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RESEARCH PAPER

Metabolites Profiling and Fingerprinting of the Ethanolic Extract of *Zingiber zerumbet* (L.) Roscoe ex Sm.

Nur Zahratun Najah Mohd Razali[1], Nurunajah Ab Ghani[1,2]*, Nurulfazlina Edayah Rasol[1,2]

[1]Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Level 9, FF3 Building, Universiti Teknologi MARA, UiTM Selangor, Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor, MALAYSIA
[2]Faculty of Applied Science, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, MALAYSIA

*Corresponding author: nurunajah@uitm.edu.my

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Abstract

Zingiber zerumbet (L.) Roscoe ex Sm. is a herbaceous medicinal plant from the Zingiberaceae family. Previous studies have mainly focused on the essential oils of the plant with little attention to its secondary metabolites. LCMS-Orbitrap is used to analyze ethanolic extract of *Z. zerumbet* rhizome. Dereplication is a technique applied to identify chemical constituents based on their accurate mass and comparison with custom databases, Dictionary of Natural Products (DNP), and literature using an operating system named MZmine 2.53. In this study, we have identified chemical constituents related to several classes, including flavonoids, terpenoids, diarylheptanoids, terpenes, phenolic compounds, and fatty acids. Our results provide useful information for further isolation and identification of other unknown compounds and mass spectroscopy-based metabolite profiling of *Zingiber* species.

Keywords: metabolites profiling, fingerprinting, extract, *Zingiber zerumbet*, bitter ginger

INTRODUCTION

Natural products are organic compounds produced by living organisms' metabolism, characterized by their chemical diversity and versatile bioactivity (Erb and Kliebenstein, 2020). According to Shen (2015), natural products are evolutionarily optimized as drug-like molecules as they own enormous structural and chemical diversity. Thus, the discovery of natural products has significantly impacted different chemistry, biology, and medicinal research areas and particularly has inspired drug discovery and therapy. Natural products are grouped into three categories based on their chemical class, biosynthetic origin, and functional groups. (Koga et al., 2016; Prakash et al., 2011). These include primary metabolites, which are essential for the plant growth process. Secondary metabolites mediate the interactions of the plant with its environment. Lastly, plant hormones modulate its organismal processes and metabolism.

Z. zerumbet, commonly known as bitter ginger, is a species from the Zingiberaceae family. It is traditionally used as herbal medicine, food flavoring, and appetizer in Asian, Indian, Chinese, and Arabic cuisines. There has been an enormous interest in folk medicines, mainly in herbal remedies and their conjunction with modern medicine in recent decades. Medicinal plants and their secondary metabolites have a long history of treating various diseases, illnesses, and infections (Rajkumari and Sanatombi, 2018).

Dereplication is a fast and efficient process to identify novel biologically active compounds and distinguish between known and unknown compounds based on previously characterized compounds. Various software is used for natural product dereplication and untargeted data processing, including open-source software such as XCMS, MZmine, OpenMS, MetAlign, Metabo and Analyst, and commercial software such as MarkerView, MetaboScape, and Compound Discoverer (CD). The current study uses MZmine because it is a user-friendly, adaptable, and easily expandable software that includes a complete set of modules covering the entire LC-MS data analysis workflow.

Previous studies on *Z. zerumbet* have mainly focused on mixtures of volatiles and on its essential oils, which only cover the plant's surface. In contrast, there is little research conducted on the secondary metabolites of *Z. zerumbet*. The high diversity of natural products in these complex biological matrices makes their dereplication, detection and early identification difficult. Modern chromatography and spectroscopic techniques have been used to identify components in *Z. zerumbet.* (Singh et al., 2012; Sharifi-Rad et al., 2017).

The current study aims to identify phytoconstituents present in *Z. zerumbet* rhizome in order to build a compound database of *Z. zerumbet*. We have used the Dictionary of Natural Products (DNP) online database and literature search. A dereplicate of *Z. zerumbet* ethanolic extract using LC-MS-orbitrap was also analyzed and determined. Considering the importance of *Z. zerumbet* in daily use and medicinal application, this study will provide necessary information and secondary metabolites profiling of medicinally important herbal plants for future reference.

