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Exploring the Potential of Subcritical Water Extraction for Zerumbone and the Antioxidant Activity of *Zingiber Zerumbet* Rhizome

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ABSTRACT

Traditional extraction methods for obtaining bioactive compounds from the rhizome of *Zingiber zerumbet* are associated with long extraction times and the use of hazardous organic solvents, posing significant health and environmental risks. In this study, a modern approach was used in which the fresh rhizome of *Z. zerumbet* was extracted by subcritical water extraction (SWE). Zerumbone concentration and antioxidant activity of the extract generally increase with longer extraction time. The highest zerumbone concentration (57.278 ± 6.066 mg/g) was obtained at an extraction time of 25 minutes with a crude concentration of 10 mg/mL. However, the antioxidant activity value (92.234 ± 3.132 %) was highest at an extraction time of 10 minutes, slightly higher than the value at 25 minutes (89.489 ± 1.913 %) at the same concentration. The IC_{50} values decreased significantly with longer extraction times, indicating an increased antioxidant potential. The 25 minutes extraction showed a robust IC_{50} value of 0.041 ± 0.022 mg/mL, indicating a strong 50 % inhibition of free radicals. Statistical analysis showed no significant differences in zerumbone concentration ($p = 0.930$) and antioxidant activity ($p = 0.851$) between the different extraction times and extract concentrations. Correlation analysis showed a bidirectional relationship between zerumbone concentration and antioxidant activity ($R^2 = 0.9316$, $r = 0.703$), indicating a moderate to strong relationship. The consistent trends in this study support SWE as an effective method for extracting fresh rhizomes, yielding extracts with significant zerumbone concentration and antioxidant activity. Due to the large sample size (N = 36), this study was credible and provides valuable insights for various industries, including pharmaceuticals, dietary supplements, food, and natural health products.

Keywords:

Antioxidant activity; IC_{50}; OFAT; Subcritical water extraction; Zerumbone; Zingiber zerumbet

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1. Introduction

Zingiber zerumbet, known as "lempoyang" in Malaysia (Figure 1), is a fragrant herbaceous plant that has attracted attention due to its pharmacological potential, e.g., as an antioxidant, as reported in the literature [1-3]. Antioxidants are the first line of defence against the harmful effects of free radical damage such as reactive oxidative stress (ROS) [4], and they are essential for maintaining optimal health via various mechanisms of action [5]. Since 1944, researchers have extracted bioactive compounds from the rhizome using various methods and have found that the main constituent of the rhizome, zerumbone, exists in a variety of forms, including crude extracts, isolated bioactive compounds, and essential oils with different concentrations. Zerumbone is a multifunctional compound with antioxidant, antimicrobial, antitumor, hyperalgesic, and anti-inflammatory properties and represents a starting molecule for future synthesis of derivatives with improved efficiency [6]. In Malaysia, a one-week ethanolic maceration was used and a zerumbone concentration of more than 95.37 % was obtained [7]. In a study using six-hour hydro distillation, zerumbone concentration of 87.93 % was found in Brazilian samples [8]. Studies on the island of Tahiti and in Japan found that zerumbone content was 65.30 % [9] and 48.13 % [10], respectively. Variations in zerumbone composition are due to factors such as the age of the plant, processing, and geographic conditions [11,12]. This variability highlights the influence of numerous factors on the formation of zerumbone in *Z. zerumbet*. This divergence is a strong reason for researchers to study the plant and its compounds more extensively.

Fig. 1. *Zingiber zerumbet* rhizome (Lempoyang)

Meanwhile, the selection of an optimal extraction method is crucial to unlock the full potential of *Z. zerumbet* rhizomes. Various conventional solid-liquid extraction methods have been used to extract the bioactive compounds from this rhizome such as percolation [13], reflux [14], hydro distillation [12,15-17], maceration with methanol or ethanol [18-20], and Soxhlet method with chloroform and methanol [21,22]. Conventional extraction methods, despite their simplicity, are often laborious and use potentially hazardous solvents, resulting in a large waste of organic solvents and polluting the environment if not properly managed [23]. In the search for bioactive chemicals, the trend is increasingly toward advanced and environmentally friendly techniques that promote safety and health [24]. This change highlights the need for environmentally friendly methods to obtain important compounds from natural sources.

Therefore, subcritical water extraction (SWE), which uses water as a solvent and operates optimally at 100 to 374 °C and 1 to 220 bars, is a viable method for extracting bioactive compounds that enables sustainable use and production patterns in line with the Sustainable Development Goal (SDG 12). It has evolved in response to the challenges of traditional solvent extraction and aims to improve the quality of extraction [25]. In addition, SWE has proven to be efficient, economical, environmentally-friendly, harmless, and fast process [26]. SWE has become a viable alternative for the extraction of bioactive compounds from plant materials, such as apple rice bran protein [27], pomace pectin [28], *Ganoderma lucidum* [29] and *Z. zerumbet* [30,31]. Recent studies using dried and ground rhizomes of *Z. zerumbet* on a laboratory scale have shown comparable results to traditional methods [30,32]. These results highlight the efficiency and potential of SWE in extracting bioactive compounds from *Z. zerumbet* rhizomes.

