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GAMMA IRRADIATION FOR PRESERVATION OF SURUHAN HERBS (*Peperomia pellucida* L. Kunth.) AND ITS BIOACTIVITY AGAINST L1210 LEUKEMIA CELLS

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ABSTRACT

Peperomia pellucida includes the Piperaceae family that has anti-cancer, anti-inflammatory, antioxidant, and anti-microbial activities. Microbes easily contaminate dry herbs during storage, so special handling is needed. One of the preservation techniques is using irradiation technology. This technique can be used to extend the shelf life of herbal medicinal ingredients. This research aimed to study the effects of irradiation on the anti-cancer activity of *P. pellucida* leaves. *P. pellucida* leaves' dried powder was irradiated at doses of 5, 7.5, 10, and 15 kGy. The anti-cancer activity test was carried out against L1210 leukemia cells. The un-irradiated irradiated samples were macerated successively with *n*-hexane, ethyl acetate, and ethanol, then be concentrated. The ethyl acetate extract was fractionated using silica gel column chromatography, obtained seven fractions. Their IC_{50} values < 20 μg/mL, with fraction F4 was the most active fraction (IC_{50} 1.9 μg/mL). The result showed that gamma radiation at doses of 5 - 15 kGy reduced their cytotoxic activity significantly. However, the fractions were still in the active category as anti-cancer (IC_{50} values were < 20 μg/mL).

Keywords: *suruhan, Peperomia pellucida, gamma irradiation, L1210 leukemia cells*

INTRODUCTION

As an agricultural country with thousands of types of plants, including medicinal plants, Indonesia has been used by their ancestors from generation to generation to improve public health. One of the Indonesian people's herbal medicinal ingredients is *suruhan* leaves (*Peperomia pellucida* L. Kunth) to cure illnesses. *P. pellucida* is a plant that is grown in Asian countries, used as a medicinal plant. This plant is known to benefit from analgesics, anti-inflammatory, antipyretic, antioxidant, antihyperglycemic, anti-hyper-uricemia, burn medication, and depressant effect, gastro-protective, hypotensive, anti-microbial, a lipase inhibitor, fibrinolytic and thrombocytic, anti-diarrhoea, headache due to fever, vertigo, and belly paint [1]. *P. pellucida* is also known to have been used to treat stomach upset, abscesses, acne, ulcers, colic, fatigue, gout [2][3]. The results of another study [4] showed that the ethanol extract and aqueous extract of P. pellucida leaves contained antioxidants (297 and 257

µg / mL) and flavonoids (4.1 and 0.67 QE / g, respectively).

Researchers are now developing cytotoxic agents' discovery. The *P. pellucida* plant can be a chemopreventive agent based on its phytol content [5] [6]. The water extract of *P. pellucida* leaves contained antioxidants of 42.6 mg/g DW [7]. Isolated 13 compounds from *P. pellucida* leaves; five of them were new compounds. Further, they tested the bioactivity of compound 1 and peperomin E against HL-60, MCF-7, and HeLa cells showed that those compounds had an inhibitory effect on the growth of three cancer cells with IC_{50} values ranging from 1.4 – 11.1 µM [8].

the methanolic extract of *P. pellucida* leaves had the activity in inhibiting the growth of MCF-7 breast cancer and HeLa cervical cancer cells with IC_{50} values of 10.4 and 2.9 µg/mL[9]. Another study [10] showed that the ethanolic extract and water extract of *P. pellucida* leaves could inhibit the growth of HT-29 colon cancer cells with IC_{50} values of 129 and 170 µg/mL, respectively [10]. Successive maceration of the dried powder of *P. pellucida* leaves with *n*-hexane, ethyl acetate, and ethanol showed that the three extracts inhibited the growth of L1210 leukemia cells with IC_{50} values of 10.9, 7.8, and 5.1 µg/mL, respectively [11]. Based on the above studies results, *P. pellucida* leaves have big potential as an anti-cancer agent.