MATERIALS AND METHODS

Plant materials

Z. zerumbet plant sample was bought from Sungai Udang, Melaka in October 2021 and was deposited at Atta-ur Rahman (Aurins) Institute Herbarium, Universiti Teknologi MARA (UiTM) with Voucher number of 0211/2021. Samples were washed with tap water to remove any dirt or contaminants.

Plant Extraction

Z. zerumbet rhizomes were air-dried, chopped, and placed in an oven at 40 ºC for 48 hours. Dried samples were coarsely grounded using an electrical grinder to increase their surface area. The dried sample (20 g) was soaked in a conical flask filled with ethanol (200 mL) while stirring for 24 h. The ethanolic solution was filtered using Whatman filter paper No. 1. This extraction process was repeated three times. The filtrate was combined and concentrated using a rotary evaporator at 40ºC, and the weight of the crude was recorded.

Ultra-High Performance Liquid Chromatography (UHPLC)

Ethanolic extract was transferred into an Eppendorf tube, dissolved in 1 mL of a solvent mixture (methanol: water with a ratio 95:5), and was sonicated for a few seconds before filtration into an HPLC vial through a 0.20 µm UHPLC filter. The extract separation was optimized by changing

the solvent system, elution gradient, flow rate, and column type. UHPLC was carried out using Luna Omega 1.6 µ C18 10 (100 × 2.1 mm) column and a diode array detector (DAD). The column temperature was set at 36ºC, and the system flow rate was set up to 0.2 mL/min with an injection volume of 1.0 µL. Gradient elution was conducted using deionized water (%A) and methanol (%B).

Table 1 shows the elution system of the extract that was developed for the first time and the profile was observed to obtain the best peak width and resolution. Figure 1 shows the chromatogram based on the first elution system developed. The elution system had to be modified as the profile obtained was not the most preferred. Table 2 shows the second elution system developed, and Figure 2 shows the spectrum of the mentioned elution system needing to be modified again. Table 3 shows the final solvent system developed as Figure 3 shows the chromatogram with the most preferred peak width and resolution of the peaks detected. Samples were monitored at different ultra-violet (UV) absorptions (210 nm, 225 nm, 254 nm, 270 nm, and 360 nm) to identify different classes of compounds.

Table 1. The first elution system developed.

Time	%B (Methanol)
0	10
30	100
35	100
37	10
42	10

Table 2. The second elution system developed.

Time	%B (Methanol)
0	40
25	100
30	100
32	40
35	40

Table 3. Solvent system used for *Z. zerumbet.*

Time	%B (Methanol)
0	40
15	100
20	100
22	40
25	40

Liquid Chromatography-Mass Spectrometry (LC-MS)

A Thermo Scientific Vanquish Horizon UHPLC system with AccucoteTM Vanquish C18 column of 2.1 × 100 nm, 1.5 µm was used in this study. The column temperature was set at 30 ºC. Flow rate was set at 0.2 mL/min, and the injection volume was 2 µL. The sample previously used for UHPLC was diluted to 250 ppm before injected into LC-MS. Gradient elution was set as stated in Table 3. Thermo ScientificTM Orbitrap FushionTM TribridTM mass spectrometer was applied together with electrospray ionization (ESI) to identify molecular ions. The samples were screened using positive and negative ionization modes and analysed using multi-stage mass spectrometry (MS^n) data analysis, where the product ions were subjected to re-fragmentation.

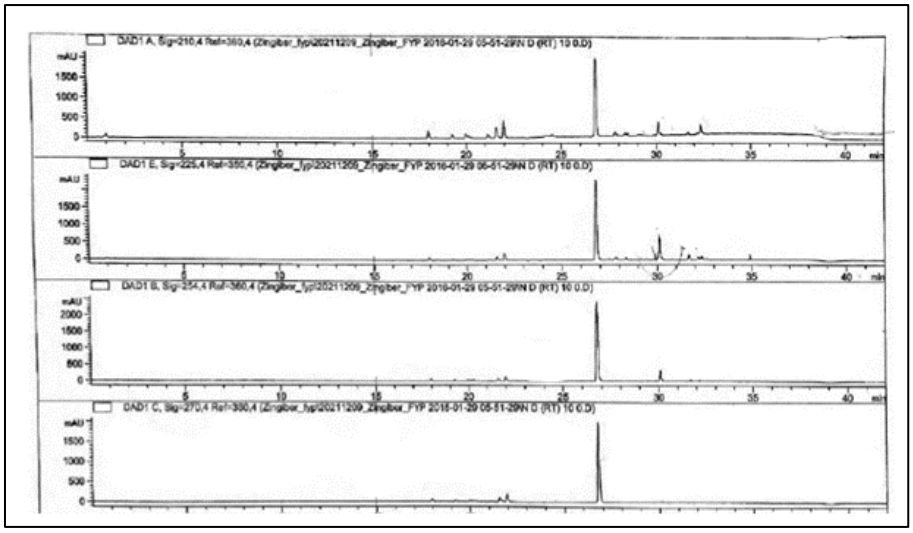


Figure 1. Chromatogram of first elution system developed.

