Effectiveness of Gamma Irradiation for Decontamination of Microbes on Tea Parasite Herb *Scurrula atropurpurea* (Bl.) Dans.

E. Katrin[1*], M. Yulianti[2] and H. Winarno[1]

[1] *Center for Application of Isotope and Radiation Technology, National Nuclear Energy Agency*
Jl.Lebak Bulus Raya No. 49, Pasar Jumat, Jakarta, Indonesia

[2] *Faculty of Pharmacy, University of Pancasila*
Srengseng Sawah, Jagakarsa 12640, Jakarta, Indonesia

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ABSTRACT

The purpose of this study was to find the minimum and maximum dose of gamma irradiation on dried tea parasite herb that can reduce the number of microbes without reducing the inhibitory activity against leukemia L1210 cells. Samples of tea parasite herbs were irradiated by gamma rays with doses of 0, 5, 7.5, 10, 15 and 20 kGy. The microbial contamination, cytotoxic activities and the chromatogram profiles of irradiated and unirradiated samples were observed. The results revealed that the bacteria contaminants of 7.57 x 10^9 cfu/g were eliminated after irradiation of the samples with dose of $\geq$ 7.5 kGy, meanwhile the mold-yeast contaminants of 5.68 x 10^8 cfu/g were eliminated after irradiation of the samples with dose of $\geq$ 5 kGy. Ethyl acetate extracts of irradiated samples until the dose of 10 kGy were still able to maintain its cytotoxic activity against L1210 leukemia cells proliferation with IC_{50} values of < 30 µg/ml. Fractionation of ethyl acetate extracts of each sample by open column chromatograph was obtained four fractions from each extract. Among the fractions, fraction 2 was the most active fraction which had a potent as anticancer agent. Cytotoxic activities assay of fraction 2's showed that this fractions significantly decreased after irradiation of samples with doses up to 10 kGy. The thin layer chromatogram profiles of fraction 2 from irradiated and unirradiated samples showed that spot 2 and 3 gradually tended to fade. It is suggested that 7.5 and 10 kGy were the minimum and maximum irradiation dose for bacterial and mold/yeast decontamination with the initial contamination of 7.57 x 10^9 cfu/g and 5.68 x 10^8 cfu/g respectively. At this condition, the bacteria and mold/yeast have been killed, whereas the cytotoxic activities of active components (ethyl acetate extract and fraction 2) in tea parasite herbs decreased, but the decrease was not significant and did not remove these cytotoxic activities.

INTRODUCTION

Indonesia has a variety of plants which is very useful both in the field of food and in the field of health. Those plants can be used as a phytotherapy medicine and expected to be used in formal health treatments, whereas in the long term they may have economic potential because they can reduce the import of raw materials for synthetic drugs. On the other hand with the availability of abundant natural resources, the engineering with right technology will generate greater added value and benefit to the people greatly.

Indonesian traditional medicines known as *jamu* are a concoction of various medicinal herbs. One of the medicinal herbs that can be developed is tea parasite herb *Scurrula atropurpurea* (Bl.) Dans., which is parasitic on tea plant (*Thea sinensis* L.). Traditionally, *Scurrula atropurpurea* (Bl.) Dans. can be used for treatments of cowpox, chickenpox, diarrhea, hookworm, measles, hepatitis, and cancer [1]. The tea parasite contains alkaloids, flavonoids, saponins and tannins [2]. Ohashi *et al.* [3] reported that an unsaturated fatty acid octadeca-8,10,12-triynoic acid isolated from tea parasite herb had bioactivity against MM1 cancer cell invasion with an IC_{50} of 2.7 µg/ml.

* Corresponding author.
E-mail address: erminkk@batan.go.id

In order to support the medicinal herb industries, preservation handling of medicinal herbs is necessary to avoid mold and bacterial contamination. According to The National Agency of Drugs and Food Controls (NADFC = BPOM) [4], the maximum contamination limit of raw material of medicinal herbs for microbes and fungal is 10^6 cfu/g and 10^4 cfu/g, respectively. To preserve medicinal herbs remain hygienic to the consumer, a treatment is necessary to reduce bacterial or mold-yeast contamination. The various techniques have been used for preservation, such as cooling, heating, salting, addition of preservatives, freezing, drying, and gamma irradiation.

