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EFFICIENT RECOVERY OF ZERUMBONE AND A-HUMULENE FROM *ZINGIBER ZERUMBET* RHIZOME FOR VALUE-ADDED AGRICULTURAL APPLICATIONS

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Abstract

The crude extract of bioactive compounds, zerumbone and α-humulene, from Lempoyang ginger (*Z. zerumbet* (L.) Smith) rhizome biomass faces challenges in achieving optimal yields due to variations in extraction parameters. Current methods lack standardized conditions, leading to inconsistent recovery rates of these valuable compounds. This study aimed to improve the Soxhlet extraction parameters, including the type of extraction solvent, solid-to-solvent ratio, average particle size, and thermal extraction duration, to maximize the yield of zerumbone and α-humulene. Results showed that optimal recovery of zerumbone (49.24 ± 0.85 mg/g yield) was achieved using dichloromethane (DCM) as the solvent, with biomass particle size of <180 µm, and an extraction duration of 2 hours. The highest yield of α-humulene (12.47 ± 0.14 mg/g) was obtained with ethanol as the solvent, with a particle size of <180 µm and a 6-hour extraction duration. These findings establish optimal extraction parameters that enhance the recovery of zerumbone and α-humulene from *Z. .zerumbet* dried biomass. The optimized method enables scalable post-harvest processing, supporting the integration of *Z. zerumbet* into the agri-based and herbal industry to strengthen the phytochemical supply chain.

Key words: Biomass; Crude extract; Phytochemicals; Optimization; Soxhlet extraction

Introduction

Zerumbone (5.88–28.51 mg/g) (Ahmad *et al.,* 2023), the primary constituent and other compounds such as α-humulene (3.5-3.8 mg/g) (Alwakil *et al.,* 2022), Gallic acid (4.47 mg/g), Quercetin (2.65 mg/g) and Kaempferol (1.48 mg/g) (Gandhi and Saravanan, 2018) from *Z. zerumbet* (L.) Roscoe ex Sm. rhizomes have attracted significant scientific interest (Rawat *et al.,* 2023; Ahmad *et al.,* 2025). Cultivated widely across diverse regions and renowned for its extensive use in traditional medicine, this plant holds high-value commercial potential (Ibáñez *et al.,* 2022). Zerumbone can be extracted as an essential oil often used as a natural food preservative (Deepika *et al.,* 2020) and as a crude extract applied in cosmetics for hydration and anti-inflammatory effects (Yang *et al.,* 2023). Zerumbone also shows antimicrobial, anticancer, and immune-boosting properties, with uses in yogurt and antifungal mouthwash (Padalia *et al.,* 2018; Huanbutta *et al.,* 2024). In agriculture, it protects crops and serves as a natural antibiotic in livestock feed (Rafiqi *et al.,* 2022). Different extraction methods yield varying amounts of zerumbone (Table 1). Soxhlet extraction is the most effective due to its continuous process (Ibrahim *et al.,* 2023), cost-effective, simple to operate, and capable of processing large sample sizes, making it a preferred choice over methods like microwave, subcritical water extraction and maceration (Osorio-Tobón, 2020).

Previous studies have reported the extraction of zerumbone and other sesquiterpenes from *Z. zerumbet* using Soxhlet extraction (Wahab *et al.,* 2022; Ahmad *et al.,* 2023). While Soxhlet extraction is widely used, it typically requires prolonged extraction times, large volumes of organic solvents, and high energy consumption (Alfaro *et al.,* 2003). Therefore, the present study focuses on optimizing a more efficient extraction approach to improve the recovery of zerumbone and α-humulene while reducing extraction time and solvent usage. Additionally, this work quantitatively evaluates both compounds under optimized conditions, highlighting the potential of this approach for value-added agricultural and phytochemical applications. The study specifically investigates the effects of solvent type, particle size, solid-to-solvent ratio, and extraction duration on extraction efficiency.