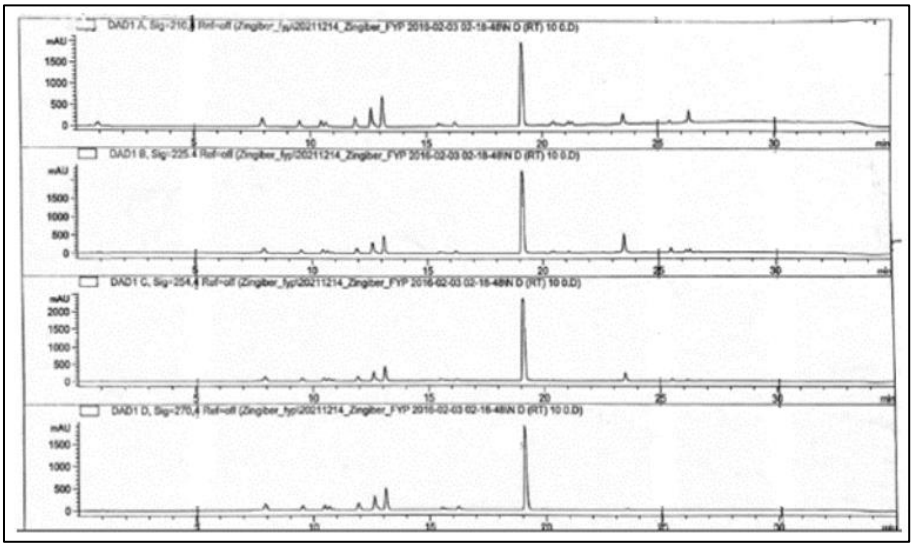


Figure 2. Chromatogram of second elution system developed.

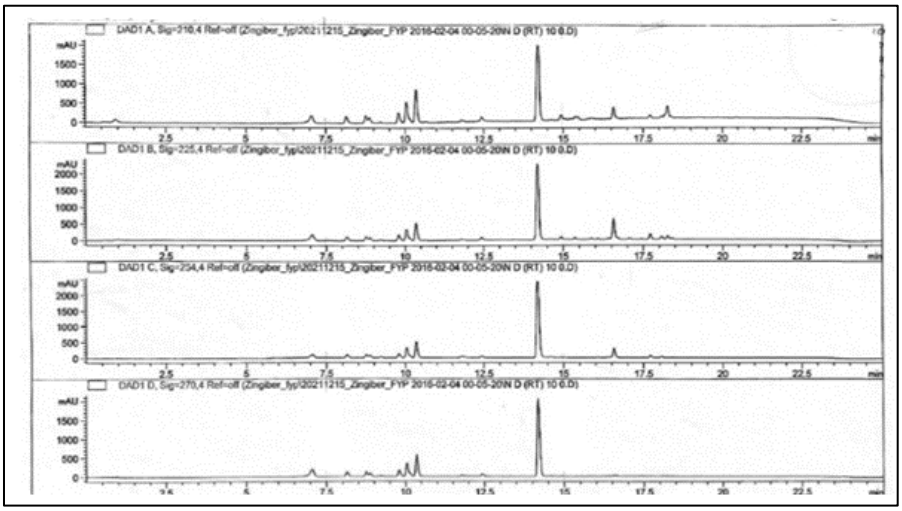


Figure 3. Chromatogram of the final solvent system developed.

System Requirements and Performance of MZmine

MZmine is an open-source software for LC-MS data processing available on its official website. MZmine 2.53 (64-bit Windows version) was used for analysis on a 64-bit CPU, 4GB RAM.x, and 1.7 Java SE runtime (JRE) version.

