

# Chromatogram Profiles and Cytotoxic Activity of Irradiated *Mahkota Dewa* (*Phaleria Macrocarpa* (Scheff.) Boerl) Leaves

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## ABSTRACT

Gamma irradiation has been used by the industries for preservation of herbal medicine, but it has not been studied the effect of gamma irradiation on their efficacy, especially their bioactivity as anticancer substances. The purpose of this research was to study the effect of gamma irradiation on the *mahkota dewa* leaves which has been claimed to contain potent anticancer substances. Maceration of dried *mahkota dewa* leaves successively with *n*-hexane, ethyl acetate, and ethanol gave crude extracts which the ethyl acetate was the most cytotoxic extract against leukemia L1210 cells with an inhibition concentration fifty (IC<sub>50</sub>) value of 10.3 µg/ml. Further separation of ethyl acetate extract by column chromatograph gave 7 fractions, and fraction 2 showed the most cytotoxic fraction exhibited the most cytotoxic extract against leukemia L1210 cells with an IC<sub>50</sub> value of 1.9 µg/ml. Since, the fraction 2 of ethyl acetate extract was the most potent fraction, the irradiated samples were treated with the same procedure as treatment of fraction 2 from control sample. Cytotoxic activity test of fractions 2 from irradiated samples showed that the cytotoxic activity decreased depending on increasing of irradiation dose. Gamma irradiation dose up to 7.5 kGy on *mahkota dewa* leaves could decreased the cytotoxic activity of fraction 2 as the most cytotoxic-potential fraction against leukemia L1210 cells, but decreasing the cytotoxic activity has not exceeded the limit of the fraction declared inactive. So that the irradiation dose up to 7.5 kGy can be used for decontamination of bacteria and fungus/yeast without eliminating the cytotoxic activity. Gamma irradiation also caused changes in the thin layer chromatograph (TLC) spots and HPLC chromatograms profiles of fraction 2 which was the most cytotoxic fraction in ethyl acetate extract of *mahkota dewa* leaves against leukemia L1210 cells. One of the main peaks (peak 1) on HPLC chromatograms decreased with increasing the irradiation dose. In agree with the decreasing peak 1 in chromatogram of fraction 2 due to irradiation, cytotoxic activity of fraction 2 also decreased along with increasing doses of irradiation. It suggested that peak 1 is the component that contributes to the cytotoxic activity of fraction 2 in ethyl acetate extract of *mahkota dewa* leaves.

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## INTRODUCTION

*Mahkota dewa*, *Phaleria macrocarpa* (Scheff.) Boerl (Thymelaeaceae) is one of the Indonesian medicinal plants which is recently popular due to its ability for treating various diseases, namely cancer, tumors, diabetes mellitus, hypertension, hepatitis, rheumatism, gout, skin diseases, kidney disorders, allergies, asthma, cold, haemorrhoid, stroke, and migraine [1,2,3]. Leaves and bark of *mahkota dewa* contained alkaloids,

terpenoids, flavonoids, polyphenols, saponins, resins, lignin, and benzophenones [1,4,5].

The stems of *mahkota dewa* believed to be able to heal bone cancer, meanwhile eggshell of the fruits or seeds more efficacious compared with the bark and fruit flesh. Seeds of *mahkota dewa* is only used for external treatment especially skin diseases [6]. Some several benefits in the plant of *mahkota dewa* has been discovered, moreover the research to explore active compounds from *mahkota dewa* has been started. In 2003, it has been reported that one of the fraction of methanol extract from the leaves of *mahkota dewa* showed the cytotoxic activity against *Artemia salina* with the LC<sub>50</sub>

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(lethal concentration fifty) value of 1.04  $\mu\text{g/ml}$ . Furthermore, the same fraction also exhibited inhibitory activity against myeloma cells (NS-1) with an  $\text{IC}_{50}$  value of 81  $\mu\text{g/ml}$  [7]. An  $\text{IC}_{50}$  is the concentration of the tested samples that can inhibit cell proliferation as much as 50%. The same researcher has isolated 4,5-dihydroxy-4'-methoxybenzophenone-3-*O*- $\beta$ -D-glucoside so called phalerin from the methanol extract of *mahkota dewa* leaves [8]. Recently this structure has been revised by Oshimi [9] as 2,4,6-trihydroxy-4-methoxybenzophenone-2-*O*- $\beta$ -D-glucoside.