In Indonesia, the preservation techniques of medicinal herbs and their ingredients using gamma irradiation has been developed by Center for the Application of Isotopes and Radiation Technology National Nuclear Energy Agency since 1980 [5]. Since 1984 until now it has dozens of herbal medicine industries use gamma irradiation techniques to extend the shelf life of cosmetic ingredients and traditional medicines [5]. Some companies such as Kimia Farma, Mustika Ratu, Bintang Toejoe, Mahkota Dewa and Karya Sari have been already used gamma irradiation technique for preservation of their products [6]. Irradiation dose of 5 kGy was able to reduce the number of microbes to four log cycles and the number of molds to five log cycles. Irradiation doses below 10 kGy are safe because there is neither chemical residue nor change of the content and characterization of materials. Moreover, irradiation can extend shelf life of perishable commodities in the storage packaging, because microbes on the material can be eliminated [7]. It has been observed that increasing irradiation dose from 2.5 to 10 kGy on medicinal herbs and/or its raw materials caused the materials more durable [7]. However, there is no report about the effects of gamma irradiation on tea parasite plant as the herbal drugs, which is claimed as anticancer. The objective of this research is to study the effect of gamma irradiation on tea parasite herb without losing its efficacy, by observing the cytotoxic activity against leukemia L1210 cells. In addition, the numbers of microbial contamination as well as the chromatogram profiles of TLC were also observed.

EXPERIMENTAL METHODS

Materials and apparatus

Fresh *Scurrula atropurpurea* (Bl.) Dans. was collected from Gunung Mas Plantation, Bogor in January 2008. The authentication of the plant material was carried out at the Herbarium Bogoriensis - Research Center for Biology, Indonesian Institute of Sciences, Cibinong and the voucher specimen of the plant was deposited at the Herbarium Bogoriensis and Center for the Application of Isotopes and Radiation Technology.

The leukemia L210 cells used for cytotoxicity test was tumor cell line isolated from DBA strain mouse induced by methylcholanthrene.

n-Hexane, ethyl acetate, ethanol, 0.4% tryphan blue, RPMI medium-1640, fetal bovine serum (FBS), liquid nitrogen, distilled water, aquabidest, chloroform, methanol, cerium sulfate, sulfuric acid, silica gel 60 (70-230 mesh), celite 545, and GF_{254} silica plate were used in this research.

Apparatus used were Gamma Irradiator "*Karet Alam*" from cobalt-60 source with an activity of 118.6 kCi in September 2008, vacuum rotary evaporator (Büchi), oven (Prolabo), column chromatograph (diameter: 8 cm, length: 90 cm), hot plate, ultrasonicator, oven desiccator equipped with vacuum pump, analytical balance (Mettler Toledo), autoclave (S4-ON), incubator, microscope, chromatograph chamber, multi-well plate tissue's culture, haemocytometer Neubauer (0.100 mm x 0.0025 mm).

Preparation and irradiation of samples

Dried tea parasite herbs were milled, weighed (110 g), packed in polyethylene bag, then the polyethylene bag was sealed using sealer machine. They were prepared as many 12 bags, and then each bag irradiated at doses of 0 (control), 5 kGy, 7.5 kGy, 10 kGy, 15 kGy and 20 kGy with dose rate of 10 kGy/h. Irradiation at each dose was done in duplicate.

Determination of microbial contaminations

Total Plate Count (TPC) determination was performed according to SNI 01-2897-1992 [8]. A total of 10 g tea parasite herb powder were added into 90 ml of sterilized 0.1% peptone solution, thus obtained the dilution of 1:10, homogenized, then gradually diluted to 10^{-2} , 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. A total of 1 ml of sample was poured into a sterile Petri dish, 15-20 ml of nutrient agar medium was added, shaken gently until evenly mixed sample, silenced until frozen, and then the Petri dish was reversed and incubated at 30^{o}C for 48 h. The number of bacteria was calculated by multiplying average number of colonies by dilution factor.

Determination of the numbers of mold and yeast was also performed according to SNI 01-2897-1992 [8]. Similar as described in the determination of TPC, a total of 1 ml of sample was poured into a sterile Petri dish, 15-20 ml of potato dextrose agar medium was poured, shaken gently until evenly mixed sample, silenced until frozen, then the Petri dish was reversed and incubated at 20-24^{o}C for 5 days. The number of mold and yeast were calculated by multiplying the average number of colonies by dilution factor.

