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Isolation and Characterization of Zerumbone Isolated from *Zingiber aromaticum*

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ABSTRACT

Zerumbone, a monocyclic sesquiterpene compound predominantly found in the rhizomes of lempuyang (*Zingiber aromaticum*), exhibits diverse biological activities including anticancer, antibacterial, anti-inflammatory, immunomodulatory, hepatoprotective, and gastroprotective effects. This study presents an optimized method for isolation, characterization, and purity analysis of zerumbone from the ethanolic extract of *Z. aromaticum*. The isolation process employed column chromatography with a gradient eluent system (n-hexane:ethyl acetate in ratios of 19:1 and 9:1) to separate compounds based on polarity differences. The extract and fractions were monitored using thin layer chromatography (TLC) under UV light at 254 nm and 366 nm. Characterization of isolated zerumbone was performed using multiple spectroscopic techniques, including TLC against reference standard, UV-Vis spectrophotometry, infrared (IR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS/MS). The purified isolate demonstrated a yield of 11.2%, representing a significant improvement over previously reported methods (0.87–2.26%). TLC analysis with three different solvent systems consistently showed single spots, confirming high purity. UV-Vis analysis revealed maximum absorption at 252 nm, identical to the zerumbone standard. LC-MS/MS analysis identified a molecular ion peak at m/z 219.26 $[M+H]^+$ with matching retention time and fragmentation pattern to the reference standard. IR spectroscopy identified functional groups characteristic of zerumbone, such as carbonyl and double-bond stretches. This efficient isolation method, yielding a highly purified compound, demonstrate its potential to support zerumbone's application in pharmaceutical development.

Keywords: Zerumbone; Lempuyang wangi; Isolation

INTRODUCTION

Zerumbone (2,6,9,9-tetramethyl-[2E,6E,10E]-cycloundeca-2,6,10-trien-1-one) is a monocyclic sesquiterpene belonging to the terpene group with significant bioactive properties (Ahmad et al., 2023). Structurally, zerumbone features three double bonds: one isolated at the C-6 position and two within the dienone system at the C-2 and C-10 positions, with the C-10 double bond cross-conjugated with a carbonyl group, forming an 11-membered ring. This hydrophobic compound possesses a characteristic bitter taste (Kitayama et al., 2001). The therapeutic potential of zerumbone has attracted considerable attention in recent years. Its anticancer properties are particularly noteworthy, with studies demonstrating its anti-angiogenic effects through the inhibition of thymidine phosphorylase with an IC_{50} value of 230 µM and the generation of reactive oxygen species (ROS) in lung cancer cells (Albaayit & Maharjan, 2018). Additionally, zerumbone targets key proteins involved in cancer progression, including AKT1 and MDM2, showing promising efficacy against colorectal cancer (Fauziyya et al., 2023). Its

suppression of cell migration and invasion via multiple signalling pathways further highlights its potential as a cancer therapeutic agent (Jamil et al., 2023). Beyond its anticancer properties, zerumbone exhibits remarkable skin-lightening properties due to its anti-melanogenic activity. Clinical trials have shown a substantial reduction in melanin levels after just one week of topical application, with no reported adverse effects (Kuek et al., 2024). Furthermore, zerumbone demonstrates potent anti-inflammatory properties, reducing inflammatory cytokines and oxidative stress markers in conditions such as rheumatoid arthritis (Alsaffar et al., 2023) and promoting macrophage polarization toward the protective M2 phenotype (Yeh et al., 2022).