Post-harvest handling, until it is processed into dried herbal ingredients, is the important step so that the traditional medicines remain in a hygienic condition when they reach consumers. Various methods for preserving the herbal medicinal ingredients to extend their shelf life are cooling, freezing, heating, smoking, salting, adding additives, fumigation, and radiation techniques. Currently, radiation techniques by using ionizing radiation are proven to effectively reduce pathogenic microorganisms, prevent damage, prevent insect attack, delay germination, and inhibit the ripening process of fruits, vegetables, and other products have been routinely applied [12].

As part of nuclear technology, gamma radiation techniques have been developed to improve food safety, including herbal medicine. In recent years, gamma-ray irradiation techniques to extend herbal ingredients / herbal medicines' shelf life have increased. More than 30 herbal medicine companies have utilized gamma irradiation to pasteurize herbal ingredients to extend their shelf life [13]. According to the Regulation of The National Agency for Food and Drug Administration and Control (NAFDAC) No. 3 the year 2018, irradiation at a dose up to 10 kGy can reduce certain pathogenic microbes and insect disinfestation [14]. Throughout 2017, 200 tons of commodities classified as herbal ingredients/ herbal medicines irradiated at the Gamma Merah Putih Irradiator belong to BATAN - Indonesia, 531 tons in 2018 426 tons in 2019. Although many herbal medicine industries have long used gamma irradiation, unfortunately, there is limited information on the radiation effect on dried *P. pellucida* leaves. There have been no previous research results that studied especially the effect of gamma irradiation on P. pellucida leaves' efficacy.

In this study, *P. pellucida* was chosen because it has been widely used as herbal medicine. In the previous [15], gamma irradiation has been reported at doses of 2.5-

10 kGy on the angiotensin-converting enzyme (ACE) inhibitory activity, antioxidant activity, total phenolic, and flavonoid contents in dried *P. pellucida* leave. The result showed that irradiation at a dose of 7.5 kGy has no significant difference in antioxidant activity and ACE inhibitory activity, and total phenolic and flavonoid contents. Nevertheless, there were no data and information about gamma irradiation's effect on P. pellucida herbs' anti-cancer properties.

The research aimed to study the effects of irradiation toward its anti-cancer activity on the dried powder of *P. pellucida* leaves preserved by gamma irradiation. The anti-cancer activity changed due to gamma irradiation, which was studied based on the cytotoxic activity expressed as the half-maximum inhibitory concentration (IC_{50}) value of irradiated dried powder *P. pellucida* leaves against L1210 leukemia cells compared to the control sample. The extractor fraction is declared to has a cytotoxic activity if it has an IC_{50} value of ≤ 20 µg/mL [16]. This data and information are necessary for clarifying whether the gamma irradiation can eliminate its cytotoxic activity or not, since it is beneficial for Herbal Medicine Industries to decide the suitable irradiation doses, NAFDAC to formulate the regulation, and consumer protection.

METHODS

1. Materials

The fresh *suruhan* leaves (*Peperomia pellucida* L. Kunth.) was obtained from Indonesian Research Institute for Medicinal Crops and Aromatic (Balai Penelitian Tanaman Obat dan Aromatik, BALITTRO) Jl. Tentara Pelajar No. 3A, Bogor - West Java and plant determination was done by Research Center for Biology-The Indonesian Institute of Science (Pusat Penelitian Biologi - LIPI), Cibinong, Bogor.

Other materials were *n*-hexane, ethyl acetate, ethanol, methanol, distilled water, $NaHCO_3$, calf bovine serum (Gibco), L1210 leukemia cells, which originally obtained from The Institute of Physical and Chemical Research (RIKEN) Japan, RPMI 1640 (Media Roswell Park Memorial Institute 1640), tryphan blue 1%, celite 545, chloroform, silica gel 70-230 mesh size (Merck).

2. Equipment

Column Chromatography, thin layer chromatography chamber, of the rotary evaporator (Buchi R-22 SE; five L capacity) and (Buchi R-111; one L capacity), ultra-sonicator (NEY), CO_2 incubator, light micros-cope, Neubauer Improved hemocytometer, tissue culture multiwall plate, analytical scales (Mettler Taledo), sero cluster plate, blender (Miyako), UV lamps 254 and 366 nm (Camag), oven, aluminum foil, micropipettes, electric heater, vacuum desiccator, filter paper, and glassware.