Extraction and fractionation of ethyl acetate extracts

A total of approximately 100 g of irradiated and unirradiated tea parasite herbs were macerated with 600 ml of acetone for 48 hours and the filtrates were filtered. The maceration was repeated 3 times, then the combined filtrates were evaporated with rotary evaporator to obtain concentrated extract. According to previous work [3], the obtained extracts were partitioned into ethyl acetate-water mixture (1:1). The ethyl acetate layer was separated and concentrated to obtained ethyl acetate extract. A total of 1.0 g of ethyl acetate extract was separated using a glass column containing 30 g of silica gel 60 as adsorbent, eluted using *n*-hexane - ethyl acetate (10:1), chloroform - methanol - water (10:1:0, 7:3:1), then methanol. The separated fractions were analyzed by thin layer chromatograph (TLC) using *n*-hexane - ethyl acetate (3:1) as eluent. The spots were observed and marked under UV 254 nm light cabinet, then sprayed with 1% cerium sulfate reagent in 10% sulfuric acid and dried on the hot plate to form visible spots. The fractions which had the same spots pattern were combined, evaporated by rotary evaporator followed by drying in oven desiccator *in vacuo* at 45^{o}C until the fix weight of the fractions were obtained.

Cytotoxic activity test against mouse leukemia L1210 cell line

The cytotoxic activity of ethyl acetate extracts from irradiated and unirradiated tea parasite herbs were tested against L1210 leukemia cells growth by *in vitro* technique. The media used were RPMI-1640 containing glutamine and sodium bicarbonate. A total of 15 ml fetal bovine serum was added into 85 ml of the medium. All activities were done in a sterile room. A total of media containing 2 x 10^5 cells/ml were incorporated into every hole in tissue culture plate. The ethyl acetate extracts were serially diluted in dimethyl sulfoxide to produce 6 concentrations and each concentration was added to plates in 2 replicates to obtain final ethyl acetate extract concentration of 0 (control), 5, 10, 20, 40, and 80 µg/ml for extract, while for the concentration of active fractions were adjusted as 0, 1, 2, 4, 8 and 16 µg/ml. The plates were incubated for 48 h in 5% CO_2 incubator at 37°C, then the number of cells were counted using Neubauer haemocytometer under a microscope. The percentages of inhibition were calculated by comparison of live cells in the samples with live cells in the control as described in previous work [9]. The IC_{50} value (*inhibitory concentration fifty*) is the concentration of sample that inhibits the proliferation of leukemia L1210 cells in 50% can be obtained from linear regression of logarithmic of sample concentration and probit of inhibition percentage. The smaller the IC_{50} value of a sample, the higher its cytotoxic activity.

RESULTS AND DISCUSSION

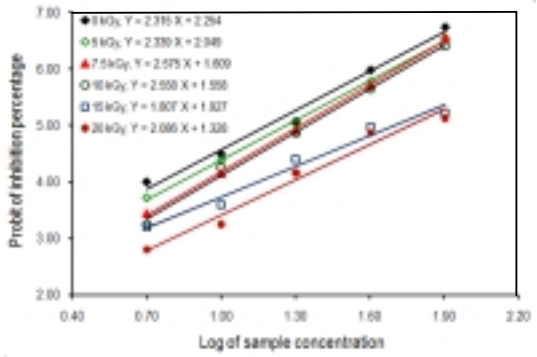
The microbial contamination of irradiated tea parasite herbs can be seen in Table 1. An irradiation dose of 5 kGy on the tea parasite herb could eliminate mold-yeast from initial contamination of 5.68 x 10^8 cfu/g, but this dose only reduced the number of bacterial contamination of one log cycle from 7.57 x 10^9 cfu/g to 6.62 x 10^8 cfu/g. To remove bacterial contamination completely, the higher dose is needed. Bacterial contamination can be removed completely by irradiation dose of $\geq$ 7.5 kGy.

Table 1. Microbial contamination of irradiated and unirradiated tea parasite herbs.

