CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITY FROM THE RHIZOMES OF *ZINGIBER CASSUMUNAR*

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Abstract

Zingiber cassumunar is one of the species belongs to the genus of *Zingiber* in the Zingiberaceace family. Hydrodistillation of fresh rhizomes afforded 2.83% of essential oil as a pale yellow liquid with pungent smell. Chemical compositions of the oils were determined by gas chromatography and gas chromatography-mass spectrometry techniques. Twenty-nine compounds were found in the rhizome essential oil which contributed to 78.74% of the total oil. The compounds have been classified as monoterpenes (75.9%), sesquiterpenes (1.8%) and ester (0.8%). The major constituents were found to be terpinen-4-ol (31.35%) **(1)** and sabinene (23.37%)**(2)**. The dried rhizome of *Z. cassumunar* was also extracted with chloroform using Soxhlet extraction for twenty hours. Fractionation of the crude extract using silica gel vacuum liquid chromatography, followed by repeated purification using silica gel gravity column chromatography afforded two compounds. The structure of these compounds were elucidated by infrared and nuclear magnetic resonance spectroscopies as *trans*-1-(3,4-dimethoxyphenyl)butadiene **(3)**and *trans*-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate**(4)**. DPPH antioxidant screening using DPPH free radical scavenging activity showed that the essential oil of the rhizome of *Z. cassumunar* gave weak antioxidant property and chloroform crude extract gave positive antioxidant result at higher concentration.

Keywords: Zingiberaceace, Zingiber cassumunar, essential oil, crude extract, DPPH

INTRODUCTION

Herbs have long been used in traditional medicine in various cultures throughout the world [1]. The phytochemicals constituents of herb plants play an important role as a prevention and therapy in human health and development [2]. Zingiberaceace is one of the largest families of plant kingdom that established traditional forms of medicine to treat various kinds of diseases [3, 4]. This species is native to East Asia and it is one of the herbaceous ground floras of the rainforest component in Peninsular Malaysia [5]. The members from this family provide many useful food products, perfumes, medicines, ornamentals and other economic uses [4].

*Zingibercassumunar*is one of the genus fromZingiberaceace familythat has been widely known as a medicinal plant and folklore remedies [6]. This plant is native plant of India and is now widely cultivated in tropical Asia. It occurs widely as a home-garden plant in Southeast Asia [7]. *Z. cassumunar* has a pungent odour, and foul-smelling flowers. It is common to be known as *'bonglai'* in peninsular Malaysia, *'bangle'* in Java and *'Plai'* in Thailand [8]. The rhizomes are very rich in essential oil. The oil has a pale amber color, cool scent and a green peppery odour with a touch of a bite [9]. *Z. cassumunar*was claimed to have various functions in folklore medicine as its essential oil has medicinal properties which is used in medical field for various treatment. The pungent components are proven beneficial in treating health problems and protection against certain diseases. Another pharmacological study had discovered that the rhizome of *Z. cassumuna r*provide an antioxidant activity which gave useful usage in medical field [10].Within the scope of continuation search for bioactive compounds from natural plants, the rhizomes of *Z. cassumunar* were further investigated. In this paper, we report the isolation and structural elucidation of chemical constituents of the essential oil as well as the crude extract from the rhizome of *Z. cassumunar*. In addition, both essential oil and the crude extract were evaluated for their antioxidant activity.

