyses after a fixed extraction duration (Dehariya *et al*., 2021;

[1]Faculty of Engineering and Food Technology, University of Agriculture and Forestry, Hue University, 102 Phung Hung Street, Hue 49000, Vietnam.

[2]Department of Food Biotechnologies and Agricultural Product Quality, Faculty of Agriculture and Technology, University of South Bohemia in Èeské Budìjovice, Studentska 1668, 37005 Èeské Budìjovice, Czech Republic.

Corresponding Author: Nguyen Thi Van Anh, Faculty of Engineering and Food Technology, University of Agriculture and Forestry, Hue University, 102 Phung Hung Street, Hue 49000, Vietnam. Email: ntvanh@hueuni.edu.vn

ORCIDs: 0009-0009-0798-7841, 0000-0003-2242-8094

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Tran *et al*., 2024). Such single-time-point approaches often yield conflicting optimal conditions and fail to capture the dynamic kinetic transformations of the extract. This is particularly critical for *Allium* volatile fractions, where heat-sensitive precursors like allicin degrade rapidly into various transient polysulfides under continuous thermal stress (Zhou *et al*., 2025). Consequently, focusing solely on yield plateaus without tracking these time-dependent chemical shifts can lead to over-processing and the loss of potent bioactive intermediates.

This study pursues two primary objectives. First, it systematically evaluates the comparative efficacy of mechanical, ultrasound-assisted and microwave-assisted pretreatments on the hydrodistillation of *A. sativum* and *A. schoenoprasum* volatile fractions. Second, it investigates how extraction duration influences not only yield but also the kinetic evolution of the chemical profile. By analyzing fractions at 2, 3 and 4-hour intervals via GC-MS, the research aims to elucidate the interplay between thermal processing time, chemical composition and biological activity against *Escherichia coli* and *Staphylococcus aureus*. Ultimately, these findings provide a scientific basis for optimizing extraction protocols to prioritize specific bioactive profiles over mere volume maximization.

MATERIALS AND METHODS

Plant materials

Fresh garlic (from Son La) and chives (from Quang Tri) bulbs were harvested (April-August, 2024) and identified according to the Vietnamese Pharmacopoeia. The raw materials were cleaned, skinned and air-dried to moisture contents of approximately 58% and 71%, respectively. Samples were stored in sealed polyethylene bags at 4°C and utilized for extraction within two weeks.

Bacterial strains

Two representative pathogenic bacteria, comprising Gram-negative *Escherichia coli* ATCC 35218 and Gram-positive *Staphylococcus aureus* ATCC 12493, were used in this study.

Volatile fractions extraction

General hydrodistillation protocol

Fresh materials (100 g) were finely crushed and thoroughly mixed with varying volumes of distilled water to achieve the specified material-to-water ratios. The mixture was then transferred to a 1000 mL round-bottom flask connected to a Clevenger-type apparatus for hydrodistillation. To prevent boil-over and ensure efficient mass transfer, the total working volume within the flask was strictly maintained at an optimal level. The flask was heated to the boiling point. The mixture of volatile fractions and water vapor was condensed and collected in a separating funnel. Finally, the volatile fractions were dried with anhydrous Na_2SO_4 to eliminate residual moisture and stored in sealed amber glass bottles at 5°C until analysis.

Experimental design

The optimization of the extraction process was carried out through three sequential single-factor experiments:

Experiment 1: Effect of pretreatment technologies

To evaluate the impact of physical intensification on extraction efficiency, mixtures underwent specific pretreatments prior to hydrodistillation. Microwave-assisted pretreatment involved irradiation at 700 W for 5 min, while ultrasound-assisted pretreatment utilized a Kingsonic bath (Model MC-040S, 300 × 240 × 150 mm) at 40 kHz for 10 min at 35°C. These experimental conditions were subsequently compared against a control group subjected solely to grinding (conventional hydrodistillation).

Experiment 2: Effect of material-to-water ratio

The influence of the solvent volume was investigated by varying the material-to-water ratio at three levels: 1:2, 1:3 and 1:4 (w/w). During this stage, the pretreatment method was kept constant based on the results from Experiment 1, while the extraction time was fixed at 2 hours.

