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ISOLATION AND IDENTIFICATION OF ACTIVE COUMPOUND FROM BENGLE RHIZOME (*ZINGIBER CASSUMUNAR* ROXB) AS A STIMULANT IN PHAGOCYTOSIS BY MACROPHAGES

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ABSTRACT
Immunomodulators are pharmacological agents that modify or regulate the immune system through stimulating the functioning of the immune system and, at the same time, inhibiting excessive immune responses. This study was conducted to determine the active compound in *Z. cassumunar* that is responsible for increasing the immune system based on the parameters of phagocytic activity. The isolation method began with fractionation, which involved extraction with ethanol and successive fractionation with hexane and chloroform. *Z. cassumunar* extract, hexane fraction, and chloroform fraction were tested on mice macrophage cells for their phagocytic functions. The phagocytic activity of macrophages was measured by active phagocytic cells (averagely 39.194 ±1.597, 27.923 ±2.941, and 62.090 ±6.947) and phagocytic index (in a row, averagely 47.513 ±2.844, 41.129 ±7.195, and 101.527 ±10.555). The results showed that the *Z. cassumunar* extract, hexane fraction, and chloroform fraction exhibited more significant phagocytic activities of macrophages (p <0.05) compared with the normal group. Since the chloroform fraction showed the best result, this fraction was further separated by column chromatography. This procedure yielded five sub-fractions, namely F1, F2, F2C, F3, and F4. Based on the phagocytic activity testing, the results were as follows: (1) the active phagocytic cells of F1, F2, F2C, F3 and F4 were 18.860 ±3.191, 27.077 ±4.482, 15.749 ±3.026, 64.333 ±1.780, and 44.943 ±2.944, respectively, and (2) the phagocytic indices were 30.0249 ±3.4231, 44.5969 ±8.3646, 24.5597 ±5.4487, 102.7447 ±1.0806, and 76.5007 ±4.7293. Because F3 produced the best result, this subfraction was then identified using 1H-NMR and 13C-NMR. The identification results showed that F3 was (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol as an active compound.

Keywords: *Zingiber cassumunar*; phagocytosis macrophage; (E)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol

INTRODUCTION
Through various studies on effects and efficacy, traditional medicine has been increasingly developed for its use in overcoming health problems. Around 59.1% of the Indonesian population use traditional medicine, and 95.60% of which claim to perceive the benefits. This figure is supported by the abundant natural resources in Indonesia and the willingness to start following WHO's recommendations to incorporate natural ingredients in treatment and lifestyle. To substitute for immune enhancers, people tend to prefer traditional medicine **(Indonesian Health Ministry, 2013)**. The immune system consists of organs, tissues, cells, and chemical compounds that are used by the body to protect it from the danger of foreign objects or cells that potentially cause abnormal internal changes. The immune system is divided into two types, namely specific and non-specific. The non-specific immune system is the body's first defense against the attacks of foreign bodies because it responds directly to antigens. whereas, a specific immune system is a system that can recognize foreign substances that enter the body and can stimulate the development of specific immune responses to these substances **(Baratawidjaja and Rengganis, 2014; Hooper, Littman, and Macpherson, 2015; Shiland, 2014)**. In this system, macrophages play the most crucial role. They act as effectors on therapeutic bodies in the immune system against pathogens using the phagocytosis mechanism, either directly or indirectly, by releasing ROI and cytokines. These functions can be influenced by immunomodulators **(Baratawidjaja and Rengganis, 2014; Gabrilovich and Nagaraj, 2009)**. Immunomodulators are drugs that enhance immune systems with low function (immunostimulants), suppress excessive immune function (immunodepressant), and increase vaccine efficacy (Immunoadjuvant). In other terms, immunomodulators help the body to optimize the role of its immune system. One of the natural ingredients that have the potential to modulate the immune system is *Z. cassumunar* **(Baratawidjaja and Rengganis, 2014; Bascones-Martinez et al., 2014; Chairul, 2009; Nair, Chattopadhyay, and Saha, 2019)**.

Z. cassumunar is a type of rhizome from the Zingiberaceae family. It contains several chemical compounds, namely essential oils, starch, resin, tannins, flavonoids, and phenylbutanoic acids. This rhizome is often used as a medicine to treat various diseases, including fever and headache, and to increase immunity **(Hartati et al., 2013; Utami, 2008)**. In previous research, *Z. cassumunar* has been reported as an effective adjunctive therapy in malaria **(Hermansyah and Utami, 2015)**.