RESULTS AND DISCUSSION

Data Mining of Dictionary of Natural Products (DNP)

The dereplication technique is particularly used in this study to compare molecular mass based on MS Level 1. For this, a database of known molecular masses must be constructed. The data mining process for the custom database (DNP) was done by extracting information on chemical constituents from the Zingiberaceae family based on chemical name, synonym(s), molecular formula, and accurate mass parameters. A total of 1128 compounds were extracted from the DNP of the Zingiberaceae family, and 40 compounds were reported in the literature for *Z. zerumbet*.

Data Processing by MZmine 2.53

MZmine predictions are based on accurate mass with the value of proton weight a, 1.007276 amu to be used to calculate molecular ion mass, $[M+H]^+$. The positive ionization mode was chosen for the current data analysis. Data processing was performed by converting raw data from LC-MS to an mzML file type using MSConvertGUI (64-bit), 3.0.22015-aadd392 version (an automated build) to be imported into MZmine software with mass signal level set into 1. Filters for scanning purposes throughout the process were set, as shown in Figure 4.

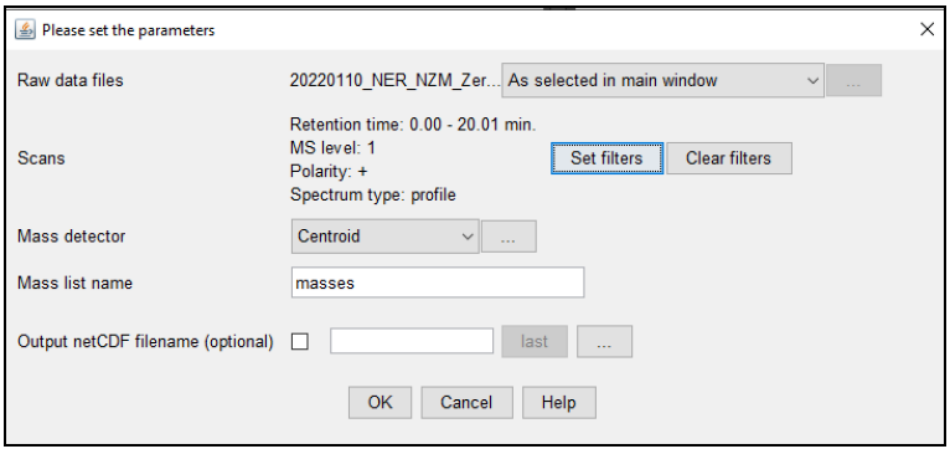


Figure 4. Scanning filters

A mass list for each scan with the identification of individual ions was built using the "mass detection" feature with a noise level set to 1.0E5, with the resulting peaks shown in red colour (Figure 5).

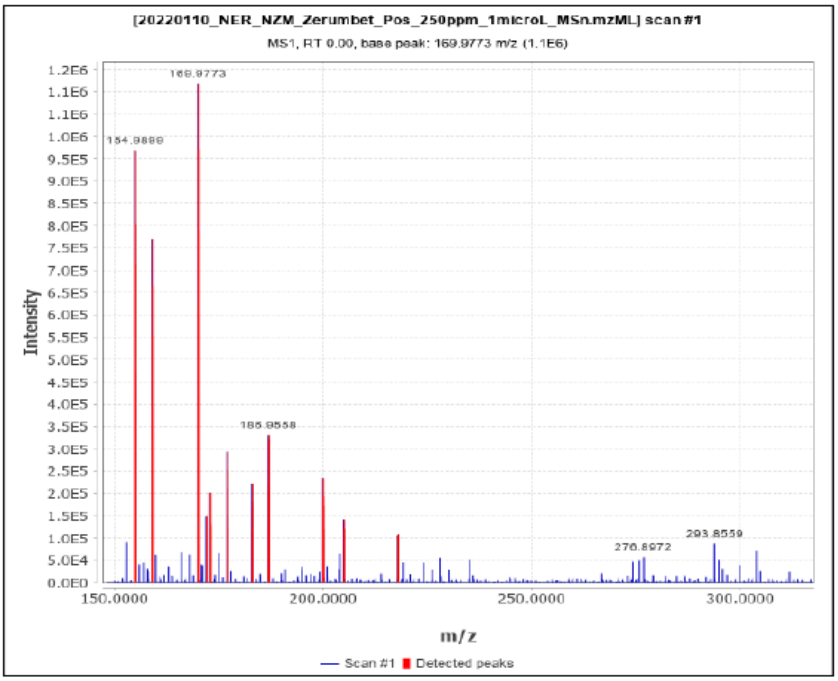


Figure 5. Preview of selected peaks at 1.0E5 noise level.

