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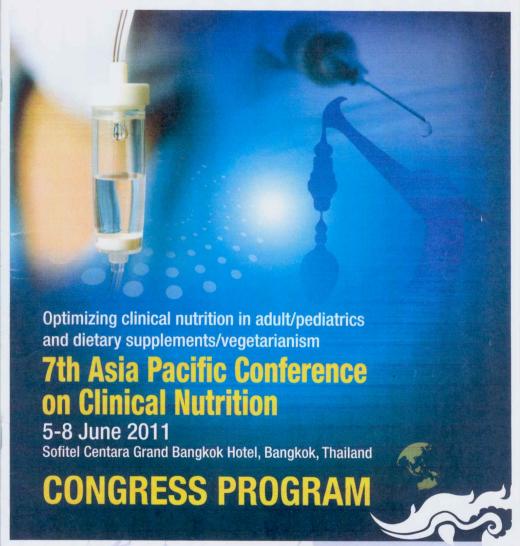
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Ginger's Bioactive Compounds Increased Intracellular Antioxidant In Vitro



Tejasari

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ABSTRACT

Glutathione (y-glutamil-sisteinil-glisin) is non protein thiol compound abundance in animal tissue and eucaryote cells, including lymphocyte. It plays important roles in many cellular functions, such as DNA and protein synthesis, xenobiotic-carcinogenic substance detoxification, and immune function maintenance. This experiment was designed for investigating the effects of ginger bioactive compounds i.e oleoresin, gingerol, and shogaol on glutathione in lymphocyte cultured in normal and under stress oxidative conditions, respectively. Using spectro photometry measurement, it was known lymphocyte used in this study had 0,43 µg glutathione /10³ cell. Effects of ginger oleoresin, gingerol, and shogaol on lymphocyte's glutathione content was depend greatly on culture conditions. In culture without stress oxidation, 50 µg/ml treatment of ginger oleoresin, gingerol, and shogaol significantly increased lymphocyte's glutathione content, respectively, by increase of 449, 188 and 172 percent. The response curve of oleoresin and shogaol was quadratic, while gingerol's was cubic. Similarly, under stress oxidation condition, oleoresin (200 µg/ml), gingerol (100 µg/ml), and shogaol (200 µg/ml) compounds increased lymphocyte's glutathione concentration significantly by increase of 563, 1553, and 531 percent. respectively. The response curve of oleoresin was cubic, while gingerol's and shogaol 's was quadratic. The effect of oleoresin, gingerol, and shogaol on the increase of glutathione content of lymphocyte cultured under stress oxidative was higher than lymphocyte cultured without stress oxidative.

Keywords: ginger's oleoresin compounds, gingerol, shogaol, glutathione, lymphocyte, intracellular antioxidant

INTRODUCTION

Ginger (Zingiber officinale Roscoe), despite contributing nutrient absolutely needed by the body, it also having bioactive compound that emerge good physiological effect for health. As mention by Tang and Eisenbrand (1992), the ginger's special properties due to its bioactive compounds. Ginger bioactive compounds that were proved its health effect scientifically, namely oleoresin, gingerol, and shogaol. Gingerol and zingeron has a sporostatic activity to Bacillus substilis (Al-Khayat and Blank, 1985). Compounds of (6)-shogaol, and (6)-gingerol had antitusive effects (Suekawa et al., 1984). Meanwhile, antioxsidative character of gingerol, shogaol, and zingeron compounds was studied by Kikuzaki and Nakatani (1993). Similarly, Tejasari (2000) studied anti oxidative effect of oleoresin, gingerol, and shogaol compounds that decreased the content of peroxide, specifically malonaldialdehide (MDA) and free radical of internal lymphocyte cultured in vitro.

Despite its anti oxidative characteristics, ginger extract was proved by Zakaria *et al.* (1996; 1999), Nurahman *et al.* (1999), and Prangdimurti *et al.* (1999) have capability in enhancing immunity in rat, and human *in vivo* and *in vitro*. The effect of ginger extract depended on culture condition, and, the dosage of the extract. It has been proved that oleographic gingerol.

and shogaol enhanced lymphocyte function as seen by increasing B cell proliferation (Tejasari *et al.*, 2002), cytolysis activity of NK cells (Tejasari and Zakaria, 2004), and the amount of T-cell surface molecules, i.e CD3+CD4+ (Tejasari, 2005). Those research findings suggested that the mechanism of lymphocyte function enhancement were thorough stimulation of lymphocyte proliferation, anti oxidative activity, and cytolysis activity of NK cells.