Table 1. Reported yields of zerumbone using different extraction methods from *Z. zerumbet.*

Extraction method	Zerumbone yield (mg/g)	Source
Microwave-assisted extraction	5.08	Hossain *et al.,* 2016
Subcritical water extraction	19.82	Wahab *et al.,* 2022
Soxhlet extraction	28.51	Wahab *et al.,* 2022
Hydrodistillation	15.8	Azelan *et al.,* 2018

Materials and Methods

Plant material: Mature rhizomes of *Z. zerumbet* were collected from Kuala Selangor, Malaysia, in January 2024, and their identity was confirmed by comparison with the Universiti Malaya Herbarium specimen (Voucher No. 041148). The rhizomes were thoroughly cleaned by

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repeated washing with distilled water and excised into 1-cm lengths prior to oven-drying for 48 hours at 50°C. The dried rhizomes were ground into powder using commercial grade grinder (Model EBM-9182, ELBA, Malaysia).

Bioactive compound extraction

Effect of extraction duration: The rhizome powder was weighed (0.1g) and loaded into a cellulose extraction thimble and extracted with 250 ml of dichloromethane (DCM) in a Soxhlet apparatus for 2, 4, 6, and 8 h to evaluate the effect of extraction time.

Effects of Solid-to-Solvent ratio: The sample was weighed (0.1g) and extracted with 50, 100, and 150 ml of DCM for 6 h under identical Soxhlet conditions to examine the influence of the solid-to-solvent ratio.

Effect of particle size and type of solvent: The ground samples were sieved using Test Sieve (Endecotts, London) to separate the samples into various average sizes (µm) *viz.* <37, <74, <180, and <425. Samples of different particle sizes were weighed (0.1g) and placed in cellulose extraction thimble. Each particle size fraction (0.1 g) was extracted using 100 mL of DCM, methanol, and ethanol for 2, 4, 6, and 8 hours. Each analysis was repeated three times. The crude extract was evaporated using a rotary evaporator at the respective boiling points of the solvents (BÜCHI Rotavapor R-114, USA). The remaining residual was dissolved in 10 ml acetonitrile (HPLC grade, Merck, USA) and kept at 4ºC for further analysis.

HPLC analysis of zerumbone and α-humulene: High performance liquid chromatography (HPLC) analysis was performed using Agilent 1220 Infinity LC system, Chromolith RP-18 endcapped column (100 × 4.6 mm) and equipped with the Agilent OpenLab ChemStation software (Agilent Technologies, Santa Clara, CA, USA) software. HPLC analysis was performed using HPLC-grade solvents. Solvents used in the mobile phase including ultrapure water (solvent A) and acetonitrile (solvent B), delivered in a gradient elution according Alwakil *et al*., (2022). The flow rate was 1.0 mL/min with a total run time of 20 min. The detection was performed at 243 nm and temperature of the column was set to 35°C. Samples were filtered through 0.25 µm membrane filters prior to injection. Identification of zerumbone and α-humulene was based on matching retention times and chromatographic profiles with those of authentic standards; zerumbone (Medchem Express, USA) and α-humulene (ChemCruz, USA). Each sample was injected in triplicate, and quantification was based on peak areas calibrated against external standard curves.

Calibration equation for zerumbone concentration,
C_Z (µg/ml): $C_Z = 1.9075 \cdot Peak\ Area - 34$; $R^2 = 0.9957$
and α-humulene concentration,
C_H (µg/ml): $C_H = 1.2875 \cdot Peak\ Area + 0.4548$; $R^2 = 0.999$

Statistical analysis

Data were statistically analysed by one-way ANOVA followed by Tukey's multiple range tests at $p<0.05$ level using SPSS version 16.0.1 (SPSS Inc., Chicago). All experiments were performed using three independent biological replicates, and each measurement was carried out in technical triplicate.

Results and Discussions

Effect of extraction time: The effect of extraction time was evaluated using dichloromethane (DCM, 250 mL) as the solvent for all samples, with extraction conducted at its boiling point (39.6°C) for 2, 4, 6 and 8 hours. For zerumbone, the yield increased significantly when the extraction time was extended from 4 to 6 hours, reaching 39.08 ± 1.01 mg/g (Fig. 1). However, a lower yield was recorded at 8 hours, although it was not significantly different from that obtained at 6 hours. The zerumbone yields obtained with DCM at 6 and 8 hours were slightly higher than the previously reported yield of 28.51 ± 0.08 mg/g from an 8-hour Soxhlet extraction of *Z. zerumbet* rhizome using ethanol (Wahab *et al.,* 2022).

