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CYTOTOXIC ACTIVITY AGAINST L1210 LEUKEMIA CELLS FROM THE ETHYL ACETATE FRACTION OF KENIKIR LEAVES (*Cosmos. Caudatus*) PRESERVED BY GAMMA IRRADIATION

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ABSTRACT

Kenikir leaves (*Cosmos caudatus*) Has been used as traditional medicine, especially as an anti-cancer, this plant has been in Indonesia both in herbs and capsules. Microbial contamination of herbal medicinal raw materials occurs when the storage process is done. One of the preservation techniques used in the industry is using gamma irradiation techniques to reduce microbial and fungal contamination. The purpose of this research was to study the effect of gamma irradiation for the preservation of *kenikir* leaves (*C. caudatus*) as an anti-cancer based on cytotoxic activity using L1210 leukemia cells. The simplicia was gamma-irradiated by Co-60 source with variation doses of 0 (control); 5; 7.5; 10; and 15 kGy. Then the irradiated and control samples were macerated successively using *n*-hexane, ethyl acetate, and ethanol. The active extract (ethyl acetate) was further fractionated using column chromatography, obtained seven fractions (F1 - F7). The seven fractions' cytotoxic activity against L1210 leukemia cells showed that fraction 3 (F3) was the most active fraction with an IC_{50} of 1.26 µg/mL. Each dose's F3 cytotoxic activity showed that the IC50 7.5 kGy irradiation sample did not change significantly with control (0 kGy) based on ANOVA analysis using SPSS 24 with a 95% confidence level. In comparison, irradiation samples of 10 and 15 kGy showed a change in the IC_{50} value is significant with the control (0 kGy). These results indicate that gamma irradiation can be used as an alternative for preserving *C. caudatus* with a maximum dose of 7.5 kGy, so that its anti-cancer properties do not change with those without irradiation.

***Keywords**: Kenikir, Cosmos caudatus, anti-cancer, L1210 leukemia cells*

INTRODUCTION

Indonesia has an abundant natural wealth of plants, and nearly 75% of the world's plants are in Indonesia. About 90% of the total number of medicinal plants in Asia grows in Indonesia [1]. The many types of medicinal plants that grow in Indonesia resulted from the Indonesian population have been using medicinal plants to treat various diseases. This herb commonly consumed by Indonesian people is known as *jamu*; this herbal medicine has been consumed by Indonesians from generation to generation to treat various diseases [2].

One of the medicinal plants used by Indonesian people is kenikir (*Cosmos caudatus*). *C. caudatus* has properties healing high blood pressure, anti-diabetes, anti-inflammatory, anti-bacterial and anti-cancer [3]. The secondary metabolite of *C.*

caudatus leaf extract consists of flavonoids, especially quercitrin glycosides. The results revealed that secondary metabolites of flavonoid compounds act as anti-cancer, which induce apoptosis [4]. Methanol extract from kenikir leaves has potential as chemo-therapy derived from natural ingredients. The methanol extract of kenikir leaves can against T47D breast cancer cells through apoptotic mechanisms to develop as an anti-cancer drug [5]. The ethanol extract of kenikir leaves has a high antioxidant content 181.64 µg chatecin/mg extract, so it can prevent free radicals and can inhibit the development of hella cancer cells with IC^{50} 89.90 µg/mL [6]

C. caudatus, as traditional medicine has been widely used in Indonesia either in herbs or capsules. Microbial contamination in herbal medicinal products is common and usually comes from raw materials, processing to the equipment used [6]. Although the number of bacteria and fungi are found still under the recommendation and regulation of BPOM No. 12 of 2014 that the number of bacteria should not be more than 10^7 colonies/gram, and the number of fungi should not be more than 10^4 colonies/gram. Can be dangerous if the bacteria and fungi are aflatoxins [7]. The unirradiated temu putih (*Curcuma zedoaria*) capsule product has the number of bacteria and fungi $28.5x10^2$ colonies/gram and $19x10^2$ colonies/gram. After irradiated gamma of 5 kGy the number of bacteria and fungi decreased to $18.6x10^2$ colonies/gram and $0.57x10^2$ colonies/gram [8].

One of the preservation techniques used in the industry is the gamma irradiation technique. According to the BPOM regulation, no 18 of 2019 concerning irradiate food, preservation using gamma irradiation techniques can reduce the number of bacteria, fungi, and storage time longer than before [9]. Preservation techniques using gamma irradiation can reduce the number of bacteria and fungi and extend these products' shelf life. Preservation techniques are currently widely used because it has many advantages such as more efficient, easy to control, does not cause residues, and is very environmentally friendly. Therefore the cost of production is cheaper [10]. Irradiation of peach fruit using a dose 5 kGy increased to 17 days longer shelf life than unirradiated samples [11]. Research on the simplicia of herbal plants has been done a lot, even preservation techniques using gamma-ray irradiation in herbal medicinal plants have been used by industry. Research on the effect of gamma irradiation of *C. caudatus* leaves still needs a lot to do with this.