Zerumbone is predominantly distributed in lempuyang species belonging to the Zingiberaceae family, with Indonesia recognized as one of the primary regions where these plants are widely found. Several species have been identified as important natural sources of zerumbone, including *Zingiber amaricans* BL (locally known as lempuyang emprit), *Zingiber aromaticum* Vahl (lempuyang wangi), and *Zingiber zerumbet* (L.) (lempuyang gajah), each exhibiting distinct morphological characteristics. Lempuyang emprit is characterized by relatively small, yellow-colored rhizomes with a pronounced bitter taste, whereas lempuyang wangi possesses whitish rhizomes with a notable aromatic fragrance. In contrast, lempuyang gajah produces the largest rhizomes among the three species, which are typically yellow in color and more robust in appearance (Sutardi et al., 2015). These plants are generally characterized by a relatively short life cycle, typically lasting approximately one year, and by the presence of pseudo-stems that are formed from tightly overlapping leaf sheaths rather than true woody stems. In addition, the leaves are arranged alternately along the pseudo-stem and are elongated to oval in shape, with a green coloration, pointed apices, and smooth, entire margins. The flower clusters, which emerge from the underground portion of the stem, are typically green or reddish-green (Wahyuni et al., 2013).

The rhizome is the most valuable part of the lempuyang plant, traditionally used to treat various conditions including stomach discomfort, respiratory ailments, colds, intestinal inflammation, nerve weakness, and as a blood enhancer and appetite stimulat. Rich in bioactive compounds such as essential oils, saponins, flavonoids, and tannins, the zerumbone contains zerumbone as its primary constituent, with concentrations ranging from 36% to 49% in extracts and essential oils of lempuyang wangi. Other significant compounds include α-humulene, humulene oxide, β-endemol, β-selinene, linalool, 12-oxabicyclo, caryophyllene oxide, 3-octadecyne, hexadecenoic acid, and 3-octyne 5-methyl (Wahyuni et al., 2013).

Various techniques have been employed for zerumbone isolation, including vacuum liquid chromatography (VLC) from *Z. zerumbet* (Hanwar et al., 2013), column chromatography using with n-hexane : ethyl acetate (10.0–7:3 ratios) from *Z. aromaticum* (Akhtar et al., 2019), and overnight crystallization with n-hexane (Noor & Sirat, 2016). However, these methods present limitations: VLC, while efficient under low pressure, lacks accuracy in separating structurally similar compounds and typically requires further purification; n-hexane crystallization, though straightforward, yields poor results due to incomplete precipitation; and previous column chromatography procedures, despite their effectiveness, utilized solvent ratios that produced relatively low percentages of pure zerumbone (≤2.26%). This study addresses these limitations by presenting an optimized column chromatography technique for isolating higher amounts of pure zerumbone. Our approach emplyes specific gradient ratios of solvents (n-hexane:ethyl acetate, 19:1 and 9:1) to maximize compound separation based on polarity differences. During elution, compounds with lower affinity for the stationary phase elute first, followed by those with higher affinity, resulting in a more efficient purification process (Nugroho, 2017). Additionally, our method incorporates a cold ethanol washing step to increase the final isolate's purity.

The development of this improved isolation technique is particularly timely given the growing interest in zerumbone derivatives, which have demonstrated enhanced biological activity compared to zerumbone itself. Certain derivatives exhibit stronger interactions with key proteins, including XIAPBIR3, AKT1, JAK2, HSP90AA, MDM2, and XIAPBIR2, with Compound 4 showing particular promise due to its lowest binding energy against AKT1 (Fauziyya et al., 2023). The continued development and production of these derivatives require a higher yield of pure zerumbone, underscoring the importance of an improved isolation process to ensure sufficient raw material supply for further pharmaceutical studies and applications.

MATERIALS AND METHODS

Zerumbone isolation

Zingiber aromaticum extract was obtained from PT. Lansida Group. The extract was prepared by macerating the rhizome powder with 70% ethanol in a ratio of 1:5 (powder:ethanol). Reference standard zerumbone was purchased from Sigma Aldrich. Fractionation was performed using 5 grams of *Z. aromaticum* thick extract for each cycle, with the process repeated three times. The extract was initially dissolved in acetone and impregnated onto silica gel 60 (70–230 mesh ASTM) in a 1:1 ratio. The impregnated sample was then loaded into a chromatography column (4 cm diameter) with a sample-to-stationary phase ratio of 1:30. The column was packed using the wet method with n-hexane as the solvent.