Experiment 3: Effect of extraction time

Using the optimized pretreatment and material-to-water ratio, the effect of extraction time was investigated at 2, 3 and 4 hours. Volatile fractions were collected at these intervals to evaluate variations in both yield and chemical composition.

The volatile fractions yield (%) was calculated using the following equation:

$$\text{Volatile fractions (\%)} = \frac{m_{\text{volatile fractions}}}{m_{\text{sample}}\left(1-\frac{\%\ \text{moisture content}}{100}\right)} \times 100$$

Where,

- Volatile fractions (%): Volatile fractions yield (%).
- $m_{\text{volatile fractions}}$: Mass of obtained volatile fractions (g).
- m_{sample}: Mass of the initial raw material (g).

GC-MS analysis

GC-MS analysis was performed on an Agilent 7890B system coupled with a 5977B detector and a DB-1 column (30 m × 0.25 mm, 0.25 µm). A 1 µL sample was injected at 250°C (split 1:200) with helium carrier gas at 1 mL/min. The temperature program initiated at 45°C, ramped at 5°C/min to 150°C, then 40°C/min to 290°C (held 5 min). MS conditions included 70 eV ionization, source at 230°C, quadrupole at 150°C and a scan range of m/z 30-500. Data were processed using MassHunter. The volatile compounds were tentatively identified by comparing their mass spectra with the NIST 2017 library (match factor >85%). To support this tentative identification, the elution order of the detected compounds on the non-polar DB-1 column was cross-referenced with established literature data. Quantification of the constituents was performed by peak area normalization.

Evaluation of antioxidant activity using the free radical scavenging assay (DPPH)

The antioxidant activity was determined using the DPPH radical scavenging assay (Emami *et al*., 2007). Briefly, 0.5 mL of serially diluted volatile fraction was mixed with 2.5 mL of a 0.004% (w/v) DPPH solution prepared in ethanol. The control contained DPPH solution without volatile fraction, while pure ethanol (99.5%) served as the blank. After incubation in the dark at room temperature for 30 minutes, absorbance was measured at 517 nm against the blank. Ascorbic acid (Vitamin C, 12.5-100 µg/mL) was used as the positive reference standard to validate the assay sensitivity.

The percentage of DPPH radical scavenging activity (Inhibition Capacity - IC%) was calculated using the following equation:

$$IC\ (\%) = \frac{AC - AS}{AC} \times 100$$

Where,

- AC: Absorbance of the control reaction (containing all reagents except the volatile fraction).
- AS: Absorbance of the test sample containing the volatile fraction.

The antioxidant activity was expressed as the IC_{50} value, defined as the concentration of the volatile fraction required to scavenge 50% of the DPPH radicals. The IC_{50} value was determined by plotting the inhibition percentage against the sample concentrations using linear regression analysis. To ensure reliability and method reproducibility, all DPPH assays were performed in independent triplicates (n=3) and the resulting IC_{50} values were expressed as the mean ± standard deviation (SD).

Determination of antibacterial activity and minimum inhibitory concentration (MIC)

The antibacterial efficacy of the volatile fractions was assessed against two bacteria: *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 12493.

Antibacterial activity was screened via the agar well diffusion method (Balouiri *et al*., 2016). Briefly, 50 µL of the volatile fraction was dispensed into 6-mm wells on Mueller-Hinton agar seeded with standardized bacterial suspensions (0.5 McFarland; ~1.5×10^8 CFU/mL). Pure n-hexane served as the solvent control, while Tetracycline (10%) was utilized as the positive reference standard to validate the susceptibility of the tested strains. Inhibition zones were measured after incubation at 37°C for 24 hours. Subsequently, MIC were determined using broth microdilution (Moreira *et al*., 2007). Serial two-fold dilutions of the volatile fractions in 5% DMSO (range: 3.9-2000 µg/mL) were inoculated to a final density of 5×10^5 CFU/mL. After incubation at 37°C for 24 hours, the MIC was recorded as the lowest concentration inhibiting visible growth. To ensure reliability and method reproducibility, all antibacterial assays were performed in independent triplicates (n=3). The resulting inhibition zones were expressed as mean ± standard deviation (SD), whereas the MIC values were reported as the consistent values across the replicates.