For α-humulene, the highest yield (7.37 ± 1.32 mg/g) was observed after 8 hours of extraction (Fig. 1). Nevertheless, this value was not significantly different from those obtained at 2, 4, and 6 hours. Since prolonged Soxhlet extraction at elevated temperatures may lead to thermal degradation or oxidation of heat-sensitive bioactive compounds (Thach, 2022), extraction durations of 2, 4, and 6 hours were selected for further investigation.

Effects of Solid-to-Solvent ratio: Three solid-to-solvent ratios (g:ml) viz. 1:50, 1:100 and 1:150 on the extraction yields of zerumbone and α-humulene were compared (Fig. 2). Zerumbone and α-humulene exhibited highest yields at 1:100 ratio with value of 50.58 ± 0.96 mg/g and 17.3 ± 0.12 mg/g. The increasing yield at 1:100 ration is likely due to the enhanced concentration gradient between the sample and the solvent. The error bars indicated narrow variability, suggesting consistent measurements. The lowest recovery of zerumbone (21.51 ± 0.45 mg/g) and α-humulene (5.85± 0.18 mg/g) was observed at 1:50 ratio.

The concentration gradient that drives the mass transfer process, i.e., extraction of compounds from solid mass to the liquid solvent is sustained for longer period when the ratio is higher, and in this case at 1:100. (Bhadange *et al.,* 2024). The solid-to-solvent ratio significantly impacts the equilibrium constant and the relationship between yield and solvent usage, often showing an initial rapid increase in yield followed by a plateau at maximum efficiency. At low ratios, increasing solvent volume enhances the concentration gradient, leading to an increased in yield (Rajković *et al.,* 2020). However, once equilibrium is reached, further solvent addition results in only slight improvement on the yield and raises processing costs (Wang *et al.,* 2022; Lay and Houng, 2024).

Similar patterns were observed in other studies. For example, research on *Aquilaria crassna* found that total phenolic content (TPC) increased significantly as the solid-to-solvent ratio increased from 1:10 to 1:20 but showed negligible improvement beyond 1:60 (Tay *et al.,* 2014). In the extraction of compounds from elderberry flowers (*Sambucus nigra* L.), using solid-to-solvent ratios of 1:15 and 1:30, the higher ratio of 1:30 resulted in increased yields of (poly)phenols, beyond which no further

improvement in yield was observed (Predescu *et al.,* 2016). Optimal ratios, often between 1:10 and 1:20 depending on the system, provide high yields without excessive solvent use (Rajković *et al.,* 2020; Lay & Houng, 2024). These finding underscores the importance of optimizing the solid-to-solvent ratio to achieve maximum yield, as excessively high ratios may not improve extraction efficiency and concomitantly lead to solvent wastage.

Effects of particle size and extraction solvent on zerumbone yield: The size of solid particles is crucial for extracting bioactive chemicals from natural products using solid-liquid extraction methods. Prior to extraction, the samples were sieved to obtain various particle sizes (<37 µm, <74 µm, <180 µm, and <425 µm), as illustrated in Fig. 3.

The effects of solvent type, particle size, and extraction time (2, 4, and 6 hours) on zerumbone yield are shown in Fig. 4C. In methanol extraction (Fig. 4A), zerumbone yields were consistently lower than those obtained with less polar solvents. Although smaller particles provide greater surface area, particles <37 µm did not consistently improve yield, likely due to agglomeration that limit solvent penetration. Highest recoveries were observed with larger particle sizes (<74 µm), with maximum yield at 4 h (32.75 ± 2.04 mg/g), followed by a decline at 6 h, due to thermal or oxidative degradation of heat-sensitive zerumbone during prolonged Soxhlet extraction. Additionally, methanol's higher polarity may promote co-extraction of polar compounds, reducing the relative recovery of lipophilic zerumbone.

