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Research Article

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Study of antibacterial activity and identification of the most active fraction from ethanol extraction of *Zingiber cassumunar* Roxb. rhizomes by vacuum liquid chromatography

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ABSTRACT

The purpose of this research is to evaluate the antibacterial fractions' activity of Zingiber cassumunar Roxb. rhizomes against both Bacillus cereus bacteria and Klebsiella pneumoniae and to identify the most active fraction of them. The rhizome's powder was macerated with 96% ethanol, then the resulting extract was evaporated and fractionated by vacuum liquid chromatography with comparation of hexane : ethyl acetate as eluent. The antibacterial activities were evaluated using diffusion method and then the most active fraction was evaluated by inhibition zone. The most active fraction was evaluated for Minimum Inhibitory Concentration (MIC) and its' equivalent value compared to amoxicillin. Then, it was identified with Gas Chromatography - Mass Spectrometry (GC-MS). The F fraction showed the most active antibacterial fraction against two testing bacteria. The F fraction had MIC 0.075% against two testing bacteria. The F fraction antibacterial activity value was $5.864x10^{-3}$% compared to amoxicillin for Bacillus cereus and $3.094x10^{-3}$% for Klebsiella pneumoniae. The GC-MS result showed that the F fraction contained phenylbutenoid compounds and phenylbutenoid dimer compounds.

Keywords: *Zingiber cassumunar* Roxb., Phenylbutenoid, *Bacillus cereus, Klebsiella pneumoniae*

INTRODUCTION

Application of plants for medical purposes goes back to the early human civilization. Ability to formulate and serve herbs was hereditary and strongly taught among Indonesian family. Biodiversity is a source to explore plants with medical benefit which needs to be dug deeper in order to be able to be used for the benefit of the society. In using plants for medical activity, research needs to be done for accountability purpose to be scientifically assured for both the benefit and safety of the usage. This will help to promote the usage of herbs throughout the society.

An example of plant used for traditional medication comes from Zingiberaceae family. *Zingiber cassumunar Roxb.* is one species from Zingiberaceae family with several medical benefit out of other purposes. A single plant might have different chemical component between each structure. A part of this plant that is used for medication is its rhizome. Rhizome is a place where plants store their specific metabolism such as essential oils. Ethanol extract of *Zingiber cassumunar Roxb* rhizomes contains flavonoid, tannin, terpent, and essential oil [1]. *Zingiber cassumunar Roxb* rhizome contains resin, starch, tannin, essential oils (sineol, pinen, sesquiterpen) [2]. The usage of ethanol as solvent was because ethanol is a universal solvent and has both polar and non polar group which makes it able to solve both polar and non polar compounds.

Several researches already showed that *Zingiber cassumunar Roxb* is able to act as anti inflammatory agent, analgesic, antipyretic, mosquito repellent, carminative agent, laxative, anti dysentery, cough medicine, and used as

skin treatment [3,4,5,6]. Methanol extract of *Zingiber cassumunar Roxb* rhizomes at the level 12.5 mg/20 gram weight of mice can suppress pain induced by 5% acetic acid solution intra peritonially [7]. Ethanol extract of *Zingiber cassumunar Roxb* rhizomes has antibacterial activity to *Escherichia coli* with minimum inhibitory concentration of 12.5% and minimum killing concentration of 25% [1]. Essential oil from *Zingiber cassumunar Roxb* rhizome has antifungial activity to the live of a fungi species *Malassezia furfur* in *invitro* [2]. 2,6,9,9-tetramethyl-2,6,10-cycloundecatrien-1-one (60.77%) and α-caryophyllene (23.92%) were abundant in *Zingiber cassumunar* oil, displayed varying degrees of antimicrobial activity against all tested microorganisms [8]. Dichloromethane and methanol extracts of 13 Zingiberaceae species from the Alpinia, Costus and Zingiber genera were screened for antimicrobial activities of most of the extracts was antibacterial with only the methanol extract of Costus discolor showing very potent antifungal activity against only Aspergillus ochraceous (MID, 15.6 µg per disc) [9]. Most Zingiberaceae plant extracts exhibited antimicrobial activity against all tested food microorganisms [10]. The essential oil of Zingiber cassumunar (Plai oil) exhibits antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, dermatophytes and yeasts. The minimum bactericidal concentration (MBC) determined by the broth macrodilution method ranged from 0.62 to 2.5 vol % for Plai oil and from 52 to 79 mg/mL for the 5 wt % Plai oil gel [11].