The use of gamma-ray irradiation in Indonesia is currently widely used by herbal medicine industry players to extend raw materials and final products' shelf life. However, regulations regarding the limits for using irradiation doses have not been issued by BPOM. This research is expected to provide supporting data for BPOM to establish rules on gamma irradiation for preserving kenikir leaves. Research on the effect of gamma irradiation of *C. caudatus* leaves is very beneficial for industries currently using it to preserve raw materials because gamma irradiation can reduce its anti-cancer properties. For this reason, it is necessary to research the simplicia of *C. caudatus* leaves that have been irradiated with gamma for their properties as an anti-cancer drug by conducting cytotoxic tests of

C.caudatus leaf extract against L1210 leukemia cells.

METHODS

1. Preparation of samples

Samples (*Cosmos caudatus leaves*) came from the Indonesian Medicinal and Aromatic Plants Research Institute (Indonesian Medicinal and Aromatic Plants Research Institute, BALITTRO). Dried at room temperature, and weighed 200 g of each sample, wrapped in polyethene plastic, the samples were vacuumed and sealed.

2. Irradiation of samples

The samples were irradiated using gamma irradiator IRKA (source: Co-60) at PAIR-BATAN with the irradiation doses of 0 (control), 5, 7.5, 10 15 kGy with each irradiation dose were two replications.

3. Extraction

After irradiation, each carried out maceration of the sample successively using n-hexane, ethyl acetate, and ethanol and soaking until all components have been extracted into each solvent. The filtrate was evaporated using a Buchi rotary evaporator (35 °C) until obtained. Each extract from each solvent was concentrated again in a desiccator using a vacuum pump and then weighed. Only the ethyl acetate extract was fractionated by column chromatography, and cytotoxicity test against leukemia L1210 cells because ethyl acetate extract is the most active extract against L1210 cancer cells according to previous studies with an IC_{50} of 6.74 µg/mL [12].

4. Fractionation.

Fractionation of ethyl acetate extract was carried out using column chromatography with silica gel 60 (70-230 mesh). The separation of the fractions was carried out as a stationary phase, starting from non-polar (*n*-hexane) to polar solvents (methanol). Amount of 150 mL of each fraction was concentrated using a Buchi rotary evaporator (35 °C), followed by concentrated in a desiccator using a vacuum pump and then weighed.

5. The toxicity test against L1210 leukemia cells.

Toxicity test of ethyl acetate extract was carried out with various concentrations of 1, 2, 4, 8, and 16 µg/mL against L1210 leukemia cancer cells. The cell suspension, Roswell Park Memorial Institute 1640 (RPMI 1640) medium, 10% calf bovine serum, and the samples were placed into a 24 well multiwell plate and incubated in a 5% CO_2 incubator (37 °C, 48 h). Living cells were counted under a microscope at 4000 times magnification. The fraction was further tested for each radiation dose fraction to determine irradiation's effect on the IC_{50} value and its efficacy on L1210 leukemia cells.

RESULTS AND DISCUSSION

The moisture content of *C. caudatus* after drying for one week at room temperature (22°C) is 6.58%. This value follows the NAFDAC (Badan POM) requirements that the water content in herbal plants must be below 10% [7].

Drying is carried out to reduce the moisture content in the simplicia and prevent decay caused by microorganisms to affect the quality and efficacy of the simplicia finally.

Maceration was carried out in stages starting from non-polar (*n*-hexane) to polar solvents (ethanol) to separate the secondary metabolites in the *C. caudatus* simplicia. The yield of each extract can be seen in Table 1.

Table 1**.** The weight of n-hexane, ethyl acetate, and ethanol extract from 200 g of *C. caudatus* at varying irradiation dose (average of two replicates)

Dose (kGy)	Weight (g)			Total extract (g)	Yield (%)
	n-hexane	ethyl acetate	ethanol		
0	2.56	6.58	37.03	46.16	23.08
5	2.81	6.04	33.64	42.49	21.25
7.5	2.78	6.22	28.42	37.42	18.71
10	2.63	6.75	29.70	39.08	19.54
15	2.84	6.14	29.76	38.74	19.37

In the previous research revealed that ethyl acetate extract was the most active extract against leukemia cells L1210 compared to *n*-hexane and ethanol extract [12]. Therefore fractionation was carried out on ethyl acetate extract to determine the most active fraction to inhibit L1210 leukemia cells. The fractionation of ethyl acetate extract using column chromatography obtained seven fractions, and the weight of the fractions obtained, as shown in Table 2.