The extracted masses were grouped using the Automated Data Analysis Pipeline (ADAP) chromatogram builder feature by setting the minimum group size to the value of 5 and the group intensity threshold to 2.0E0. The *m/z* tolerance, the mass difference between experimental and calculated mass, was decided at 0.01 *m/z* or 5.0 ppm. Figure 6 shows that 842 masses were detected prior to the filtering stage. Deconvolution is the clean-up process and separating any overlapping peaks. As shown in Figure 7, the mass number has increased to 1000 peaks, and 133 peaks were identified after the deconvolution process. The deisotope process is the next filter stage in which it groups available peaks into one identity. In Figure 8, the number of mass peaks has been reduced to 831 with 164 identified peaks. The whole data mining and data processing flow chart is shown below in Figure 9.

Compounds Identification

The identification process starts by identifying common adducts such as sodium ion [M+Na-H] and potassium ion [M+K-H]. The mass data generated from the MZmine 2 was compared with a custom database (DNP) and literature within the mass error of 0.01 m/z or 5.0 ppm, which indicates the precision between the experimental and calculated mass values. The mass error (in unit ppm) is calculated as following:

$$Mass\ error = \frac{calculated\ mass - experimental\ mass}{calculated\ mass} \times 10^6$$

Figure 10 shows the total ion chromatogram (TIC) with highlighted peaks of identified compounds. 37 compounds were identified in the ethanolic extract of *Z. zerumbet* based on their mass and peak shape, with a mass error of less than ±5.0 ppm (Table 4).

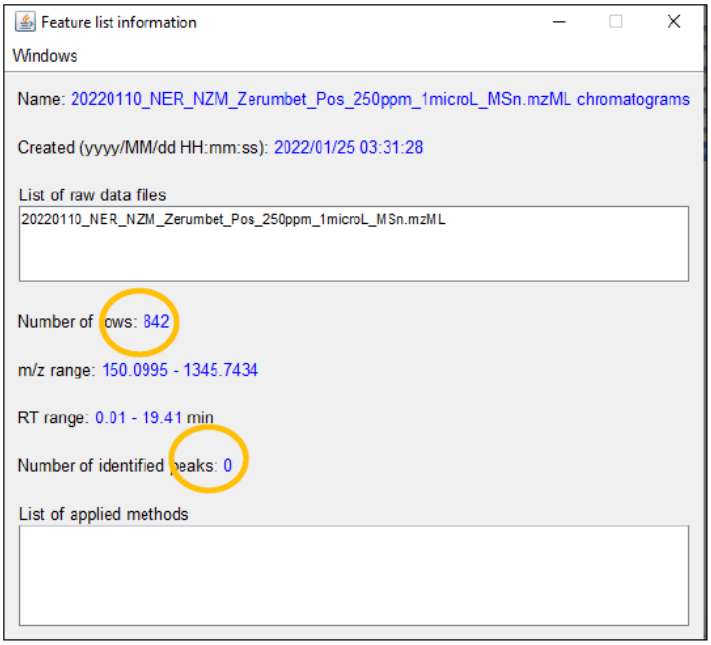


Figure 6. Number of masses detected and identified peaks before deconvolution.