For ethanol extraction (Fig. 4B), the highest yield (37.31 ± 0.47 mg/g) was obtained using particles <425 µm at 2 h, suggesting that intermediate particle sizes provide an optimal balance between surface area and solvent penetration. Similar trends have been reported for *Z. zerumbet*, where zerumbone yield increases with extraction time up to an optimum, beyond which degradation or volatilization may occur (Ghasemzadeh *et al.,* 2017). However, statistical analysis revealed that this amount was not significantly different from the yields achieved at 4 and 6 hours for the <425 µm particle size, as well as at 4 hours for the <180 µm particle size.

Dichloromethane (DCM) produced the highest zerumbone yields (Fig. 4C), particularly at larger particle sizes (<180 µm). The maximum yield (49.24 ± 0.85 mg/g) was achieved at 2 h, with no significant different at longer extraction times (6 h). This is consistent with the lipophilic nature of zerumbone and the effectiveness of less-polar solvents for extracting semi-volatile terpenoids (Kumar *et al.,* 2025). Previous studies have also demonstrated the suitability of DCM for Soxhlet extraction of lipophilic compounds (Teixeira *et al.,* 2022). Besides, larger particle sizes have been shown to enhance zerumbone recovery during hydrodistillation of *Z. zerumbet* (Azelan *et al.,* 2018). Beyond polarity, its relatively low boiling point prevents the compounds from degradation and improves process efficiency at the laboratory scale. However, the classification of DCM as a hazardous substance under the EU REACH regulation raises environmental and occupational safety concerns, including documented neurotoxic effects at elevated exposure levels (Anon., 2021; Winneke, 1981), prompting consideration of greener solvents such as ethanol, which can maintain a higher extraction yields with lower toxicity and improved biodegradability (Olakunle & Ade-Omowaye, 2020). In addition, although Soxhlet extraction provides efficient and reproducible recovery of target compounds, its high solvent consumption and prolonged extraction time may limit its scalability. While solvent recovery can partially alleviate these limitations, the optimized laboratory-scale conditions established here provide a foundation for future studies exploring greener and scalable extraction strategies.

The optimal particle size and extraction duration varied across treatments, suggesting that solvent penetration and mass transfer were highly dependent on particle size. While smaller particles are generally reported to enhance extraction yield due to increased surface area (Olakunle & Ade-Omowaye, 2020), excessively fine particles (<37 µm) in this study resulted in agglomeration, which reduced solvent accessibility and limited mass transfer. Conversely, moderately sized particles (<180 µm) provided higher extraction efficiency due to improved solvent penetration and reduced aggregation. These findings emphasize the need to optimize both physical and chemical extraction parameters to maximize process efficiency and achieve consistent zerumbone recovery from *Z. zerumbet*.

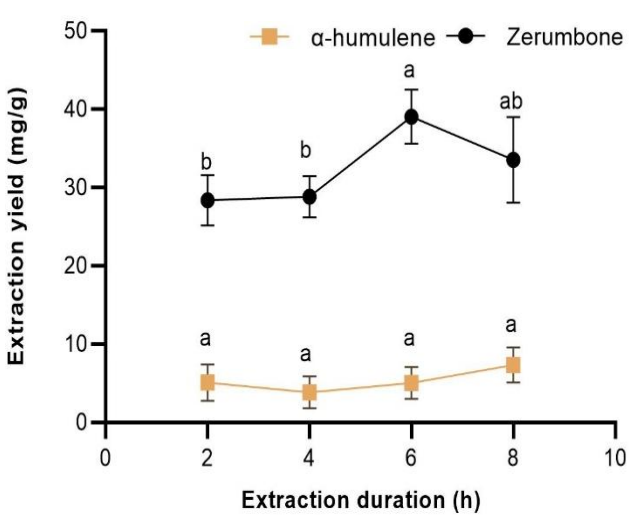

Fig. 1. Effects of extraction duration on the yield of zerumbone and α-humulene using DCM as extraction solvent. Value represent mean ± SE (n=3), and different letters show significant differences (p<0.05).