EXPERIMENTAL

Vacuum liquid chromatography (VLC) was carried out on Merck silica gel 230-400 mesh using distillated petroleum ether 60-80ºC and diethyl ether as eluent system with increasing polarity to give of fractions. Purification of the compounds were carried out by using column chromatography which used Merck silica gel 70-230 nm as the stationary phase, eluted with a eluents mixture of petroleum ether and diethyl ether and mixture of *n*-hexane and diethyl ether. Thin layer chromatography (TLC) was carried out using aluminium sheet coated with 0.20 mm Merck silica gel plates (60 F_{254}). The spots on the TLC plates were visualised by Ultraviolet (UV) radiation with wavelength of 254 nm and 365 nm. Vanillin sulphuric acid reagent was used to spray the TLC plate in the TLC analysis. This reagent was sprayed on the TLC plate and heat at 100ºC to visualise the spots. Gas chromatography was carried out using Hewlett-Packard gas chromatography with Ultra 1 column (dimethylpolysiloxane) (0.2 mm internal diameter, 0.32 µm thickness and 25 m long). The carrier gas used is Helium with rate flow 1.0 mL/min. Operating temperature for GC was 50ºC to 300ºC with rate by 4ºC

per minute. The type of detector used was Flame Ionization detector (FID). The chemical composition from the essential oil of the rhizome was identified using gas chromatography-mass spectrometry (GC-MS) equipped with Wiley/NIST Registry of Mass Spectral Library, 7th edition 1999. HP 19092A-102 Hewlett-Packard gas chromatography was used with Ultra 1 capillary column (0.2 x 0.32 x 35 mm). Helium gas is the carrier gas with flow rate 1.0 mL/min and the oven temperature was programmed to increase after 5 minutes to 50% and continuously heated until 250ºC at a rate of 5ºC /min. The end temperature was held for 5 minutes. The chromatogram was compared with the Kovats Indices reference and MS libraries. Infrared (IR) spectra were measured using Perkin-Elmer Series 1600 using (NaCl) pellet for liquid samples. The ^{1}H NMR spectra were recorded on BrukerAvance 400 MHz with $CDCl_3$ as solvent. Residual proton in $CDCl_3$ was used as standard.

Plant Material

The fresh rhizome of *Z. cassumunar* was purchased from a wet market in Larkin, Johor Bahru. The rhizomes were washed and chopped into small pieces. The rhizomes were kept in dried conditions.

Extraction of Essential Oil

The fresh rhizomes of *Z. cassumunar*(310 g) were hydrodistilled in an all glass apparatus using Dean-Stark apparatus for 8 hours. The essential oil collected was extracted with ether (3 x 5 mL), dried over anhydrous magnesium sulphate and filtered. The ether was then evaporated at room temperature overnight to give essential oil (8.78 g) as pale yellow liquid with pungent smell. The essential oil was stored at 4ºC in the air- tight container until further analysis.

Extraction of Chloroform Crude Extract

The air-dried rhizomes (214.6 g) were ground and extracted in a 2 L Soxhlet extractor using chloroform (1.5 L) for 20 hours. The resulting extract was filtered and concentrated using rotary evaporator to yield crude extract (27.6 g) as brownish yellow viscous liquid with pungent smell. The extract *Z. cassumunar*(14.1 g) was fractionated using vacuum liquid chromatography on Si gel (200 g) and eluted with increasing polarity of the solventsto give twenty six fractions. Fraction 10 (0.28 g) from the VLC was further purified using column chromatography with Si gel 70-230 (7.0 g) and eluted using *n*-hexane and ether with increasing polarity to give 60 fractions. Fractions with similar TLC profile were combinedto afford**(3)**(0.025 g) as a pale yellow viscous liquid with R_f value 0.70 in *n*-hexane: ether (1:1). Fractions 19 to 21 (1.4) g were combined and further purified by column chromatography using petroleum ether: ether with increasing polarity to give 190 fractions. Fractions that showed similar TLC profile were recombined and evaporated to afford**(4)** (0.106 g) as a pale brown viscous liquid, $R_f$value0.38 in PE: E (3:2).