Several studies have succeeded in isolating phenylbutanoid derivatives from *Z. cassumunar*, namely (E) -4- (3 ', 4'-dimethoxyphenyl) but-3-en-1-ol; (E) -4- (2 ', 4', 5 '-trimethoxyphenyl) but-3-en-1-ol; (E) -4- (3 ', 4', 1-trimethoxyphenyl) but-3-en-1-ol; (E) -4- (3 ', 4'-dimethoxyphenyl) but-3-en-1-il-acetate, and methoxy-8-(3,4-dimethoxyphenyl) -1,4-napthoquinone **(Chairul, 2009; Hartati et al., 2013; Kuroyanagi et al,. 1980)**.

Scientific hypothesis

Z. cassumunar has compounds that are responsible for the phagocytic activity of macrophages.

MATERIAL AND METHODOLOGY

Plant Material

Bengle rhizomes (*Z. cassumunar*) were purchased from the Beringharjo Market, Yogyakarta, Indonesia. Before use, these materials had been identified in the biology laboratory, University of Ahmad Dahlan, with an identification number 036/Lab.Bio/B/IV/2018.

Material

Ethanol 96% (E-Merck, pro-analysis quality), Etanol 70% (E-Merck, pro-analysis quality), n-Hexane (E-Merck, pro-analysis quality), Dichloromethane (E-Merck, pro-analysis quality), Chloroform (E-Merck, pro-analysis quality). TLC plate with silica Gel GF_{254} (Merck), Silica powder for Column (Merck), NMR JNM-ECX500R (JEOL ltd) and Complete medium (MACS® media)

Procedure

Animal Preparation

The animal care procedure in this study has been approved by the research ethics committee of the University of Ahmad Dahlan, with approval number 011804063. The test animals were male BALB mice aged eight weeks old.

Extraction and Fractionation

The rhizomes of *Z. cassumunar* were washed with water, then dried in an oven at 50 °C. The produced *simplisia* (i.e., natural ingredients used as a medicine that have not been exposed to any processing and, unless stated otherwise, are in the form of dried materials) were ground into a powder and subsequently macerated using 96% ethanol at a ratio of 1:4. Afterward, the produced macerate was evaporated in a rotary evaporator to obtain a thick extract. Fractionation was carried out by successive fractionation. Thirty grams of the ethanol extract of *Z. cassumunar* were fractionated with n-hexane until no more compounds were dissolved in n-hexane. Then, the non-soluble part was fractionated with chloroform. The fractions obtained were further examined by thin-layer chromatography (TLC).

Macrophage Isolation

After fasted for 12 hours, the test mice were anesthetized using chloroform. The abdominal skin was cut open, and the peritoneal was cleaned using 70% ethanol. Ten ml of complete medium was injected into the peritoneal cavity, which was then massaged for 3 – 5 minutes. The cavity was pressed by two fingers to remove the peritoneal fluid; this fluid was aspirated using a syringe injection (by selecting parts that had no fat and far from the intestine). The number of cells were counted and added with complete medium to adjust the number of macrophages to 2.5×10^6 mL^{-1} cells.

Testing of Phagocytic Activity

Cells in 200 μL suspension were grown in each well and incubated using an incubator that was set at 5% CO_2 and a temperature of 37°C for 24 hours. The medium was removed and added with 50 μL latex and, then incubated using an incubator at 5% CO_2 and a temperature of 37 ° C for 1 hour. The suspension was removed and fixed with methanol for 3 minutes, then the methanol was removed. This mixture was colored with 10% Giemsa for 30 minutes and the coverslips were washed in distilled water. Finally, the cells were viewed under a microscope with 400x magnification (Nurkhasanah, Santoso, and Fauziah 2017).

Column Chromatography

Column chromatography was performed using silica powder with 230 – 400 mesh particle size in combination with the elute of n-hexane: dichloromethane (1:1). One gram of the chloroform fraction was ground with 5 grams of silica powder and stored at the top of the silica column. This process involved gradient elution for the mobile phase (i.e., a mixture of n-hexane: dichloromethane) and was examined using thin-layer chromatography.

Identification

The subfraction that showed the best phagocytic activity of macrophages was then prepared for 1H-NMR and 13C-NMR analyses. This procedure used a JNM-ECX500R spectrometer operated at 500MHz Superconducting Magnet.