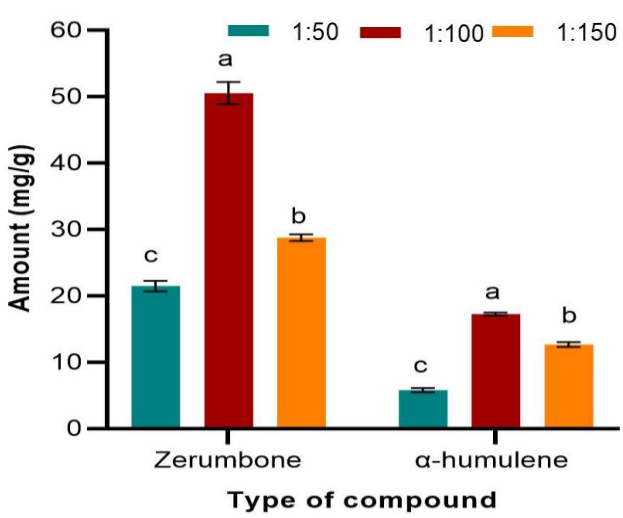

Fig. 2. Effects of solid-to-solvent ratio on the extraction yield of zerumbone and α-humulene after 6 hours of extraction using DCM. Value represent mean ± SE (n=3), and different letters show significant differences (p<0.05).

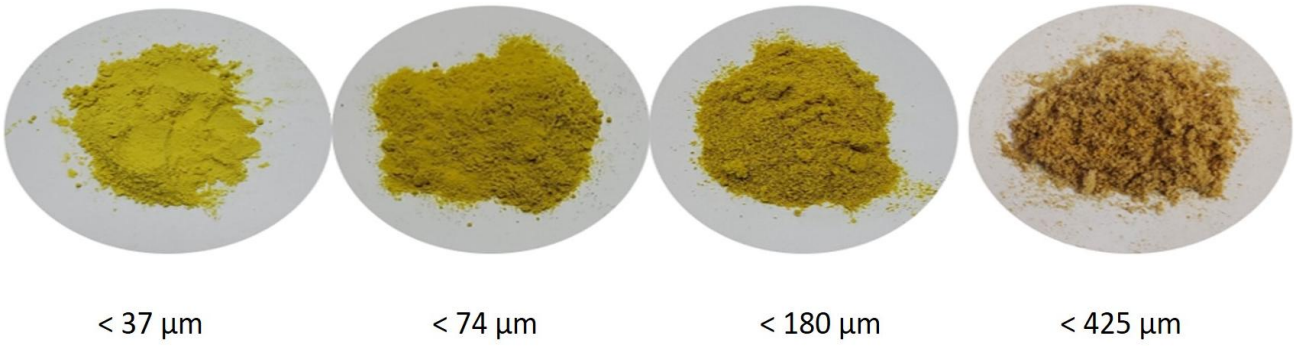

Fig. 3. Powder form of *Z. zerumbet* biomass with different average particle sizes.