Statistical analysis

All experiments were performed in triplicate (n = 3). Data were analyzed using IBM SPSS Statistics 25.0 and expressed as mean ± standard deviation (SD). Prior to one-way ANOVA, data normality and variance homogeneity were verified *via* the Shapiro-Wilk and Levene's tests, respectively. Duncan's multiple range test (DMRT) was selected as the post hoc test for its high sensitivity in detecting differences among multiple groups, with significance set at $p \leq 0.05$.

RESULTS AND DISCUSSION

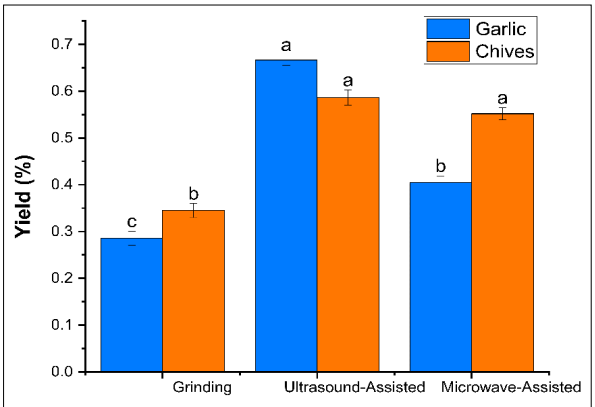
Optimization of extraction conditions

Effect of pretreatments

Fig 1 illustrates that physical intensification significantly enhanced extraction efficiency relative to conventional methods ($p \leq 0.05$). For garlic, yields rose from a baseline of 0.29% (grinding) to 0.40% with microwave treatment, peaking at 0.67% under ultrasound. Chives exhibited a similar trend, improving from 0.34% to 0.55% and 0.59% for microwave and ultrasound-assisted pretreatments, respectively. Consequently, ultrasound was established as the optimal protocol for subsequent trials.

Effect of material-to-water ratio

Following the ultrasound-assisted pretreatment, the influence of the material-to-water ratio on the volatile fraction yield was investigated. Fig 2 reveals a non-linear trend for both plant species. Garlic yield peaked at 0.83% (1:3) before declining to 0.74% (1:4). Similarly, chives yield rose from 0.59% (1:2) to 1.07% (1:3), then dropped



Data are expressed as mean±SD (n = 3). Different letters above the bars indicate statistically significant differences ($p \leq 0.05$) according to Duncan's multiple range test.

Fig 1: Effect of pretreatment methods on the yield of garlic and chives volatile fractions.

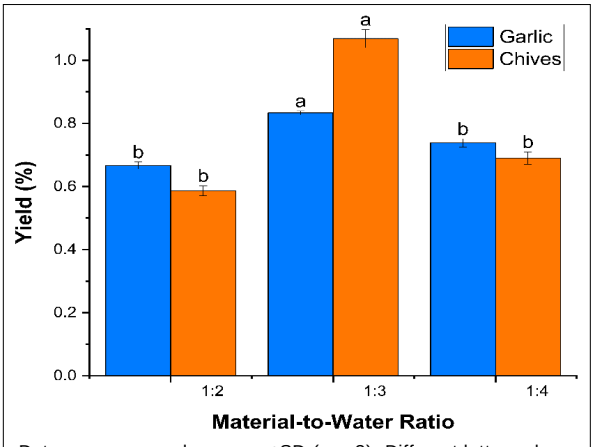
significantly to 0.69% (1:4). These results suggest that a material-to-water ratio of 1:3 provides the ideal equilibrium for extraction efficiency.