Research on the most active fraction of ethanol extract from *Zingiber cassumunar Roxb* rhizome has never been done before, therefore this research is done to isolate, identify and test the most active antibacterial activity of ethanol extract from *Zingiber cassumunar Roxb* rhizome. Hopefully, this research can increase the usage of *Zingiber cassumunar Roxb* and inform the society about application of *Zingiber cassumunar Roxb* as herb.

EXPERIMENTAL SECTION

Materials

Zingiber cassumunar Roxb rhizome from around Boyolali area Indonesia, ethanol 96%, n-hexane (*redistilled*), ethyl acetate (*redistilled*), methanol (*redistilled*), alcohol 70%, dimethyl sulfoxide solution (DMSO), aceton (*redistilled*), silica plate Merck Kieselgel 60 GF_{254} 0.25 mm, silica gel Merck Kieselgel 60 (0.2-0.5 mm), silica gel Merck Si-gel $G_{60,}$ ethanol absolut, acetic acid anhydrite, H_2SO_4, $FeCl_3$, HCl, aquades, Nutrient agar (NA), amoxicillin (Merck), *Bacillus cereus* and *Klebsiella pneumonia* bacteria (Microbiology laboratory of USB Surakarta).

Preparation and sample extraction

Zingiber cassumunar Roxb rhizomes was washed, sliced and dried for 5 days in ambient air then put to the oven (Memmert Model 500) for 3 days at 40°C then ground to form powder. 1.5 Kg of *Zingiber cassumunar Roxb* rhizome then macerated using 4.5 L of ethanol 96% for 3x24 jam at room temperature. The resulting extract was then thickened using rotary evaporator (Bibby RE 200B).

Fractionation of *Zingiber cassumunar Roxb* rhizome ethanol extract

40 grams of extract was fractionated using Vacuum Liquid Chromatography (VLC) with the column diameter of 9 cm. The fractionation was done twice with 20 grams sample each. The VLC column was dry prepared with 125 gram static phase (silica Gel 60 GF_{254}). 20 grams of ethanol extract sample was mixed with 40 gram of silica adsorb Merck Kieselgel 60 (0.2-0.5 mm), ground until the extract dried, then put above the static phase and eluted with 150 ml eluen which polarity ascends with n-hexane : EtOAc comparison. The resulting eluen then collected and evaporated to get thicker fraction.

Antibacterial activity testing

The antibacterial activity was tested by diffusion (perforation) method to *Bacillus cereus* and *Klebsiella pneumonia* bacteria. The media used was perforated gelatin natrium with 6 mm diameter holes. Each hole was filled with 20 µL of sample with pre-defined concentration using DMSO as solvent. This step was repeated 3 times, and then sample was incubated in incubator (Hotcold M P-Selecta) for 18-24 hours at 37°C. After that, the inhibition area diameter was measured using calipers.

Minimum Inhibitory Concentration (MIC) and equivalent value determination

MIC determination was done to the fraction giving the highest inhibition to the three testing bacteria. The method used was the same as the previous antibacterial activity testing method. The MIC determination of the most active fraction was done with various declining concentration: 12.5%, 6.25%, 3.125%, 1.0%, 0.75%, 0.5%, 0.25%, 0.125%, 0.075%, 0.05%.

The standard used for comparison was amoxicillin with similar treatment as testing samples. Amoxicillin MIC was done in various concentration as well: 100 ppm, 25 ppm, 20 ppm, 15 ppm, 10 ppm, 5 ppm, and so on until amoxicillin MIC was found.

