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Evaluation on The Supplementation of Fermented Garlic Extract in Protecting Human Lymphocytes *In Vitro* From The Negative Impact of Gamma-Rays

Evaluasi Penambahan Ekstrak Bawang Fermentasi Dalam Melindungi Sel Limfosit Manusia Secara In Vitro Dari Dampak Negatif Sinar Gamma

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ABSTRACT

Garlic (*Allium sativum*) contains a wide range of phytocompounds that produce various responses in human body. However the knowledge on the potential of fermented form of garlic in protecting negative impacts of radiation is very limited. In this research *in vitro* efficacy of fermented garlic in protecting negative impact of gamma ray was studied using cytogenetic test. A set of culture of human lymphocytes was irradiated with ^{60}Co gamma rays at dose of 2 Gy (dose rate of 2 Gy/min) and fermented garlic extract at four working concentrations of 0, 125, 250 and 500 mg/mL were added to these cells and then were incubated at 37^{o}C for 48 hrs. Colcemid was added at 3 hr before harvest to collect metaphase cells and it was done by standard methodology for cytogenetic analysis. The fermented garlic extract significantly ($p<0.05$) did not exhibited antigenotoxic effect of gamma rays and its effectiveness was same as in control (without extract treatment) group. In contrary all concentration of chemicals (125, 250 and 500 mg/mL) were seemingly tend to induce higher number of dicentric and fragment chromosomes than control under microscopic observation. Mitotic index of the cell that was determined with programmed metaphase finder also did not influenced by garlic addition. It was concluded that aqueous garlic extract did not possesses its efficacy in protecting impact of ionizing radiation.

Keywords : fermented garlic, chromosome aberrations, genotoxic, ionizing radiation.

ABSTRAK

Bawang (*Allium sativum*) mengandung berbagai macam senyawa fito yang menghasilkan bermacam respon dalam tubuh manusia. Akan tetapi pengetahuan tentang potensi bawang dalam bentuk fermentasi untuk melindungi efek negatif radiasi masih sangat terbatas. Dalam makalah ini kemampuan bawang fermentasi dalam melindungi efek negatif sinar gamma secara *in vitro* telah dipelajari menggunakan uji sitogenetik. Satu set kultur limfosit manusia diiradiasi sinar gamma ^{60}Co pada dosis 2 Gy (laju dosis 2 Gy/min) dan ekstrak bawang fermentasi pada empat konsentrasi kerja (0, 125, 250 dan 500 mg/mL) ditambahkan pada sel tersebut dan kemudian diinkubasi pada 37^{o}C selama 48 jam. Colcemid ditambahkan 3 jam sebelum pemanenan untuk mendapatkan sel metafase dan dilakukan dengan metodologi baku untuk analisis sitogenetik. Bawang fermentasi secara nyata ($p<0.05$) tidak menunjukkan kemampuan menekan dampak negatif sinar gamma dan efektivitasnya sama seperti dalam kelompok kontrol (tanpa pemberian ekstrak). Sebaliknya semua konsentrasi senyawa kimia (125, 250 dan 500 mg/mL) diduga cenderung menyebabkan jumlah kromosom disentrik dan fragmen yang lebih tinggi daripada kontrol pada pengamatan mikroskopis. Indeks mitosis sel yang ditentukan dengan *programmed metaphase finder* juga tidak dipengaruhi oleh penambahan bawang. Disimpulkan bahwa larutan ekstrak bawang tidak memiliki sifat melindungi dampak radiasi pengion.

Kata kunci : bawang fermentasi, aberasi kromosom, genotoksik, radiasi pengion

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INTRODUCTION

Garlic (*Allium sativum*), that is a bulbous root closely related to the onion, contains a wide range of phytocompounds that act together to produce a wide variety of responses in human body. Garlic is rich in manganese, calcium, phosphorus, selenium, and vitamins B6 and C so that it is important for bones as well as thyroid [1]. In general, its benefits fall into four main categories: reducing inflammation; boosting immune function (antibacterial, antifungal, antiviral, and antiparasitic properties); improving cardiovascular health and circulation; and toxic to around 14 kinds of cancer cells (including brain, lung, breast, and pancreatic) that probably related to its potent antioxidant effects. This garlic includes onions, chives, leeks, and scallions, and is used for flavoring in cooking and is unique because of its high sulfur content, also arginine, oligosaccharides, flavonoids, and selenium, all of which may be beneficial to health [2]. Their extracts have been recently reported to be effective in cardiovascular disease, because of their hypocholesterolemic, hypolipidemic, anti-hypertensive, anti-diabetic, antithrombotic and anti-hyperhomocysteinemia effects, and to possess many other biological activities including antimicrobial, antioxidant, anticarcinogenic, antimutagenic, antiasthmatic, immunomodulatory and prebiotic activities [3].

