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Evaluation of Ginger (*Zingiber officinale* Roscoe) Bioactive Compounds in Increasing the Ratio of T-cell Surface Molecules of CD3+CD4+:CD3+CD8+ *In-Vitro*

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ABSTRACT

The potential ability of ginger bioactive compounds in increasing the ratio of T-cell surface molecules of CD3+CD4+:CD3+CD8+ was investigated using dual tagging FITC and PE of monoclonal antibody anti-human with its fluorescence measured by flow cytometer. Oleoresin was extracted using sinkhole distillation technique. Its components namely, gingerol in fraction-1, shogaol in fraction 2 and zingeron in fraction-3 were separated by column vacuum chromatography method. The doses of oleoresin, gingerol, shogaol, and zingeron tested were 50, 100,150, 200, and 250 µg/ml. Lymphocytes ($2x10^6$ cell/ml) from human peripheral blood were isolated using ficoll density gradient technique, and cultured in the presence of the compounds in RPMI-1640 medium and phytohemaglutinin (PHA) mitogen for 96 h under normal conditions. Percentages of T-cell surface molecules (CD4+ and CD8+) were determined using dual-tagging FITC and PE fluorescents labeled on monoclonal antibody anti human. The fluorescence-labeled bands on the T-cell surface molecules were counted using flow cytometer. The experiment revealed that oleoresin and its three fractions increased the percentage of CD3+CD4+. The compound in fraction 3 of oleoresin at 200 µg/ml increased by the highest percentage of CD3+CD4+ of 9%, but slightly decreased the percentage of CD3+CD8+. These ginger bioactive compounds increased the ratio of CD3+CD4:CD3+CD8+ T-cells with the highest increment of 30% from effects of 200 µg/ml fraction 3 of oleoresin. This *in vitro* finding revealed that ginger bioactive compounds potentially increased cellular and humoral immune response. Further clinical studies are needed to confirm the benefits of these ginger bioactive compounds as a potential functional food for testing on HIV infected patients.

INTRODUCTION

Increasingly, foods are considered to be able to modulate the human body's biochemical and physiological processes, including its immunity system. In light of this belief, research on functional foods is widely conducted, including identification of bioactive compounds, evaluation on the interactions between food compounds and specific functions in the body, and its immune modulation capability. Natural bioactive compounds especially from plant sources, including spices such as

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ginger (*Zingiber officinale* Roscoe), have been investigated for their characteristics and health effects.

Some of the bioactive compounds of ginger have been identified and isolated (Kikuzaki & Nakatani, 1993; Tejasari & Zakaria, 2002). Furthermore, ginger's non-volatile bioactive compounds have been isolated and found to have antioxidative, anti-microbial, and immune-stimulating characteristics. Ginger's bioactive compounds with high anti oxidative activity include gingerol, shogaol, and zingeron. All gingerol-related compounds (fractions 1-6) namely, (6)-gingerol, (6)-shogaol and gingerdiol isolated from ginger dichloromethane extract have anti oxidative activity higher than a-tocopherol (Jitoe *et al*., 1992; Kikuzaki & Nakatani, 1993). Meanwhile, other bioactive compounds of ginger have been found to have sporostatic characteristics (Al–Khayat & Blank, 1983), suppressed bacterial growth (Davidson, 1993), and enhanced lymphocyte proliferation capability *in vitro* (Tejasari & Zakaria, 2002; Tejasari, 2006).

Evidence of the immune-stimulating capability of ginger's non volatile bioactive compounds in increasing the amount of CD4+ T-cells surface molecules was shown by previous studies (Tejasari, 2005). The increase could be due to the capability of ginger bioactive compounds in enhancing the cellular and humoral immune response. If the bioactive compounds could also increase the ratio of CD4+ : CD8+ T-cells, these compounds may have the potential to be tested on immune deficiency conditions, such as Acquired Immune Deficiency Syndrome (AIDS) caused by Human Immuno-deficiency Virus (HIV) infection. The CD4 antigen on T-cell surface molecules of CD3+CD4+ is a receptor for HIV, and hence the virus could attach, enter, and destroy the T-cells. Consequently, the amount of CD4+ (T helper) T-cells and the ratio of CD4+:CD8+ T-cells in the HIV/AIDS patient might decrease drastically.