Gas Chromatography – Mass Spectrophotometry (GC–MS)
Resulting fraction from VLC with the highest antibacterial activity was then analyzed and identified using GC-MS (QP2010S SHIMADZHU). The equipment condition including ionizer type EI (Electron Impact), column type AGILENT DB-1, column length 30 meter, column diameter 0.25 milimeter, column temperature 120°C, injector temperature 310°C.

RESULTS AND DISCUSSION

Preparation and sample extraction
Aqueous extract acquired was then evaporated using *rotary evaporator* resulting 250 mL thick extract. The extract drying process using desiccators yielded 126 grams of thick extract with 8.4% yield.

Ethanol extract separation
40 grams of ethanol extract was then fractionated twice using VLC with the same solvent which is *n*-hexane : EtOAc (9.5:5) (2x); (9:1) (2x); (8:2) (2x); (7:3) (2x); (6:4) (2x); (5:5) (2x); (4:6) (2x); and (0:10). The first and second VLC result which has similar diffraction pattern was combined to get 6 fraction (A-F) with each fraction weigh 0.076 gram (A), 0.305 gram (B), 1.067 gram(C), 7.042 gram(D), 6.674 gram(E), 11.163 gram(F). From the result, we took fraction C to F for next bacterial testing because these fractions have sufficient weight.

Antibacterial activity testing of fractions from VLC result
Thick fractions from VLC was then tested its antibacterial activity using *Bacillus cereus* and *Klebsiella pneumoniae* through perforation method with 6 mm holes in diameter. The concentration of each fraction in the testing activity was 100%, 75%, 50%, and 25% using DMSO as solvent. The concentration was set similar in order to get the most active fraction to inhibit the growth of both testing bacteria. The result can be seen in Table 1.

Table 1. Result of antibacterial activity testing from fractions of VLC result with the concentration 100% to 25% to 2 testing bacteria

Fraction	Concentration (%)	Average inhibition zone diameter (mm)*	
		B. cereus	*K. pneumoniae*
C	100	11.02	12.36
	75	10.85	11.08
	50	10.51	10.38
	25	9.11	10.29
D	100	11.03	11.41
	75	10.72	11.26
	50	10.54	11.05
	25	9.12	10.56
E	100	11.23	11.74
	75	10.85	9.90
	50	10.66	9.17
	25	9.30	8.06
F	100	13.60	19.13
	75	13.56	15.61
	50	12.76	13.81
	25	10.50	10.81

*Remark : pores diameter 6 mm, *data from 3 tests*

The result shows that fraction F has the highest activity in two tests compared to other fractions from VLC yield. It shows that the compounds in fraction F has the most potency to inhibit the growth of *B. cereus* and *K. pneumoniae.*

MIC and equivalent value determination of fraction F to amoxicillin
Amoxicillin MIC determination
MIC determination was done with various concentrations starting from 1.10^{-3}% to 5.10^{-5}%. The amoxicillin MIC result can be seen in Table 2.

Table 2. Result of amoxicillin MIC testing to 2 testing bacteria

Concentration ($x10^{-5}$%)	Average inhibition diameter (mm)*	
	B. cereus	*K.pneumoniae*
100	9.02	11.80
50	8.01	9.78
25	7.12	8.06
12.5	6.86	7.22
10	6.72	6.66
7.5	6.58	6.40
5	6.00	6.00

*Remark : pores diameter 6 mm,*data from 3 tests*

Based on the data on table 2, we can get the amoxicillin MIC value to 2 testing bacteria. Amoxicillin MIC value is $7.5.10^{-5}$% to *B. cereus* with Inhibition Area Diameter (IAD) 6.58 mm and *K. pneumonia* with IAD 6,40 mm.

Equivalent value determination of amoxicillin

Table 3. Result of equivalent value determination from F fraction to 2 testing bacteria against amoxicillin

Bacteria	IAD (mm)	F Fraction Concentration (%)	Amoxicillin concentration (%)	Equivalent value (%)
B. cereus	10.50	25	$1.466.10^{-3}$	$5.864.10^{-3}$
K. pneumoniae	10.81	25	$7.726.10^{-4}$	$3.094.10^{-3}$

GC-MS result

F fraction that showed the highest inhibition ability to two testing bacteria was then analyzed using GC-MS to find out the component inside. The GC-MS analysis will generate 2 data which are chromatogram from GC and mass spectra from MS. The chromatogram result shows 23 peaks. GC Chromatogram from fraction F can be seen in Figure 1.