Analysis

The macrophage activity was observed from the cells exhibiting active phagocytosis and the phagocytic index. Active macrophage cells are the percentage (x100%) of the number of latex cells among the total macrophages in the field of view **(Chairul, 2009)**, while the index is the percentage of a total number of macrophages that phagocyted latex particles among the total macrophages in the field of view **(Hartini, Wahyuono, and Widyarini, 2013)**.

$$\text{Active phagocytic cells (\%)} = \frac{Number\ of\ active\ macrophage\ cells}{total\ of\ macophage\ cell} \times 100\%$$

$$\text{Index of phagocytosis} = \frac{total\ antigen\ phagocyted}{total\ of\ active\ macophage\ cell}$$

Statistical analysis

The results were analysed statistically using Normality, Homogeneity, ANOVA, and LSD (Least Significant Differences); all of which were processed in SPSS version 22. The normality test result is accepted if the *p*-value is >0.05, which means that the data are normally distributed. The homogeneity test result is accepted if the *p-value* is >0.05, which represents homogeneously distributed data. The ANOVA result is accepted if the *p*-value is <0.05, which signifies a significant difference in the treatment group. Meanwhile, the LSD test is a test performed as a reference to determine whether or not the averages of two treatments are statistically different.

RESULTS AND DISCUSSION

An immunomodulator is a drug or a pharmacological agent that regulates the immune system. The ethanol extract of *Z. cassumunar* has been reported to exhibit immunomodulatory activity by increasing the secretion of ROI. ROI secretion shows the increased activity of macrophage cells. Macrophages with their phagocytosis mechanism are the main phagocytic cells that ward off or deflect pathogens **(Abbas, Litchman and Pillai, 2012; Akrom, 2013; Baratawidjaja and Rengganis, 2014; Hartini et al., 2013; Nurkhasanah et al., 2017)**.

Based on Figure 2, Figure 3, Table 1 and Table 2, the

Figure 1 *Z.cassumunar* rhizome.

Table 1 RF values and colors of the spots on TLC.

Spot	RF	Curcumin		n-Hexan		Ekstrak		Kloroform	
		UV 254	UV 366	UV 254	UV 366	UV 254	UV 366	UV 254	UV 366
1	0.027	Orange	Orange	-	-	-	-		-
2	0.088	Orange	Orange	-	-	-	-		-
3	0.150	-	-	-	-	Blue	-	Blue	-
4	0.244	Orange	Orange	-	-	Orange	Orange	Orange	Orange
5	0.522	-	-	Blue	-	Blue	-	Blue	-
6	0.577	-	-	Blue	-	Blue	-	Blue	-
7	0.844	-	-	Blue	-	Blue	-	Blue	-

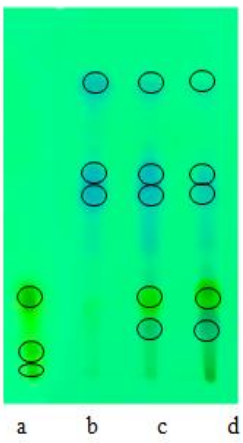

Figure 2 Profile TLC using the mobile phase chloroform: dichloromethane and stationary phase silica gel F254: a. curcumin b. n-hexan fraction c. bengle extract d. chloroform fraction.

Z. cassumunar extract, hexane fraction, and chloroform fraction significantly increased the active phagocytic cells and phagocytic index (p <0.05). Of the five test results, the highest increase in phagocytic activity was exhibited by the chloroform fraction, as indicated by active phagocytic cells (SFA) = 62.090 ±6.947 and phagocytic index (IF) = 1.638 ±0.079. Meanwhile, the lowest increase was shown by the hexane fraction, with SFA = 27.923 ±2.941 and IF = 1.476 ±0.227. These results are influenced by differences in the compounds of the fractions and *Z. cassumunar* extract. For instance, the hexane fraction only produced three spots with RF values = 0.522, 0.577, and 0.844 during the TLC procedure. Meanwhile, the extract and the chloroform fraction each had five spots with RF values= 0.15, 0.244, 0.522, 0.577, and 0.844.

The increase in phagocytic activity can occur in two ways, namely by the process of oxidative and non-oxidative. Oxidative processes increase the use of oxygen, myeloperoxidase, hydrogen peroxide, and hexose monophosphate that destroy bacteria. Hydrogen peroxide, superoxide anion, and nitric oxide secreted within the phagolysosome generate toxic oxygen metabolites that can be used to kill bacteria. Non-oxidative processes occur due to the influence of various proteins, such as hydrolytic enzymes, cationic proteins, lysozyme, lactoferrin, and nitric oxide synthase (NOS). Nitric oxide synthase can increase NO production from macrophages in the spleen with the help of IFN-γ and TNF-α **(Bermudez and Young, 1989; Ferrari, 2011; Ishimoto et al., 2008)**.

