B I O D I V E R S I T A S
Volume 10, Number 1, January 2009
Halaman: 40-43

ISSN: 1412-033X (printed edition)
ISSN: 2085-4722 (electronic)
DOI: 10.13057/biodiv/d100108

Phagocytosis Effectivity Test of Phenylbutenoid Compounds Isolated from Bangle (*Zingiber cassumunar* Roxb.) Rhizome

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Received: 3rd November 2008. Accepted: 20th December 2008.

ABSTRACT

This study was conducted to determine the immunostimulant activity of several phenylbutenoid derivatives isolated from bangle (*Zingiber cassumunar* Roxb) rhizome: **[1]** [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol], **[2]** [(*E*)-4-(2',4',5'-tri-methoxyphenyl)but-3-en-1-ol] and **[3]** [(E)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol]. Immunostimulant activity was done by stimulating macrophage cells of mouse peritoneum. The result showed that [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol] compound had highest immunostimulant activity (99.0%) compared to compound 2 (93.7%) and 3 (80.0%).

Key words: *Zingiber cassumunar* Roxb, immunostimulant, phenylbutenoid derivative, [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol], [(*E*)-4-(2',4',5'-trimethoxyphenyl)but-3-en-1-ol]and [(E)-4-(3',4',1-trimethoxy phenyl)but-3-en-1-ol].

INTRODUCTION

Phagocytosis process is one of non-specific defense mechanism of the body against some of foreign agent/body including pathogen microorganism. The primary cell that take important role in phagocytosis (phagocyte cell) is mononuclear (monocyte and macrophage) and polymorphonuclear or granulocyte (neutrophil). Effective phagocytosis process in early microorganism invasion can prevent illness. Microorganism destruction in the process of body defense mechanism is divided into several steps which are chemotaxis or phagocyte cells movement to the infection site, and then phagocyte cells bind it through non-specific receptor. If the microorganism already in the phagocyte cells, lysosomes fused with phagosome to form phagolysosome and microorganism is destroyed by microbicidal mechanism (Baratawijaya, 1991). Several plant species have been known as immunostimulant such as *Echinaceae angustifolia* and *Z. officinale.* The common natural immunostimulants are interleukin, interferon (INF), monoclonal antibody, crestin and lentinan, while the synthetic immunostimulant are levamisol, isoprinosin dipeptide muramil.

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Several studies of phytochemical compounds and biological activity of *Zingiber cassumunar* Roxb had been done previously. The main component of *Z. cassumunar* rhizome essential oil were triquinacene 1,4-bis (methoxy), (Z)-ocimene and terpinen-4-ol (Bhuiyan et al., 2008). The result of the isolation work showed that its rhizome contained several phenylbutenoid compounds, curcuminoid, and sesquiterpene (zerumbon). Curcuminoid (cassumunin A and cassumunin B) isolated from *Z. cassumunar* may possess a potent protective action on living cells suffering from oxidative stress (Nagano et al., 1997). Some of the phenylbutenoid had the biological activity as cyclooxygenase inhibitor, cytotoxic, antiinflammation (Ozaki et al., 1991; Han et al., 2003, 2005; Fachriya et al., 2007), phenylbutenoid dimmer proved to have cytotoxic activity (Han et al., 2004), and acted as antioxidant, antiinflammation and anticancer (cytotoxic) (Murakami et al., 2002), and curcuminoid is potential as antioxidant (Nagano et al., 1997). Other study concluded that *Z. cassumunar* rhizome performs biological activity as bowel/ intestine relaxant (Kanjanapothi et al., 1987), lipase enzyme activator (Darusman et al., 2001) and hepatoprotector (Arafah, 2005), but its role for phagocytosis activity has not been studied yet.

The aim of this study was to determine the potential of several phenylbutenoid compounds isolated from bangle (*Z. cassumunar* Roxb) rhizome: **[1]** [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol], **[2]** [(*E*)-4-(2',4',5'-tri-methoxyphenyl)but-3-en-1-ol] and **[3]** [(E)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol] as immunostimulant.