Considering the potential of SWE for the extraction of plant material, this study focused on the use of a 70-litre plant-scale SWE for the extraction of fresh rhizome of *Z. zerumbet* to obtain a crude extract loaded with bioactive constituents known for their free radical scavenging properties. The main objectives included quantifying the concentration of zerumbone in the crude extract, evaluation of its antioxidant activity, and determination of IC_{50} value of the extract. In addition, a comprehensive analysis was performed using SPSS version 20 (SPSS Inc., USA) to investigate a plausible relationship between the concentration of zerumbone and the observed antioxidant activity at different extraction times. This study highlights the feasibility of SWE as a promising method for extracting plant material while retaining bioactive compounds rich in antioxidant properties.

2. Methodology

2.1 Acquisition of Z. zerumbet Rhizome and Plant Preparation

The fresh rhizomes of *Z. zerumbet* were obtained from Naturemedic Laboratories Sdn Bhd, Chendering, Terengganu, Malaysia. The plant was identified and authenticated by the Herbarium Universiti Sultan Zainal Abidin with voucher specimen UniSZA/A/000000006. The plants were thoroughly cleaned under running tap water to remove soil and dirt, and carefully examined to exclude spoiled or infested rhizomes. The cleaned rhizomes were sliced into approximately 1 to 2 mm thick slices using a high-powered food processor, and all samples were immediately used for the extraction process to ensure maximum freshness.

2.2 Subcritical Water Extraction

SWE was performed statically on an industrial scale in a 70-litre stainless steel batch tank built by NatXtract, AM Zaideen Ventures Sdn Bhd, Malaysia. The temperature and pressure during subcritical extraction were maintained at 100 to 110 °C and 10 to 12 bar, respectively. To ensure proper cooling, the cooling tank was precooled to a temperature between 25 and 27 °C by activating the coolant tank (Lauda, Alpha RA 12) before the extraction started. Eight kilograms of cleaned rhizomes were placed in a stainless-steel mesh basket, which was then immersed in the extraction tank at a ratio of 1:4 reverse osmosis water to rhizomes (solid:solvent). At the start of the process, nitrogen was supplied to the SWE system as the designated pressure control gas, carefully reaching a pressure of 1.4 bar before the process began. The extraction process was carried out according to the one-factor-at-a-time (OFAT) procedure for various extraction times of 10, 15, 20, and 25 min, with the other operating conditions remaining constant throughout the experiment. The sample was stirred at 1300 rpm using a stirrer with four blades. Once the temperature and pressure reached the subcritical range, the

heater was turned off while the stirrer remained in operation. The extraction time was measured with a stopwatch from this point on. After the extraction was completed, the stirrer was turned off and the crude extract was collected after it reached approximately 40 to 50 °C. This experiment was repeated with different extraction times, while the other variables remained constant.

2.3 Freeze Drying Process

Freeze-drying was performed according to the previous method with modifications [33]. After completion of SWE, the crude extracts obtained were evenly distributed on trays and positioned in the single product chamber of a pilot scale freeze dryer (CUDDON FD80, New Zealand). The drying process comprised three different stages: Initial freezing, primary drying and secondary drying. The cooling parameters were set as follows: a freezing temperature of -20 °C, a sublimation temperature of -20 °C to 0 °C and a vacuum pressure of 0.34 mbar. The isothermal desorption process took place in a temperature range of 0 to 60 °C and in a vacuum environment of 0.060 mbar. The entire freeze-drying process took about 34 hours and ended at a final temperature of 40 °C. The freeze-dried extracts were then stored in a tight bottle for further analysis.

2.4 Preparation of Serial Dilution Extract

A methanol solvent (HPLC grade) was used to prepare a freeze-dried extract stock solution with a concentration of 10 mg/mL. The solution was sonicated with an ultrasonic basin (Sukinbo, China) and vigorously shaken with a vortex mixer (Heidolph, Germany) to ensure effective extraction and dissolution. The stock solution was then serially diluted to produce extract solutions with concentrations of 8, 6, 4, 2, 1, 0.5, 0.25 and 0.1 mg/mL. Prior to subsequent analysis, each diluted extract was filtered through a nylon syringe membrane (13 mm x 0.45 µm) to remove visible suspended solids.

2.5 Zerumbone Concentration Calibration Curve

The linearity of the calibration for the zerumbone standard was determined for the quantification of zerumbone content in the extract. For this purpose, a standard calibration curve was formulated in the present study, covering the preparation of nine different concentrations of zerumbone standard solutions. First, a precisely measured amount of zerumbone standard was dissolved in HPLC methanol to prepare a stock solution with a concentration of 0.1 mg/mL. This stock solution was then successively diluted to obtain the target working concentrations: 0.1, 0.05, 0.02, 0.01, 0.005, 0.002, 0.001, 0.0005, and 0.0002 mg/mL. Approximately 10 µL of each zerumbone standard solution was added to the HPLC instrument to facilitate peak area determination.