Chloroform extract of *mahkota dewa* leaf showed an antiproliferation activity against cervical cancer cells HeLa ( $\text{IC}_{50}$  40.2  $\mu\text{g/ml}$ ), melanoma skin cancer cells HM3KO ( $\text{IC}_{50}$  62.9  $\mu\text{g/ml}$ ) and breast cancer cell MCF-7 ( $\text{IC}_{50}$  70.8  $\mu\text{g/ml}$ ) [10]. In the other hand, ethyl acetate extract of *mahkota dewa* bark showed the highest cytotoxic activity against L1210 leukemia cells compared with *n*-hexane extract and ethanol with an  $\text{IC}_{50}$  value of 10.2  $\mu\text{g/ml}$ . Further fractionation of this ethyl acetate extract gave fraction 2 was the most active fraction with the  $\text{IC}_{50}$  value of 8.3  $\mu\text{g/ml}$  [11]. The phalerin has also been isolated from the *mahkota dewa* bark [12].

In the recent years, the use of herbal medicines for complementary treatments of some diseases has been popular. While, dry herbs easily damaged during storage due to microbial contamination, cause reducing the efficacy and quality of herbal medicines. To overcome this problem, gamma irradiation can be used. Codex Alimentarius Commission stating that ionizing radiation has been used to decontaminate the pathogenic microbes and to extend the shelf life of herbs, foods, and spices with the maximum absorbed dose should not exceed 10 kGy, except when necessary to achieve legitimate technological purposes [13]. In Indonesia the regulation of the ionizing radiation for sterilization of products has been regulated by the Minister of Health No. 701/MENKES/PER/VIII/2009 [14]. Irradiation dose up to 10 kGy can reduce the number of bacteria on *mahkota dewa* dried powder from  $10^{10}$  cfu/g to  $10^5$  cfu/g [15], this value is qualified according to The National Agency of Drug and Food Control (NADFC = BPOM) [16]. Gamma irradiation technique has several advantages including high penetrating energy, is processed at room temperature, leaving no residue, environmentally friendly, and can be packaged in the end products [13,16].

In spite of gamma irradiation has been used by some of the herbal medicine industries, but the effect of gamma irradiation on the efficacies has not been studied yet. In previous study, gamma

irradiation with doses up to 7.5 kGy on the *mahkota dewa* bark could be used for decontamination of pathogenic microbes and to extend the self life without affecting the cytotoxic activity on L1210 leukemia cancer cells [15]. In this report, the samples which had been irradiated at the dose of 7.5 kGy were examined the bioactivity in vitro against human cancer cell lines, namely: cervical cancer cells HeLa, leukemia THP-1, lymphoma HUT-78, and lung carcinoma A-549. The aim of this study was to examine whether the irradiation dose of 7.5 kGy does not eliminate there efficacies as anticancer based on their bioactivity against human cancer cell lines.

## EXPERIMENTAL METHODS

### General information

Flush column chromatography was carried out on silica gel 60 (70 to 230 mesh ASTM, Merck). Thin-layer chromatography on silica gel 60 F<sub>254</sub> plate (Merck) was used for monitoring and checking the fractions collected from column chromatography. The spots on TLC were detected by short (254 nm) and long wavelength (366 nm) ultraviolet light and visualized by spraying the plates with 1% Ce (SO<sub>4</sub>)<sub>2</sub> in 10% aqueous sulfuric acid followed by heating. Concentrating of extracts and fractions were done by using a rotary evaporator *in vacuo* in temperature of 35°C followed by drying in oven desiccator *in vacuo* at 40°C until fix weight of the fractions were obtained. Analysis components of fractions by HPLC was done on a Shimadzu LC-6A equipped with UV-detector at 210 nm, C-18 reverse phase coloumn of varian microsorb MV 100-5 with dimension of 250 mm length and 4.6 mm inner diameter, and the comparison of mobile phase methanol to water was 7:3.

### Materials and apparatus

The leaves of *mahkota dewa Phaleria macrocarpa* (Scheff.) Boerl were collected from Cibuteung village, Parung, West Java, Indonesia in May 2006 as described in previous report, and determined by Herbarium Bogoriensis, Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia [11]. The leaves of *mahkota dewa* were then dried, sliced and blended.