MATERIALS AND METHODS

Materials

Rhizome of bangle (*Z. cassumunar*) was obtained from Bogor Botanical Garden and identified for its scientific name in Herbarium Bogoriense, Research Center for Biology, Cibinong-Bogor, West Java, Indonesia. The rhizome was thin sliced and dried in the oven with temperature of 40-50°C. Dried rhizome was powdered with grinder. Macrophage cells were obtained from peritoneum of *Swiss Webster* male mouse (*Mus musculus*) 2 month old, 20-30 g body weight. *Staphylococcus epidermidis* isolates as foreign agent that will be phagocyted by macrophage cell from mouse peritoneum *in-vitro.* Bacteria growth medium: *Mueller-Hinton Agar* (MHA) (Sigma) and Nutrient Broth (NB) (Sigma). Reagent and solvent: Phosphate Buffer Saline (PBS) pH ± 7.4, dimethyl sulfoxide (DMSO), Na_2EDTA 0.2 M, Giemsa, tripan blue, methanol, ethanol, ethyl acetate, *n*-hexane, and aquadest. Levamisol HCl as control positive (200 L)

Equipments

Equipments used in this study were incubator, autoclave for sterilization, laminar air flow, centrifuge, touch mixer, hemocytometer Nechbauer, spectrophotometer microscope, animal cages and animal balance, pH meter, analytical balance, rotary vacuum evaporator, percolator, Eppendorf pipette , petridish, ose, vial, object glass, sterilized glassware.

Isolation and purification of bioactive compounds

Dried rhizome powder of *Z. cassumunar* (1 kg) macerated in methanol 80% for 24 hours, the filtrate was separated and placed in the erlenmeyer and composited. The composed filtrate was evaporated with rotary evaporator with the temperature of 60°C. The evaporation continued with water bath (petridish had been weighed previously). Maceration and percolation was done until the filtrate was clear. This crude extract was dissolved in methanol 50%, and placed in the separating funnel. Fractionation was done based on solvent polarity of *n*-hexane, ethyl acetate and residue (MeOH/water) consecutively. Every fraction was shacked several times until the fraction was clear. Every fraction was concentrated with rotary evaporator with the temperature of 35°C. The evaporation was continued with until the fraction was concentrated. Three fractions were obtained, n-hexane, ethyl acetate and MeOH/water. Every fraction was tested for phagocytosis activity and capacity of mouse peritoneum macrophage cells. High effectivity was purified by chromatography technique.

Chromatography column of ethyl acetate fraction was used as stationary phase SiO_2 and mobile phase in gradient system with the eluent of *n*-hexane/ethyl acetate in the composition of 9: 1, 8: 2 up to 4: 6 respectively, each 2,5 L. Based on the result of Thin Layer Chromatography (TLC), this extract composed of 4 fractions. Every fraction was composited and evaporated. The dried fraction was tested for its phagocytosis activity and capacity of mouse peritoneum macrophage cells. Purification of bioactive compounds of the fraction by HPLC was done based on its immunostimulant activity; this was done by stationary phase SiO_2 and mobile phase *n*-hexane/ethyl acetate (4: 6). Lead us to four isolated compounds and three of them were pure compounds (**[1]**, **[2]** and **[3]**) which can be elucidated and to be determined as phenylbutanoids derivatives.

(*E*)-4(3',4'-dimethoxyphenyl)but-3-en-1-ol (**[1]**) isolated as a pale yellow oil, UV $\lambda_{max:}$ 259.70, 214.20 nm, IR v_{max}: 3491-3334 (broad, OH), 1514 (aromatic), 2997, 2932, 2836 cm^{-1}(weak, short chain aliphatic). MS m/z 208 $[M]^+$, 190, 177, 146 ,77. ^{1}H-NMR; δ 2.46 (q, 2H), 3.74 (t, 2H), 3.86 (s, 3H), 3.88 (s, 3H), 6.06 (m, 1H), 6.42 (d, 1H), 6.79 (d, 1H), 6.88 (dd, 1H), and 6.91 (d, 1H). ^{13}C-NMR; δ 36.49 (2), 55.95 (3), 56.06 (3), 62.24 (2), 108.87 (1), 111.27 (1), 119.27 (1), 124.50 (1), 130.54 (0), 132.58 (1), 148.69 (0), and 149.14 (0).