On the other hand ionizing radiations produce deleterious effects in the living organisms, and the rapid technological advancement has increased human exposure to this ionizing radiation enormously. Attemptsto protect against these deleterious effects of ionizing radiation by pharmacological interventions were made as early as 1949 and there have been continuous efforts to search for radio-protectors that are of great help for human application [4]. This physical agent interacts with organic compounds and water in cells and would results in the generation of free radicals, which attack deoxyribonucleic acid (DNA) and act as genotoxins. DNA lesions lead to gene mutation and subsequent cancerous transformations. Based on this description, DNA is the main target of ionizing radiation and the magnitude of DNA lesions determines the probability of cancer development [5].

Radioprotective, anti-oxidative efficacy of garlic extract has been investigated intensively and their results were presented in many reports [6,7]. Onion contains quercetin that is believed to have anticancer, anticholesteroland antioxidant properties. Administration of the dried bulb Allium cepa at a concentration of 20 mg/kg was active against X-irradiation [6]. However the knowledge on the potential of fermented form of onion in protecting negative effects of radiation is very limited. The present study investigated the possible protective effects of fermented garlic extract on genotoxic activities of gamma rays as one of ionizing radiations. Garlic is used in fermented form with its own specific properties. However, as garlic is usually consumed raw, the effects of different form of garlic on the magnitude of damages were evaluated.

MATERIALS AND METHODS

Fermented garlic of (BGL-60) production

Garlic obtained from local market (without rotting and germinating) was put in a pan and stored in a special apparatus with controlled temperature of 65-70^{o}C and 40-60% humidity for 30 days. This process resulted in new compound which has pharmacologic properties. Its taste is acetic-sweet, the texture is shoft and without raw garlic aroma.

Preparation of fermented garlic

Fermented black garlic (0.5 mg of wight) as a paste was dissolved in steril water (1 mL) and was vortexed to obtain a mixture and then was centrifuged at 6000 rpm for 20 minutes at room temperature. The volume of supernatant was taken accordingly to obtain four concentrations (0, 125, 250 and 500 mg/mL) and the remaining extracts were quickly stored in refrigerator until next used.

Culture and harvest

Venous blood for lymphocyte culture was taken from three healthy and non smooking volunteers (20-39 years old, two males and 1 female) with standard procedure (informed consents were provided). The bloods were put in BD heparinized vacutainer tube and then irradiated with gamma rays of ^{60}Co with the prescribed dose of 2 grays (at a dose rate of 2 Gy/minute) at Panoramic Irradiator Facility of the Center for Application of Isotope and Radiation, BATAN in Jakarta. Fermented garlic extract at concentrations of 0, 125, 250 and 500 mg/mL

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were added to the cells. The blood were cultured according to a modification of the method of International Atomic Energy Agency (IAEA). Cultures were stimulated by fetal calf serum (15%) (Cultilab), phytohemagglutinin (2%) (Gibco), L-glutamine 200 nM (1%) (Gibco) and Roswell Park Memorial Institute medium (RPMI) without folic acid (Cultilab). Phytohaemagglutinin (Difco) was added to each 10 mL culture volume. After a two-day incubation periodat 37^{o}C for 48 hr and then 100 µl colcemid solution at a concentration of 0.05 mg/mL (Sigma®) was added to each tube 3 hours before the 48th hour to collect mitotic/metaphase cells. The cells were processed for chromosome studies by the standard 'Colcemid'-hypotonic citrate-fixative sequence. Cells were centrifuged at 1,000 rpm for five minutes, submitted to a hypotonic solution of KCl (0.0075 M), and fixed twice with fixative solution (3 methanol: 1 acetic acid) until a white precipitate suspension was obtained.

