

IN VITRO BIOACTIVITY TEST OF IRRADIATED MAHKOTA DEWA BARK (*Phaleria macrocarpa* (Scheff.) Boerl.) AGAINST HUMAN CANCER CELL LINES

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ABSTRACT

Gamma irradiation has been used to preserve an herbal medicine, but it has not been known the effects of gamma irradiation on their bioactivity as an anticancer agent yet. In the previous study, the gamma irradiation on mahkota dewa bark with the optimum dose of 7.5 kGy could be used for decontamination of bacteria and fungus/yeast. In this report, the effect of gamma irradiation with the dose of 7.5 kGy on the bioactivities of mahkota dewa (*Phaleria macrocarpa* (Scheff.) Boerl.) bark against leukemia L1210 cells was studied. The control and irradiated samples were successively macerated with *n*-hexane and ethyl acetate. In the previous results, silica gel column chromatography of ethyl acetate extract of non irradiated sample (control) gave 8 fractions. Among these fractions, fraction 6 indicated the most cytotoxic-potential fraction, so that in this experiment, the ethyl acetate extract of irradiated and non irradiated sample were fractionated with the same manner as previous fractionation. The fraction 6 obtained both from control and irradiated samples were then assayed their inhibitory activities against 4 kinds of human cancer lines, i.e. HeLa, THP-1, HUT-78 and A-549. The results showed that the fraction 6 from control sample gave IC_{50} values of 3.65, 5.59, 3.55, and 4.06 $\mu\text{g/mL}$, against HeLa, THP-1, HUT-78 and A-549, respectively, meanwhile fraction 6 from irradiated sample gave IC_{50} values of 8.26, 7.02, 5.03, and 5.59 $\mu\text{g/mL}$, respectively. Gamma irradiation dose of 7.5 kGy on mahkota dewa bark could decreased the cytotoxic activity of fraction 6 as the most cytotoxic-potential fraction against HeLa, THP-1, HUT-78 and A-549 cancer cell lines, but decreasing the cytotoxic activity has not exceeded the limit of an extract and the fraction declared inactive. So that the irradiation dose of 7.5 kGy can be use for decontamination of bacteria and fungus/yeast without eliminating the cytotoxic activity.

Keywords: gamma irradiation; cytotoxicity; human cancer cell lines; *Phaleria macrocarpa*; mahkota dewa bark

ABSTRAK

Iradiasi gamma telah digunakan untuk pengawetan obat herbal, tetapi belum diketahui pengaruh iradiasi gamma terhadap bioaktivitasnya sebagai antikanker. Pada penelitian pendahuluan, iradiasi gamma pada batang mahkota dewa dengan dosis optimum 7,5 kGy dapat digunakan untuk dekontaminasi bakteri dan kapang kamir. Dalam laporan ini, pengaruh iradiasi gamma dosis 7,5 kGy pada bioaktivitas batang mahkota dewa (*Phaleria macrocarpa* (Scheff.) Boerl.) terhadap sel leukemia L1210 telah dipelajari. Sampel kontrol dan yang diiradiasi dimaserasi dengan *n*-heksan dan etil asetat. Pada penelitian sebelumnya, dari difraksinasi ekstrak etil asetat sampel kontrol (yang tidak diiradiasi) dengan kromatografi kolom silika diperoleh 8 fraksi. Diantara fraksi-frasi ini, fraksi 6 menunjukkan fraksi yang paling berpotensi sitotoksik, maka pada penelitian ini, ekstrak etil asetat dari sampel yang tidak dan yang diiradiasi difraksinasi dengan cara yang sama seperti fraksinasi sebelumnya. Fraksi 6 diperoleh dari kedua sampel kontrol dan yang diiradiasi diuji aktivitas penghambatannya terhadap 4 jenis sel kanker manusia, yaitu HeLa, THP-1, HUT-78 dan A-549. Hasilnya menunjukkan bahwa fraksi 6 dari sampel kontrol memberikan nilai IC_{50} 3,65; 5,59; 3,55; dan 4,06 $\mu\text{g/mL}$ masing-masing terhadap sel kanker HeLa, THP-1, HUT-78 dan A-549, sedangkan fraksi 6 dari sampel yang diiradiasi memberikan nilai IC_{50} masing-masing yaitu 8,26; 7,02; 5,03; dan 5,59 $\mu\text{g/mL}$. Iradiasi gamma dosis of 7,5 kGy pada batang mahkota dewa dapat menurunkan aktivitas toksisitas fraksi 6 sebagai fraksi yang berpotensi sitotoksik terhadap sel kanker HeLa, THP-1, HUT-78 dan A-549, tetapi penurunan aktivitas sitotoksik tersebut tidak melebihi batas suatu ekstrak dan fraksi dinyatakan tidak aktif. Dosis iradiasi 7,5 kGy dapat digunakan untuk dekontaminasi bakteri dan kapang kamir tanpa mengurangi aktivitas sitotoksiknya.