A linear regression equation (y = mx) was constructed for a standard calibration curve to correlate the relationship between peak area (y-axis) and zerumbone concentration (x-axis). A linear regression equation (y = mx) was constructed for a standard calibration curve to correlate the relationship between peak area (y-axis) and zerumbone concentration (x-axis). Peak area, represented as absorbance (AU), was plotted against concentration (mg/mL) to obtain a linear equation. Zerumbone calibration curve was generated for standard zerumbone with a coefficient of determination (R^2) of 0.999 or less [34]. Figure 2 was the standard calibration curve developed with a linear trend line.

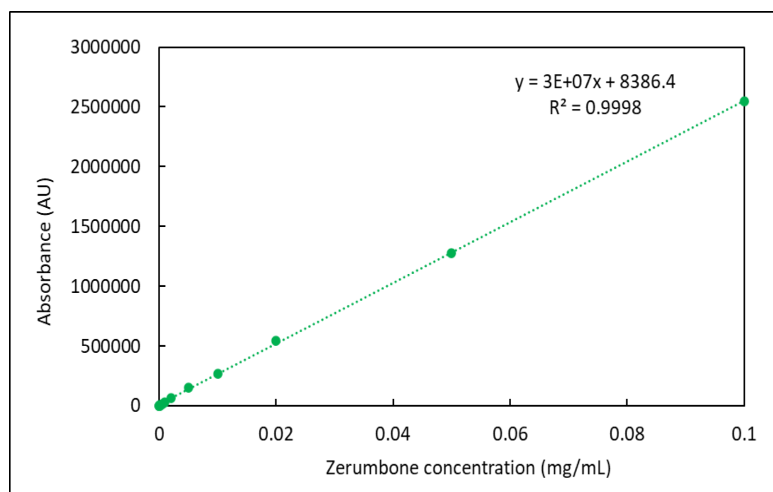


Fig. 2. Zerumbone absorbance versus zerumbone concentration

2.6 Zerumbone Concentration

The method for determining the concentration of zerumbone was adopted from previous studies [30,32]. The experiment was performed using a high-performance liquid chromatography (HPLC) (Altus, Perkin Elmer MS, USA) equipped with a photodiode array detector (PDA) (Altus, Perkin Elmer MS, USA) and analytical column C18, 5 µm, 150 x 4.6 mm (Perkin Elmer, USA). EmpowerTM software was used to quantify the compound (Waters, Milford, MA, USA). The mobile phase consisted of 100 % methanol HPLC grade (solvent A) and 100 % acetonitrile HPLC grade (solvent B). It was prepared daily and filtered through a nylon syringe membrane (25 mm x 0.45 µm) before sonication. Separation was performed by isocratic elution at 35 % (solvent A) and 65 % (solvent B), with a total run time of 3 min. The PDA detector examined the eluate at a wavelength of 254 nm and a flow rate of 1 mL/min. Approximately 10 µL of each sample aliquot was injected and zerumbone peak area was determined. Zerumbone concentrations (mg/mL) were calculated using the standard calibration curve shown. Consequently, the amount of zerumbone compound was determined in milligrams per gram (mg/g) according to Eq. (1).

$$C = \frac{(C_{ZER})(DF)(V_S)}{W_S} \quad (1)$$

Where C represents the zerumbone compound (mg/g), C_{ZER} stands for the zerumbone concentration obtained from the standard calibration curve (mg/mL), DF denotes the dilution factor, V_s represents the sample volume (mL), and W_s symbolizes the sample weight (g).

2.7 Antioxidant Activity by DPPH Assay

The antioxidant activity or 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the crude extract was determined according to previous methods [35-39] with modifications. 0.1 mM DPPH methanolic solution was prepared by dissolving DPPH (Sigma-Aldrich, Germany) in methanol (HPLC grade). 2 mL of each diluted extract was added to 2 mL of DPPH solution and vigorously shaken with a vortex to ensure optimal reaction. The solution was then incubated for 30 min at room temperature in a dark environment. The absorbance of the sample was measured at 517 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) with a cuvette (optical path length, 1 cm). The absorbance of these solutions was measured at the same wavelength as that of the control

solution. The same procedure was repeated for the standard antioxidant with a series of dilute ascorbic acid of 0.01, 0.08, 0.06, 0.001, and 0.0005 mg/mL. Ascorbic acid was used as a positive control. All experiments were performed in triplicate, and the results were expressed as mean values. Antioxidant activity was calculated according to Eq. (2):

$$Antioxidant\ activity\ (\%) = \frac{(A-B)}{A} x\ 100 \quad (2)$$

Where A is the absorbance of the control (methanolic DPPH solution without extract) and B is the absorbance of the sample (methanolic DPPH solution in the presence of extract). Antioxidant activity was also expressed as the inhibitory concentration at 50 % of the antioxidant activity value (IC_{50}). IC_{50} represents the concentration of the test solution required to reach 50 % of the antioxidant activity, expressed in mg/mL. To express the antioxidant capacity of the sample in a more familiar and easily understood way, the ascorbic acid equivalent antioxidant capacity (AAEAC) in mg AA/100 g was expressed using Eq. (3):

$$AAEAC = \frac{IC_{50(AA)}}{IC_{50(Sample)}} x\ 10^5 \quad (3)$$

Where $IC_{50(AA)}$ was determined with an ascorbic acid calibration curve.