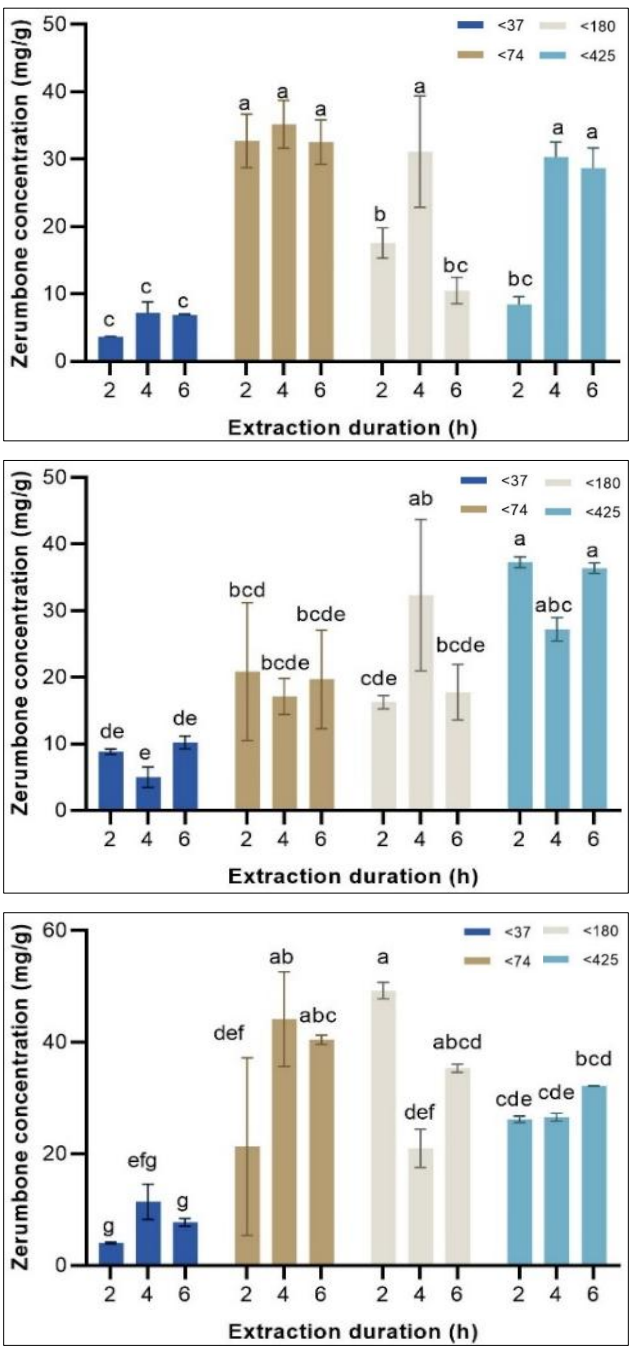

Fig. 4. Yield of zerumbone extracted using different solvents, particle sizes and durations of extraction (A) Methanol (B) Ethanol, and (C) DCM. Value represent mean ± SE (n=3), and different letters show significant differences (p<0.05).