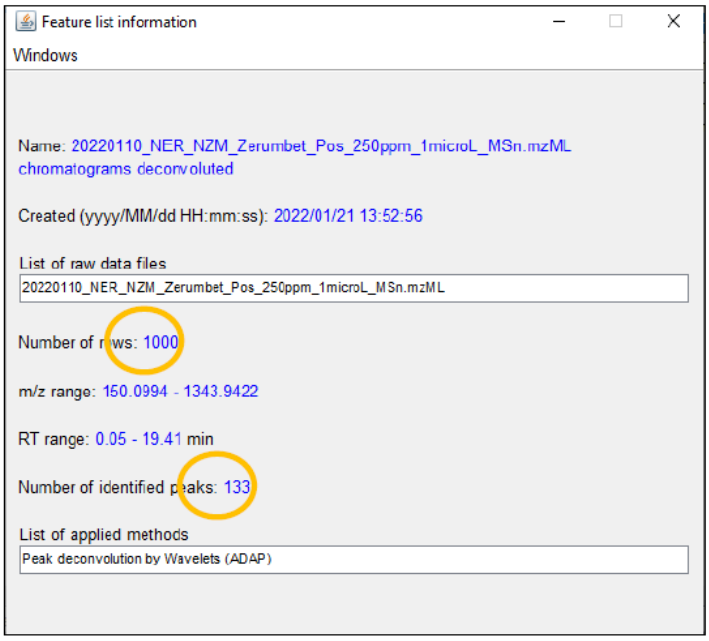


Figure 7. Number of masses detected and identified peaks after deconvolution.

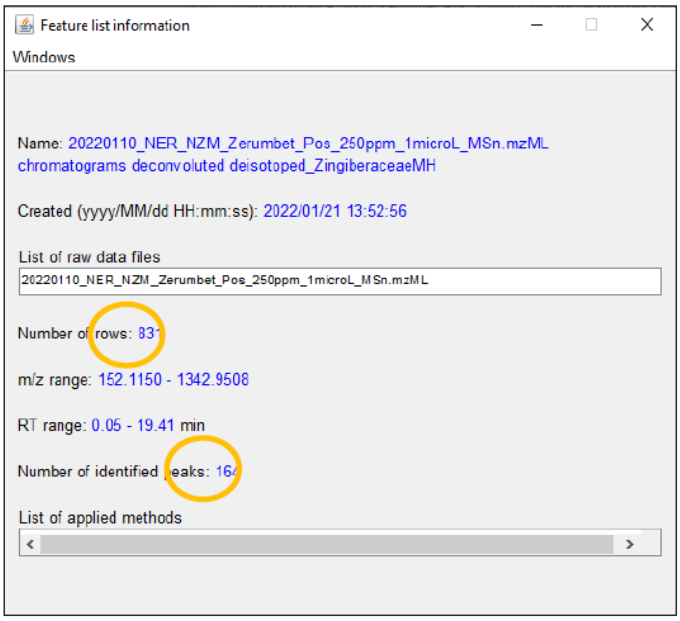


Figure 8. Number of masses detected and identified peaks after deisotoped.

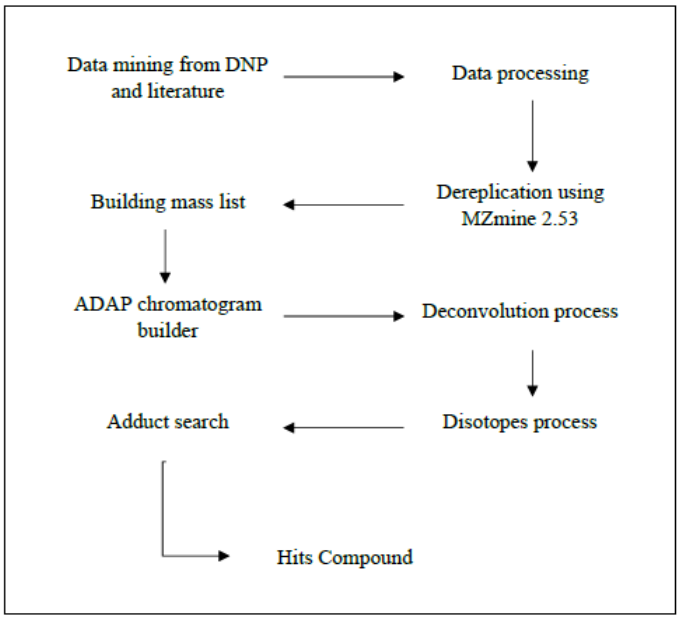


Figure 9. Data mining and data processing flow chart.