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

Chemicals used in this study were *n*-hexane, ethyl acetate, ethanol, tryphan blue 0.4 %, RPMI Medium-1640, fetal bovine serum (FBS), liquid nitrogen, distilled water, aquabidest, chloroform, methanol, cerium sulfate, H<sub>2</sub>SO<sub>4</sub>, silica gel 60 (70-230 mesh), celite 545, GF<sub>254</sub> silica plate.

Apparatus used were Gamma Irradiator from cobalt-60 with an activity of 140 kCi, high performance liquid chromatograph (Shimadzu LC 6-A), column chromatograph (diameter 8 cm and length 90 cm), vacuum rotary evaporator (Büchi), oven (Prolabo), multi-well plate tissue's culture, haemocytometer Neubauer (0.100 mm × 0.0025 mm<sup>2</sup>), hot plate, ultrasonicator, oven desiccator, analytical balance (Mettler Toledo), autoclave (S4-ON), incubator, microscope.

### Preparation and irradiation of samples

The dried *mahkota dewa* leaves was powdered, then was weighed each 100 g and put in polyethylene bags and sealed with a sealer machine. Ten samples were preferred. The each two samples were irradiated at a dose of 0 (control), 5, 7.5, 10, and 15 kGy with dose rate of 10 kGy/h. Determination of the dose was done by Fricke solution as a primary standard dosimeter consisting of 0.001 M of ferrous ammonium sulfate and 0.001 M of sulfuric acid.

### Preparation and separation of extracts by column chromatograph

The control and irradiated samples were macerated as much as four times, each time with 1.4 L of *n*-hexane. The residue was again extracted with ethyl acetate and then with ethanol with the same procedure as maceration with *n*-hexane. The each filtrate obtained was concentrated using a rotary evaporator followed by drying in oven desiccators *in vacuo*.

The most active extract against leukemia L1210 cell line was fractionated using silica gel column chromatograph. Amount of 1.0 g of extract was homogenized with celite, then subjected into column chromatograph and was gradiently eluted by *n*-hexane - ethyl acetate - methanol (1:0:0, 5:1:0, 3:1:0, 0:1:0, 0:1:1, 0:0:1). Each fraction was collected with a volume of 150 ml. The fraction obtained was concentrated using a rotary evaporator followed by drying in oven desiccator *in vacuo*. The obtained

fractions were analyzed by TLC and the fractions that had a similar spot pattern were merged.

### Analysis of fractions by HPLC

The most active fractions from control and irradiated samples which have been examined against leukemia L1210 were then analyzed by high performance liquid chromatograph (HPLC). Amount of 1.6 mg fraction was dissolved in 1 ml methanol, filtered with membrane filter (0.45 µm), then 20 µl of solution was injected into the HPLC.

### Cytotoxic activity test against mouse leukemia L1210 cell line [12]

Exponentially growing leukemia L1210 cells at a density of  $2 \times 10^5$  cells/ml suspended in RPMI-1640 medium was placed into serocluster 24 wells plate, the sample solution of extracts or fractions in various concentrations were added then incubated at 37°C for 48 h under 5% CO<sub>2</sub> condition. The varying concentration of extracts were 0 (control), 5, 10, 20, 40, and 80 µg/ml, meanwhile the varying concentration of fractions were 0 (control), 1, 2, 4, 8, and 16 µg/ml. Control consisted of exposed to fresh medium only. Duplicate wells were prepared for each concentration of extracts or fractions and control. Ninety µl of cell suspension in every well was pipetted and put into serocluster plate and 10 µl of tryphan blue was added and the mixture was homogenized. Furthermore, 10 µl of suspension was put in haemocytometer and the amount of viable cells of the samples and control were enumerated by microscope. The inhibition percentage was calculated as the following equation.

$$\% \text{ inhibition} = \frac{[\sum \text{ cells in control}] - [\sum \text{ cells in sample}]}{[\sum \text{ cells in control}]} \times 100 \%$$

By making the graph of sample concentration in logarithm (*X* axes) versus probit of % inhibition (*Y* axes), the linear regression equation  $Y = aX + b$  is obtained. The inhibition concentration-fifty (IC<sub>50</sub>) which expresses the ability of the samples to inhibit 50% of cancer cell proliferation is calculated by the substitution of *Y* by 5 (probit value of 50) to the linear regression equation  $Y = aX + b$ . Subsequently, IC<sub>50</sub> value = antilogarithm of  $X = \{\text{antilogarithm of } (5-b)/a\}$  can be determined.