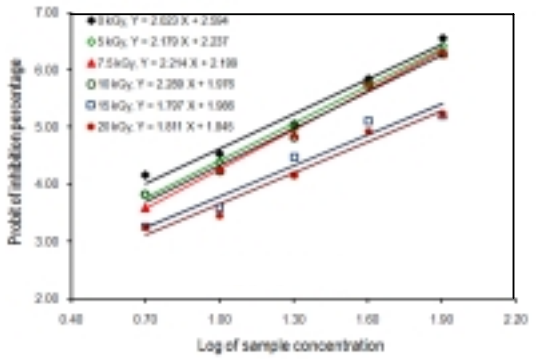
Irradiation dose (kGy)	Microbial contamination (cfu/g)	
	Bacteria	Mold-yeast
0	7.57 x 10^9	5.68 x 10^8
5	6.62 x 10^8	0
7.5	0	0
10	0	0
15	0	0
20	0	0

Extraction of irradiated and unirradiated tea parasite herbs each 100 g, got 4.6% of dried extract each sample. Subsequently, the cytotoxic activity assay of ethyl acetate extracts against leukemia L1210 cells which was implemented in linear regression Y = a X + b between logarithmic of sample concentration *versus* probit of percent

inhibition were shown in Fig. 1. The inhibition concentration-fifty (IC_{50}) which expresses the ability of the samples to inhibit 50% of leukemia L1210 cells proliferation calculated by the substituting Y by 5 for each linear regression curve, in order to obtain the IC_{50} values as shown in Table 2.



(a)



(b)

Fig. 1. Curve of logarithmic of ethyl acetate extract concentration *versus* probit of inhibition percentage; (a) replicate 1 and (b) replicate 2.

Table 2. The IC_{50} value of ethyl acetate extracts from irradiated and unirradiated tea parasite herbs.

Irradiation dose (kGy)	IC_{50} value of ethyl acetate extracts (µg/ml)		
	replicate 1	replicate 2	average
0	15.35	15.46	15.41^{a}
5	18.27	18.55	18.41^{ab}
7.5	20.76	20.95	20.869^{ab}
10	22.16	20.48	21.32^{b}
15	48.35	47.56	47.96^{c}
20	57.35	55.39	56.37^{d}

Note: Numbers followed by the same letters as superscript are not significantly different.

It showed that the increase in radiation dose caused the increase in the IC_{50} value, which means that the cytotoxic activity decreased. The significant increase in IC_{50} value occurred after irradiation at the dose of $\geq$ 7.5 kGy. However, the increase did not eliminate its cytotoxic activity. According to the American National Cancer Institute, the extract is declared to have a cytotoxic activity if the IC_{50} value $\leq$ 30 µg/ml [10,11]. This fact suggested that the irradiation dose until 10 kGy is still feasible for the microbial decontamination of tea parasite herb without reducing its cytotoxic activity.

Furthermore, fractionation of 1.0 g of ethyl acetate extracts from irradiated and unirradiated tea parasite herbs obtained four fractions respectively, which were classified according to the spot fraction of TLC. The weight of fractions obtained was shown in Table 3.

Table 3. Weight of fractions from ethyl acetate extracts of irradiated and unirradiated tea parasite herbs.

Irradiation dose (kGy)	Weight of fraction (mg)				Sum	
	Fr-1	Fr-2	Fr-3	Fr-4	(mg)	(%)
0	42^{a}	313^{cd}	247^{g}	328^{i}	930	93.0
5	48^{b}	318^{c}	222^{h}	317^{i}	905	90.5
7.5	50^{b}	303^{e}	249^{g}	320^{ij}	922	92.2
10	52^{b}	302^{e}	244^{g}	330^{ij}	928	92.8
15	47^{b}	309^{de}	240^{gh}	323^{j}	919	91.9
20	49^{b}	282^{f}	250^{h}	359^{k}	940	94.0
Average	48±2.3	304.5±8.8	242±7.3	329.5±10	924±8.7	92.4±9

Note: Numbers followed by the same letters as superscript are not significantly different.

Based on the result of statistic analysis by one way ANOVA, the weight of fractions obtained were not similar. The weight of fraction 1 increased after irradiation at the dose $\geq$ 5 kGy, the fraction 2 decreased after irradiation dose $\geq$ 7.5 kGy, and the fraction 3 was decreased at dose of 5 up to 15 kGy. Meanwhile, the fraction 4 was not almost change after irradiation at the dose up to 15 kGy, but it was decrease at the dose of 20 kGy. The increase of gamma irradiation dose caused the decrease and increase of the fractions weight. However, it was no uniformity of each fraction weight, but the overall yield of each sample was > 90%, which indicated that the fractionation was successful.

Among the four obtained fractions, the fraction 2 was selected for further analysis because the fraction 2 was the most active fraction as reported in previous work [3]. Based on the TLC profiles there were at least 6 spots observed in fraction 2 (Fig. 2).