Other possible mechanism of enhancing lymphocyte function was thru increasing the cellular anti oxidant defense, such as intra cellular anti oxidant glutathione. Glutathione (yglutamil-sisteinil-glisin) is a non protein thiol compound that function as an important intra cellular anti oxidant, since it acts in variety cellular function such as exogenous and endogenous carcinogen detoxification, synthesis of DNA and protein, transportation of amino acid (Meister and Anderson, 1983), enzyme activation (Fanger et al., 1970), cell protection against radiation and free radical expose, and maintenance of immune function (Meydani et al., 1995). The three peptide compounds was also proved that it functions as radical quencher (Sies, 1991). plays a role in repetitive tocopherol radical cycle (Sies and Murphy, 1991), and regulation of immune function thru its effects on trans membrane signaling transduction and activation of nuclear transcription factor (Kavanagh et al., 1993). Glutathione protect signal transduction from obstruction induced by oxidative stress on epithelial pulmonary type II. Other study proved that enough glutathione (GSH) was needed for proliferation (Noelle and Lawrence, 1981). Cell with high glutathione content was able to enter cell cycle compare to cell with low glutathione. Supplementation of GSH on rat diet significantly increased lymphocyte proliferation (Furukawa et al., 1987), since depletion of GSH reduced mytogenic response. The obstruction of glutathione content caused the change of CMI response (Meydani et al., 1995)

This study investigated *in vitro* the capability of ginger non volatile bioactive compounds, namely oleoresin, gingerol in fraction-1, shogaol in fraction-2, and zingeron in fraction-3 oleoresin in increasing the body anti oxidative defense. This study is aimed at providing scientific evidence for some health effects of the ginger, especially in increasing intracellular anti oxidant in lymphocyte. In this way, the tuber root of ginger could be used in the formulation of a functional food for person highly expose to oxidative stress condition.

MATERIALS AND METHODS

Experimental Design

This laboratory experiment was performed in three stages namely, 1) analyses and extraction of oleoresin, 2) oleoresin fractionation, and 3) *in vitro* assays of the capability of ginger oleoresin in maintaining or increasing intra cellular anti oxidant glutathione, but decreasing

malonaldehide and free radical in lymphocyte. The first and second phases were conducted in the Chemical and Biochemical Laboratory, Faculty of Agricultural Technology, and Faculty of Science and Mathematic, Jember University. Meanwhile the final phase was performed in the Microbiology Laboratory, Primate Research Center, Hayati Laboratory, and Biochemistry Laboratory at Bogor Agricultural University, and in the Immunology Laboratory US NAMRU-2 (Navy Army Research Unit-2 United States) in Jakarta.

The experiment was designed as complete randomized design, two factors with 60 treatment combinations, and 3 controls for every variable tested. There were 4 kinds of ginger bioactive compounds (factor I) tested, namely oleoresin, fraction-1 (gingerol), fraction-2 (shogaol), and fraction-3. Each compound was tested for 5 concentration levels (factor II) namely, 50, 100,150, 200, and 250 μ g /ml. These two factorial treatments were done in three replications. The variables tested consisted of (i) amount of intra cellular glutathione (ii) amount of malonaldehide, and (iii) amount of total free radical in lymphocyte.

Oleoresin Extraction and Fractionation

Oleoresin compound was extracted from dry ginger powder by soxhlet distillation method as explained in Tejasari (2006). Ginger slices after freeze dried were ground to powder. About 20 gram of ginger powder was packaged by filter paper, and tied and soaked in ethanol in a tube at 70°C for 4-8 hours. Ethanol in oleoresin extract was totally evaporated to obtain the oleoresin. Fraction-1 (gingerol), fraction-2 (shogaol), and fraction-3 were obtained by column vacuum chromatography technique, using silica gel G60 230-400 mesh, and hexane: ether solvent (3:7). Every fraction was confirmed by TLC technique. The oleoresin, gingerol, and shogaol obtained were diluted in RPMI-1640, and 4 concentrations were prepared namely, 250, 500, 1000, 2000, and 2500 µg/mL. The solutions were sterilized using 0,22 µm (Millipore).