Free Radical Scavenving Activity (DPPH) Assay

The radical scavenging activity using1,1-diphenyl-2-picryhydrazyl (DPPH) was carried out on the sample essential oil and $CHCl_3$ crude extract with the ascorbic acid as the positive standard. Sample stock solution (1.0 mg/mL) of sample essential oil and $CHCl_3$ crude extract were diluted to final concentration using methanol (CH_3OH) solvent. Methanolic solution was added to different concentration sample solutions and was to react for 30 minutes at room temperature.After 30 minutes, the absorbance of the reaction mixtures was recorded at 517nm. All test and analysis were run triplicates and average values were calculated. Percentage of antioxidant activity was calculated using the following equation:

$$\% \text{ Scavenging} = \frac{[\text{Abs (control)} - \text{Abs (DPPH)} + \text{sample}]}{\text{Abs (control)}} \times 100\%$$

RESULTS AND DISCUSSIONS

Chemical Composition of essential oil

The hydrodistillation extraction of the fresh rhizome of *Z. cassumunar* (310 g) using Dean-Stark apparatus for 8 hours gave the essential oil (8.78 g) a pale-yellow liquid. The essential oil was analysed using the GC and GC-

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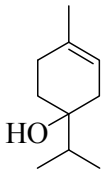
MS with an Ultra 1 GC capillary column. The mass spectrum of each peak was compared with Wiley Library Data and Kovat Indices with literature values. Table 1 shows twenty four constituents in the rhizome oil which contributed 78.47% of the oil. The oil comprised of monoterpenes 75.9%, sesquiterpenes 1.8% and ester 0.8%. The major constituents were found to be terpinen-4-ol **(1)**(31.35%) and sabinene**(2)**(23.37%).



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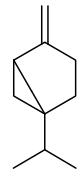

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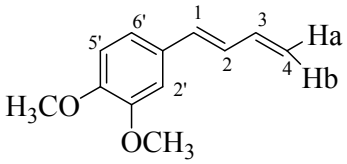

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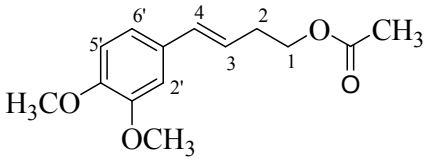

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Table 1: Chemical composition of essential *Zingibercassumunar* from GC

No	Retention Time,t_R	KI	Compound	MW	Molecular Formula	% Area,
1	9.68	923	*α*-Thujene	136	$C_{10}H_{16}$	0.49
2	9.87	929	*α*-Pinene	136	$C_{10}H_{16}$	1.20
3	10.28	942	Camphene	136	$C_{10}H_{16}$	0.06
4	11.20	968	Sabinene	136	$C_{10}H_{16}$	24.37
5	11.26	970	β-Pinene	136	$C_{10}H_{16}$	2.72
6	11.81	985	Myrcene	136	$C_{10}H_{16}$	0.58
7	12.59	1006	α-Terpinene	136	$C_{10}H_{16}$	2.92
8	12.71	1011	*p*-Cymene	136	$C_{10}H_{16}$	0.98
9	12.92	1018	β-Phellandrene	136	$C_{10}H_{16}$	0.74
10	12.20	1020	Limonene	136	$C_{10}H_{16}$	0.61
11	13.96	1051	γ-Terpinene	136	$C_{10}H_{16}$	5.58
12	14.08	1054	*cis*-Sabinene hydrate	154	$C_{10}H_{18}O$	1.46
13	14.90	1079	Terpinolene	136	$C_{10}H_{16}$	0.58
14	15.02	1082	2,6-dimethyl-2,7-octadiene-6-ol	154	$C_{10}H_{18}O$	1.09
15	15.79	1105	*trans*-Menth-2-en-1-ol	154	$C_{10}H_{18}O$	0.65
16	16.32	1124	*cis*-Menth-2-en-1-ol	154	$C_{10}H_{18}O$	0.43
17	17.57	1166	Terpene-4-ol	154	$C_{10}H_{18}O$	31.35
18	17.92	1177	(*Z*)-4-Hexenyl Butyrate	170	$C_{10}H_{18}O_2$	0.02
19	17.96	1179	α-Thujanol	150	$C_{10}H_{14}O$	0.02
20	28.09	1572	Caryophellene Oxide	220	$C_{15}H_{24}O$	0.14
21	26.19	1491	Zingibrene	204	$C_{15}H_{24}$	0.56
22	26.72	1513	β-Sesquiphellandrene	204	$C_{15}H_{24}$	1.11
23	27.77	1559	(*E*)-1-(3,4-dimethoxyphenyl)but-1-ene)	190	$C_{12}H_{14}O_2$	0.04
24	28.709	1598	(*E*)-1-(3,4-dimethoxyphenyl)buta-1,3-diene)	190	$C_{12}H_{14}O_2$	0.78