The fractionation process involved two distinct elution steps using different solvent mixtures. In the first step, a total of 800 mL of *n*-hexane : ethyl acetate (19:1) was passed through the column to elute the initial fractions. Once the first elution was completed, a second elution was carried out using 400 mL of a new solvent mixture, n-hexane:ethyl acetate (9:1), to continue the separation process. The eluates obtained from both elution steps were collected in fractions and allowed to stand at room temperature until the solvents evaporated (Haque et al., 2018). The main goal of this step was to separate compounds based on their polarity. The first eluent, was used to elute less polar compounds, while the second eluent, targeted slightly more polar compounds. The resulting isolates from the fractionation process were washed with cold 96% ethanol to ensure purity.

Extract monitoring

The extract was monitorid using TLC to determine the optimal solvent system for column chromatography. The concentrated extract was spotted onto silica gel-coated TLC plates (F254). The plates were developed using various solvent mixtures: n-hexane : ethyl acetate (19:1), n-hexane:ethyl acetate (9:1), n-hexane:ethyl acetate (8:2), and n-hexane:ethyl acetate (7:3). Developed plates were examined under UV light at wavelengths of 254 nm and 366 nm (Haque et al., 2018).

Fraction monitoring

The zerumbone compound was isolated through column chromatography, using silica gel 60 as the stationary phase. The column was packed by the wet method with n-hexane as the solvent, maintaining a sample-to-stationary phase ratio of 1:30. The concentrated extract, dissolved in acetone and mixed with silica gel 60 at a 1:1 ratio, was then applied to the column. The fractionation process involved a gradient elution system with three sequential eluents to achieve effective separation. First, 200 mL of 100% n-hexane was passed through the column to elute non-polar compounds. This was followed by 800 mL of n-hexane:ethyl acetate (19:1), and finally, 500 mL of n-hexane:ethyl acetate (9:1) was used to elute more polar compounds. Each eluent was applied sequentially, and the eluates were collected in vials and left to evaporate at room temperature, resulting in the formation of crystals at the bottom of the vials. The two-step fractionation process is crucial for achieving a more precise separation of compounds with similar chemical properties. In the second step, three different eluents are used sequentially with increasing polarity. The use of these three eluents allows for more effective separation based on the varying polarity of the compounds. This step is more selective, as progressively more polar solvents are used in a controlled manner to separate compounds with similar chemical characteristics, ultimately resulting in purer isolates.

The crystals obtained from each elution step were monitored using TLC to confirm the presence of zerumbone. The TLC analysis was performed using n-hexane:ethyl acetate (9:1) as the eluent, and the plates were observed under UV light at 254 nm and 366 nm. Additionally, the plates were sprayed with 10% H_2SO_4 reagent to enhance visualization of the separated compounds. After monitoring, the crystals were washed with cold ethanol 96% to obtain purified zerumbone crystals (Haque et al., 2018).

Identification, characterization, and purity assessment

The isolate was identified and characterized through TLC and UV-Vis spectrophotometry. For TLC identification, both the isolate and zerumbone reference standard were analyzed using n-hexane : ethyl acetate (9:1) as the eluent. Identification was confirmed when the isolate and reference compound showed identical Rf value (Haque et al., 2018). The purity of the isolate was assessed using TLC with three different solvent systems: n-hexane:ethyl acetate (19:1), n-hexane:ethyl acetate:ethanol (18:1:1), and ethyl acetate:methanol (95:5). The TLC plates were

examined under UV light at 254 nm and 366 nm. A single spot observed in each solvent system indicated purity the isolate.

UV-visible spectroscopy

UV-Vis spectrophotometric analysis was perfomed using a Thermo Scientific, Genesys 150 840-300000 spectrophotometer. The isolated crystals and zerumbone reference standard were dissolved in methanol, and their absorbance profiles was measured over a wavelength range of 200–800 nm (Lallo et al., 2018). Comparison of the absorption maxima and spectral patterns between the isolate and reference standard provided confirmation of compound identity and assessment of purity.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed using a Thermo HPLC DIONEX ULTIMATE-TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer. Chromatographic separation was achieved on a Hypersil GOLD™ C-18 Selectivity HPLC column (Thermo Scientific). The mobile phase consisted of: water with the addition of 0.1% formic acid (solvent A) and acetonitrile with the addition of 0.1% formic acid (solvent B).