3. Sample Preparation

Fresh *P. pellucida* leaves were washed and dried at room temperature until their water content 3.3%. The dried leaves were made into a coarse powder, wrapped in plastic bags, and weighed 200 g, respect-tively.

4. Samples Irradiation

The dried powder of *P. pellucida* leaves wrapped in a PE plastic bag was placed in a cardboard box then irradiated with gamma rays at various doses of 5, 7.5, 10, and 15 kGy (2 replicates for each dose). The irradiation was conducted at the Center for Isotopes, and Radiation Application-BATAN, Jakarta-Indonesia, with a gamma source was Co-60.

5. Extract preparation

The un-irradiated (control) and irradiated samples @ 200 g were successively macerated with *n*-hexane, ethyl acetate, and ethanol. Each sample was macerated repeatedly five times for each type of solvent to ensure that all components are extracted into the solvent. The macerate is filtered, evaporated, dried with a vacuum pump in a desiccator, and then weighed. In this study, only ethyl acetate extract was selected, then further fractionated by column chromatography.

6. Fractionation of ethyl acetate extract

A total amount of 1.0 g of ethyl acetate extract from an unirradiated (control) sample was dissolved in ethyl acetate, then mixed with 30 g of cellite 545, evaporated to get a dry powder, then fractionated using column chromatography with the stationary phase of silica gel 70-230 mesh (30 g). The used mobile phase was started from n-hexane, a mixture of n-hexane-ethyl acetate, and finally methanol. The fractions with a similar spot pattern on the TLC silica gel plat were combined, evaporated by the vacuum-rotary evaporator, and then further dried by a vacuum pump in the desiccator. Furthermore, the ethyl acetate extracts from irradiated *P. pellucida* leaves with various doses were also fractionated in the same manner as fractionation of the control sample.

7. Bioassay on L1210 leukemia cells

The cytotoxic activity test of the ethyl acetate extract fraction against leukemia L1210 cells was carried out with previous studies [17] [18]. The sample concentration variation was 0.5; 1, 2, 4. and 8 µg / mL using Roswell Park Memorial Institute 1640 media (RPMI 1640) with 5% bovine fetal serum. Incubation was carried out in a 5% CO_2 incubator at 37^{o}C for 48 hours.

8. Calculation of IC_{50} value and data analysis

The number of living cells was counted under the microscope, then converted to the number of dead cells to get the inhibition percentage. The half value of the maximum inhibitory concentration (IC50), which is the sample's concentration, states the ability to inhibit 50% of cancer cell proliferation. It can be calculated using a linear regression equation between the probit percentage of inhibition (Y-axis) versus the sample concentration logarithm (X-axis). Analyzed statistically using one-way analysis of variance (ANOVA)[17][18].

RESULTS AND DISCUSSION

1. Extract and fraction

From a total amount of 200 g of dried powder of *P. pellucida* leaves with a water content of 3.3%, the obtained extracts from

unirradiated and irradiated samples are presented in Table 1.

Table 1. The yield of extract from the un-irradiated (control) and irradiated *P. pellucida* leaves

Radiation dose (kGy)	Weight of extract			Weight of total extract (g)	Rende-men (%)
	n-Hexane	Ethyl acetate	Ethanol		
0	4.5	8.5	10.1	23.1	11.6
5	3.1	7.7	7.7	18.4	9.2
7.5	3.8	4.5	9.6	17.8	8.9
10	3.5	6.2	9.5	19.2	9.6
15	3.7	8.3	8.1	20.0	10.0

Table 1 shows that all of the extract's weight tends to decrease compared to the extract from the control/un-irradiated sample. In the total yield, it was also seen that the irradiated samples tended to decrease compared to the control sample. Based on the previous study [11], *n*-hexane, ethyl acetate, and ethanol extracts from un-irradiated *P. pellucida* leave had cytotoxic activity against L1210 leukemia cells with IC_{50} values of 10.9, 7.8, and 5.1 µg/mL. Accordingly, in this study, the ethyl acetate extract of *P. pellucida* leaves was selected to be fractionated using column chromato-graphy and obtained seven fractions (F1 - F7) with the weight of each fraction are presented in Table 2.