Lymphocyte Isolation

Lymphocytes were isolated from human peripheral blood by centrifugation and separation using ficoll (Sigma 1077-1) density $(1.77\pm0.001~g/ml)$ gradient technique (Freshney, 1994). Cellular components separation was performed by centrifugation on 514 x g for 10 minutes, and yielded a buffy coat layer with high content of lymphocytes. The buffy coat layer was passed on ficoll-hypaque solution slowly, and then centrifuged on 1430 x g for 30 minutes. The upper layer containing lymphocytes, monocytes and platelets was washed twice with basal medium, followed by centrifugation at 288 x g for 10 minutes. Lymphocytes (in precipitate)

separated from the platelets, monocytes, plasma, and ficoll (in supernatant). Lymphocytes were counted by tryphan blue dye on hemacytometer (Neuberger). Lymphocyte suspension with high viability (>95%) 2x10⁶ cell/ml was prepared by addition of basal medium.

Lymphocyte incubation

randomly to 96- micro plate wells. Then to each well was added 20 μL oleoresin, fr-1 (gingerol), fr-2 (shogaol), and fr-3 (zingeron) at 500, 1000, 1500, 2000, and 2500 μg/ml concentrations for each of three of them. To each well also added by 40 μL mitogen PHA and LPS 12.5 μg/ml for each of them. Therefore, the final concentration for each compound were 50, 100, 150, 200, 250 μg/mL, and 5 μg/mL for mitogen. The PHA mitogen was used for stimulating T cells proliferation, LPS for stimulating B cells proliferation. For the control, to each well was added RPMI-1640 medium. Incubation was performed under the conditions of 37°C, 95% CO₂, 5% O₂, 95% RH for 24 hours. Paraquate dichloride (Sigma, M-2254) (BM=257,2) 10 mM was used for stress oxidative conditioning of the cell culture.

A 100 µl lymphocyte suspension (2x10⁵ cell/ml) in complete medium was distributed

Analyses of Intracellular Glutathione in Lymphocyte (modified from Bergmeyer, 1990)

After 24 hours of incubation, micro titer plates were centrifuged at $1180 \times g$ for 30 minutes. The pellet wash was perform three times with 0,01 M PBS, and centrifuged at $423 \times g$ for 10 minutes. Lymphocyte were lysed in 0.5 ml lyses solution (0.2 ml Triton X-100 0.2 % and 2.3 ml sulfosalicilic acid 50% and free ion water 97,5%) for 2 ml lymphocyte suspension. Protein was agglutinated thru centrifugation at $5000 \times g$ for 5 minutes and the supernatant was counted for its glutathione content by spectrophotometer. A 1000 µl supernatant or glutathione solution standard was added by 1.5 ml PBS and 0.5 ml DTNB. Yellow colour intensity from thio- dinitro benzoate acid that released at Ellman reaction, ie reduction of 5.5 '-ditio-bis-2-nitrobenzoate (DTNB) by glutathione was counted by spectrophotometer at λ =412 nm. Concentration of reduction glutathione (GSH) in lymphocytes was analyzed based on

Statistical analysis

curve standard.

The results were expressed as mean \pm SD. One-way ANOVA and Duncan's Multiple Range Test were used to test for differences in the glutathione, malondialdehide, and free radical between treatments. P value <0.05 was used to indicate statistical significance.

RESULTS AND DISCUSSION

Ginger's oleoresin, fr-1 (gingerol), fr-2 (shogaol), and fr-3 (zingeron)

Ginger oleoresin, commercially known as zingerin, with a phenol group is a non volatile compound, brown color and hot taste. In this study, oleoresin was extracted from ginger powder using ethanol solvent since it has a higher polarity than hexane, diethyl ether, and acetone. Therefore, using ethanol solvent yielded a higher amount of oleoresin at relatively low boiling point, and was not toxic for the cells. On a dry basis, from 100 gram ginger, about 10.2 gram of oleoresin was obtained or 10.2 w/w %.