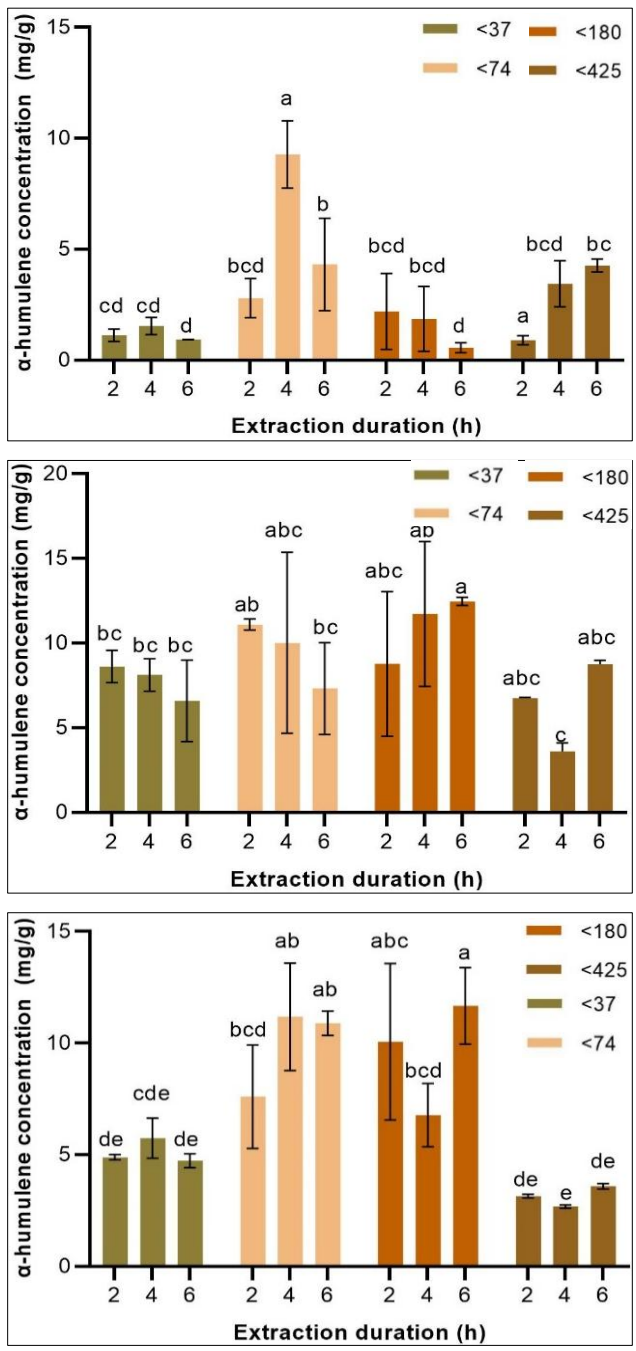

Fig. 5. Yield of α-humulene extracted using different solvents, particle sizes and durations of extraction (A) Methanol (B) Ethanol, and (C) DCM. Value represent mean ± SE (n=3), and different letters show significant differences (p<0.05).

Effects of particle size and extraction solvent on α-humulene yield: The yield of α-humulene based on the solvent type, particle size, and extraction duration (2, 4, and 6 hours) is shown in Fig. 5. α-Humulene showed a distinct extraction profile relative to zerumbone, indicating differences in polarity and solvent–matrix interactions. Methanol shows a lower extraction yield for particles <180 µm at 6 hours (Fig. 5A) than ethanol and DCM, due to its high polarity and lower compatibility with lipophilic constituents like α-humulene. While polar solvents like methanol favour hydrophilic phytochemicals, less polar solvents such as ethanol and dichloromethane (DCM) recover more non-polar, mono- and sesquiterpenes due to superior solubility and organic phase partitioning (Nabi *et al.,* 2025).

Ethanol extraction yielded the highest amount of α-humulene (12.47 ± 0.14 mg/g) with a particle size <180 µm and a 6-hour extraction time (Fig. 5B) suggesting that the compound has higher solubility in ethanol. Since there was no significant increase in α-humulene yield between 2 and 6 hours, it can be inferred that the majority of extractable α-humulene was recovered within the first 2 hours. The reported yield is greater than that obtained from *Z. nimmonii* achieved through hydrodistillation using a Clevenger-type apparatus for 8 hours (Govindarajan *et al.*, 2016; Dalavaye *et al.*, 2024). The combination of ethanol and small particle size came out as the most effective for maximizing α-humulene extraction yield, highlighting the importance of both solvent selection and material preparation in optimizing extraction efficiency of targeted compound.

Dichloromethane extraction (Fig. 5C) showed comparable α-humulene yields to ethanol with the maximum yield (11.67 ± 0.99 mg/g) obtained at 6h and <180 µm. This yield is not significantly different from the yield extracted either at 2 hours using the same particle size or the 4 and 6 hours yields obtained at <74 µm. This may be attributed to variations in solvent–matrix interactions and penetration efficiency, despite the scarcity of direct solubility data for α-humulene in DCM (Alemdar *et al.,* 2017). Although α-humulene is a non-polar sesquiterpene, its recovery in ethanol was nominally higher, due to its solubility and extraction efficiency in ethanol at specific parameters. Nevertheless, a lower yield of α-humulene recoveries across all solvents with lowest particles (<37 µm) confirming that excessive size reduction promotes agglomeration and mass-transfer limitations instead of enhancing extraction efficiency.

Conclusion

In conclusion, the optimization of soxhlet extraction parameters enhanced the recovery of zerumbone and α-humulene from *Z. zerumbet* rhizome. The optimal conditions for zerumbone extraction were dichloromethane (DCM) as the solvent, particle size of <180 µm, and a 2-hour extraction period, yielding 49.24 ± 0.85 mg/g. Meanwhile, the best parameters for α-humulene extraction were ethanol as the solvent, particle size of <180 µm, and a 6-hour extraction time, yielding 12.47 ± 0.14mg/g.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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