Table 2. Result of the column fractionation of ethyl acetate extract *C.caudatus* (1 g) for varying irradiation dose

Dose (kGy)	Weight (mg)						
	F1	F2	F3	F4	F5	F6	F7
0	41.3	30.8	92.1	20.3	141.4	149.7	340.1
5	27.6	52.4	108.3	31.2	75.4	102.7	594.5
7.5	25.5	31.6	102.9	24.4	72,7	202.9	448.3
10	21.8	25.6	107.9	65.6	46.7	89.2	470.3
15	19.8	21.7	97.5	95.4	66.8	79.8	461.2

Cytotoxic tests were performed to predict the presence of herbal medicines that can act as anti-cancer drugs. Cytotoxic testing will provide information about the concentration of drugs that can kill cancer cells. *C.caudatus* leaves have high anti-oxidant properties with an IC_{50} value of 70 µg/mL, besides the flavonoids isolated from the ethyl acetate fraction can inhibit cancer cell growth [4]. Cytotoxic test results on ethyl acetate extract of *C. caudatus* showed that the most active fraction was fraction 3 (F3) with the smallest IC_{50} was 1.26 µg/mL compared to the others (Figure 1).

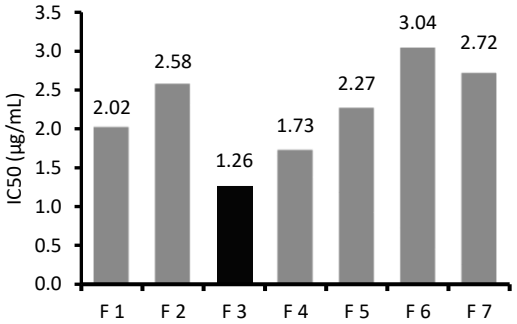

Figure 1. Cytotoxicity of fraction F1-F7 from ethyl acetate extract *C.caudatus* against L1210 leukemia cells

The cytotoxic test on ethyl acetate extract's fractions showed that F1-F7 had strong anti-cancer properties because it had an IC_{50} ≤ 20 µg/mL [13]. It was supported by another study that ethyl acetate fraction of *C. caudatus* contains flavonoids which could inhibit the development of T47D cancer cells [4]. The content of flavonoids, especially quercetin glycosides in *C. caudatus* leaves extract, can regulate cancer cells' life cycle by binding to several targets. Also, except for regulating the cell life cycle, quercetin can cause apoptosis cancer cells with the mitochondrial pathway where cancer cells release cytochrome C in the cytoplasm and activate caspase [5]. Apoptosis is programmed cell death and plays an important role in the development of cancer cells. Quercetin in *C. caudatus* leaves extract can induce apoptosis of cancer cells to kill cancer cells [14]. The apoptosis mechanism is due to the presence of DNA fragmentation that begins with the release of DNA chains by reactive oxygen compounds such as hydroxyl radicals, another effect of flavonoids can inhibit cancer cell because flavonoids can inhibit protein kinase activity by blocking signals from the membrane to the nucleus [15].

The cytotoxic fraction results were used to determine the effect of gamma irradiation of the most active fraction (F3) of all irradiated samples and tested their cytotoxic activity. The results are presented in Figure 2. Gamma irradiation will affect the secondary metabolites in the plant sample. At dose 10 kGy, it will cause a decrease in the number of secondary metabolite components due to changes in the active compound's chemical bond structure of the active compound [16]. A change in the chemical bond structure can lead to new compounds' formation due to their degradation [17].

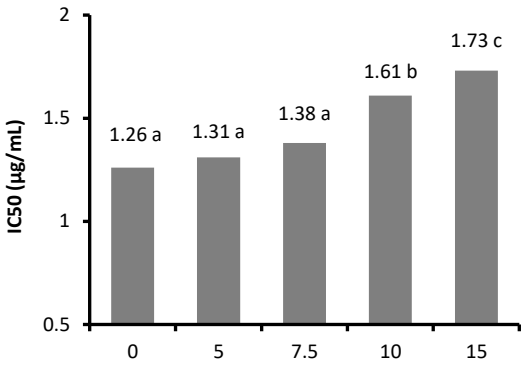

Figure 2. Cytotoxic activity against L1210 leukemia cells of F3 from ethyl acetate extract at a varying irradiation dose

Statistical analysis using one-way SPSS 24 ANOVA with a 95% confidence level showed that irradiation at doses of 5 and 7.5 kGy had no significant difference in the IC_{50} values than controls (without irradiation). Still, the IC_{50} values were a significant increase at irradiation doses of 10 and 15 kGy. These results indicated that preserving the simplicia of *kenikir* leaves (*C. caudatus*) without reducing its anti-cancer properties could be done using gamma irradiation up to the dose of 7.5 kGy. However, irradiation up to 15 kGy can still be used. According to the National Cancer Institute, a substance can be anti-cancer if it has an IC value of ≤ 20 µg/mL [18]. Based on these criteria, it can be stated that fraction three (F3) of the ethyl acetate extract of kenikir leaves (*C. caudatus*) which irradiated at doses between 5 - 15 kGy is still potential as an anti-cancer because it has an IC value of <20 µg/mL.

CONCLUSION

The maximum dose for preserving *kenikir* (*C.caudatus*) leaves using gamma

irradiation was 7.5 kGy. Irradiation up to this dose, cytotoxic activity against leukemia L1210 cells was not significantly different from controls. Meanwhile, irradiation doses of 10 and 15 kGy can still be used, although it can reduce the cytotoxic activity, because irradiation at the two doses still has IC_{50} values <20 µg/mL.

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