Z. cassumunar is a Zingiberaceae family that has curcumin. Curcumin is known to give antioxidant and immunostimulant effects by increasing T-cell proliferation, natural killer (NK) cell activity, and the activity of NO.

One compound marker of *Z. cassumunar* is phenylbutanoid, which contains a phenyl group that initiates antioxidant effects and can support the immune system. Anti-oxidants are often associated with the immunostimulatory potential **(Ak, 2008; Chairul, 2009; Jayaprakasha, Jaganmohan, and Sakariah, 2006; Lu et al., 2008; Taechowisan, Suttichokthakorn, and Phutdhawong, 2018; Yadav et al., 2005)**.

Table 2 The phagocytic activity of the extract and fractions of *Zingiber cassumunar*.

Group	Active Phagocytic Cells (%)	Phagocytic Index
Control	8.731 ±4.080	1.641 ±0.387
n-Hexane Fraction	27.923 ±2.941*	1.476 ±0.227
Chloroform Fraction	62.090 ±6.947*	1.638 ±0.079
Z.cassumunar Extract	39.194 ±1.597*	1.215 ±0.105*

Note: *Showing a significant difference (p <0.05).

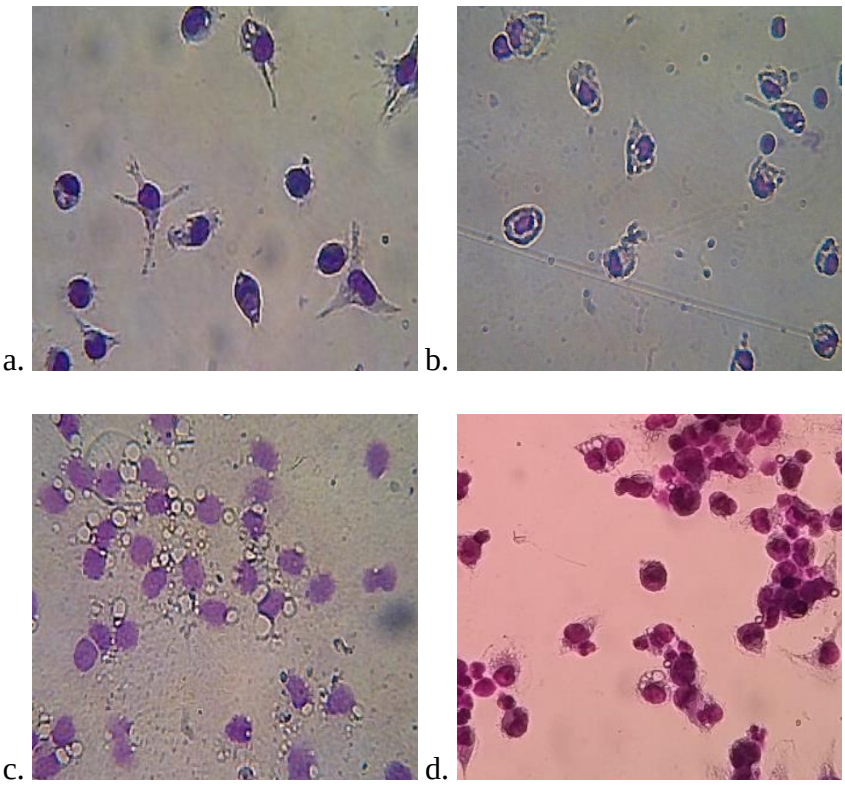

Figure 3 The Phagocytic activity of macrophage cells treated with: a. Normal group, b. Fraction of n-hexane, c. Fraction of Chloroform, d. Bengle extract.

The chloroform fraction was separated using column chromatography, and it produced five subfractions, as shown in Figure 4. These subfractions were then tested for their phagocytic activities, and the results are presented in Table 3. Table 3 shows that F1, F2, F2C, F3, and F4 influenced the phagocytic activity of macrophages. The highest activity was exhibited by subfraction F3 with SFA= 64.333 ±1.780 and IF= 1.598 ±0.059, followed by F4, with SFA= 44.943 ±2.944 and IF= 1.702 ±0.043. Meanwhile F1, F2 and F2C exhibited poor phagocytic activities, as evidenced by their SFA (18.860 ±3.191, 27.077 ±4.482, and 15.749 ±3.026, respectively) and IF (1.608 ±0.19,0 1.646 ±0.113, 1.557 ±0.167.