(*E*)-4(2',4',5'-trimethoxyphenyl)but-3-en-1-ol (**[2]**) isolated as a pale yellow oil, UV $\lambda_{max:}$ 314.20, 258.70, 210.80 nm, IR v_{max}: 3539-3320 (broad, OH), 1510 (aromatic), 2996, 2928, 2849 cm^{-1} (weak, short chain aliphatic). MS m/z 238 $[M]^+$, 207, 192, 161, 151, 77. ^{1}H-NMR: δ 2.51 (q, 2H), 3.76 (t, 2H), 3.82 (s, 3H), 3.87 (s, 3H), 3.89 (s, 3H), 6.06 (m, 1H), 6.49 (s, 1H) , 6.75 (d, 1H) and 6.97 (s, 1H). ^{13}C-NMR: δ 36.99 (2), 56.81 (3), 56.66 (3), 57.27 (3), 62.37 (2), 97.82 (1), 109.78 (1), 118.21 (1), 124.75 (1), 127.19 (1), 143.45 (0), 149.34 (0) and 151.11 (0).

(*E*)-4(3',4'-dimethoxy phenyl)but-3-en-1-methoxy-1-ol (**[3]**) isolated as a pale yellow oil, UV $\lambda_{max:}$ 262.90, 212.20 nm, IR v_{max}: 3387 (broad, OH), 1514 (aromatic), 2996, 2928, 2835 cm^{-1}(weak, short chain aliphatic). MS m/z 238 $[M]^+$, 207, 190, 177, 161, 46, 77. ^{1}H-NMR; δ 3.64 (m, 2H), 3.87 (m, 1H), 3.89 (s, 3H), 3.91 (s, 3H), 5.90 (q, 1H), 6.59 (d, 1H), 6.83 (d, 1H), 6.94 (dd, 1H), and 6.96 (s, 1H). ^{13}C-NMR; δ 56.02 (3), 56.13 (3), 56,72 (3), 65.76 (2), 83.87 (2), 108.87 (1), 111.21 (1), 120.15 (1), 123.85 (1), 129.37 (1), 134.36 (0), 149.25 (0) and 149.37 (0).

Immunostimulant activity test

Immunostimulant activity was determined by phagocytosis activity and capacity of mouse peritoneum macrophage cells. Phagocytosis activity value is the percentage of active macrophage cells in 100 macrophage cells.

$$\text{Phagocytosis activity (\%)} = \frac{\text{Number of active macrophage cells}}{\text{Total of macrophage cells}} \times 100\%$$

Phagocytosis capacity = the number of bacteria phagocyted by 50 macrophage cells.

Before phagocytosis test was carried out, viability test was conducted previously to determine the macrophage cells viability and the total number of cells. Viability was expressed by the percentage of viable macrophage cells to total number of macrophage cells. Viability value should not be less than 95%.

Phagocytosis test

200 μL of bacteria suspension, 200 μL macrophage cells and 200 μL sample were placed in the test tube, and then incubated for 30 minutes, 37^0C. After incubation period, the sample was added 50 μL Na_2EDTA 0.2 M to stop phagocytosis process. The control positive was 200 μL levamisol HCl, and negative control was 200 μL phosphate buffer saline (PBS). After incubation period, the thin slides was made by dropping 100 μL suspension to object glass and spread with the spreader, dried, and then fixed with MeOH for 6 minutes. Thin slides were stained with Giemsa 2% and left in the stain for 45 minutes. Put in the acetic acid 1% 4 times, washed under tap water and allowed to air-dried. The thin slides were observed under the microscope with the magnification of 10x100 to determine macrophage cells activity and capacity (Wagner and Jurcic, 1991).

RESULTS AND DISCUSSION

The most active fraction of *Z. cassumunar* rhizome was screened for its immunostimulant properties lead to 4 compounds, but one of them was not pure (*impurity*), so that the structure elucidation was done toward 3 compounds. The result of structure elucidation showed that the compounds identified as: **[1]** [(*E*)-4-(3',4'-dimethoxy phenyl)but-3-en-1-ol], **[2]** [(*E*)-4-(2',4',5'-tri-methoxy phenyl)-but-3-en-1-ol] dan **[3]** [(E)-4-(3',4',1-trimethoxy phenyl)but-3-en-1-ol] and the first compound was the major compound (Figure 1). All of the three compounds were oily liquid.

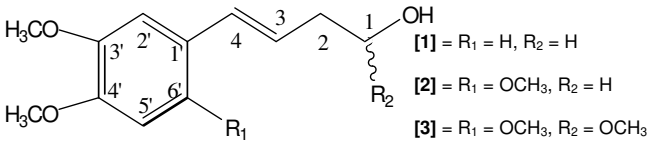

Figure 1. Structure of phenylbutenoid derivatives of bangle rhizome.