2.8 Statistical Analysis

Each sample measurement was performed in triplicate. Results were expressed as means with corresponding standard deviations (SD). All descriptive statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA) for Windows. This was a one-way analysis of variance (ANOVA). Tests were used to detect significant differences between means within a 99 % confidence interval. A p value of less than 0.01 ($p <0.01$) indicates a higher level of significance. To put this problem into the usual format for hypothesis testing (Statement of Work), the null hypothesis and the alternative hypothesis were formulated as follows: Null hypothesis H_0: "*There was no statistically significant difference in the activity of the response variables between extraction times for all crude extracts studied*", while the alternative hypothesis H_A: "*There was a statistically significant difference in the activity of the response variables between extraction times for all crude extract concentrations studied*". Thus, if the p-value of the statistical analysis is less than 0.01 ($p < 0.01$), the null hypothesis is rejected.

For the correlations between zerumbone concentration and the antioxidant activity of the samples, the Bivariate and Pearson correlation with a two-sided significance test were used. Pearson's correlation coefficient, r, measures the strength of the linear relationship between two variables. It has a value between -1 and 1, with a value of -1 indicating a completely negative linear correlation, 0 indicating no correlation, and +1 indicating a completely positive correlation [40]. The absolute criterion was used, with values of the correlation coefficient r from 0 to 0.19 indicating no correlation, 0.2 to 0.39 indicating low correlation, 0.40 to 0.59 indicating moderate correlation, 0.60 to 0.79 indicating moderately high correlation, and values of 0.80 or more indicating high correlation [41,42].

3. Results and Discussion

3.1 Zerumbone Concentration

Figure 3(a) and 3(b) shows the relationship between the different concentrations of crude extract and the corresponding content of zerumbone over a range of extraction times from 10 to 25 min. The observed relationship shows that as the extraction time increased, the concentration of zerumbone also increased. This recurring trend indicates a continuous absorption process over time that applies to all extract concentrations studied. As expected, the results confirmed the idea that an increase in the concentration of the crude extract was accompanied by a proportional increase in the quantitative presence of the zerumbone compound.

In Figure 3(a), at the lower range of initial concentrations of crude extract (between 0.1 and 2 mg/mL), the recorded zerumbone concentrations remained at a minimum across the different time intervals, ranging from 0.0009 ± 0.0009 to 2.102 ± 0. 0995 mg/g. In contrast, higher initial concentrations of crude extract (between 4 and 10 mg/mL) resulted in a progressively increasing trend in zerumbone content over the course of extraction. The peak of this concentration increase was observed during the 25 min extraction time, particularly evident at a crude extract concentration of 10 mg/mL. At this point, the zerumbone concentration reached an impressive 57.278 ± 6.066 mg/g. This crucial observation underlines that the 25 min time point represents the period during which the greatest amount of zerumbone was successfully extracted.

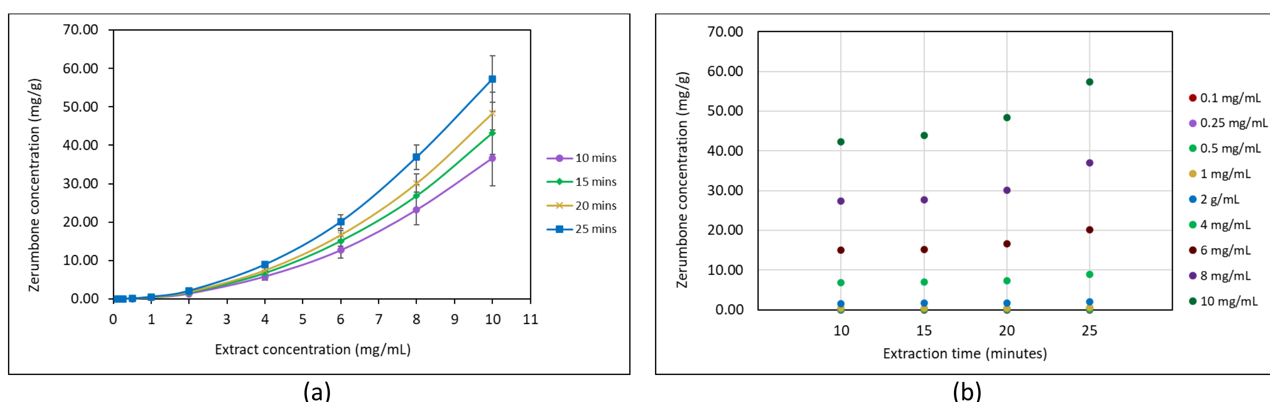


Fig. 3. Zerumbone concentration (a) at several crude extract concentrations (b) at different extract times

In this study, increasing the extraction time allowed better exposure of the active constituents, which led to better results. Conversely, insufficient or short extraction times resulted in less effective procedures, while longer extraction times led to the degradation of the desired bioactive compounds [43]. Remarkably, a 25 min extraction yielded the highest concentration of the target compound, zerumbone. This raised the question of whether extending the extraction time to 30 min would further increase or decrease zerumbone. It is worth noting that previous research has indicated that SWE takes less time compared to conventional methods, which usually require at least hours [44]. Therefore, the duration of extraction plays an important role. This is consistent with previous findings that a short duration is sufficient to complete the extraction process [45].