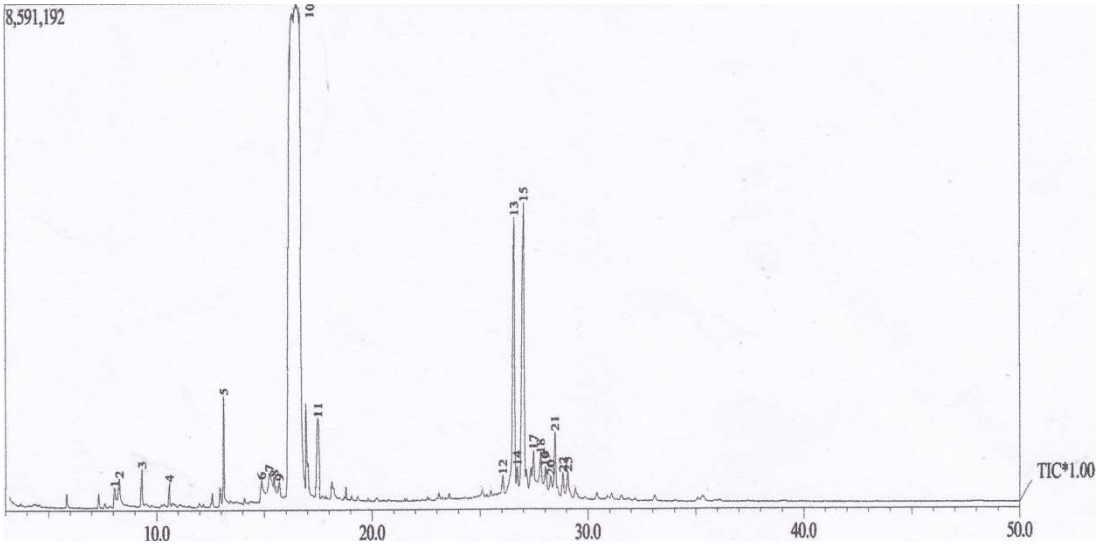

Fig.1. GC Chromatogram from fraction F of *Zingiber cassumunar Roxb* rhizome

Further identification was done using MS which will generate mass spectra from each peak in the chromatogram. The mass spectra analysis was based on *Similiarity Indeks* (SI) value, *base peak*, and mass spectra diffraction compared to the spectral database in *Wiley 229.LIB* and *NIST62.LIB.*

Table 4. GC-MS data analysis of *Zingiber cassumunar Roxb* rhizome

Peak	SI	Time Retention (minute)	Surface area (%)	MW	Advised compound	Group
3	94	9.319	0.55	151	Vanillin	BA
4	87	10.585	0.28	168	$C_9H_{10}O_3$	BA
5	76	13.112	1.40	190	(E)-1-(3,4-dimetoxi phenyl)butadiene	PB [12]
6	71	14.860	0.83	218	$C_{12}H_{10}O_4$	PB [13]
7	62	15.226	1.42	210	$C_{12}H_{18}O_3$	PB [13]
8	64	15.442	0.60	220	-	PB [13]
9	83	15.675	0.63	192	-	AC
10	72	16.523	67.09	208	(E)-4-(3,4- dimetoxi phenyl)but-3-en-1-ol	PB [13]
11	70	17.479	2.30	250	(E)-4-(3,4- dimetoxi phenyl)but-3-ene-1-il acetate	PB [13]
13	70	26.584	6.95	380	$C_{24}H_{38}O_4$	DPB[14]
14	47	26.708	0.53	382	$C_{24}H_{40}O_4$	DPB[14]
15	72	27.022	8.80	380	$C_{24}H_{38}O_4$	DPB[14]
16	67	27.342	0.63	382	$C_{24}H_{40}O_4$	DPB[14]
18	67	27.792	1.38	378	$C_{24}H_{36}O_4$	DPB[14]
21	68	28.465	1.52	-	-	DPB[14]
22	67	28.839	0.48	-	-	DPB[14]
23	60	29.057	0.64	383	$C_{24}H_{41}O_4$	DPB[14]