Table 4 shows that F3 is an active compound that is responsible for the immunomodulatory activity, which increased the activity of the murine macrophage cells. Increased macrophage activity is influenced by the production of NO, the ROI of intra phagosome killer, phagosome acidification, the fusion of phagosomes, and Fe supply reduction. The activation of macrophages can increase phagocytosis due to the increased expression of the gene transcription. The gene of macrophages has specific functions that the same cells cannot perform while in the rest position. Phagocytosis is affected by ROS. Through the ROI, ROS kills antigens that enter macrophages. Superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen belong to the group of ROS. ROS is a highly reactive species that can kill bacteria and destroy the cells. Nitric Oxide Synthase molecules bind the cofactor tetrahydrobiopterin, then this NOS-cofactors bond produces NO, which is highly toxic to bacteria. This pathway is activated by INF-γ and is triggered by the presence of TNF-α, which increases the production of NO in the spleen **(Abbas et al., 2012; Baratawidjaja and Rengganis, 2014; Ismail, Stevenson, and Walker, 2006; Parslow et al., 2001; Tzianabos, 2000)**.

IFN-γ is a potential cytokine macrophage activator. IFN-γ will increase phagocytosis and stimulate the expressions of MHC-I and MHC-II and costimulator APC. IFN work on B cells in the transfer of IgG subclasses that enable FcyR in phagocytes and activate complement. IL-10 inhibits macrophage activity and controls non-specific immune responses and cellular immunity. The main function of IL-10 is to inhibit the production of several cytokines (TNF, IL-1, chemokines, and IL-12) and the function of macrophages in helping the activation of T cells **(Brummer, Hanson, and Stevens, 1988; Lo et al., 2019; Ravindran et al., 2019; Salim, Sershen, and May 2016)**.

Table 3 The phagocytic activity of the extract and fractions of *Zingiber cassumunar*.

Group	Active Phagocytic Cell (%)	phagocytosis Index
F1	18.860 ±3.191	1.608 ±0.190
F2	27.077 ±4.482	1.646 ±0.113
F2c	15.749 ±3,026	1.557 ±0.167
F3	64.333 ±1.780*	1.598 ±0.059
F4	44.943 ±2.944	1.702 ±0.043

Note: *Shows the best result (p <0.05).

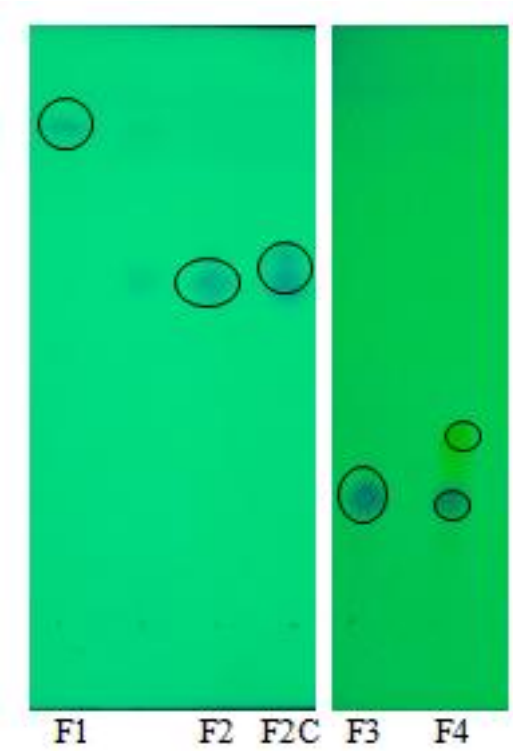

Figure 4 The TLC profile using chloroform: diklоromethan as the mobile phase and silica gel F254 as the stationary phase.

Table 4 The comparison of the NMR peaks of the active compound with other studies.