Qualitative analysis of oleoresin by TLC identified five (5) fractions shown by distinct spots with rf values as follows: fraction (1) = 0.24, fraction (2) = 0.42, fraction (3) = 0.54, fraction (4) = 0.60, and fraction (5) = 0.68 (Table 1). Fraction 1 and fraction 2 were gingerol and shogaol respectively (Chen, *et al.*, 1986), with high anti oxidative activity (Kikuzaki & Nakatani, 1993). Gingerol [1(4-hidroxy-3 metoxyphenyl)-hydroxyalkan-3-one] and shogaol [1(4-hydroxy-3-metoxyphenyl)-4-dekena-3-one] concentrations were 0.52 and 0.24 % (dry weight) Gingerol, shogaol, and zingeron are simple phenol compounds with one aromatic ring as shown in Figure 1. Oleoresin, Fr-1 (gingerol), fr-2 (shogaol), and fr-3 (zingeron) concentration tested were identified based on calculation of the consumption of one glass ginger beverage made from 25 g fresh ginger, i.e. 50 μ g/ml. The other three levels compound concentrations also tested i.e. 100, 150, 200, and 250 μ g/ml.

Simple phenol compounds have many hydroxyl groups, hence have polar characteristics and high antioxidant activity (Hudson, 1990). Kikuzaki & Nakatani (1993) showed that (6)-(gingerol), (6)-shogaol, and (6)-gingerdiol had antioxidant activity higher than that of α-tocopherol. The anti oxidative effects of gingerol, shogaol, and oleoresin protected lymphocyte from oxidative damages (Tejasari, 2003). Ginger bioactive compounds had ability in increasing B cells proliferation (Tejasari, 2001), and T cells proliferation of (Tejasari, 2006). Furthermore, the compounds also increased CD4+ T cells that is, T cells surface receptor molecules or Thelper cells, which function in cellular and humoral immune responds (Tejasari, 2005)

The Effects of Ginger Oleoresin, fr-1 (gingerol), fr-2 (shogaol) and fr-3 (zingeron) on Intracellular Glutathione in Lymphocyte

Glutathione (γ -glutamil-sisteinil-glisin) is abundance in animal tissue, ie hepar and spleen and eucaryote cells, including lymphocyte. It functions as cytoplasmic radical quencher and in electrophilic xenobiotic metabolism stage II. About 98 percent of total glutathione in reduction

form or GSH (Hoppenkamps *et al.*, 1984). In this study, it was known that total glutathione content in lymphocyte is $0.43 \mu g/10^3$ cells.

Compounds of ginger oleoresin, gingerol, and shogaol respectively affected (p=0,0001) the total glutathione content in lymphocyte (μ mol/10³ cells). The concentration influence of ginger oleoresin, gingerol, and shogaol compounds on the content of total glutathione lymphocyte really depend on culture conditions. At condition without oxidative stress, compounds of ginger oleoresin, gingerol, and shogaol significantly increased total glutathione content in lymphocyte by increase of 449, 188, and 172 percent respectively, and occurred at the same concentration level, i.e 50 μ g/ml. Curve effect of ginger oleoresin and shogaol on content of total glutathione lymphocyte characterized by quadratic, while gingerol effect was cubic (**Figure 1**, and **Table 1**).

Table 1. Regression equation of ginger oleoresin, gingerol, and shogaol on total content of glutathione in lymphocyte at two culture conditions

Culture conditions	Ginger oleoresin	F-sign	R ²	Regression equation
Without oxidative stress	 Oleoresin Gingerol Shogaol 	0,000 0,000 0,000	0,68 0,78 0,78	Y=-0,6489+0,0243X-0,00014X ² Y=-0,952+0,0479X+0,00058X ² +(1,84x10 ⁻⁶) X ³ Y=-0,880+0,0226X-0,00012X ²
Oxidative stress	1. Oleoresin 2. Gingerol 3. Shogaol	0,000 0,000 0,000	0,92 0,84 0,90	$ \begin{aligned} Y &= -1,704 + 0,0714X - 0,00088X^2 + (2,87x10^{-6}) \ X^3 \\ Y &= -1,651 + 0,0476X - 0,00024X^2 \\ Y &= -1,826 + 0,0222X - (6,07x10^{-5})X^2 \end{aligned} $