### Data analysis

The data of analysis IC<sub>50</sub> values and HPLC chromatogram for studying the effect of

gamma irradiation toward cytotoxic activity of the samples, was performed by one-way ANOVA using SPSS 17.0 for windows in the confidence level of 95%.

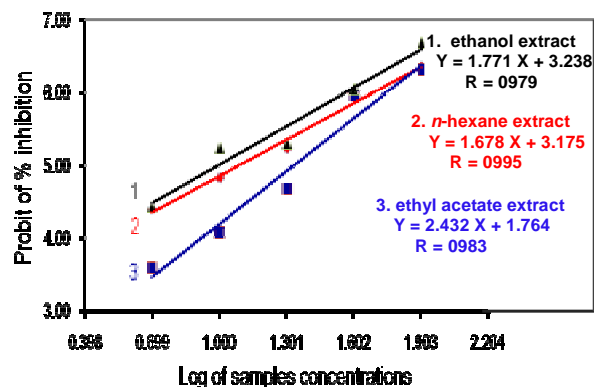
## RESULTS AND DISCUSSION

Maceration from 100 g of control and irradiated samples by *n*-hexane, ethyl acetate, and ethanol was shown in Table 1. It can be seen that the yield of *n*-hexane extracts from control and irradiated samples did not significantly change, but the yield of ethyl acetate extracts as well as ethanol extracts from irradiated samples at doses of 10 and 15 kGy, slightly increased. This is apparently due to radiation energy causing the samples become soft and brittle, making the chemical components was more easily extracted.

**Table 1.** The yield of extracts from control and irradiated mahkota dewa leaves.

Dose (kGy)	yield of <i>n</i> -hexane extract (%)	yield of ethyl acetate extract (%)	yield of ethanol extract (%)
0	2.3	5.9	18.8
5	2.4	5.9	19.2
7.5	2.4	5.3	19.2
10	2.3	<b>7.1</b>	<b>19.5</b>
15	2.4	<b>7.1</b>	<b>19.7</b>

Furthermore, the extracts from control sample (non irradiated sample) then were assayed their cytotoxic activity against leukemia L1210 cells line. The obtained inhibition percentage value was converted to probit value and then was performed in linear curve versus log of ethyl acetate extract concentration as seen in Fig. 1. Inhibition concentration fifty (IC<sub>50</sub>) value was calculated based on the linear equation  $Y = 5$ , then it was obtained the concentration as IC<sub>50</sub>. The results showed that all extracts exhibited cytotoxic activity with an IC<sub>50</sub> < 30 µg/ml (Table 2).



**Fig. 1.** Curve of log of samples concentrations versus probit of inhibition percentage.

**Table 2.** Inhibition percentage of ethanol, *n*-hexane, and ethyl acetate extracts from control sample against leukemia L1210 cells and its IC<sub>50</sub> value.

Sample	Sample concentration (µg/ml)	Inhibition (%)	IC <sub>50</sub> (µg/ml); regression eq.; linearity value, R
ethanol extract	5	28.2	$Y = 1.771 X + 3.238$ R = 0.979
	10	59.7	
	20	61.8	
	40	85.4	
	80	95.4	
<i>n</i> -hexane extract	5	27.4	$Y = 1.678 X + 3.175$ R = 0.995
	10	43.4	
	20	59.7	
	40	83.3	
	80	91.3	
ethyl acetate extract	5	8.2	$Y = 2.432 X + 1.764$ R = 0.983
	10	18.0	
	20	37.6	
	40	64.5	
	80	90.6	

According to the American National Cancer Institute quoted by Nor Aini *et al.* [17] and Magalhaes *et al.* [18], an extract exhibits a potent anticancer if the IC<sub>50</sub> value < 30 µg/ml, while according to Mans *et al.* [19], an extract exhibits a potent anticancer if the IC<sub>50</sub> value < 50 µg/ml. Among the extracts, the ethyl acetate extract gave highest cytotoxicity against leukemia L1210 cells (IC<sub>50</sub> 10.3 µg/ml) compared to *n*-hexane extract (12.4 µg/ml) and ethanol extract (IC<sub>50</sub> 24.1 µg/ml), so in this experiment, ethyl acetate extract has been chosen to be further separation using column

chromatograph. Subsequently, to study the effect of irradiation onto the leaves of *mahkota dewa*, the ethyl acetate extract from irradiated sample were then assayed and also further separation using column chromatograph as the same manner as treatment of the control sample.