A consistent trend was observed in zerumbone concentration, which increased with increasing extraction time, especially up to 25 min. Although zerumbone concentration could theoretically continue to increase beyond this point, the study was intentionally limited to this time frame to ensure that the results remained relevant and practical for industrial applications. This decision was intended to reduce potential adverse effects of prolonged extraction, such as a reduction in active compounds and other practical issues. In this way, the study primarily provided relevant findings

while minimizing the risk of unfavourable results in an industrial setting. Furthermore, in practice, extraction times of about 15 min are currently used in plants using a 70 L SWE system for production. Increasing the extraction time to 30 min would double the labour and time required for the SWE system. This has implications not only for production efficiency, but also for resource allocation.

Figure 3(b) is a scatter plot showing the relationship between zerumbone concentration and the extraction time. To determine the dependence of zerumbone concentration around the mean on the extraction time, a comprehensive statistical analysis was performed using SPSS. Table 1 summarizes the results of the one-way analysis ANOVA which confirmed that the null hypothesis was not rejected ($p = 0.930 > 0.01$), meaning that there was no statistically significant difference in zerumbone concentration between extraction times at all extract concentrations studied. This result was strongly supported by the calculated F-value (the ratio of between-group variance to within-group variance) of 0.149. The F-distribution of the upper tail point of the $F_{0.01,3.35}$ distribution is 3.925. Based on the F-value from the ANOVA analysis, the F-value was not large enough ($F = 0.149 < 3.925$) to reject the null hypothesis at the selected significance level ($p < 0.01$). Therefore, the null hypothesis was accepted in this study and it was concluded that the different extraction times did not have a statistically significant effect on the mean zerumbone concentration.

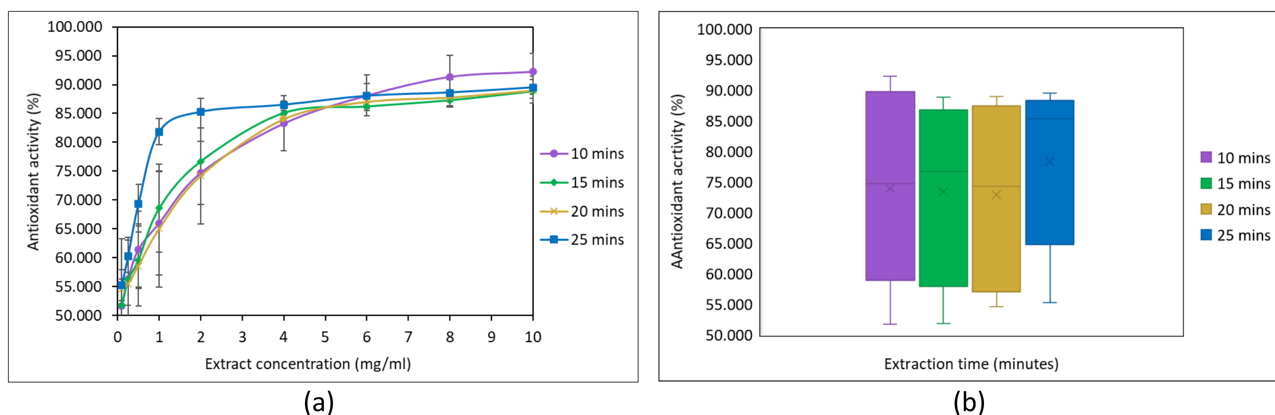
Table 1
Analysis of variance (ANOVA) for zerumbone concentrations at different extraction times

Source of variation	Sum of Squares	df	Mean Square	F	P-value
Between Groups	125.165	3	41.722	0.149	0.930
Within Groups	8979.094	32	280.597		
Total	9104.259	35			

The observed standard deviations (SD) in zerumbone concentrations are due in part to the natural variability that can occur in chemical analysis. In addition, the nature of plant extracts can inherently lead to variations in compound concentrations, which is reflected in the SD. In addition, the HPLC method can detect even small variations in concentration that contribute to the calculated SD. This sensitivity is advantageous for accurate detection of concentration changes, especially when studying different extraction times and concentrations. It is worth noting that despite the relatively high SD, the observed trends, and differences in zerumbone concentrations between different conditions (extraction times and concentrations) remain statistically significant. This underlines the robustness of the results.

3.2 Antioxidant Activity

Figure 4(a) and 4(b) shows the antioxidant activity of the crude extract obtained from the fresh rhizome of *Z. zerumbet* by SWE with different extraction times and different extract concentrations. The results, which are presented as mean values with standard deviations (SD), show that all samples exhibited radical scavenging activity that varied at different extraction times and concentrations. Overall, the results in Figure 4(a) showed a gradual increase in antioxidant activity at higher concentrations. At the lowest concentration of 0.1 mg/mL, the antioxidant activity ranged from 51.709 ± 4.453% to 55.232 ± 2.673% over different time periods. With increasing concentration, the antioxidant activity also showed an upward trend, reaching a range of 88.489 ± 0.1913 % to 92.234 ± 3.132 % at the highest tested concentration of 10 mg/mL. For each extraction time, a higher concentration means that more antioxidant compounds were present and thus there was a higher potential to neutralize free radicals.