The sample was prepared by dissolving 5 mg of isolate in 5 mL of HPLC-grade methanol and filtering through a 0.22 µm 51 nylon filter. The injection volume was 2 µL, with a retention time of 10 minutes. Mass spectrometric analysis was conducted in positive ionization mode (MS+) (Parasuraman et al., 2014)

Infrared spectrophotometer (IR)

IR analysis was conducted using a Shimadzu FTIR IRSPIRIT-T with QATR-S and Dehumidifier. For sample preparation, about 2 mg of zerumbone isolate was ground with 98 mg of potassium bromide (previously dried for 24 hours at 105° C). The background was established using potassium bromide, and the sample was analyzed across a wave number of 4000 cm to 400 cm^{-1}. Zerumbone characterization was based on distinctive absorption peaks at 2963 cm^{-1} C-H stretch and 1650 cm^{-1} (C=O stretch), with a margin of error of ±0.5 for each value (Sulistyani, 2017). These specific absorbtion patterns correspond to the functional groups present in the zerumbone structure and serve as a fingerprint for identification.

Data analysis

Identification of zerumbone was established through comparison of TLC Rf values, UV-Vis spectra, IR absorption patterns, and LC-MS/MS fragmentation profiles with those of the reference standard. Purity was determined based on the presence of a single spot in multiple TLC solvent systems, consistent spectroscopic profiles, and absence of significant impurity peaks in the LC-MS/MS analysis. All experiments were performed in triplicate to ensure reproducibility of results.

RESULTS AND DISCUSSION

This study focused on isolation and characterization of zerumbone from *Z. aromaticum* rhizomes. The analysis encompassed yield percentage, organoleptic properties, TLC fractionation patterns, as well as spectroscopic characterization using UV-Vis, IR, and LC-MS/MS techniques.

Percentage yield

Zerumbone was isolated from from *Z. aromaticum* extract an optimized column chromatography method. The fractionation process employed a gradient mobile phase consisting of n-hexane and ethyl acetate in ratios of 19:1 and 9:1. The yield was calculated by comparing the final weight of the isolate with the initial weight of the extract and then multiplying by 100% (Dewatisari et al., 2018). After three repetitions, we obtained 0.56 grams of pure zerumbone from 5 grams of extract, corresponding to a yield of 11.2%. This represents a significant improvement compared to previous studies that reported yields of 0.87% (Akhtar et al., 2019) and 2.26% (Noor & Sirat, 2016) achieved using the unmodified method. The active compound zerumbone shows significant potential for development as a candidate for cancer therapeutics. As there are no prior reports on the synthetic process of zerumbone, various studies have already carried out modifications and optimizations to achieve high zerumbone yield, but none have a yield as high as ours (Kaur et al., 2025). This modification study to the isolation procedure and the substantial improvement in yield provide crucial information for obtaining zerumbone isolate from its plant source. Moreover, the improved efficiency demonstrated in this study can serve as a practical reference for future large-scale isolation strategies aimed at supporting downstream pharmacological investigations.

Organoleptic properties

The isolated zerumbone exhibited organoleptic characteristics that were consistent with those of the reference zerumbone standard (Figure 1). In terms of appearance, the isolate was obtained in the form of a transparent crystalline powder. Upon visual examination under ambient lighting conditions, the crystals displayed a faint yellowish hue, which was clearly observable and comparable to the standard material.