Kata Kunci: iradiasi gamma; sitotoksitas; sel kanker manusia; *Phaleria macrocarpa*; batang mahkota dewa

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INTRODUCTION

Indonesia is one of country that hereditary use medicinal plants for treating some diseases. The use of herbal medicines is believed to have small side effects compared to the use of synthetic drugs. Herbal medicines are not only used by people in rural areas but also used by communities in urban areas. Therefore, searching for sources of new drugs in order to give an added value of these medicinal plants has been encouraged.

Mahkota dewa (*Phaleria macrocarpa* (Scheff.) Boerl) is one of the medicinal plants which grow in Indonesia. Empirically, mahkota dewa are used for herbal treatment. The stems are used for treatment of bone cancer; the leaves are used for treatments of impotency, blood diseases, allergies, diabetes mellitus, and tumor. Eggshell of seeds are used for breast cancer, cervix cancer, lung diseases, liver and heart diseases [1]. Fruits are consisting of alkaloid, saponin, flavanoid, and polyphenol [2].

In recent years, chemical and biological studies on constituents of mahkota dewa have continuously encouraged. Wahyuningsih et al. [3] found a benzophenone glucoside from the leaves of mahkota dewa, so called phalerin (4,5-dihydroxy,4'-methoxybenzophenone-3-O- β -D-glucoside, recently this structure has been revised by Oshimi [4] as 4',6-dihydroxy-4-metoxibenzophenone-2-O- β -D-glucoside. This compound has been isolated from *Gnidia involucreta* by Ferrari et al. [5] in 2000. The same compound have also isolated from the bark of mahkota dewa [6]. Biological activity test on phalerin showed that this compound was non toxic and has a potent immunostimulant [7], meanwhile inhibitory activity test against mouse leukemia L1210 cell line showed that this compound exhibited inhibitory activity with an IC_{50} 5.1 μ g/mL [6]. The new benzophenone glucoside derivative, 4,4'-dihydroxy-6-methoxybenzophenone-2-O- β -glucopyranoside so called mahkoside A has also been isolated by Zhang et al. besides six known compounds including mangiferin, kaempferol-3-O- β -D-glucoside, dodecanoic acid, palmitic acid, ethyl stearate and sucrose [8].

Although the search for new compounds have continuously been done, but herbal medicines in the form of tablets or capsules have been used for treatments of some diseases. Since, dry herbs easily damaged during storage due to microbial contamination, it cause reducing the efficacy and quality of herbal medicines. To overcome this problem, gamma irradiation up to 10 kGy dose reduced the number of bacteria on mahkota dewa dried powder from 10^{10} colonies/g to 10^5 colonies/g [9], this value is qualified according to the Food and Drug Administration (BPOM) [10]. Gamma

irradiation for the decontamination of pathogenic microbes and to extend the shelf life has been declared safe and it is regulated by the Ministry of Health No. 701/MENKES/PER/VIII/2009. 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 [11-12].

Effect of γ -radiation on the herbal medicines observed by some researchers, including its effects on active components, antimicrobial, and antioxidants activity. Irradiation treatment (10 – 50 kGy) and storage time did not give a significant change on curcumin content of *Curcuma domestica* rhizome [13]. Gamma irradiation treatment up to 20 kGy does not affect the antifungal and antibacterial activity of *Glycyrrhiza glabra* root, however, phenolic contents increased in samples treated with 20 and 25 kGy doses [14]. Gamma irradiation (10 – 60 kGy) decreased tannin and antioxidants activity of pistachio (*Pistachia vera*) hull [15]. In spite of gamma irradiation has been used by some of the herbal medicine industries, but the effect of gamma irradiation on the antiproliferation activity has not been studied yet. The effect of γ -irradiation on the bioactivity of herbal medicine such as mahkota dewa bark was interesting to be observed. 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 causing changes in cytotoxic activity on L1210 leukemia cancer cells [16]. In this report, the mahkota dewa bark 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 bioactivities against human cancer cell lines.

EXPERIMENTAL SECTION

General Information

Flash column chromatography was carried out on silica gel 60 (70–230 mesh ASTM, Merck). The removal of solvents of extracts, fractions and isolates were done by rotary evaporator under reduced pressure, followed by drying in oven desiccator *in vacuo*. Thin-layer chromatography on silica gel 60 F₂₅₄ plate (Merck) was used for monitoring of reaction kinetic and checking the fractions collected from column chromatograph. The spots on TLC were detected by short and long wavelength ultraviolet light

and visualized by spraying the plates with 1% $\text{Ce}(\text{SO}_4)_2$ in 10% aqueous sulfuric acid followed by heating.