Remark :Total identified=96.03%, Total unidentified= 3.97%, BA =Benzoic acid, PB=phenylbutanoid, AC=aliphatic compounds, DPB= Dimer phanylbutanoid

The most active fraction of *Zingiber cassumunar Roxb* rhizome consists of benzoic acid derivative (vanillin and $C_9H_{10}O_3$), phenylbutenoid compounds:(E)-1-(3,4-dimetoxiphenyl) butadiene (DMPBD); $C_{12}H_{10}O_4$; $C_{12}H_{18}O_3$; (E)-4(3,4-dimetoxiphenyl)but-3-en-1-ol; (E)-4-(3,4-dimetoxiphenyl)but-3-en-1-il acetate, aliphatic compounds and dimer phenylbutanoid group: $C_{24}H_{38}O_4$; $C_{24}H_{40}O_4$; $C_{24}H_{36}O_4$; $C_{24}H_{41}O_4$.

Below are examples of mass spectra analysis from several dominant compounds in the GC-MS result from fraction F of *Zingiber cassumunar Roxb* rhizome:

Peak 10 compound

Peak 10 compound has retention time of 16.525 minutes. This compound is the most dominant of all because of its abundance among all peaks which was 67.09%. The peak 10 compound's base peak was 177 and molecular weight 208.

The compound (E)-4(3,4-dimetoxiphenyl)but-3-en-1-ol in *Zingiber cassumunar Roxb* rhizome hasmolecular weight 208 and data EI-MS,m/z (rel.int.): 208$[M]^+$ (49),177(100),146(43) [13]. So it's highly possible that peak 10 compound is (E)-4(3,4- dimetoxiphenyl)but-3-en-1-ol which belongs to phenylbutenoid group. The compound (E)-4(3,4- dimetoxiphenyl)but-3-en-1-ol is shown on Figure 2.

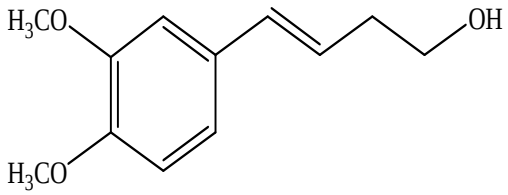

Fig. 2. (E)-4(3,4- dimetoxiphenyl)but-3-en-1-ol

Peak 11 compound

Peak 11 compound has retention time of 17.475 minutes. This compounds was 2.30% in abundance. The base peak of this compound is 159, and molecular weight 250.

The peak 11 compound does not comply with the data from *WILEY229.LIB* and *NIST62.LIB*. The compound (E)-4-(3,4- dimetoxiphenyl)But-3-ene-1-il acetate on *Zingiber cassumunar Roxb* rhizome extract has the following data: EI-MS, m/z (rel. int.): 250 $[M]^+$, 190, 159 [13]. The peak 11 compound is (E)-4-(3,4- dimetoxiphenyl)But-3-ene-1-il acetate which belongs to phenylbutenoid groups and shown in Figure 3.