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 ratio of CD4+:CD8+ T-cells. This study is aimed at providing scientific evidence for some health effects of the ginger, especially in increasing the ratio of CD4+:CD8+ T-cells. In this way, the tuber root of ginger could be used in the formulation of a functional food for HIV infected patients.

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 the ratio of T cells surface molecules CD4+:CD8+. The first and second phases were conducted in the Chemical and Biochemical Laboratory, Faculty of Agricultural Technology, and Faculty of Science and Mathematics, Jember University. Meanwhile the final phase was performed in the Microbiology Laboratory, Primate Research Center, 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 µg /ml. These two factorial treatments were done in three replications. The variables tested consisted of (i) the amount of T-cell

receptor molecules of CD3+CD4+, (ii) CD4+ T-cell surface molecules, and (iii) the ratio of CD4+:CD8+ T-cells. The T-cells population expressed by CD3+ molecules was identified by the usage of PHA mitogen.

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±0.001 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 hemacytometre (Neuberger). Lymphocyte suspension with high viability (>95%) $2x10^6$ cell/ml was prepared by addition of basal medium.

Lymphocyte incubation

A 100 ml lymphocyte suspension (($2x10^6$ cell/ml) was distributed randomly to 96 micro-plate wells. Then to each well was added 20 µL oleoresin, fr-1 (gingerol), fr-2 (shogaol), or fr-3 (zingeron) at 500, 1000, 1500, 2000, and 2500 µg/ml concentrations. Every concentration for each compound was prepared in triplicate. To each well was also added 80 mL PHA mitogen at a concentration of 12.5 µg/ml. Thus, the final concentration for each compound was 50, 100, 150, 200, 250 µg/mL, with 5 µg/mL PHA mitogen. The PHA mitogen was added for stimulating T-cells proliferation. For the control, to each well was added RPMI-1640 medium. Incubation was performed under the conditions of 37°C, 95% CO_2, 5% O_2, 95% RH for 96 hours.

Analyses of T cell surface molecules (CD3+CD4+ and CD3+CD8+)

After 96 hours of incubation, micro titer plates were centrifuged at 514 x g for 10 minutes. The pellet obtained was added to 100 µl semi-complete medium, and placed in a Falcon tube. Into the Falcon tube was added 10 µl monoclonal anti–human CD3-FITC/CD4-PE conjugates, and monoclonal anti-human CD3-FITC/CD8-PE conjugates. The conjugate was not added to the control tube. The mixture was incubated for 30 minutes at 22°C. The wash was performed with 0.01 M PBS at pH 7.4 and centrifuged at 228 x g and 5°C for 5 minutes. The lymphocytes were fixed with 0.5 ml 0.1 % paraformaldehyde in PBS. Percentage of

Table 1. Comparison of Rf values between this study and the references

Fraction number	*This study*	*Rf value*	
		Reference 1	*Reference 2*
1	0.24	0.20 - 0.24	0.15 – 0.22
2	0.42	-	0.42
3	0.54	0.41 - 0.48	0.48 – 0.55
4	0.60	0.51 - 0.57	0.58 – 0.62
5	0.68	0.65 - 0.67	0.68 – 0.72

Reference 1 = Wikandari (1994)
Reference 2 = Chen et al. (1986)

CD4+ T-cells and CD8+ T cells were analyzed by flow cytometer with software of Fluorescence Activated Cell Sorter (FACS) (Becton Dickinson, Cat. No. 340048) (Rose *et al.,* 1994; Freshney, 1994).

Statistical analysis

The results were expressed as mean ± SD. One-way ANOVA and Duncan's Multiple Range Test were used to test for differences in the ratio of CD4+:CD8+ T-cells 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 antioxidative 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) concentrations tested were identified based on calculation of the consumption of one glass jahe beverage made from 25 g fresh ginger, i.e. 50 µg/ml. The other three levels compound concentrations were also tested i.e. 100, 150, 200 and 250 µg/ml.