Chemical components of chloroform crude extract

The dried powder rhizomes of *Z. cassumunar* (214.6 g) were extracted by Soxhlet extraction using chloroform as the solvents for 20 hours. Evaporation of the solvent yielded (27.6 g, 12.8%) as a brown viscous liquid with pungent smell crude extract.

Trans-1-(3,4-dimethoxyphenyl)butadiene **(3)**was obtained from the purification of the chloroform crude extract as a pale yellow viscous liquid with R_f values 0.70 in *n*-hexane: ether (1:1) which suggested as a non-polar compound. The IR spectrum of the *trans*-1-(3,4-dimethoxyphenyl)butadiene **(3)** showed characteristic bands for sp^2 =CH at 3019 cm^{-1}, sp^3 C-H stretching at 2962 and 2936 cm^{-1} strong absorption of C-O stretching at 1265 and 1216 cm^{-1}, strong absorption at 1597 cm^{-1} showed conjugation of C=C alkenes stretching, 1508 and 1465 cm^{-1} aromatic stretching. Analysis of ^{1}H NMR spectrum revealed the characteristic signals of two methoxyl groups (OCH_3) in compound **(3)** as two singlet signals at δ 3.90 and 3.93. The peaks for H-1 and H-3 were overlapping to each other at δ 6.51 which showed the coupling between olefenic protons. A doublet signals (J = 9.6 Hz) at δ 5.15 indicated the *cis*position H-4a and δ 5.31 (J= 16.5 Hz) H-4b to the H-3 resonance at δ 6.45. The signal for H-2 which is *trans* position to H-1 (J = 15.9 Hz) at δ 6.52 and coupling in acyclic dienes to H-3 (J = 10.8 Hz) at δ 6.45 was demonstrated by a doublet of doublet multiplicity at δ 6.70. A doublet with (J= 8.1 Hz) at δ 6.85 was assigned to H-5' that showed an *ortho*-coupling with H-6'. A doublet of doublet with (J= 8.1 and 1.8 Hz) at δ 6.95 was attributed to H-6', while *meta*-coupled proton with (J= 1.8 Hz) was assigned to H-2.