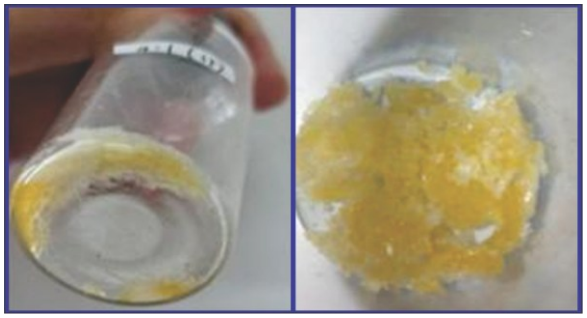

Figure 1. Isolated crystal of zerumbone

Extract monitoring

Initial extract monitoring was conducted using TLC with four different mobile phase compositions: *n*-hexane and ethyl acetate in ratios of 19:1, 9:1, 8:2, and 7:3. This initial screening allowed rapid identification of solvent systems capable of resolving the target compound effectively. The differences in Rf values across mobile phases also provided insight into the polarity-dependent behavior of the analytes within the extract. Visualization under UV light at 254 nm and 366 nm revealed varying Rf values across the different mobile phases. The Rf values were 0.2 for the 19:1 ratio (matching the standard zerumbone), 0.49 for the 9:1 ratio, 0.68 for the 8:2 ratio, and 0.85 for the 7:3 ratio. The Rf values obtained with the 19:1, 9:1, and 8:2 mobile phases were within the optimal range of 0.2–0.8, making these solvent systems suitable for further analysis.

Fractionation monitoring

The fractions collected during column chromatography were monitored TLC with n-hexane and ethyl acetate (9:1) as the mobile phase. This monitoring step ensured consistent tracking of compound distribution across the eluates and helped identify fractions with similar chromatographic behavior. Visualization under UV light at 254 nm showed that fractions 13–20 contained a single spot with an average Rf values of 0.5. The presence of a single, well-defined band in these fractions also indicated a high degree of preliminary purity. Comparison with the standard zerumbone confirmed that these factions contained the target compound, as evidenced by the identical Rf values and spot characteristics (Dash et al., 2021). These fractions were subsequently combined for further purification and characterization.

Purity and identity confirmation

Thin layer chromatography (TLC)

The purity of the isolated zerumbone was evaluated using TLC with three distinct eluent systems of varying polarities: n-hexane:ethyl acetate (19:1), n-hexane:ethyl acetate:ethanol (18:1:1), and ethyl acetate:methanol (95:5). The use of eluents with gradually increasing polarity allowed evaluation of how the compound migrated under different separation conditions, helping reveal any impurities that might travel alongside it. A single spot was observed in each system, with Rf values of 0.21, 0.41, and 0.7, respectively. The consistent migration pattern across these solvent systems further supports the absence of interfering compounds within the isolate. The presence of a single spot across three different sovents systems with distinct polarities indicates the high purity of the isolated compound (Claudea & Yuswi, 2017).

The identity of the purified isolate was confirmed by comparing it with the zerumbone standard using TLC with *n*-hexane : ethyl acetate (9:1) as the mobile phase. Both the isolate and the standard exhibited spots at the same Rf value of 0.45, confirming the identity of the isolated compound as zerumbone. This confirmation step reinforces the reliability of TLC as a rapid and effective method for verifying compound identity during the isolation workflow.

UV-Vis spectrophotometry

UV-Vis spectrophotometric analysis displayed overlapping absorption spectra between the isolate and the zerumbone standard, with both exhibiting maximum absorption at 252 nm (Figure 2). This finding aligns with previous research, which reported the characteristic maximum absorption of zerumbone at 252 nm (Suhartati, 2017). The resemblance in both spectral profile and peak intensity provides additional confirmation that the isolate shares the same structural characteristics as the reference compound. This agreement in UV absorption patterns further verifies the presence of the chromophoric groups typical of zerumbone.