Fractionation of ethyl acetate extract from control sample gave 7 fractions (Fr.1-Fr.7). Furthermore the Fr.1-Fr.7 were then assayed against leukemia L1210 cells as the same manner as bioassay of extracts, gave  $IC_{50}$  values as presented in Table 3. Since, Fr.2 was showed the lowest  $IC_{50}$  value (1.9  $\mu\text{g/ml}$ ) (Table 3), it indicated the Fr.2 was the most active fraction which exhibited cytotoxic activity against leukemia L1210 cells, so assayed the fractions from irradiated samples was done toward only Fr.2.

**Table 3.** The yield and  $IC_{50}$  value Fr.1-Fr.7 of ethyl acetate extract from control (non irradiated) sample against leukemia L1210 cells.

Sample	Yield		$IC_{50}$ ( $\mu\text{g/ml}$ )
	mg	%	
Fr.1	6	0.6	4.2
<b>Fr.2</b>	21	2.1	<b>1.9</b>
Fr.3	18	1.8	4.3
Fr.4	174	17.4	4.4
Fr.5	72	7.2	5.6
Fr.6	676	67.6	5.0
Fr.7	30	3.0	5.0

Table 4 showed the comparison of  $IC_{50}$  values of ethyl acetate extracts and Fr.2 from control and irradiated samples. Based on the  $IC_{50}$  values of ethyl acetate extract and Fr.2, it appeared that gamma irradiation tends to cause the  $IC_{50}$  value increased, which means the cytotoxic activity decreased, but the increasing of  $IC_{50}$  value at irradiation dose until 15 kGy has not exceeded the limit of an extract and the fraction was declared inactive [17]. Nevertheless, irradiation at the dose of 7.5 kGy caused the  $IC_{50}$  values of Fr.2 significantly increased. So it is recommended

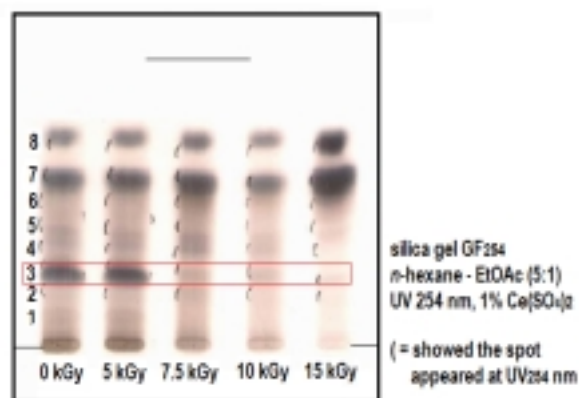
that the irradiation of mahkota dewa leaves for decontamination of bacteria and fungus/yeast and for preserving and extending its self life without eliminating the cytotoxic activity was no more than 7.5 kGy.

**Table 4.**  $IC_{50}$  comparison of ethyl acetate extract and Fr.2 from control and from irradiated samples.

Sample	$IC_{50}$ ( $\mu\text{g/ml}$ )				
	control	5 kGy	7.5 kGy	10 kGy	15 kGy
ethyl acetate extract	10.3 <sup>a</sup>	14.7 <sup>b,c</sup>	14.2 <sup>b,c</sup>	13.2 <sup>a,b</sup>	17.1 <sup>c</sup>
Fr.2	1.9 <sup>a</sup>	2.4 <sup>ab</sup>	3.1 <sup>b</sup>	4.0 <sup>c</sup>	5.8 <sup>d</sup>

Note: Similar letters as superscript indicate that they were not significantly difference.

Furthermore, to study the effect of gamma irradiation on the components presence in the Fr.2, the TLC spot and HPLC chromatogram profiles of Fr.2 from control and irradiated samples were compared. Figure 2 shows the TLC spots profile of Fr.2 from control and irradiated samples. It appeared that at least 8 spots included in the Fr.2 from control sample (0 kGy). Irradiation dose of 7.5, 10, and 15 kGy caused spot 3 significantly decreased, meanwhile spots 7 and 8 seem to be increased at irradiation dose of 15 kGy. The decreasing of spot 3 means those compounds degraded to another compounds, probably become compound included in the spots 7 and 8. It suggested that the spot 3 contained the active substances which have cytotoxic activity against leukemia L1210 cells.