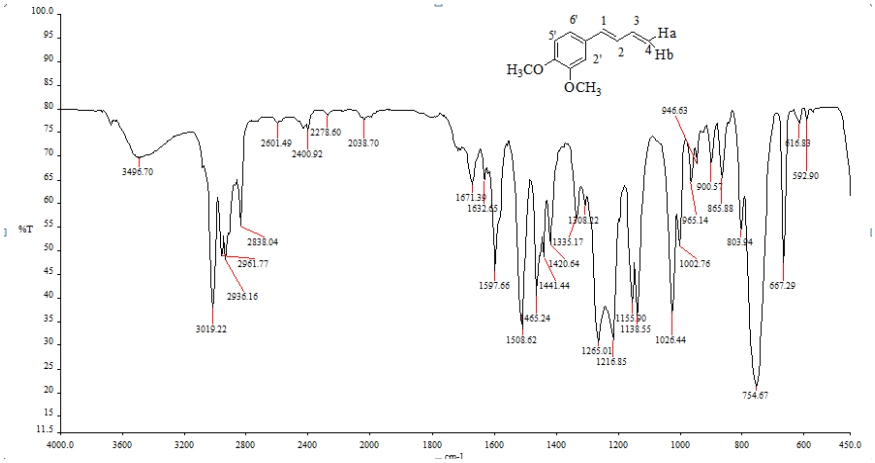

Figure 1: IR spectrum for trans-1-(3,4-dimethoxyphenyl)butadiene **(3)**

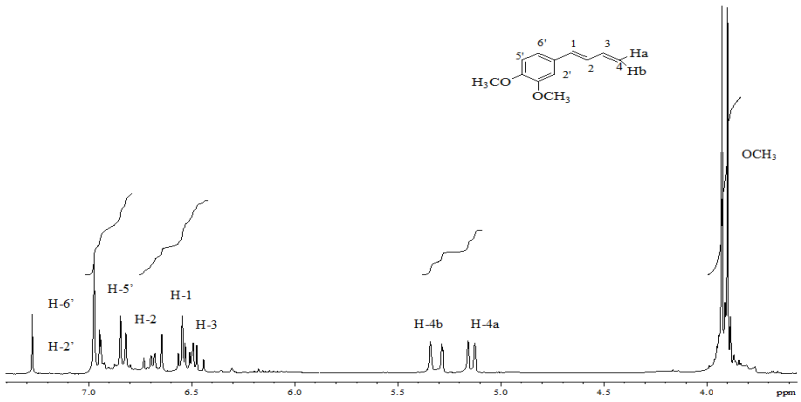

Figure 2: ^{1}H NMR spectrum for trans-1-(3,4-dimethoxyphenyl)butadiene **(3)**

Trans-4-(3,4-dimethoxyphenyl)but-3-1-yl acetate **(4)** was obtained from the purification of the chloroform crude extract as a pale yellow viscous liquid with R_f value 0.38 in PE: E (3:2). The IR spectrum of *trans*-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate **(4)** showed characteristic bands for =CH sp^2 at 3020 cm^{-1}, C-H sp^3 stretching at 2960 and 2838 cm^{-1}. A strong absorption bend at 1734 cm^{-1} for C=O stretching together with C-O stretching bands at 1240 and 1216 cm^{-1} suggested the presence of ester moiety. The IR also showed stretching υ for conjugated alkene C=C at 1586 cm^{-1} and 1465 cm^{-1}. The analysis of ^{1}H NMR spectrum of *trans*-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate **(4)** revealed the presence of a singlet signal integrating for three proton at δ 2.08 was assigned to acetoxyl group (CH_3CO) group. A quartet signal at δ 2.53 with J = 6.9 Hz integrating for two protons was attributed to methylene proton of carbon two for H-2. Two singlet signals integrating for

three protons each at δ 3.90 and 3.98 were attributed to methoxyl groups (OCH_3). A triplet peak with *J*= 6.9 Hz at δ 4.19 was assigned to methylene protons bearing the acetoxy group. Two olefinic protons at δ 6.04 and 6.42 were mutually coupled with a coupling constant of *J*= 15.9Hz, which indicated that the geometry of the double bond was in *trans* configuration. A doublet of doublets with *J*= 8.1 and *J*= 1.8 Hz at δ 6.89 was assigned to H-6' showing an *ortho*-coupling with H-5', while a *meta*-coupled proton *J*= 1.8 Hz at δ 6.92 was assigned to H-2'. The comparison of the *trans*-1-(3,4-dimethoxyphenyl)butadiene **(3)**and*trans*-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate **(4)** obtained from the experiment showed a similarities in pattern of ^{1}H NMR spectrum of compound from the previous research based on the ^{1}H NMR spectrum and IR data [11]. Figure 1, Figure 2, Figure 3 and Figure 4 showed the IR and ^{1}H NMR spectrum for both compound.