Scoring chromosome aberrations

The metaphase cells obtained were then stored at -20°C for at least one night. After the cells were dropped on cleaned slides and stained with Giemsa at 2% solution, the slides were observed under microscope at 1000 times magnification with immersion oil. Chromosomal aberrations are scored on 100–120 cells for each treatment. Aberrations scored consist of dicentric, fragment, and ring chromosomes. Other forms of aberration such as double minutes (DM), chromatid and chromosome breaks and gaps in which the discontinuity was at least equal to the width of the chromatid were also observed.

Mitotic index

Mitotic index of the cell that was treated with irradiation and all doses of garlic was determined with programmed/automated metaphase finder (Metafer-MSearch, Germany) according to the Manual of the machine.

Table 1. The individual results of chromosome aberration observed in all sample of cultured blood treated with fermented garlic at concentration of 0 (control), 125, 250 and 500 mg/mL, either irradiated with 2 Gy (R) or not (K, control).

No.	Code of Sample	No. of chromosome aberration		
		Dicentric	Fragment acentric	Ring
1.	dX0-K1 (control)	-	-	-
2.	dX0-K2 (control)	-	-	-
3.	dX0-RA1	5	4	1
4.	dX125-KA1	-	1	-
5.	dX125-RA2	3	4	1
6.	dX250-KA1	-	-	-
7.	dX250-Ra1	-	2	-
8.	dX500-KA2	-	-	-
9.	dX500-RA1	2	10	-
10.	dY0-K1 (control)	-	-	-
11.	dY0-RA1	2	3	-
12.	dY125-KA1	-	1	-
13.	dY125-RA1	2	4	-
14.	dY250-KA1	-	-	-
15.	dY250-RA1	6	3	1
16.	dY500-KA1	-	-	-
17.	dY500-RA1	5	9	-
18.	dZ0-K1 (control)	-	-	-
19.	dZ0-R1	5	7	-
20.	dZ125-KA1	-	-	-
21.	dZ125-RA1	1	8	-
22.	dZ250-KA1	-	-	-
23.	dZ250-RA1	1	4	-
24.	dZ500-KA1	-	-	-
25.	dZ500-Ra1	3	3	-

Note : X, Y and Z represents the code for each volunteers.

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RESULTS AND DISCUSSION

The results of evaluation showed that a number of dicentric and fragment chromosomes were found under microscopic observation either in treated sample for all concentration of garlic tested (125, 250 and 500 mg/mL) or in control (without extract treatment) and were depicted in Table 1. It was found that even ring chromosomes were observed in some samples although it was very rarely. DM, chromatid and chromosome breaks and gaps were not observed. It means that the fermented garlic treatment did not exhibited its efficacy in supressing the negative impact of gamma rays and this showing that there is a lack of garlic extract at all doses studied in suppressing the genotoxic effects of gamma rays. Moreover it seems that in our experiment garlic extract was not an effective radioprotector but oppositely it act as radiosensitizer due to higher number of chromosome aberration in garlic treated sample compared to control. An example of radiation induced ring chromosome found (there were 3 rings for all samples) in this evaluation is presented in Figure 1.

Statistical analysis results with SPSS Program showing that black garlic addition into culture after radiation exposure at all concentrations tested did not affecting the induced chromosomal aberration significantly ($p<0.05$).

Mitotic index of the cell treated with gamma ray at 2 Gy that was determined with programmed metaphase finder machine also did not influenced by garlic addition. Only in X donor/volunteer the mitotic index of irradiated cells was higher than that of control and irradiation reduced mitotic index in two other donors, showing different individual response. The results for three individuals were presented in Figure 2.

We studied the chromosome damage induced by gamma radiation and measured by the chromosome aberration technique such as dicentric to assess the radiation dose absorbed by cells. However, this technique has some disadvantages. One of them is scoring that is difficult and requires skill and experience which of these lead low number of cell counts [9]. Other simpler technique is needed such as micronuclei which are relatively faster in counting the cells and simpler form compared to dicentric chromosome [10,11].