Effect of extraction time

Using the optimized pretreatment (ultrasound) and material-to-water ratio (1:3), the kinetics of volatile fraction recovery were evaluated over a period of 4 hours. The time-dependent yield profiles are presented in Fig 3. For garlic, the yield increased progressively from 0.83% at 2 hours to a maximum of 1.00% at 3 hours. However, extending the distillation further to 4 hours resulted in a notable decline in oil recovery to 0.79%. A similar trend was observed for chives, where the yield peaked at 1.34% after 3 hours (up from 1.07% at 2 hours) before slightly decreasing to 1.28% at the 4-hour mark. These results indicate that 3 hours is the optimal duration for the hydrodistillation of these *Allium* species.

Effect of time on chemical compositions

Chemometric analysis confirmed that volatile profiles were driven primarily by species differentiation rather than extraction duration. As shown in the PCA (Fig 5), species formed distinct clusters along PC1 (58.3%), with garlic occupying the positive quadrant and chives the negative quadrant. This separation was corroborated by heatmap data (Fig 6), which contrasted the allyl-sulfide dominance in garlic against the propyl- and methyl-rich signatures of chives. Kinetically, sulfur distribution followed a non-linear trajectory (Fig 4), with bioactive diallyl sulfides peaking at 3 hours across both species; specifically, garlic reached a maximum concentration of >25% before declining. Extending the process to 4 hours triggered a qualitative shift, marked by the conversion of acyclic sulfides to vinyldithiins in garlic and the near depletion of diallyl sulfides in chives (sample 4HC).

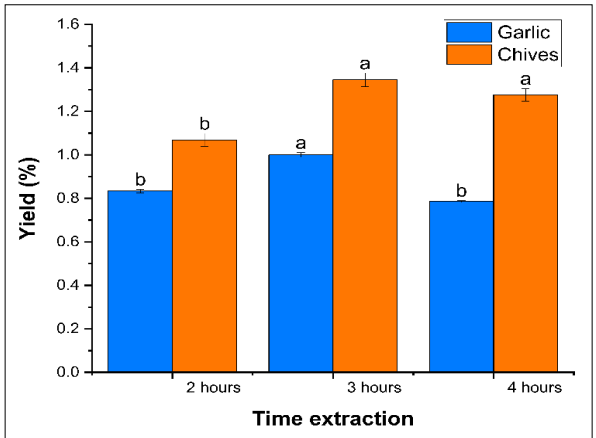


Data are expressed as mean±SD (n = 3). Different letters above the bars indicate statistically significant differences (p≤0.05) according to Duncan's multiple range test.

Fig 2: Effect of material-to-water ratio on the yield of garlic and chives volatile fractions.

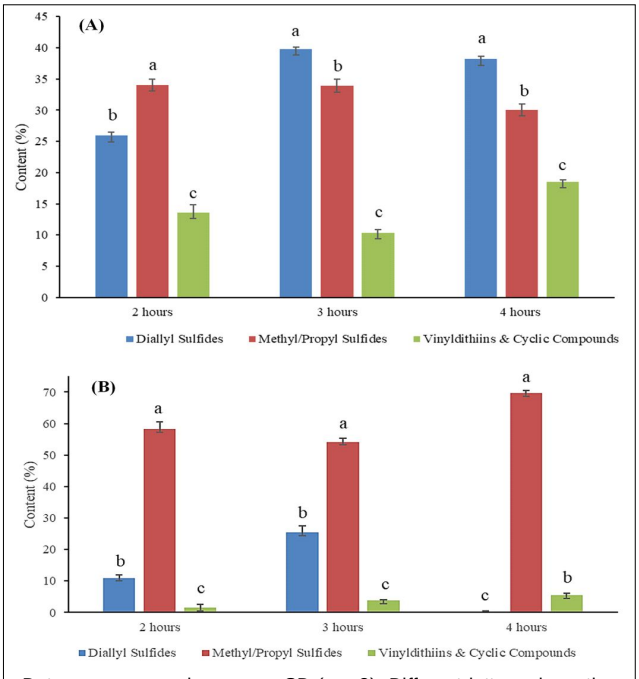
Antioxidant and antimicrobial activity of optimized volatile fractions

The biological assays revealed a significant contrast in the antimicrobial efficacy of the two *Allium* volatile profiles. To validate the assay reliability, standard controls were evaluated: the positive control (10% Tetracycline) exhibited strong inhibition against *E. coli* (25.5±0.35 mm) and



Data are expressed as mean±SD (n = 3). Different letters above the bars indicate statistically significant differences (p≤0.05) according to Duncan's multiple range test.