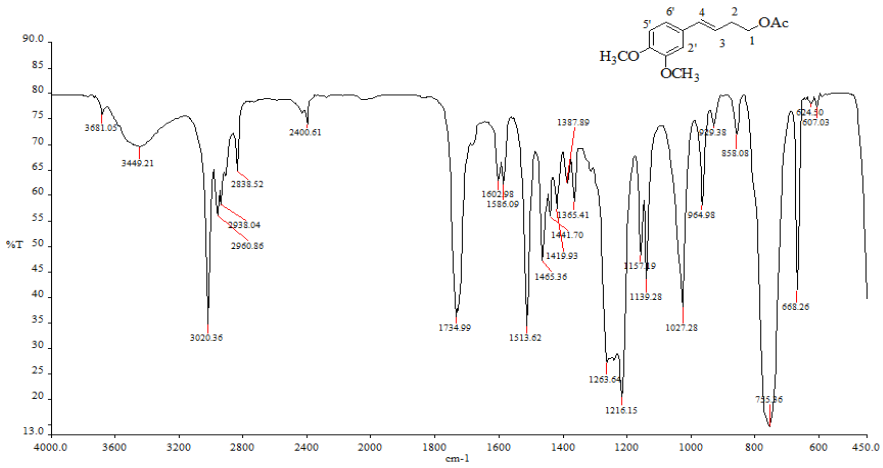

Figure 3: IR spectrum for *trans*-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate **(4)**

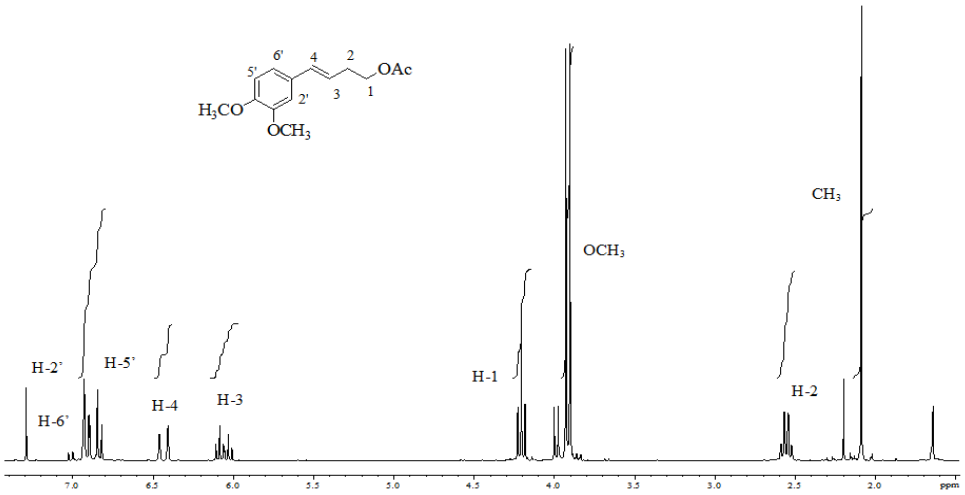

Figure 4: ^{1}H NMR spectrum for *trans*-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate **(4)**

DPPH Free radical Scavenging Activity Assay

The DPPH system is a stable radical scavenging-generating procedure because it can accommodate a large number of solid or liquid samples in a short period, and it is sensitive enough to detect active principles at low concentration [10]. The structure of DPPH and its reduction by an antioxidant are shown in **Scheme 1.**

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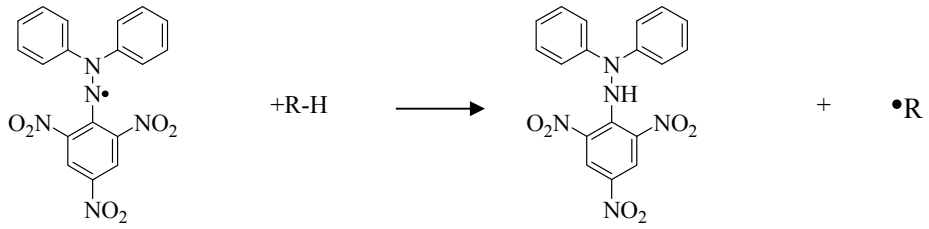