Materials

The plant of *mahkota dewa* is collected from Cibuteung village, Parung, West Java, Indonesia in May 2006, and determined by Herbarium Bogoriensis, Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia [17]. The bark of *mahkota dewa* was then dried, sliced and blended.

The chemicals used in the research were *n*-hexane, ethyl acetate, methanol, human cancer cell lines, namely cervical cancer cells HeLa (human cervix epitheloid carcinoma), leukemia THP-1 (human peripheral blood acute monocyte), lymphoma HUT-78 (human cutaneous T-cell lymphoma), and carcinoma A-549 (human lung carcinoma) were obtained from Cell Culture Laboratory, Division of Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor. Other materials were Dulbecco's modified Eagle's medium (DMEM/F-12), fetal bovine serum (FBS), 10% of phosphate buffer saline (PBS), dimethyl sulfoxide (DMSO), 0.4% of trypan blue, liquid nitrogen.

Instrumentation

The equipments used in this research were cobalt-60 gamma irradiator, rotary evaporator, column chromatograph, analytical balance, incubator CO_2 , and microscope.

Procedure

Irradiation of samples

The blended of dried *mahkota dewa* bark (100 g) was put in polyethylene bag, sealed using sealer machine, then was irradiated by cobalt-60 gamma irradiator with the dose of 7.5 kGy at dose rate of 10 kGy/h and another polyethylene bag containing 100 g sample was not irradiated and used as control. Each treatment was carried out in duplicates.

Extraction of samples

The control and irradiated samples (each 100 g) were macerated in 700 mL of *n*-hexane, the residues were then macerated by 700 mL of ethyl acetate. Each extraction was repeated three times. The each collected filtrates were concentrated by rotary evaporator then drying in oven desiccator *in vacuo* to give *n*-hexane and ethyl acetate extracts.

Column fractionation of ethyl acetate extract

Amount of 1.0 g ethyl acetate extract from control and irradiated samples were dissolved in chloroform-

methanol, subsequently 6.5 g of *celite* 545 was added, homogenized then the solvent was removed using rotary evaporator. The dried powder of extract-celite was subjected into silica gel column chromatograph (30 g), then gradiently eluted by *n*-hexane - ethyl acetate - methanol (3:1:0 to 0:0:1). Every 150 mL of eluate was collected and then the solvent was removed by rotary evaporator. The fractions which had the similar spot pattern were combined, gave 8 fractions, Fr.1 ~ Fr.8.

Bioassay method

Cancer cell lines in DMEM/F-12 medium containing 2×10^5 cell/mL was placed into the serocluster 24 wells plate, then the sample solution (in DMSO) of ethyl acetate extract from irradiated and non irradiated sample was added in 6 various concentrations, i.e.: 0 $\mu\text{g/mL}$ (control), 5, 10, 20, 40, and 80 $\mu\text{g/mL}$. Doxorubicin (0.6 $\mu\text{g/mL}$) was used as positive control. Each concentration was carried out in triplicates. The plates were then incubated at 37 °C for 72 h under 5% CO_2 condition. Ninety μL of cell suspension in every well was pipetted and put into serocluster plate (96 well) and 10 μL of trypan blue was added and the mixture was homogenized. Furthermore, 10 μL of suspension was put in haemocytometer and the amount of viable cells and death cells were enumerated by microscope. The bioactivity was calculated as the following equation;

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

By making the graph of sample concentration in logarithm (X axes) versus probit of % bioactivity (Y axes), the linear regression equation, $Y = aX + b$ is obtained. The inhibition concentration-fifty (IC_{50}) 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_{50} value = antilogarithm of X {= antilogarithm of $(5-b)/a$ } can be determined.

RESULT AND DISCUSSION

Extract Preparation and Column Fractionation

Maceration of control and irradiated samples by *n*-hexane gave 1.2 g (1.2%) and 1.2 g (1.2%). Furthermore, maceration of residues by ethyl acetate gave ethyl acetate extracts in the same yield (2.0 g, 2%). Maceration of irradiated and control samples using ethyl acetate did not give the different yield (Table 1). Since, in the previous work, the ethyl acetate extract gave highest cytotoxicity against leukemia L1210

Table 1. The extract yield of non irradiated (control) and irradiated *mahkota dewa* bark

Extract	Yield of control sample		Yield of irradiated sample	
	g	%	g	%
<i>n</i> -hexane	1.2	1.2%	1.2	1.2%
ethyl acetate	2.0	2.0%	2.0	2.0%

Table 2. The yield of column fractionation of 1.0 g ethyl acetate extract of irradiated *mahkota dewa* bark and control

Fraction	color	Yield of control sample (%) ^a	Yield of irradiated sample (%) ^a
Fr.1	dark green	3.10	3.05
Fr.2	dark green	2.70	2.70
Fr.3	dark green	2.90	2.95
Fr.4	dark green	3.30	3.30
Fr.5	dark green	5.60	5.60
Fr.6	blackish green	52.10	52.05
Fr.7	dark brown	18.30	18.30
Fr.8	dark brown	10.2	10.2