Simple phenol compounds have several hydroxyl groups giving rise to 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 a-tocopherol. The antioxidative effects of gingerol, shogaol, and oleoresin protected lymphocyte from oxidative damages

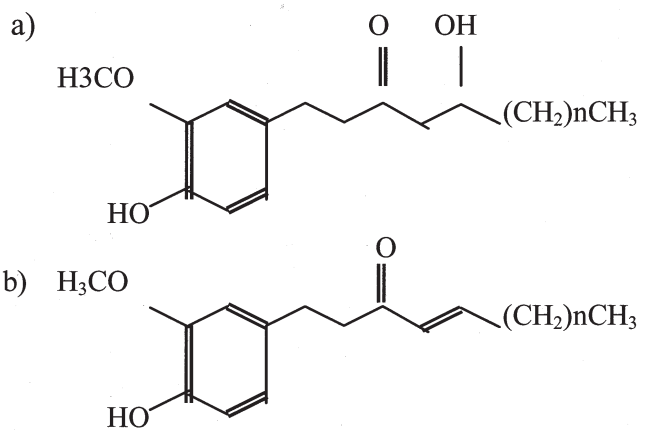

Figure 1. Molecule structure of gingerol (a) and shogaol (b) (Source : Kikuzaki and Nakatani, 1993)

(Tejasari, 2003). Ginger bioactive compounds had ability in increasing B cells proliferation (Tejasari, 2004), and T-cells proliferation of (Tejasari, 2006). Furthermore, the compounds also increased CD4+ T cells that is, T-cell surface receptor molecules or T helper 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 CD4+T Cells (Thelper) and CD8+ T cells (Tcytotoxic)

T-lymphocyte or T-cell is a non-phagocytic leukocyte responsible for cellular immune response, especially cell mediated immune response that functions in cleaning up intracellular pathogens, virus-infected cells and malignant cells. T-cells have membrane surface receptor molecules or T-cell receptors (TCR) with CD3, CD4, and CD8 accessory molecules (Kuby, 1992; Roitt, 1991). Molecules of CD3+CD4+ or CD4+ T-cells molecules are glycoprotein signaling for T helper (Th) cells sub population. Identification of this sub-population was done by using of antibody monoclonal anti-human CD3-FITC/CD4-PE fluorescence conjugates. Meanwhile, molecules of CD3+CD8+ or T cells CD8+ are glycoprotein signaling for T cytotoxic (Tc) cells marker. This surface receptor molecules were identified by using dual tagging antibody, namely antibody monoclonal anti-human CD3-FITC/CD8-PE conjugates, which were added to lymphocytes culture. The two antibodies were separately bound to CD4+ and CD8+ T-cell surface hence, the fluorescence counted was the percentage of CD3+CD4+ and CD3+CD8+ .

Oleoresin and its three fractions increased the percentage of CD3+CD4+ sub population. Compared to the control, the percentage increase of CD3+CD4+ occurred in the treatment of 200 µg/ml fraction- 2 by 3 %, in the 200 µg/ml fraction-3 by 9 %, the100 µg/ml oleoresin by 4 %, and the 50 µg/ml fraction-1 by 7 % (Figure 2). The active compounds in fraction -3 oleoresin increased the CD3+CD4+ percentage higher than that of gingerol in fraction-1, oleoresin, and shogaol in fraction-2 of oleoresin. The increase can be explained by the ligand characteristics of the ginger oleoresin compounds that easily bound to the protein receptors on the T-cell surface. The binding stimulated the membrane system enzyme responsible for T-cell enzymatic proliferation. The