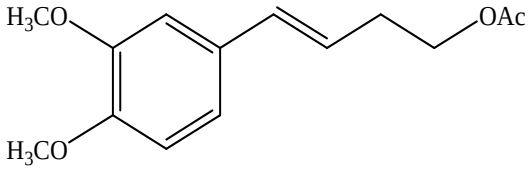

Fig. 3. (E)-4-(3,4- dimetoxiphenyl)But-3-ene-1-il acetate

Peak 13 and 15 compounds

The peak 13 compound has retention time of 26.583 minutes and 6.95% abundance, while peak 15 has retention time of 27.025 minutes and 8.80% abundance. The base peak and molecular weight of these two compounds are the same which are 190 and 380. Looking at the SI number, peak 13 and 15 compounds are not comply with *WILEY229.LIB* and *NIST62.LIB*. The dimer phenylbutenoid group isolated from *Zingiber cassumunar Roxb* rhizome has the following data: EI-MS m/z 380 $[M]^+$, 190, 175, 159. This phenylbutenoid compound has molecular formula $C_{24}H_{38}O_4$. Several compounds that belong to this groups are (±)-cis-1,2-bis[(E)-3,4-dimetoxystiril]cyclobutene (1); (±)-cis-3-(3,4- dimetoxiphenyl)-4-[(E)-3,4-dimetoxystiril]cyclohexene (2); (±)-trans-3-(3,4- dimetoxiphenyl)-4-[(E)-3,4- dimetoxystiril]cyclohexene (3) [14]. The possibility of compound 13 and 15 is shown on Figure 4.

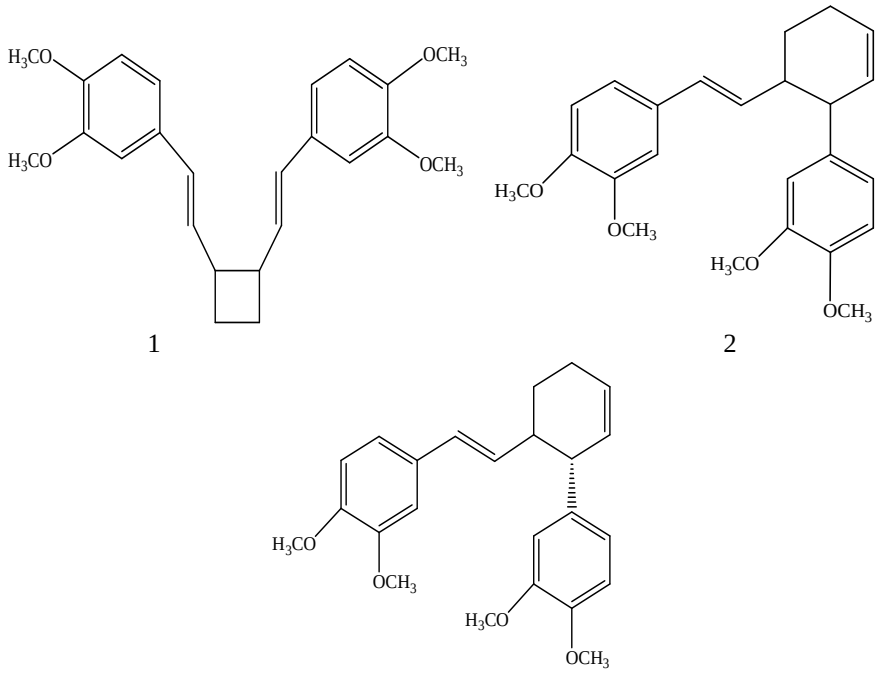

Fig. 4. (±)-cis-1,2-bis[(E)-3,4-dimetoxystiril]cyclobutane (1); (±)-cis-3-(3,4- dimetoxiphenyl)-4-[(E)-3,4- dimetoxystiril]cyclohexane (2); (±)-trans-3-(3,4- dimetoxiphenyl)-4-[(E)-3,4- dimetoxystiril]cyclohexane (3).

From the previous research, it was known that phenylbutenoid group is analgetic, anti inflammatory, anti fever and insecticide. The phenylbutenoid compound isolated from $CHCl_3$ extract of *Zingiber cassumunar Roxb* rhizome has inhibitor cyclooxygenation activity (Han et al., 2005). The phenylbutenoid compounds from *Zingiber cassumunar Roxb* rhizome (E)-4-(3,4-dimetoxyphenyl)but-3-en-1-ol; [(E)-4-(2,4,5-trimetoxyphenyl)but-3-en-1-ol]; and [(E)-4-(3,4,1-trimetoxyphenyl)but-3-en-1-ol] have pagositosis effect that has immunostimulant activity to macrophage cell in mice's peritonium [15]. The dimer phenylbutenoid compound from *Zingiber cassumunar Roxb* rhizome has cytotoxic activity [16], and also act as antioxidant, anti inflammatory and anti cancer [17]. The dominant compounds from F fraction of *Zingiber cassumunar Roxb* rhizome both the phenylbutenoid or dimer phenylbutenoid can resist the growth of *Bacillus cereus* and *Klebsiella pneumonia*. Phenylbutenoid was isolated from *Z. cassumunar* which is a derivative from phenol with one or more hydroxyl substitution [15]. The hydroxyl compound from the phenylbutenoid group is the one that possibly interacts with the bacteria.