We also evaluated the potential of garlic in suppressing the deleterious/negative effects of gamma rays. Raw garlic by itself has many potential health benefits, but there are different possibilities when raw garlic is aged and fermented, of which it has been shown to improve antioxidant potency. This process that results in black garlic that has often times doubled or more,

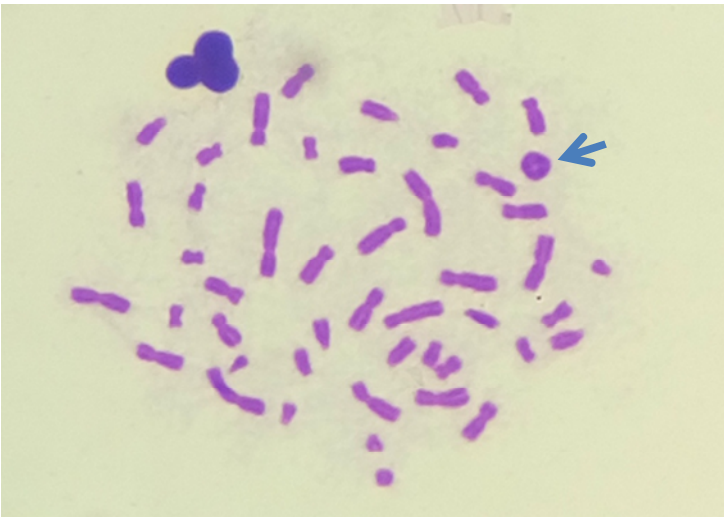

Figure 1. A ring chromosome (arrow) induced by gamma rays was found in a sample of this experiment. No any fragments were seen in this slide observation that may be already combined to the end of chromosome which could be detected with fluorescence in situ hybridization (FISH) method.

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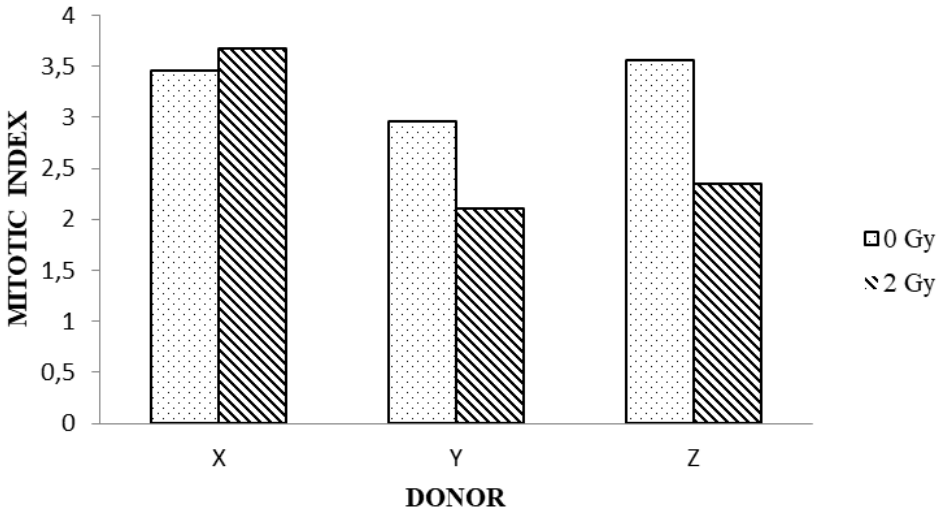

Figure 2. Mitotic indexes of control (0 Gy) and irradiated (2 Gy) cells for each volunteer (X, Y and Z) determined with programmed metaphase finder.

the oxygen radical absorption capacity value of the raw garlic. Black garlic is packed with high concentrations of highly beneficial sulfur compounds, one in particular is S-allyl-cysteine. Protective effects from garlic may also arise from its antibacterial properties or its ability to block the formation of cancer-causing substances, halt the activation of cancer-causing substances, enhance DNA repair, reduce cell proliferation, or induce cell death [12].