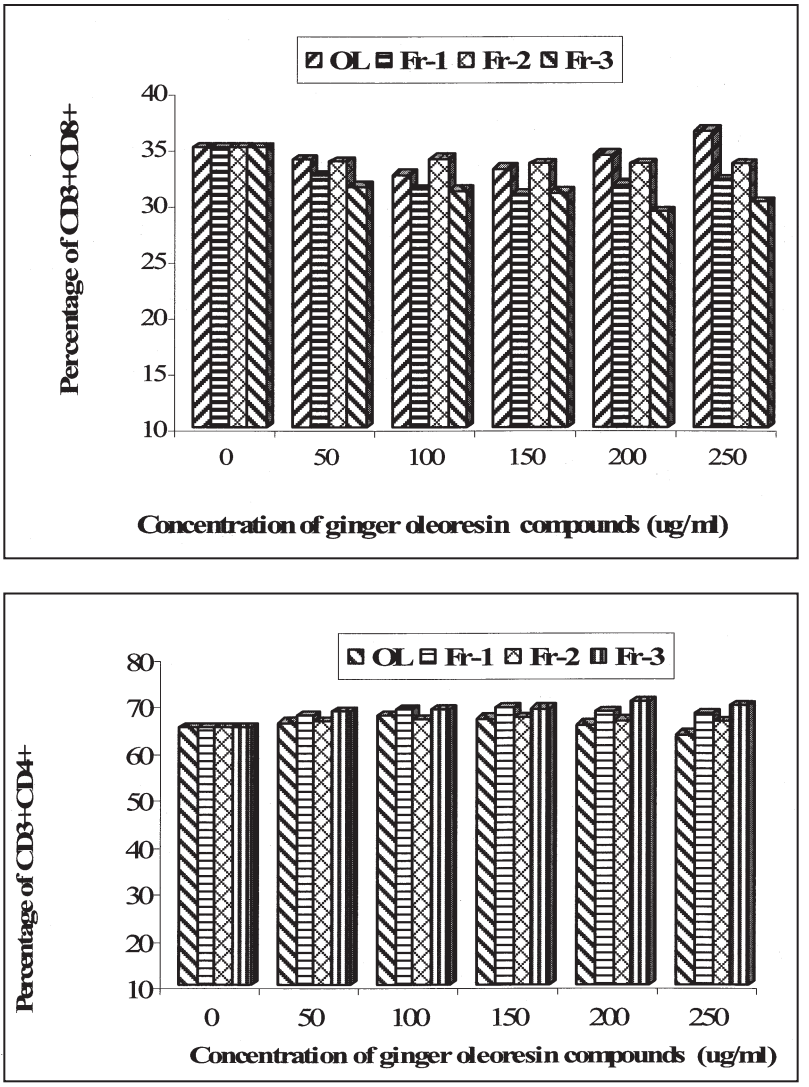

Figure 2. The percentage of CD3+CD4+ and CD3+CD8+ as respond of oleoresin and its three fractions treatment on several levels concentration tested

increase of T-cells indicates an increase in the T-cell surface molecules. It was assumed that the compound in fraction-3 has binding capability on T-cell surface, especially CD3+CD4 + molecules that was higher than that of gingerol and shogaol.

On the other hand, the compound in oleoresin fractions slightly decreased the percentage of CD8+ T-cells. The decrease could be explained by the characteristic of the ginger oleoresin compounds, which bound more quickly to the CD3+CD4+ than to the CD3+CD8+ T-cells. Therefore, there was not much compounds left for binding to the CD3+CD8+ molecules.

Phenol compounds have the ability to attach to protein through hydrogen bond (Suradikusumah, 1989). This makes possible for ginger phenol to bind to protein receptor membrane of T lymphocytes cells. In this way it activates enzymatic membrane system for proliferation.