(a) (b)

Fig. 4. Antioxidant activity (a) at several crude extract concentrations (b) at different extraction times

In general, a significant acceleration of antioxidant activity occurred in the concentration range of 0.1 to 2 mg/mL. The rate of increase became more pronounced with increasing concentration. The steeper increase may indicate that at higher concentrations, the active compounds of the extract interact more actively with reactive oxygen species (ROS), resulting in more efficient neutralization of ROS through the release of electrons, thus contributing to more rapid alleviation of oxidative stress [46]. Between the concentrations of 2 to 6 mg/mL, the data set shows a concentration range that seems to be optimal for achieving the highest level of antioxidant activity. The efficacy of the extract continues to increase, but at a slightly slower rate than in the previous concentration range. This observation could be taken as a sign that the scavenging mechanisms of the active compounds are approaching a saturation point. In this concentration range, there seems to be an appropriate balance between the amount of available antioxidants and the abundance of ROS that allows effective neutralization of ROS without entering a phase of diminishing returns [47].

When the concentration of the extracts exceeded 6 mg/mL and reached 10 mg/mL, the trend indicated a possible plateau or diminishing return of antioxidant activity. This phenomenon could indicate that the antioxidant mechanisms of the extract were saturated and that there was an upper limit to its ability to neutralize ROS. Increasing the concentration beyond this point may not have resulted in a proportional increase in antioxidant activity, as the interaction of active ingredients with ROS may have reached their full potential [47].

Figure 4(b) shows a boxplot illustrating the relationship between antioxidant activity and extraction time. It shows the five-digit summary of a data set: the minimum, the first quartile, the median, the third quartile, and the maximum. The graph shows the distribution of antioxidant activity at each extraction time. However, there is no clear indication that the variability of antioxidant activity around the average depends on the extraction time.

Table 2 summarizes the results of the one-way analysis ANOVA, which confirms that the null hypothesis was accepted ($p = 0.851 > 0.01$), meaning that there was no statistically significant difference in antioxidant activity between extraction times at all extract concentrations studied. This means that the mean values of the groups were not significantly different from each other. This result was strongly supported by the calculated F-value (the ratio of the variance between groups to the variance within groups) of 0.263. Based on the F-value from the ANOVA analysis, the F-value was not large enough ($F = 0.263 < 3.925$) to reject the null hypothesis at the selected significance level ($p < 0.01$). Therefore, the null hypothesis was accepted in this study and it was concluded that the different extraction times had no statistically significant effect on the mean antioxidant activity.

Table 2
Analysis of variance (ANOVA) for antioxidant activity of crude extract at different extraction times

Source of variation	Sum of Squares	df	Mean Square	F	P-value
Between Groups	167.192	3	55.731	0.263	0.851
Within Groups	6778.155	32	211.817		
Total	6945.347	35			

In a separate study with SWE, the different operating conditions were used in the extraction which allowed the evaluation of both zerumbone content and antioxidant activity. The concentration of zerumbone and its antioxidant activity increase significantly when moving from 1 L and 5 L to 70 L SWE capacity as shown in Table 3. Larger SWE capacities along with a lower solid-to-solvent ratio (as in the case of the 70 L capacity with a 1:4 ratio used in the study) can result in significantly higher zerumbone concentrations and improved antioxidant activities. However, it is also important to note that various operational factors also influence the extraction process.

Table 3
Zerumbone concentration and antioxidant activity of *Z. zerumbet* rhizome using SWE

SWE capacity	1 L [31]	5 L [48]	70 L (current study)
Raw material	Dried rhizome	Dried rhizome	Fresh rhizome
Temperature (°C)	170	170	110
Pressure (bar)	20	20	11
Extraction time (min)	40	20	15
Solid:solvent	1:20	1:20	1:4
Sample extract tested	Liquid	Liquid	Powder
Zerumbone concentration (mg/g)	19.82 ± 0.004	20.82 ± 0.42	43.20 ± 5.61
Antioxidant activity (%)	60.70 ± 0.024	63.26 ± 0.79	88.86 ± 2.01

Several studies indicate significant benefits associated with the use of fresh rhizomes. The use of fresh rhizomes eliminates the need to dry, grind, or pulverize the rhizome prior to extraction, thus preserving the bioactive constituents. In addition, drying aromatic plants could reduce their biological activity [49]. This is consistent with the observation that drying lemon balm, oregano, and peppermint leads to a significant decrease in ascorbic acid and carotenoids [50]. In addition, the water in the sample acts as a co-solvent during the extraction process and facilitates the recovery of polar components [51]. Previous studies have shown that extracts from fresh and dried rhizomes of *Z. zerumbet* have different but competing properties. For example, it was reported that the content of zerumbone in fresh and dried rhizomes ranged from 8.1% to 84.8% and from 1.2% to 35.5%, respectively [12]. This significant difference highlights that the essential oil extracted from fresh rhizomes was richer and had better antibacterial and cytotoxic properties compared to dried rhizomes.