Table 2. Weight of fractions of ethyl acetate extract from *P.pellucida* leaves

Radiation Dose	Fraction weight (mg) of ethyl acetate extract						
	F1	F2	F3	F4	f5	F6	F7
0 kGy	42	107	182	180	47	254	65
5 kGy	22	32	29	36	181	40	274
7.5 kGy	16	30	19	65	183	146	107
10 kGy	30	43	23	41	64	122	127
15 kGy	13	23	17	16	33	74	97

From Table 2, it can be seen that generally, the fraction weight tended to decrease due to increasing the irradiation doses. Still, oppositely for fractions 5 and 6, the weight increased at doses of 5 and 7.5 kGy, while for fraction seven, the weight increases at doses of 5, 7.5, and 10 kGy. This change in the solubility of components was easy or otherwise difficult to disperse into the solvents during maceration.

2. Cytotoxic activity of fraction

The cytotoxic activity test of F1 - F7 of ethyl acetate extract from the control sample against L1210 leukemia cells were shown in Figure 1. Based on the linear regression.

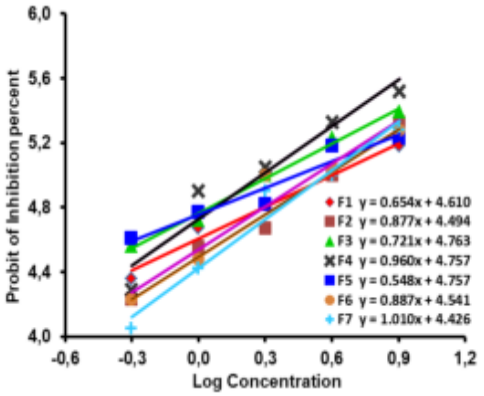

Figure 1. Linear regression curve between probit of inhibition percentage vs. log concentration of F1-F7 of ethyl acetate from un-irradiated *P. pellucida* leaves

The equation in Figure 1, then the IC_{50} value was calculated, and the results were presented in Table 3. Table 3 shows that the IC_{50} values of all fractions (F1 - F7) had < 20 µg/mL. Based on the provisions of the US NCI [19]; the extract has the potential to be anti-cancer if the IC50 value is less than 20 µg / ml. means to be the result that all fractions.

Table 3. The IC_{50} value of F1-F7 of ethyl acetate extract from unirradiated *P. pellucida* leaves

Fraction	Linier regression	IC_{50} values (mg/ml)
F1	y = 0.654 x + 4.610	4.0
F2	y = 0.877 x + 4.494	3.8
F3	y = 0.721 x + 4.763	2.1
F4	y = 0.960 x + 4.729	1.9
F5	y = 0,548 x + 4.747	2.8
F6	y = 0.887 x + 4.541	3.3
F7	y = 1.010 x + 4.428	3.7

Ethyl acetate extract of *P. pellucida* leaves was very active in inhibiting L1210 leukemia cells; this meant that all fractions had potential as anti-cancer. The qualitative identification of secondary metabolite content was also supported by Andriani et al. [20] that the plants contained flavonoids, triterpenoids, steroids, and saponins tannins are believed to affect their cytotoxic activity. Meanwhile, Yusuf et al. [15] founded that irradiation at a dose of 10 kGy could cause significant changes in antioxidant activity, phenolic levels, and flavonoids levels in *P. pellucida* leaves. The bioactivity of these *P. pellucida* leaves was also supported by the study of Xu *et al*. that the peperomin E compound isolated from *P. pellucida* leaves could inhibit cancer cells' growth HL-60, MCF-7, and HeLa with IC_{50} values range from 1.8 to 11.1 µg/mL [8].