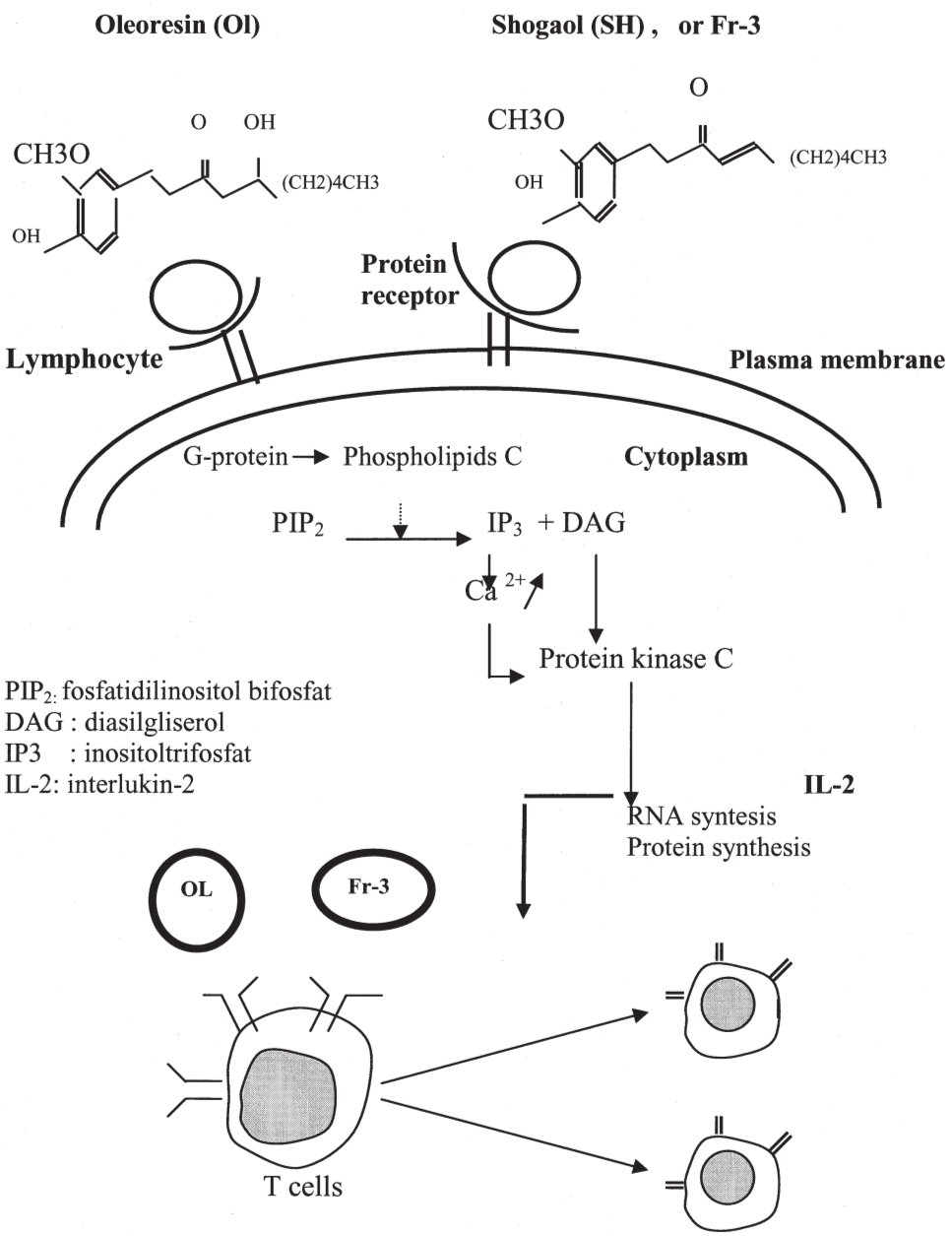

Figure 3. Schematic biochemistry pathway of T cells proliferation response stimulated by ginger bioactive compound
(Source : Tejasari & Zakaria, 2002)

Stimulation mechanism on cellular and humoral body defense system by oleoresin and its three fractions was possible through binding to protein surface molecule on T-cells (Figure 3). The mechanism has to be investigated further, especially the use of antibody of anti receptor protein surface of T-cells. If the antibody used is attached on the protein receptor, this may mean that the phenol compound is not attached to the protein, and the T cells proliferation may decrease. Another way

is by measuring the activity of protein kinase C that stimulates the lymphocyte proliferation caused by the ginger compounds.

The increase of CD4+ T-cells or T helper cells will support cell mediated immune response since with more CD4+ T-cells, the higher is the stimulation of other lymphocytes, such as B cells in producing antibody, NK cells in increasing its cytolysis activities, and macrophage in increasing phagocyte ability. In term of HIV infections, the increase of CD4+ T-cells, which are attached on HIV receptor protein molecules on the cells surfaces, will avoid the virus from being attached on the receptor molecules. As a consequence, the HIV virus might not be able to enter the lymphocytes.

Results of this study were supported by other studies. Prangdimurti (1999) reported an increase of T-cells in mice given ginger extract The increase of T-cells was also found in human subjects fed one glass of ginger extract beverage daily for one months (Nurrahman, 1998).