3.3 Estimation of IC_{50}

The IC_{50} value is a critical parameter that indicates the concentration of a substance required to achieve 50 % inhibition or response in a biological or biochemical assay. In this context, IC_{50} values serve as indicators of the antioxidant activity of the various samples. Table 4 shows the results of the estimation of IC_{50} values for different samples obtained by the experimental procedure. The IC_{50} values were expressed in both milligrams per millilitre (mg/mL) and micrograms per millilitre (µg/mL), along with the corresponding average coefficient of determination, R^2, ranging from 0.91 to 0.99. The samples were characterized by different extraction times, ranging from 10 min to 25 min, and an

additional reference sample, ascorbic acid, was included in the analysis. The results show a trend in IC_{50} values across the different extraction time intervals. It was estimated that the IC_{50} value for 10, 15, 20, and 25 min of extraction times was 0.080 ± 0.022, 0.063 ± 0.019, 0.206 ± 0.041, and 0.041 ± 0.012 mg/mL, respectively. The IC_{50} values appear to decrease with increasing extraction time. This suggests that longer extraction times result in the release of more active compounds or an increase in the concentration of active compounds present to achieve 50 % inhibition. Longer extraction times probably facilitated the release of bioactive compounds with stronger antioxidant properties from the fresh rhizome of *Z. zerumbet*.

Table 4
Estimation of IC_{50} of different samples

Sample	IC_{50} (mg/mL)[a]	IC_{50} (µg/mL)[a]	AAEAC (mg/100 g)[b]
10 min	0.080 ± 0.022	80 ± 22	750.00
15 min	0.063 ± 0.019	63 ± 19	952.38
20 min	0.206 ± 0.041	206 ± 41	291.262
25 min	0.041 ± 0.012	41 ± 12	1463.414
Ascorbic acid[c]	0.0006 ± 0.00008	0.6 ± 0.08	

[a] IC_{50}: the concentration of sample that affords a 50% reduction in the assay, expressed as the means ± SD of triplicate experiments.
[b] AAEAC (ascorbic acid equivalent antioxidant capacity) = $(IC_{50(AA)}/IC_{50(Sample)}) \times 10^5$.
[c] Ascorbic acid as positive control

This phenomenon was supported by the consistent trend observed across the different time intervals. In contrast, the IC_{50} value for ascorbic acid was exceptionally low which was 0.0006 ± 0.00008 mg/mL, indicating its robust antioxidant potential. Ascorbic acid is a well-known antioxidant compound that is often used as a standard of comparison in such studies due to its proven antioxidant properties and to verify the correctness of the procedure. It has been dissolved in methanol to avoid decomposition process like it happen in the aqueous environment. The factors such as pH, temperature, oxygen, and the presence of catalysts (iron, copper) influence this process as well [52]. The observed antioxidant activity of the samples reflects the findings of previous studies zerumbone concentration in fresh rhizomes of *Z. zerumbet* and the assessment of antioxidant capabilities using the DPPH assay.

Previous studies in which the essential oil was isolated from fresh rhizomes by hydro distillation showed a high antioxidant activity of about 78.88 ± 9.35 % along with a zerumbone content of 16.00 ± 5.21 mg/mL [53]. Other studies focused on IC_{50} values also provided relevant results and reported values of 1.6 µg/mL with a 75.2 % zerumbone concentration by the same hydro distillation technique [54]. In another study, a concentration of 75.0 % zerumbone with an IC_{50} value of 32.39 µg/mL was obtained by the same method [12]. Differences in the composition of zerumbone are due to various factors that include the age of the plant, processing methods, and geographic conditions [11,12]. This variability highlights the influence of numerous factors on the formation of zerumbone in *Z. zerumbet*. In addition, the presence of other compounds or interactions between the extracted antioxidants and other components of the rhizome extract could influence the observed trends [55-57].

3.4 Correlation Between Zerumbone Concentration and Antioxidant Activity

Table 5 shows the results of the analysis to investigate the correlation between zerumbone concentration of zerumbone and antioxidant activity. The table shows the Pearson correlation coefficients, significance levels (two-sided), and sample sizes (N) for both zerumbone concentration

and antioxidant activity. The Pearson correlation coefficient, abbreviated "r"," is a statistical measure that indicates the strength and direction of a linear relationship between two variables. It ranges from -1 to 1, with a positive value indicating a positive correlation (as one variable increases, the other tends to increase), a negative value indicating a negative correlation (as one variable increases, the other tends to decrease), and a value near 0 indicating a weak or negligible correlation.