Fig 3: Effect of time extraction on the yield of garlic and chives volatile fractions.



Data are expressed as mean±SD (n = 3). Different letters above the bars indicate statistically significant differences (p≤0.05) according to Duncan's multiple range test.

Fig 4: Effect of extraction time on the major sulfur-containing volatile groups of garlic (A) and chives (B)**.

S. aureus (29.5±0.25 mm), while the negative control (n-hexane) showed no inhibition (NI). Furthermore, the solvent control (5% DMSO) exhibited no bacteriostatic effect (MIC > 2000 µg/mL), confirming that the observed antimicrobial activities were exclusively attributed to the volatile profiles (Table 1).

The biological assays revealed a significant contrast in the antimicrobial efficacy of the two *Allium* volatile fractions. Garlic volatile fraction demonstrated potent, broad-spectrum antibacterial activity against both *E. coli* and *S. aureus,* with inhibition zones of 21.0±0.28 mm and 21.0±0.32 mm, respectively. This high potency was quantitatively confirmed by the MIC values, which were remarkably low at 12.5 µg/mL for both bacterial strains. In comparison, the chives extracts exhibited significantly weaker inhibitory effects, with zone diameters limited to 9.0±0.34 mm (*E. coli*) and 9.0±0.51 mm (*S. aureus)*. Correspondingly, the MIC values for chives reached 250 µg/mL, representing a 20-fold difference in the concentration required to achieve bacteriostasis compared to garlic.

Regarding antioxidant potential, however, both species showed strong activity. The DPPH assay revealed that garlic volatile fraction possessed high antioxidant potential with an IC_{50} value of 26.57 µg/mL, closely approaching the efficacy of the positive control, vitamin C (23.95 µg/mL). In contrast, chives volatile fraction exhibited moderate activity, with an IC_{50} of 35.28 µg/mL compared to its respective vitamin C standard (24.95 µg/mL).

Effects of extraction parameters on yield

Based on these findings, the ultrasound-assisted method was the most effective technique among those tested, resulting in a significantly higher yield compared to conventional grinding. This enhancement can be primarily attributed to the acoustic cavitation phenomenon induced by ultrasonic waves (Sindumathi *et al*., 2025). The collapse of cavitation bubbles in the vicinity of the plant matrix generates micro-jets and shock waves, which effectively disrupt the rigid cell walls of the bulbs, thereby facilitating the release of intracellular volatile fractions glands into the solvent (Panda and Manickam, 2019). Although microwaves also improved yields significantly through rapid internal heating and electroporation-like mechanisms (Hu *et al*., 2021; Thanh *et al*., 2025), it was generally less effective than sonication in this study. Kimbaris *et al.* (2006) demonstrated that ultrasound-assisted extraction outperforms traditional thermal procedures by significantly mitigating the damage to highly reactive sulfur molecules and maintaining the stability of acyclic compounds of *Allium sativum* (Kimbaris *et al*., 2006). Besides, while Putra *et al*., (2019) reported higher yields using combined microwave-ultrasound extraction, our results suggest that sonication alone provided adequate extraction efficiency for the *Allium* bulbs used in this study. The additional thermal input from microwaves did not yield proportional benefits, possibly due to the specific structural characteristics of the plant matrix and the risk of degrading thermolabile organosulfur compounds.