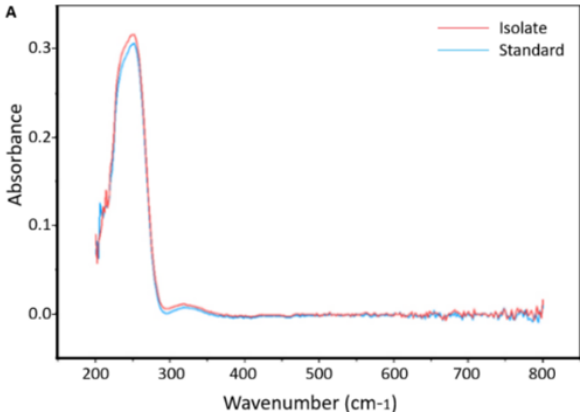

Figure 2. UV Spectra of the isolated compound and zerumbone

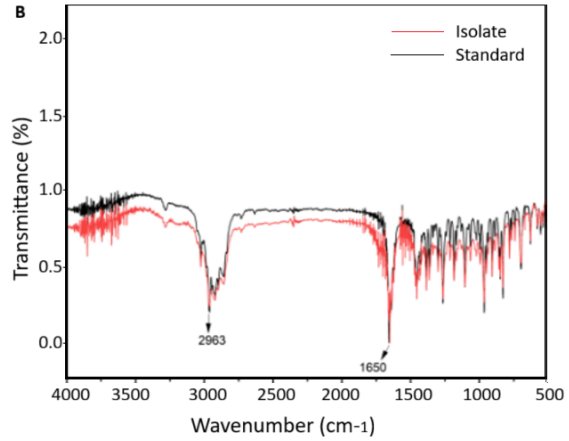

Figure 3. UV pectra of the isolated compound and zerumbone

Infrared spectroscopy (IR)

Infrared spectroscopy was employed to identify the functional groups present in the isolated compound. The IR spectra of the isolate and the zerumbone standard showed nearly identical patterns (Figure 3), particularly in the fingerprint region. Zerumbone was characterized as a sesquiterpene with an α, β-unsaturated ketone group. IR spectra of zerumbone showed significant bands at 2958.8 cm^{-1} and 2923.9 cm^{-1} for sp^3 C–H stretching, 1658.6 cm^{-1} for the conjugated C = O group, and 1735.6 cm^{-1} for a C = C bond (Kaur et al., 2025). In this study, both standard and isolate showed spectra exhibited characteristic absorption bands at 2963 cm^{-1} for CH sp^3 and 1650 cm^{-1} (C=O carbonyl group of α,β-unsaturated ketone). These spectral features correspond to the known molecular structure of zerumbone (Bayu et al., 2019; Noor & Sirat, 2016). The overlapping spectra in the fingerprint region provide further evidence of the isolate's identity and purity.

LC-MS/MS Analysis

LC-MS/MS analysis provided definitive confirmation of the identity and purity of the isolated compound. The chromatograms of both the isolate and the zerumbone standard showed peaks at the same retention time of 2.04 minutes (Figure 4a), indicating the presence of the same compound. The alignment of retention times suggests comparable interactions with the chromatographic column, which is characteristic of identical molecular structures. In addition, the mass spectra generated from both samples exhibited matching molecular ion patterns, supporting the conclusion that the isolate corresponds to zerumbone. The absence of additional significant peaks further reinforces the high purity of the isolated compound.

The mass spectra of both samples revealed identical m/z values of 219.26 g/mol (Figure 4b). While the molecular weight of zerumbone is 218.3 g/mol (Rahman et al., 2014), the observed m/z value of 219.26 g/mol can be attributed to the addition of one proton ($[M+H]^+$) in the positive ionization mode used during analysis. Positive ion mode was highly effective for detecting this sesquiterpene compound, confirming that these conditions are crucial for analyzing similar phytochemicals (Aldholmi, 2024; Vasas et al., 2021). Furthermore, the fragmentation pattern of the isolate showed a base peak at m/z 109.12 and a parent peak at m/z 219.14. When subjected to a fragmentation energy of 20 electron volts, the resulting pattern closely matched the reference spectrum from mzcloud.org, providing additional confirmation that the isolate is zerumbone.

CONCLUSION

The successful isolation zerumbone from *Zingiber aromaticum* extract with a yield of 11.2% represents a significant improvement over previously reported methods. This increased efficiency can be attributed to the optimized gradient elution strategy employed in our column chromatography procedure. Additionally, the cold ethanol washing step further increased the purity of the final isolate.