Table 5
Correlation between zerumbone concentration and antioxidant activity

		Zerumbone concentration	Antioxidant activity
Zerumbone concentration	Pearson Correlation	1	0.703**
	Sig. (2-tailed)		0.000
	N	36	36
Antioxidant activity	Pearson Correlation	0.703**	1
	Sig. (2-tailed)	0.000	
	N	36	36

**Correlation is significant at the 0.01 level (2-tailed)

In this context, the results show a Pearson correlation coefficient, r of 0.703 between zerumbone concentration and antioxidant activity. The double asterisks (**) next to the correlation coefficient mean that the correlation is statistically significant at the 0.01 level (double-tailed), indicating a high level of confidence in the result. The p-value (Sig. 2-tailed) of 0.000 further emphasizes the significance of the relationship. The magnitude or strength of the correlation coefficient r of 0.703 indicates a positive and moderately high linear relationship between zerumbone concentration and antioxidant activity ($0.6 < | r | < 0.79$) [40-42]. This result implies that as zerumbone concentration increases, the antioxidant activity also increases. The large sample size (N = 36) makes the analysis more robust and adds further credibility to the observed correlation. The correlation between zerumbone concentration and antioxidant activity exists in both directions, as shown by the symmetry of the table. The coefficient for antioxidant activity itself is 1, as expected, indicating a perfect positive correlation.

Figure 5 illustrates the correlation between zerumbone concentration and antioxidant activity. There is a non-linear relationship between zerumbone concentration and antioxidant activity. The R^2 value of 0.9316 indicates that approximately 93.16 % of the variability in antioxidant activity can be explained by the variability in zerumbone concentration. This high R^2 value indicates a robust fit for a nonlinear model. This could be due to saturation effects, in which antioxidant activity reaches a plateau with increasing zerumbone concentration. In other words: While antioxidant activity initially increased rapidly with increasing concentration, this increase eventually slowed, resulting in a flattened curve. Given the nature of antioxidants and their potential interactions, one possible explanation for this non-linear behaviour could be that there was an optimal zerumbone concentration at which antioxidant activity was maximized. Beyond this concentration, activity may have stabilized or even declined due to factors such as active site saturation or potential adverse effects.

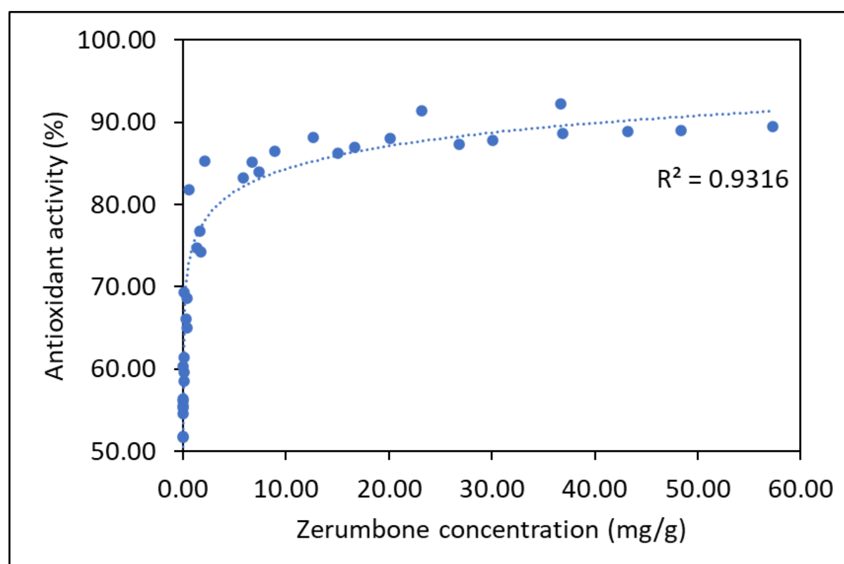


Fig. 5. Correlation of zerumbone concentration and antioxidant activity

Antioxidant activity is a complex property influenced by a variety of compounds in a plant material. Zerumbone is one bioactive compound in *Z. zerumbet* associated with antioxidant properties, but it is important to recognize that many other constituents such as phenolics, flavonoids, vitamins, and other phytochemicals may also contribute to the overall antioxidant activity of a plant extract [46]. Therefore, zerumbone may play a role in enhancing the antioxidant activity of *Z. zerumbet*, it is probably not the only factor responsible. To gain a comprehensive understanding of the factors affecting the antioxidant activity of *Z. zerumbet*, a more comprehensive analysis considering various compounds and their interactions would be required.

4. Conclusions

The successful application of the 70-litre SWE method to extract zerumbone from fresh rhizomes of *Z. zerumbet* using the one-factor-at-a-time (OFAT) approach highlights the capability of this method in this field. The primary pattern observed in this study, in which both zerumbone concentration and antioxidant activity increase with increasing extraction time, supports the efficacy of SWE. This is also confirmed by the decreasing IC_{50} values, which reflect the increased antioxidant potential with longer extraction times. Furthermore, the results using SPSS show that there were no statistically significant differences in zerumbone concentration and antioxidant activity between the different extraction times for all extract concentrations studied. Although the apparent correlation between zerumbone and increased antioxidant activity is of key importance, it is important to recognize the presence of other influencing factors that affect the antioxidant capacity of *Z. zerumbet*. It is acknowledged that the decision to complete the study at 25 min is a limitation, as discussed in the paper. Future research projects could investigate longer extraction times to further explore this possibility. However, in such studies, the potential benefits must be carefully weighed against the increased time and resources required.

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