The material-to-water ratio had a significant effect on the volatile fractions yield of both garlic and chives. At the lower ratio (1:2), the solvent volume appeared insufficient to fully immerse the plant material, leading to a higher viscosity of the slurry which hinders the convective mass transfer and the diffusion of volatile compounds from the internal matrix to the surface (Mandal *et al*., 2007; Milojević *et al*., 2008). Conversely, an excessive volume of water

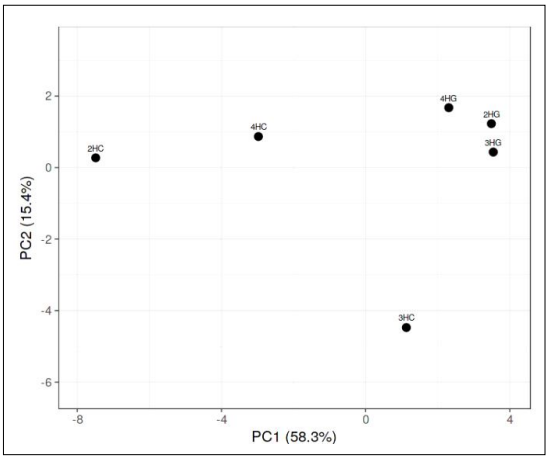
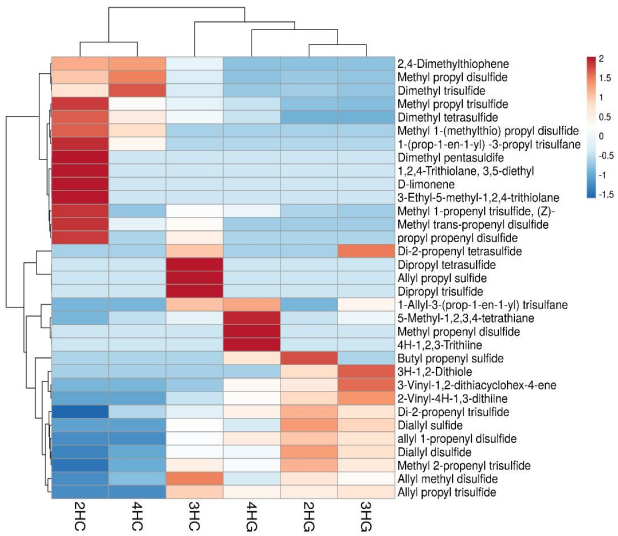


Fig 5: PCA score plot of volatile profiles in garlic (G) and chives (C) across different extraction times (2, 3 and 4 hours)**.



**Note: All analyses are based on the normalized peak areas of volatile compounds tentatively identified *via* MS library matching (NIST 2017, >85% similarity).

Fig 6: Heatmap visualization of volatile compounds in garlic and chives**.

Table 1: Antioxidant and antimicrobial activity of garlic and chives optimized volatile fractions.

Material	Antimicrobial activity				Antioxidant activity IC_{50} (µg/mL)	
	Inhibition zone diameter (mm)		MIC (µg/mL)			
	E. coli	*S. aureus*	*E. coli*	*S. aureus*	Volatile fraction	Vitamin C
Garlic	21.0±0.28	21.0±0.32	12.5	12.5	26.57±0.47	23.95±0.29
Chives	9.0±0.34	9.0±0.51	250	250	35.28±0.82	24.95±0.35
Tetracycline 10% (+)	25.5±0.35	29.5±0.25	-	-	-	-
n-hexane (-)	NI	NI	-	-	-	-
5% DMSO (Solvent)	-	-	>2000	>2000	-	-

Note: Values are expressed as mean±SD of three independent replicates (n=3); (NI): No inhibition; (-): Not tested.

(1:4) not only requires higher energy input to reach the boiling point but may also promote the hydrolysis of susceptible thiosulfinates or facilitate the solubilization of polar sulfur-containing derivatives into the hydrosol phase, thereby reducing the volume of the separated volatile fraction (Olascuaga-Castillo *et al*., 2024). Therefore, the 1:3 ratio was established as the optimal parameter for the subsequent study.