P. pellucida leaves contained phytol as a primary compound, a terpene compound, and acted as an anti-cancer [9]. Based on the description above, it was assumed that the compounds of the flavonoids, triterpenoids, steroids, saponins, and tannins, and phytol compounds caused *P. pellucida* leaves to be very active in inhibiting the growth of L1210 leukemia cells. In Table 2, from 7 fractions, F4 was the most active fraction with the lowest IC_{50} value of 1.9 µg/mL. All F4 ethyl acetate extracts from irradiated P. pellucida leaves were tested for their cytotoxic activity. The results of this test are presented in Figure 2. In contrast, the calculated IC_{50} values based on Figure 2 were shown in Table 4.

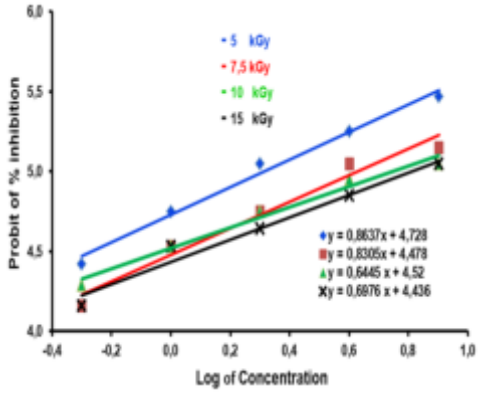

Figure 2. Linear regression curve between probit of percentage inhibition vs. log F7 concentration of ethyl acetate extract derived from irradiated samples

Analysis using one-way ANOVA with a 95% confidence level showed that gamma irradiation from the doses of 5 to 15 kGy increased the IC_{50} value significantly, which meant cytotoxic activity decreased. However, the decrease did not eliminate their activities; it can be seen that the IC_{50} values were still low (< 20 µg/ml), between 2.1 - 6.4 µg/mL (Table 4). Fraction 4 was still in the active category as anti-cancer.

Table 4. The IC_{50} value of fraction 4 of ethyl acetate extract from unirradiated and gamma-irradiated *P. pellucida* leaves

Irradiation Dose (kGy)	IC_{50} values of F4 (mg/ml)
0	1.9^{a}
5	2.1^{b}
7.5	4.3^{c}
10	5.6^{d}
15	6.4^{e}

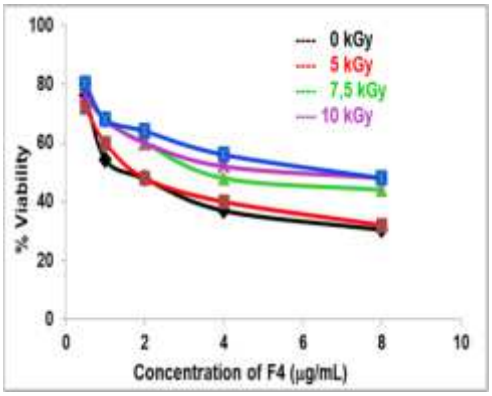

Figure 3. Graph of average viability of L1210 leukemia cell vs. F4 concentration

Furthermore, in Figure 3, it was clear that the effect of decreasing cell viability of L1210 cells by F4 at all sample concentrations decreased with increasing irradiation dose (Figure 3). However, the irradiated samples at doses of 5 - 15 kGy still had high cytotoxic activity. Based on the results, it can be stated that *P. pellucida* herbs can be preserved using the gamma irradiation technique as recommended by the NADFC [14] for dry herbs with the maximum irradiation dose of 10 kGy.

CONCLUSION

Gamma irradiation on the dried powder of *Peperomia pellucida* leaves at doses of 5 - 15 kGy has decreased ethyl acetate extract's cytotoxic activity against L1210 leukemia cells, which is shown on the increasing of IC_{50} value significantly. However, the decrease of their activity did not damage the activity of *P. pellucida* leaves, proved still to have the IC_{50} value < 20 µg/mL. It can be concluded that the use of gamma irradiation can still be done up to a dose of 10 kGy according to the NAFDAC Regulation No.3 the year 2018. Fraction 4 (F4) of the ethyl acetate extract of *P. pellucida* leaves was the most active fraction among the seven fractions with an IC_{50} value of 1.69 µg/mL, and it can be used as a comparative fraction to study the effect of radiation on the *P. pellucida* herbs.

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