**Fig. 2.** TLC spots profile of Fr.2 from control and irradiated samples.

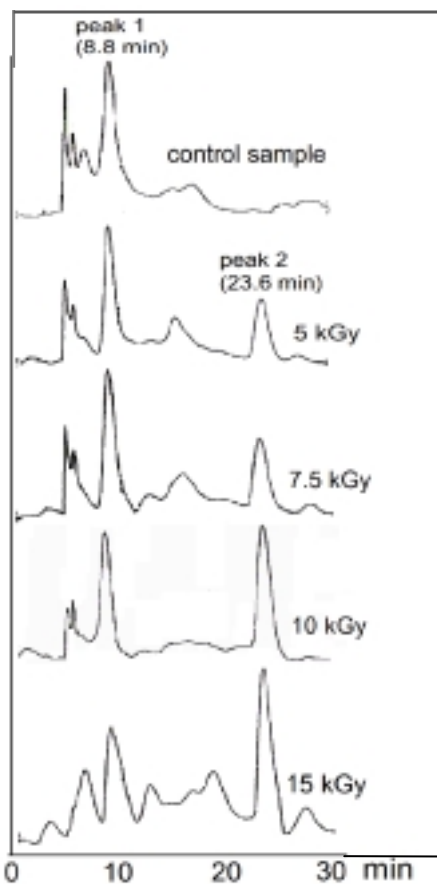


Fig. 3. HPLC chromatogram profile of Fr.2 from control and irradiated samples.

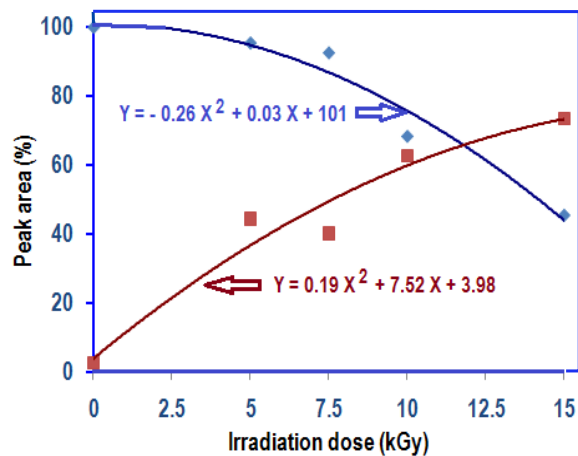


Fig. 4. The effect of irradiation dose of peak 1 and peak 2 of HPLC chromatogram profile of Fr.2.

Further analysis of Fr.2 by HPLC equipped with UV detector at 210 nm showed that there was major peak at retention time of 8.8 min (peak 1) and other minor peaks, such as peak 2 at retention time of 23.6 min (Fig. 3). Peak 1 significantly decreased with increasing the irradiation dose by follows the quadratic equation ( $Y = -0.26 X^2 + 0.03 X + 101$ ), meanwhile peak 2

was increased with increasing the irradiation dose by follows the quadratic equation ( $Y = 0.19 X^2 + 7.52 X + 3.98$ ). The changed of peaks 1 and 2 was shown in Fig. 4.

Reducing peak 1 in HPLC chromatogram of Fr.2 as well as spot 3 in TLC spot profile is likely correlated with reducing cytotoxic activity of Fr.2 due to the increasing radiation dose. It also suggested that the peak 1 was the active substance which has cytotoxic activity against leukemia L1210 cells.

## CONCLUSION

Gamma irradiation causes changes in the TLC spots and HPLC chromatograms profiles of fraction 2 (Fr.2) which was the most cytotoxic fraction in ethyl acetate extract of *mahkota dewa* leaves against leukemia L1210 cells. One of the main peak (peak 1) on HPLC chromatograms decreased, whereas the other peak (peak 2) which was a small peak in HPLC chromatogram of fraction 2 from non irradiated sample increased with increasing the irradiation dose. In agree with the decreasing peak 1 in chromatogram of fraction 2 due to irradiation, cytotoxic activity of fraction 2 also decreased along with increasing doses of irradiation. It suggested that peak 1 is the component that contributes to the cytotoxic activity of fraction 2 in ethyl acetate extract of *mahkota dewa* leaves.

Gamma irradiation dose of 7.5 kGy on *mahkota dewa* leaves could decreased the cytotoxic activity of fraction 2 as the most cytotoxic-potential fraction against leukemia L1210 cells, but decreasing the cytotoxic activity has not exceeded the limit of the fraction declared inactive. So that the irradiation dose up to 7.5 kGy can be use for decontamination of bacteria and fungus/yeast without eliminating the cytotoxic activity.

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