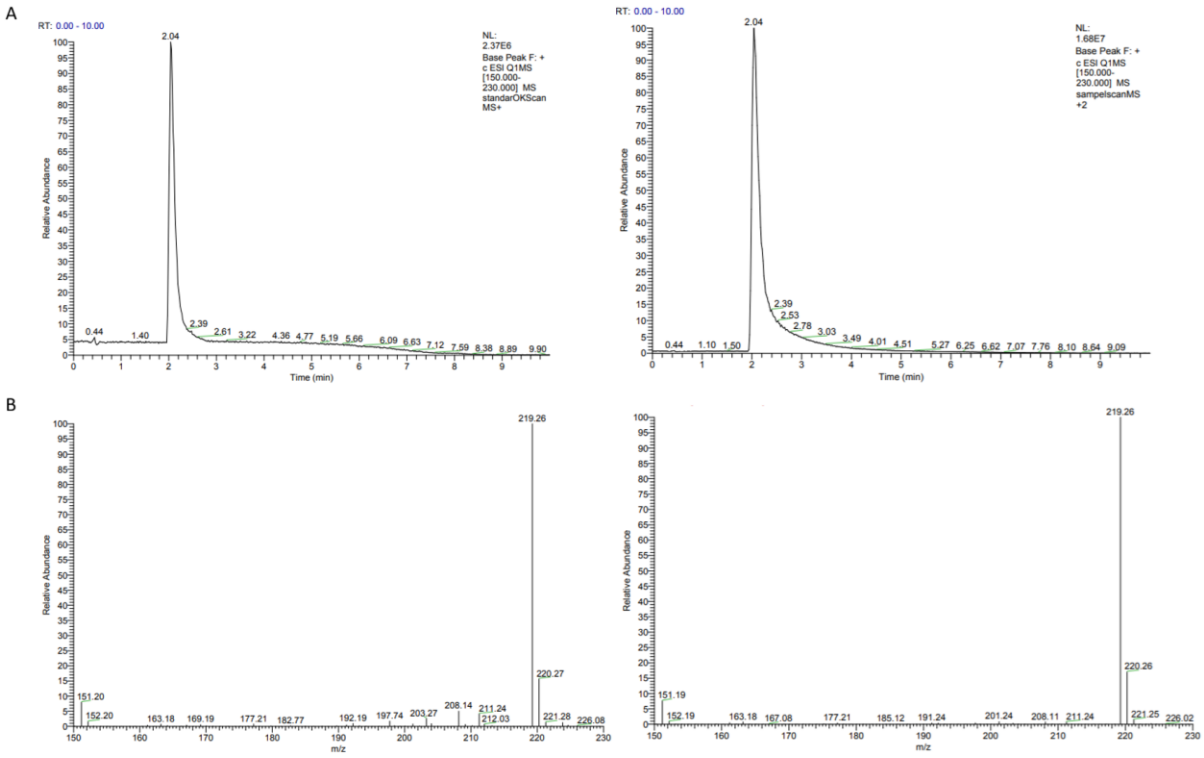

Figure 4. LC-MS/MS of zerumbone standard and isolate. (a) Chromatogram LC-MS/MS for zerumbone standard (left) and isolate (right), (b) Mass spectra of zerumbone standard (left) and isolate (right)

The identity and purity of the isolated zerumbone were comprehensively confirmed through various analytical techniques such as TLC, UV-Vis, IR spectroscopy and LC-MS/MS. These findings not only validate the effectiveness of the optimized isolation workflow but also provide a practical and reproducible approach for obtaining high-purity zerumbone from its natural source. Moreover, the improved yield and purity achieved in this study enhance the feasibility of producing zerumbone in sufficient quantities for subsequent pharmacological and preclinical evaluations. Overall, the proposed isolation strategy may serve as a valuable reference for future studies aimed at large-scale isolation and utilization of zerumbone as a bioactive compound.

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CONFLICT OF INTEREST

The authors declare no conflict of interest that might influence this study.

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