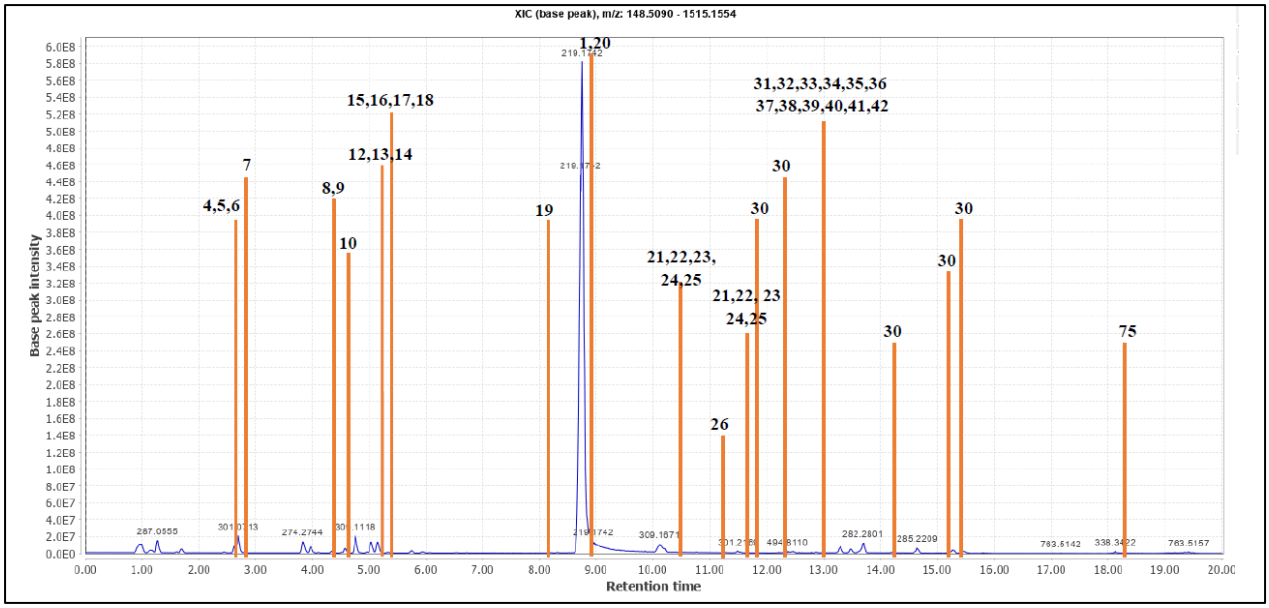
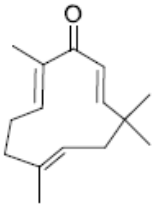
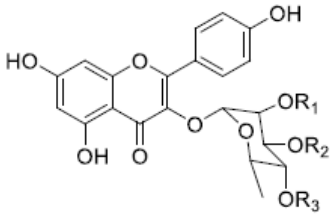


Figure 10. Total ion chromatogram (TIC) with identified compounds (highlighted peaks) in *Z. zerumbet* extract

Zerumbone **1** is the most abundant compound detected at a retention time of 8.75 minutes with a mass error of 0.643323 ppm, following findings from the literature review (Moreira et. al., 2018) as the predominant compound. Two previously isolated flavonoids from *Z. zerumbet* rhizome also match with our findings: 2",4"-diacetylafzelin **2** and 3",4"-diacetylafzelin **3** (Moreira et. al., 2018). The two flavonoids are regio-isomers detected at a retention time of 2.66 minutes with the experimental mass of *m/z* 517.1345 and mass error of -0.858578 ppm.



1



	R_1	R_2	R_3
2	CH_3CO	H	CH_3CO
3	H	CH_3CO	CH_3CO

CONCLUSION

To conclude, 37 compounds were proposed to be present in the ethanolic extract of *Z. zerumbet* rhizome. Various classes of compounds were identified, including flavonoids, terpenoids, diarylheptanoids, and phenolics. A comparison of sample mass data with DNP and literature survey following dereplication with MZmine software proved successful and efficient in identifying chemical constituents in plant samples. However, this protocol has a limitation in distinguishing isomeric compounds, which requires MS/MS full-scan prediction software.

Table 4. Identified compounds from rhizome of *Z. zerumbet*.