Temporal evolution of volatile profiles

The initial increase in yield up to 3 hours corresponds to the time required for the steam to penetrate the plant matrix and diffuse the volatile fractions from the internal glands to the surface (the diffusion-controlled phase). The subsequent decrease observed at 4 hours, particularly evident in garlic, suggests that the process is not merely reaching equilibrium but may be suffering from thermal degradation or volatilization losses (Zhou *et al*., 2025). Prolonged exposure to high temperatures can lead to the decomposition of thermolabile sulfur compounds or their conversion into water-soluble derivatives that are lost in the hydrosol, thereby reducing the final volume of the hydrophobic volatile fraction (Mungwari *et al*., 2025). Consequently, a 3-hour extraction time was deemed sufficient to maximize yield while minimizing potential thermal damage.

The distinct volatile profiles observed were primarily attributed to species-specific biosynthetic precursors. The dominance of allyl derivatives in garlic confirmed the enzymatic breakdown of alliin (Yamaguchi and Kumagai, 2019), whereas the methyl- and propyl-rich composition of chives aligned with the high levels of isoallin and methiin in the tissue matrix (Yamazaki *et al*., 2011). Regarding kinetics, extraction efficiency peaked at 3 hours, a phase that was governed by diffusion mechanisms and cellular rupture (Milojeviæ *et al*., 2008). Extending the process beyond this window shifted the kinetics toward degradation; notably, the rise in vinyldithiins at 4 hours suggested that prolonged heating triggered the rearrangement of unstable acyclic thiosulfinates into heterocyclic artifacts. This chemical transformation aligns with the observed decrease in total physical yield at 4 hours. Consequently, the 3-hour duration represented the optimal condition for maximizing bioactive recovery while minimizing thermal artifacts.

Linking chemical composition to biological activity

The stronger antimicrobial activity antimicrobial footprint of garlic volatile fraction (MIC: 12.5 µg/mL) is directly linked to the dominance of diallyl sulfides and thiosulfinates identified in its volatile profile. Mechanism-wise, these allyl-derivatives, particularly allicin, possess a highly reactive thiosulfinate group capable of penetrating bacterial membranes and irreversibly reacting with the thiol groups of essential enzymes (Borlinghaus *et al*., 2021). A strong correlation was observed between the concentration of major allyl sulfides (specifically DADS) and the antibacterial efficacy. Furthermore, the highest concentration of DADS, observed at the 3-hour mark, coincided with the strongest antimicrobial activity. Garlic volatile fraction, which possessed the higher content of DADS, compared with chives volatile fraction, exhibited significantly stronger inhibition zones and lower MIC values compared to chives volatile fraction (Fig 4). This suggests that DADS is a primary bioactive compound contributing to bactericidal activity, consistent with previous studies (Jin *et al*., 2021) and supports the selection of the 3-hour extraction time to limit the thermal degradation of this active component.

Interestingly, the radical scavenging data (DPPH assay) did not mirror the sharp contrast seen in antimicrobial testing, both volatile fractions exhibited nearly identical antioxidant profiles. This decoupling suggests that while antimicrobial activity is highly specific to the allyl-structure (found in garlic), antioxidant capacity is likely a collective property of organosulfur compounds regardless of their specific substituents. Both the diallyl polysulfides in garlic and the dipropyl polysulfides in chives appear to act as effective hydrogen donors or electron scavengers, successfully interrupting free radical chains (Osipova *et al*., 2021).

CONCLUSION

This study demonstrates that hydrodistillation with ultrasound-assisted pretreatment is a is a highly effective extraction method, achieving optimal recovery at a 1:3 material-to-water ratio over 3 hours. A clear distinction in extraction dynamics was observed: while the composition of the garlic volatile fraction remained relatively stable over the tested extraction periods, the chives fraction exhibited significant time sensitivity. This variation is likely driven by

the transient nature of mixed allyl-propyl sulfides, highlighting the need for precise temporal control during extraction. Exhibiting strong antioxidant capacity and a low MIC (12.5 µg/mL) against the tested strains, the garlic volatile fraction shows promising potential for use in natural preservative formulations. However, this study has certain limitations, including the use of a single-factor experimental design and evaluation against a limited panel of microorganisms. Future research should incorporate multivariate approaches to investigate parameter interactions, expand the microbial testing spectrum and conduct comprehensive long-term storage stability analyses to fully validate the industrial viability of these volatile fractions.

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Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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