CONCLUSION

Fractions of *Zingiber cassumunar Roxb* rhizome from VLC separation has antibacterial activity to *Bacillus cereus* and *Klebsiella pneumonia*. The most active fraction to those bacteria is the F fraction. The identification of F fraction component from rhizome (*Zingiber cassumunar* Roxb.) with KLT test contains trepenoid and phenylbutenoid group. The GC-MS analysis showed that phenylbutenoid and dimer phenylbutenoid compounds are the most dominant.

REFERENCES

[1] L Raharjoyo; Gunardi, *Jurnal Media Medika Indonesiana*, **2009**,2(3),43-67.
[2] D Ayuningtiyas, Activity bengle Essential Oils (Zingiber Cassumunar Roxb.) On growth of Malassezia furfur in vitro. Faculty Medicine, University Diponegoro, Semarang, **2008**, 34-45.
[3] Sa'roni, Anti-inflammatory Research Infusion Zingiber Cassumunar Roxb. Rhizome In White Rat. Puslitbang Farmasi Badan Litbang Kesehatan Depkes RI, **1999**,78-89.
[4] Indriatni, Analgesic effects Infusum Zingiber Cassumunar Roxb. Rhizome Against Pain That Brought Acetic Acid in Mice, Laboratory Pharmacology, Faculty Medicine, University Airlangga, Surabaya, **1999**, 1-32.
[5] Pujiastuti; Sa'roni; B Nuratmi, *Media Litbang Kesehatan*, **2001**, 11(3),23-35.
[6] MB Oliveros, Preformulation studies on terpinen-4-ol from *Zingiber purpureum* Rosc, *Proceedings of the 2nd Symposium on the Family Zingiberaceae*. Guangzhou, Zhongshan University Press, **1996**, 180-86.
[7] E Fachriyah; A Kurniawan; Meiny; Gunardi, *Jurnal Media Medika Indonesiana*, **2007**,42(1), 45-58.
[8] TSAT Kamazeri; OA Samah; M Taher; D Susanti, H Qaralleh, *Asian Pacific Journal of Tropical Medicine*, **2012**, 5(3), 202-209.

[9]M Habsah; M Amran; MM Mackeen; NH Lajis; H Kikuzaki; N Nakatani; AA Rahman; Ghafar; AM Ali, *Journal of Ethnopharmacology*, **2000**, 72(3), 403-410.
[10]IN Chen; CC Chang; CC Ng; CY Wang; YT Shyu; TL Chang, *Plant Foods for Human Nutrition*, **2008**, 63 (1), 15-20.
[11] P Pithayanukul; J Tubprasert; M Wuthi-Udomlert, *Phytotherapy Research*, **2007**, 21(2), 164-169.
[12] U Pongprayoon; P Tuchinda; P Claeson; T Sematong; V Reutrakul, *Phytomedicine*, **1996**, 3: 323.
[13] T Masuda; A Jitoe, *Phytochemistry*, **1995**, 39 (2), 459-461.
[14] A Jitoe; T Masuda; N Nakatani, *Phytochemistry*, **1993**, 32(2), 357-363.
[15] Chairul; Praptiwi; MC Sofnie, *Biodiversitas*, **2009**, 10(1), 67-76.
[16] AR Han; HY Min; T Windono; GH Jeohn; DS Jang; SK Lee; EK Seo, *Planta Medica*, **2004**, 70 (11), 1095-1097.
[17] A Murakami; D Tasahashi; T Kinoshita; K Koshimuzi; HW Kim; A Yoshihiro; Y Najamura; S Jiwajinda; J Terao; H Ohigasi, *Carcinogenesis*, **2002**, 23(5), 795-802.