Identities	tR (min)	Molecular formula	Experimental mass $[M+H]^+$	Calculated mass	Mass error (ppm)
2",3"-Diacetylafzelin **(4)**	2.66	$C_{25}H_{24}O_{12}$	517.1345	517.134056	-0.858578
2",4"-Diacetylafzelin **(5)**		$C_{25}H_{24}O_{12}$		517.134056	-0.858578
3",4"-Diacetylafzelin **(6)**		$C_{25}H_{24}O_{12}$		517.134056	-0.858578
Kaempferide **(7)**	2.70	$C_{16}H_{12}O_6$	301.0711	301.070666	-1.441522
Gingerenone B **(8)**	4.34	$C_{22}H_{26}O_6$	387.1810	387.180216	-2.024897
Isogingerenone B **(9)**		$C_{22}H_{26}O_6$		387.180216	-2.024897
Bisdemethoxydihydrocurcumin **(10)**	4.58	$C_{19}H_{18}O_4$	311.1284	311.127786	-1.973466
3-(4-Hydroxyphenyl)-2-propen-1-ol **(11)**		$C_{19}H_{18}O_4$		311.127786	-1.973466
3-O-Acetylpinobanksin **(12)**	5.15	$C_{17}H_{14}O_6$	315.0866	315.086316	-0.90134
Kumatakenin **(13)**		$C_{17}H_{14}O_6$		315.086316	-0.90134
Ermanin **(14)**		$C_{17}H_{14}O_6$		315.086316	-0.90134
4-[2-(5-Hexyl-2-furanyl)ethyl]-2-methoxyphenol **(15)**	5.34	$C_{19}H_{26}O_3$	303.1954	303.195471	0.234172
Amomaxin A **(16)**		$C_{19}H_{26}O_3$		303.195471	0.234172
Maximumin B **(17)**		$C_{19}H_{26}O_3$		303.195471	0.234172
[8]-Shogaol **(18)**		$C_{19}H_{26}O_3$		303.195471	0.234172
Acetoxy[4]-gingerol **(19)**	8.32	$C_{17}H_{24}O_5$	309.1682	309.169651	4.693216
Zerumbone **(1)**	8.75	$C_{15}H_{22}O$	219.1742	219.174341	0.643323
Longiferone B **(20)**		$C_{15}H_{22}O$	219.1742	219.174341	0.643323
Mioganal **(21)**	10.77	$C_{20}H_{28}O_2$	301.2167	301.216206	-1.640018
Zerumbetol **(22)**		$C_{20}H_{28}O_2$		301.216206	-1.640018
Coronarin A **(23)**		$C_{20}H_{28}O_2$		301.216206	-1.640018
Villosin **(24)**		$C_{20}H_{28}O_2$		301.216206	-1.640018
Ottensinin **(25)**		$C_{20}H_{28}O_2$		301.216206	-1.640018
Acetoxy[4]-gingerol **(26)**	11.07	$C_{17}H_{24}O5$	309.1682	309.169651	4.693216
Coronarin A **(27)**		$C_{20}H_{28}O_2$		301.216206	-1.640018
Villosin **(28)**		$C_{20}H_{28}O_2$		301.216206	-1.640018
Ottensinin **(29)**		$C_{20}H_{28}O_2$		301.216206	-1.640018
Coronarin E **(30)**	11.53	$C_{20}H_{28}O$	285.2219	285.221291	-2.135184
Coronarin E **(30)**	12.46	$C_{20}H_{28}O$	285.2219	285.221291	-2.135184
14,15-Epoxy-8(17),12-labdadien-16-al **(31)**	12.86	$C_{20}H_{30}O_2$	303.2322	303.231856	-1.134445
Kaempulchraol S **(32)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
15,16-Epoxy-8(17),11,13-labdatrien-16-ol **(33)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
15,16-Epoxy-8(17),13(16),14-labdatrien-12-ol **(34)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
15-Hydroxy-8(17),11,13-labdatrien-16-al **(35)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
Kaempulchraol J **(36)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
9-Hydroxy-8(14),15-isopimaradien-1-one **(37)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
8(17),12-Labdadien-16,15-olide **(38)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
8(17),12-Labdadiene-15,16-dial **(39)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
8(17),12-Labdadiene-15,16-diol**(40)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
8(17),13-Labdadien-15,16-olide **(41)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
Kaempulchraol G **(42)**		$C_{20}H_{30}O_2$		303.231856	-1.134445
Coronarin E **(30)**	14.65	$C_{20}H_{28}O$	285.2219	285.221291	-2.135184
Coronarin E **(30)**	15.27	$C_{20}H_{28}O$	285.2215	285.221291	-0.732764
Coronarin E **(30)**	15.47	$C_{20}H_{28}O$	285.2219	285.221291	-2.135184
13-Docosenamide **(43)**	18.12	$C_{22}H_{43}NO$	338.3406	338.34174	3.369374

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