Position	^{13}C-NMR (Kuroyanagi et al., 1980)	^{13}C-NMR Result	^{1}H-NMR (Kuroyanagi et al., 1980)	^{1}H-NMR Result
1	62.1	62.3	CH_2-OH 3.68 (t,J= 6) - 2.73 (s)	CH_2-OH 3.73 (t, J=6) - 2.01 (s)
2	36.4	36.5	CH_2 2.42 (q, J= 6)	CH_2 2.44 (q,J= 6)
3	124.4	124.5	CH 5.93 (d, J=16; 6)	CH 6.06 (d, J= 16; 7)
4	132.4	132.6	CH 6.35 (d, J=16)	CH 6.39 (d 16)
1’	130.5	130.6	-	-
2’	108.8	108.7	Arom. H 6.71 (d, J=1,5)	Arom. H 6.85 (d, J=2)
3’	149.1	149.2	-	-
4’	148.6	148.7	-	-
5’	111.3	111.3	Arom. H 6.64 (d, J=8)	Arom. H 6.77 (d, J=8)
6’	119.1	119.3	Arom. H 6.87 (dd, J= 8; 1,5)	Arom. H 6.87 (dd, J= 8; 2)
7’	55.9	56.1	OMe 3.79 (s)	OMe 3.85 (s)
8’	55.8	56.0	OMe 3.80 (s)	OMe 3.87 (s)

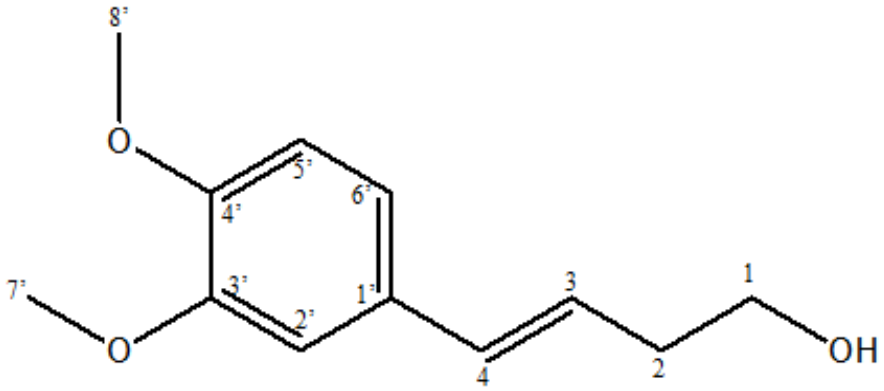

Figure 5 [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol].

TNF is the main mediator in response to a variety of antigens that cause infections. Macrophages are a major source of TNF. TNF has a biological effect that activates neutrophils, macrophages, and monocytes to eliminate the antigen and then trigger vascular cell adhesion molecule, stimulate macrophages to secrete chemokines, induce chemotaxis, leukocyte deposition, and the apoptosis of the inflammatory cells. IL-12 is a major mediator of the early nonspecific immunity against intracellular antigens. The main source of IL-12 is mononuclear phagocytes and activated dendritic cells. The biological effects of IL-12 cause the NK cells and T cells to secrete IFN **(Cavalcanti et al., 2012; Engwerda et al., 2002; Ismail et al., 2006; Salim et al., 2016)**.

The compound [(*E*)-4-(3',4’-dimethoxyphenyl)but-3-en-1-ol] is a form of light green oil with the molecular formula C12H16O3 and C6-C4 carbon framework. Table 4 shows two methoxy groups (-OCH3) at δ 3.85 (s) and 3.87 (s), which were strengthened by the presence of carbon spectra at δ 55.9 and 55.8 ppm. Moreover, aromatic carbon with ortho and meta couplings appeared as three benzene substitutions at δ 6.87 (d, J = 8, 2), 6.77 (d, J = 8) and 6.85 (d, J = 2) and was amplified at the carbon spectra δ 108.8, 111.3, and 119.1 ppm. The protons in H-7 and H-8 showed a high constant coupling of 16, which indicates a trance configuration.

Chairul (2009) suggest that the compound isolated from the identified phenylbutanoid group [(*E*)-4-(3’,4’-dimethoxyphenyl)but-3-en-1-ol] induces a more significant increase in phagocytic activity in in vivo experiments on Swiss Webster male mice than [(*E*)-4-(2', 4',5'-tri-methoxyphenyl)but-3-en-1-ol] and [(*E*)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol].

CONCLUSION

Bengle (*Zingiber cassumunar*) has the potential as immunomodulators that significantly increase phagocytic activity. The chloroform fraction has been proven to exhibit the highest phagocytic effects. Meanwhile, the subfraction that has the most substantial phagocytic effect is sub-fraction 3, which is identified as [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol].

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