The result of phagocytosis test by using 500 μg of the sample showed that the phagocytosis capacity and activity of compound **[1]** were1069.3 and 99.0%, while the compound **[2]** were (612.33 and 90.7%) and compound **[3]** (691.22 and 88.0%) (Figure 2 and 3).

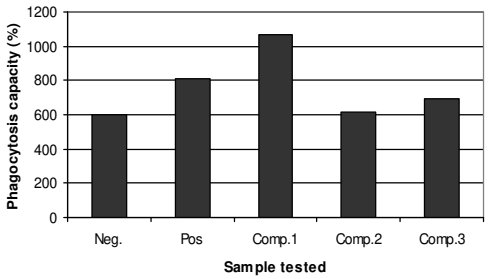

Figure 2. Phagocytosis capacity of phenylbutenoid compound from *Z.cassumunar* rhizome.

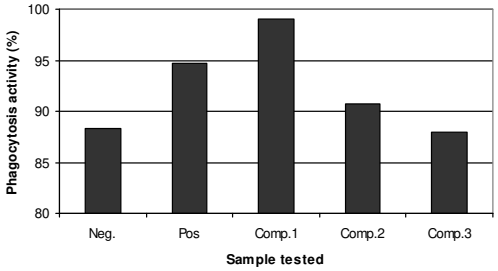

Figure 3. Phagocytosis activity of phenylbutenoid compound from *Z.cassumunar* rhizome.

Compound **[1]** did not have cyclooxygenase (COX-2) enzyme inhibitor (Han et al., 2005). Cyclooxygenase is the enzyme that has important role in arachidonic acid metabolism to prostaglandin E_2, chemical mediator that inhibit interleukin-2 and interferon γ production (NK cell activation attack tumor cell) by lymphocyte T. Prostaglandin E_2 also produced by tumor cell to suppress immune system related to tumor and cause immunodeficiency. The compound which had cyclooxygenase inhibitor properties can be used as cytotoxic, while macrophage activation for attacking tumor cell stimulated by lymphokin (chemostatic (CFM), migration inhibitor factor (MIF) and macrophage activator factor (MAF)) released by T cell stimulated by tumor antigen. The mechanism of macrophage stimulation and cyclooxygenase inhibition had different path although both of them had cytotoxic effect. Compound **[1]**. might not have cyclooxygenase inhibition properties but had the ability to stimulate macrophage cell. The study of immunostimulant activity showed that there is no correlation/relation between the structure and its activity of natural compound. Several chemical compound that had immunostimulant properties can be classified into two groups which are the compound with low molecular weight (alkilamid, phenolic compound, alkaloid, quinon, saponin, sesquiterpene, diterpene, triterpene) and compound with high molecular weight (Wagner, 1999).

Phenylbutenoid compound isolated from *Z. cassumunar* was a derivative of phenolic compound which had aromatic ring with one or more hydroxyl substitution. Phenolic compound as antioxidant

specified by its ability in capturing free radical and ROS (*reactive oxygen species*) (Huang et al., 1992). Free radical and active oxygen is one of foreign body that suppress body immune; and antioxidant properties related to the immunostimulant properties. Double bond outside the aromatic ring of phenolic compound might determine its reactivity. Besides, the more aromatic nucleus being substituted result in lowering π bond thereby lowering its reactivity and also activity and capacity of phagocytosis. Double bond in the side chain block by methoxy group subsistent in compound **[2]** and **[3]** result in decreasing reactivity so that the immunostimulant activity of both compound were lower compare to compound **[1]**.

CONCLUSION

All of the three compounds isolated from *Z. cassumunar* Roxb rhizome which were, **[1]** (*E*)-4-(3',4'-dimethoxyphe-nyl)but-3-en-1-ol), **[2]** ((*E*)-4-(2',4',5'-trimethoxyphenyl)but-3-en-1-ol dan **[3]** (*E*)-4-(3',4',1-trimethoxyphenyl)but-3-en-1-ol) showed the activity as immunostimulant through stimulation mouse macrophage cells. Compound **[1]** (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol, had higher activity compare to two other compounds (compound **[2]** and **[3]**). The *in vivo* immunostimulant activity test of active compound from *Z. cassumunar* Roxb rhizome and its relationship of structure and activity need further research.

ACKNOWLEDGEMENT

This research was sponsored by DIPA, Research Center for Biology, 2008 fiscal year.

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