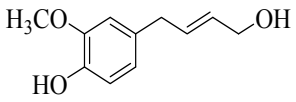
Scheme 1: The reduction reaction of DPPH

The radical electron in the DPPH free radical is purple in color and gave a strong absorption at 517 nm. The color changes when it reacts with an antioxidant compound from purple to light yellow. The color changed was due to molar absorptivity of DPPH radical at 517 nm decreased due to the odd electron of DPPH radical becomes paired with hydroxylated benzene to form the reduced DPPH [10]. Based on the experiment, the purple color of DPPH solution changed when the sample of essential oil and $CHCl_3$ crude extract were added into the DPPH solution at concentration (1000 µg/mL) and (600-1000 µg/mL) respectively. This indicated that the $CHCl_3$ crude extract was active as radical scavengers against DPPH radical at higher concentration. However, in essential oil it showed that the antioxidant activity was almost inactive. At lower concentration, both essential oil and $CHCl_3$ crude extract remain purple in colour of DPPH solution which indicated inactive radical scavengers against DPPH radical. IC_{50} shows effectiveness of the compound to inhibit biological or biochemical function and be obtained from the graph percentage inhibition against concentration in µg/mL. The percentage of scavenging (***I %***) was plotted versus concentration of sample in ppm. Ascorbic acid was used as standard ($\mathbf{r}^2 = 0.8672$) gave $IC_{50} = 10.77$ µg/mL. The IC_{50} value for ascorbic acid, essential oil and $CHCl_3$ crude extract were obtained by using equation from the most similar curve as the plot. **Table 2** showed IC_{50} value for the samples, essential oil and $CHCl_3$ crude extract.

Table 2: IC_{50} values for the Samples

Sample	IC_{50} (µg/mL)
Ascorbic Acid	10.77
Essential Oil	700.65
$CHCl_3$ crude extract	77.31

It was suggested the antioxidant property was presence in both sample but it was expected to be very weak in essential oil whereas, in crude showed higher possibilities to be a strong antioxidant. Samples acts as an antioxidant property due to the presence of phenolics groups (hydroxyl substituents next to their aromatic structures), which enable them to scavenge free radicals [10]. Therefore, it was suggested that the crude have the compounds with phenolic group such *trans*-4-(4-hydroxy-3-methoxyphenyl) but-2-en-l-ol **(33)**, *trans*-2-hydroxy-4-dimethoxyphenyl) but-3-en-l-ol **(34)**, *trans*-2-methoxy-4-(3,4dimethoxyphenyl)but-3-en-l-ol **(35)** that make it to have antioxidant property [12].



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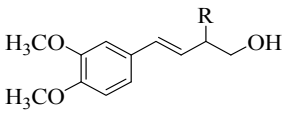

(34) R = OH

(35) R = OCH_3

CONCLUSIONS

Hydrodistillation of the rhizomes of *Zingiber cassumunar* produced essential oil in (2.83%) as a yellow pale liquid with pungent smell. A total of (78.47%) of the essential oil compositions with fifteen compounds were identified, consisting of monoterpenes (75.90%) and sesquiterpenes (1.80%) and ester 0.8%. Terpinen-4-ol **(1)** (31.35%) and sabinene **(2)** (23.37%) were found to be the main constituents in the essential oil. Soxhlet extraction of the dried rhizomes of *Z. cassumunar* using chloroform followed by purification using several chromatographic techniques afforded *trans*-1-(3,4-dimethoxyphenyl)butadiene **(3)** and *trans*-4-(3,4-dimethoxyphenyl)but-3-en-1-yl acetate **(4)**.The structures of compounds were elucidated by using spectroscopic methods as methoxyphenyl derivatives using IR and nuclear magnectic resonance (^{1}H NMR). The $CHCl_3$ crude extract gave antioxidant properties at higher concentration which more than 600 µg/mL in the DPPH assay whereas the essential oil extract showed almost inactive or very weak antioxidant activity.

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