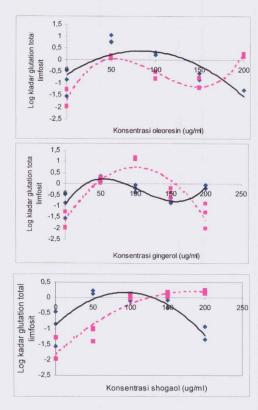


Figure 1. Curve effect of ginger oleoresin, gingerol in fraction 1, and shogaol in fraction 2 of oleoresin on total content of lymphocyte glutathione in two culture conditions

At stress oxidative culture condition, ginger oleoresin, gingerol, and shogaol increased content of total glutathione. lymphocyte significantly, by maximal increase respectively 563, 1553, and 531 percent, occurred at 200 μ g/ml oleoresin, 100 μ g/ml gingerol, 200 μ g/ml shogaol. Curve effect of ginger oleoresin on content of total glutathione. lymphocyte was cubic, meanwhile gingerol, and shogaol effect was quadratic. (Figure 1 and Table 1). The effects of ginger's oleoresin, gingerol, and shogaol on increase of the content of glutathione lymphocyte at stress oxidative culture was bigger than the effects at condition without oxidative stress.

The increase of lymphocyte glutathione was related to the high anti oxidative ability of ginger's oleoresin at low concentration. Ginger's oleoresin components were possible working together synergistically with glutathione in neutralizing endogenous free radicals, therefore the glutathione content was not declined. However, as high concentration of the ginger's oleoresin components, content of total glutathione lymphocyte starting declined. The decline of total glutathione lymphocyte content due to the decreasing of anti oxidative capability of ginger's oloreoresin components, but the glutathione content was still higher than that of in control group.

As comparison, Lin and Milner (1992) proved that active compound from garlic also increased glutathione. Phenol compounds from grape and peanut increased activity of glutation-s-transferase (Chang et al., 1985). In vivo study on mice proved that paraquate decreased glutathione content and increased activity of glutathione peroxidase (GSH-Px) (Kartikawati, 1999). However, this study proved that ginger's oleoresin components at low concentration at culture given paraquate increased glutathione total lymphocyte. This findings showed that at stress oxidative conditions, ginger's oleoresin components that having strong anti oxidative activity at low concentration (Gillard and Cormier,1980) were able in protecting lymphocyte from damage by PQ+ radicals or anion superoxide from paraquate. The possibility reaction between ginger's oleoresin components and free radicals of anion superoxide (O_2^*) from paraquate could be explained in Figure 2.

Gingerol:

Shogaol:

Figure 2. The possible reaction between ginger's oleoresin and free radical anion superoxide (O_2^*)

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At the same time, glutathione, as an important intra cellular anti oxidant, also function as cytoplasmic radical quencher and in electrophilic xenobiotik metabolism stage II. Enzimatically, glutathione as substrat for glutathione peroxidase (Se-GSH-Px) was able to hydrolyze variety of peroxide such as lipid peroxide (LOOH) and hydrogen peroxide (H₂O₂). Hydroperoxidase enzyme converted reduced glutathione (GSH) to oxidized glutathione (GSSG). Reaction of the peroxide hydrolysis by glutathione peroxidase (GSH-Px) is shown in Figure 3.

Figure 3. Reaction of peroxide hydrolysis by glutathione peroxydase (GSH-Px)

CONCLUSSIONS AND RECOMMENDATIONS

This study revealed that ginger bioactive compounds, namely oleoresin and gingerol in fraction-1 oleoresin, shogaol in fraction-2 oleoresin, and zingeron in fraction-3 oleoresin increased. The response curve of oleoresin and shogaol was quadratic, while gingerol's was cubic. Similarly, under stress oxidation condition, oleoresin (200 μ g/ml), gingerol (100 μ g/ml), and shogaol (200 μ g/ml) compounds increased lymphocyte's glutathione concentration significantly by increase of 563, 1553, and 531 percent, respectively. Therefore, the ginger oleoresin bioactive compounds increased intra cellular anti oxidants in normal and oxidative condition

These *in vitro* findings clearly supports that non volatile ginger bioactive compounds will enhance the cell from oxidative damage. In future, *in vivo* studies should be done on healthy human and patient with degerative diseases at identifying the optimal effect of the ginger bioactive compounds at different condition for supporting its use in functional food.

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