Among the spots, spot 2 and 3 gradually faded out, except at irradiation dose of 15 kGy, the density of the spot 2 and 3 were similar with an unirradiated tea parasite herb.

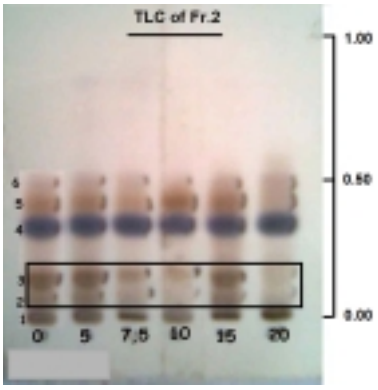
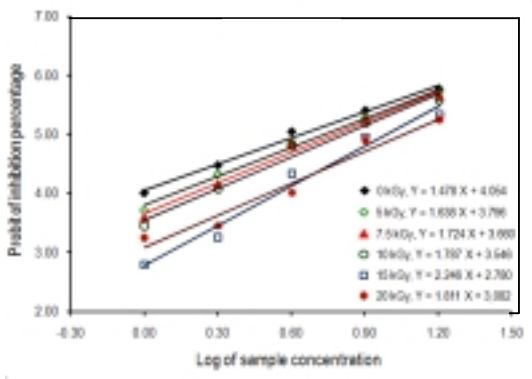
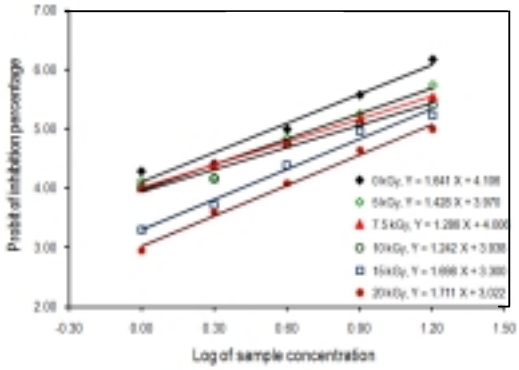


Fig. 2. TLC of fraction 2 from ethyl acetate extracts of irradiated and unirradiated tea parasite herbs (condition: silica gel GF_{254}, *n*-hexane - ethyl acetate (3:1), UV light at 254 nm, 1% cerium sulphate in 10% sulfuric acid).

Subsequently, the cytotoxic activity assay of fraction 2 against leukemia L1210 cells which was implemented in linear regression between logarithmic of sample concentration versus probit of inhibition percentage were shown in Fig. 3. Then, the calculation of IC_{50} values was shown in Table 4. As similar as ethyl acetate extract, the IC_{50} values of fraction 2 tend to increase with increasing the irradiation dose. This means that the increase in irradiation dose caused the decrease in cytotoxic activity of fraction 2. However, the IC_{50} value significantly increase at irradiation dose > 10 kGy. This fact also correlated to the spot 2 and 3 in TLC chromatogram of Fig. 3 that those spots tend to decrease with increasing the irradiation doses. It is suggested that spot 2 and 3 were the components which had the important role to the cytotoxic activity of the tea parasite herbs.



(a)



(b)

Fig. 3. Curve of logarithmic of concentration of fraction 2 *versus* probit of inhibition percentage; (a) replicate 1 and (b) replicate 2.

Table 4. The IC_{50} value of fraction 2 of ethyl acetate extracts from irradiated and unirradiated tea parasite herbs.

Irradiation dose (kGy)	IC_{50} value of ethyl acetate extracts (µg/ml)		
	replicate 1	replicate 2	average
0	4.36	3.51	3.94[a]
5	5.44	5.26	5.35[ab]
7.5	5.99	5.93	5.96[ab]
10	6.51	7.16	6.84[b]
15	9.71	9.99	9.85[c]
20	11.47	12.53	12.00[d]

Note: Numbers followed by the same letters as superscript are not significantly different

CONCLUSION

Based on the results, it could be concluded that bacterial and mold-yeast decontamination with the initial contamination of 7.57 x 10^9 cfu/g and 5.68 x 10^8 cfu/g respectively, was gamma irradiation at the minimum dose of 7.5 kGy and the maximum dose of 10 kGy. At these conditions, the bacteria and mold-yeast have been killed, whereas the cytotoxic activities of active components (ethyl acetate extract and fraction 2) in tea parasite herb decreased, but the decrease was not significant and did not remove these cytotoxic activities.

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