Ratio of CD4+:CD8+ T-cells

This study showed that ginger oleoresin and its three fractions increased the ratio of CD4+:CD8 T-cells (Figure 4). The ratio of CD4+:CD8+ T-cells was used as a parameter in HIV infection detection. Roitt (1991) stated that AIDS patients experienced decrease in cell surface molecules, especially CD3+CD4+, while CD3+CD8+ was not really affected, thus resulting in a a decrease in the ratio of CD4+:CD8+ T-cells. Other studies (Flinder, 1996) also showed that CD3+;CD8+ ratio was not affected by HIV infection.

Data on the ratio of CD3+CD4+ and CD3+CD8+ molecules are presented in Figure 4. The highest ratio occurred on the treatment of 200 µg/ml fraction-3 oleoresin by 2.41 point, with an increase of 30 %. Fraction-1 oleoresin showed the highest value of 2.25, with an increase of 21 %. The oleoresin increased the ratio with a value of 2.07 or by 11 % increase. Shogaol compound in fraction-2 also increased the ratio with a value of 1.98 by 7 %. These results

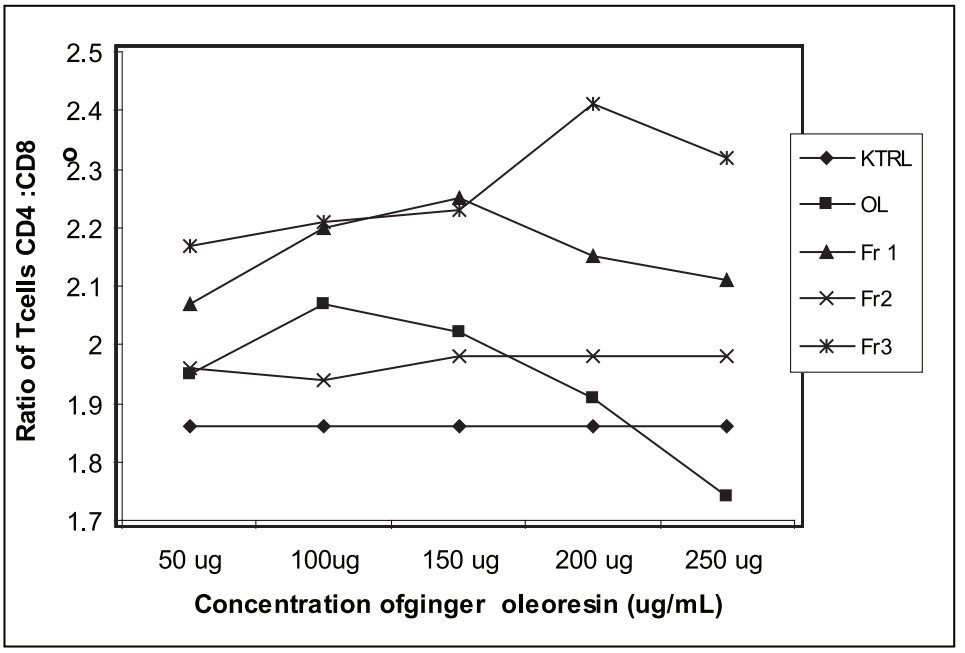

Figure 4. The ratio of T cells CD4+:CD8+ as respond to the ginger oleoresin, and its three fractions on five concentration levels

indicated that the bioactive compounds in fraction-3 oleoresin increased the ratio more than gingerol-in fraction-1, oleoresin, and shogaol in fraction-2. Overall, the increases of the ratio as a response to ginger bioactive compounds could not be considered as high. However, these findings showed that ginger has the potential ingredient for further exploitation as a functional food for use in immune deficiency illnesses.

CONCLUSIONS 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 CD4+ T-cell molecules, but decreased CD8+ T-cell molecules. Therefore, the ginger oleoresin bioactive compounds increased the ratio of CD4+:CD8+ T-cells at 100 to 200 µg/mL concentrations.

These *in vitro* findings provide evidence of cellular immune response by ginger's non volatile bioactive compounds. More studies should be undertaken to confirm these findings followed by *in vivo* studies on healthy human subjects and AIDS patients toward identifying the optimal effect of the bioactive compounds of ginger in supporting its use as a functional food.

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