In our experiment the protective properties of garlic described above were not found. This is may related to dose of the fermented garlic used in our experiment that is may be lower than that required to be protective, time of administration, and other mechanisms that underlie the actions. Here we used 125, 250 and 500 mg/mL which is predicted not appropriate in scavenging the free radical or reactive oxygen species induced by ionizing radiation, and exert a broad range of biological and physiological effects that modulate the cell's response to the action of ionizing radiation. The failure of the garlic extract may also be due to the lost of the effective substant during the process of extraction. The administration is also should be considered, as the treatment after gamma ray exposure is categorized as mitigators, of which the chemicals are administered after exposure [13]. The procedure described here is also quantitative, reproducible, and reasonably rapid. It involves assessment of chromosomal aberrations [14].

Drugs or chemicals extracted from natural products are widely used to protect against ionizing radiation used for radiotherapy or other purposes such as military. This research aimed to seek for efficient or effective radioprotective substances that alleviate various effects of ionizing radiation that has been going on for more than 6 decades. However, in our center this activities had been just started 2 decades ago. This is intended to support the task of BATAN and we have been evaluated several natural products or chemicals such as cysteine (either combined with dimethyl sulfoxide or antibiotics) [15], ginseng [16,17] or vitamin E that was done very recently [18].

Cell killing occurs as a result of unrepaired mutations located widely throughout the genome. The capacity of the cell to repair simultaneous mutations has a maximum limit. Killing is due to apoptosis/necrosis triggered in cells by a single unrepaired mutation occurring in an exposed region of the genome. It involves measurement of several parameters including chromosomal aberrations in cells without and with chemical treatment to inhibit repair [19].

This paper presents an evaluation on a chemical supplementation, which it is hoped will be useful for prevention of radiation harms. Gamma-irradiation is used as a model mutagen because of the ease and the accuracy of dose delivered and measurement, and because it produces an extremely wide range of

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chromosomal aberration events. In our study chromosomal aberration count, which is reversed by garlic, clearly reveal that doses of 2 Gy is highly mutagenic.

Different with this experiment, other researchers found that onion effectively suppressed negative impact of radiation. Chang et al. [20] evaluated radioprotective effect of sodium n-propyl thiosulfate (NPTS) and sodium 2-propenyl thiosulfate (2PTS) derived from onions and garlic, respectively, on rat hepatoma H4IIE cells and mouse lymphoma L5178Y cells by preincubated with each of these compounds for 48 hours at 37°C before receiving 10 Gy of X-ray irradiation. Cell damage caused by the irradiation was quantified as comet tail moment, which represents the degree of DNA damage. X-ray-induced DNA damage was significantly decreased in both H4IIE and L5178Y cells by micromolar concentrations of NPTS and 2PTS compared with the control without the compounds. The protective effect was more potent with 2PTS than NPTS. They concluded that onions and garlic have anti-radiation potential.

Garlic extract potential was also evaluated by Singh et al [21] in the mouse bone marrow micronucleus (MN) test for its possible protective effects against gamma-radiation-induced chromosomal damage. Three doses of freshly prepared garlic extract (125, 250 and 500 mg/kg b.w.) were orally administered to the animal for 5 consecutive days, and the animals were irradiated 2 h after the final feeding. The results of the MN test demonstrated that pre-treatment with garlic extract can lead to significant dose-related reductions in the frequencies of gamma-radiation-induced (2 Gy) micronucleated polychromatic erythrocytes. The anticlastogenic effect of garlic extract was observed against lower radiation doses of 0.5 and 1 Gy, but not 0.25 Gy. The irradiated garlic-extract pre-treated animals showed a significant reduction in sulfhydryl content and glutathione *S*-transferase activities.

The efforts of research and industry should be directed towards the improvement of processing and extraction methods in order to obtain garlic and onion and their derivatives with high quality preserving and/or improving the particular biological properties as radioprotector or mitigator. Thus, it is necessary to find the best way to elaborate high added-value foods derived from garlic and onion avoiding the adverse effects of this physical agent. High efficiency or high dose modifying factor, lack of toxicity, a convenient administration pathway (oral, subcutaneous or intramuscular administrations), low manufacturing costs, storage stability, long duration of protective effect, and the ability to alleviate effects of various types of ionizing radiation are all ideal conditions of a radioprotector [13].

CONCLUSION

All concentration of aqueous fermented garlic extract tested did not possesses its efficacy in protecting negative impact of ionizing radiation based on cytogenetics and mitotic index point of view.

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