^a Average from replicates

cancer cells compared to *n*-hexane and ethanol extracts, so in this experiment, ethyl acetate extract has been chosen as parameter for studying the effect of gamma irradiation onto bioactivity of *mahkota dewa* bark against human cancer cell lines. Column fractionated of each extract of control and irradiated samples showed no difference in yield of each fraction obtained. Table 2 showed the yield of control and irradiated samples. Among these fractions, Fr.6 represented the highest yield (52.1%) which was phalerin included in this fraction. Similarly, the color of fractions from irradiated and control samples did not difference

Bioactivity Test of Ethyl Acetate Extract against Human Cancer Cell Lines

As described in the previous work that the ethyl acetate extract gave highest cytotoxicity against leukemia L1210 cancer, so ethyl acetate extract was further examined its bioactivity against 4 kinds of human cancer cell lines, namely HeLa, leukemia THP-1, lymphoma HUT-78, and carcinoma A-549 is shown in Table 3. The IC₅₀ values of ethyl acetate extract from the irradiated *mahkota dewa* bark against all examined cancer cells seem to be decreased, nevertheless decreasing of these cytotoxic activity values has not exceeded the limit of an extract declared inactive. According to the American National Cancer Institute quoted by Aini et al. [18], an extract exhibits a potent anticancer if the IC₅₀ value < 30 µg/mL, while according to Mans et al. [19], an extract exhibits a potent anticancer if the IC₅₀ value < 50 µg/mL. As seen in Table 3, the cytotoxic activity of ethyl acetate extract from

irradiated sample was still less than 30 µg/mL. This results provided an illustration that irradiation dose up to 7.5 kGy did not damage the anticancer substances included in ethyl acetate extract of *mahkota dewa* dried powder. This means that irradiation dose up to 7.5 kGy can still be used to eliminate pathogenic microbes and to extend the shelf life of *mahkota dewa* dried powder that is claimed as anticancer herbal medicine without damaging its efficacy.

Bioactivity Test of Fr.6 against Human Cancer Cell Lines

IC₅₀ value of Fr.6 of non irradiated *mahkota dewa* bark (control) against 4 kinds of human cancer cell lines HeLa, leukemia THP-1, lymphoma HUT-78, and carcinoma A-549 were 3.7, 5.6, 3.6, and 4.1 µg/mL, respectively. Meanwhile, IC₅₀ values of Fr.6 of irradiated *mahkota dewa* bark were 8.3, 7.0, 5.0, and 5.6 µg/mL, respectively (Table 4). The IC₅₀ values of Fr.6 from irradiated sample against all cancer cells to be examined seem to be decreased, nevertheless decreasing of these cytotoxic activity values was still less than 10 µg/mL, although it is no threshold limiting value of IC₅₀ for fraction, the IC₅₀ value of fraction less than 20 µg/mL is feasible to be further exploited as a potent anticancer substances. As well as the ethyl acetate extract, this results means that irradiation treatment at the dose up to 7.5 kGy did not damage the anticancer substances included in ethyl acetate extract of *mahkota dewa* dried powder. This means that irradiation dose up to 7.5 kGy can still be used to eliminate pathogenic microbes and to extend the shelf life of *mahkota dewa* dried powder that is claimed as anticancer herbal medicine without damaging its efficacy based on the bioactivity test of Fr.6 against four kinds of human cancer cell lines, namely HeLa, leukemia THP-1, lymphoma HUT-78, and carcinoma A-549. Table 4 showed that the increasing the IC₅₀ value of Fr.6 included in the ethyl acetate extract of *mahkota dewa* bark against HeLa was the most significant. The IC₅₀ increased from 3.7 to 8.3 µg/mL (125%). This result was supported by the result of previous research [16] reported that the cytotoxic activities against leukemia L1210 cells of ethyl acetate extract from control and irradiated sample with dose of 7.5 kGy were 11.9 and 16.4 µg/mL. The Fr.6 had IC₅₀ values 7.8 for control sample and 9.1 µg/ml for irradiated 7.5 kGy sample. That research also showed that the chromatogram profiles of Fr.6 of ethyl acetate extract had 2 major peaks with retention time 12.69 and 18.64 min. The peak with retention time 12.69 min was phalerin compound, control sample had 0.025% phalerin and decreased become 0.013% in irradiated sample. The significant decreasing of phalerin in

Table 3. Bioactivity of ethyl acetate extract of non irradiated (control) and irradiated *mahkota dewa* bark against human cancer cell lines (HeLa, THP-1, HUT-78, and A-549)

human cancer cell lines	IC ₅₀ (µg/mL)		Increasing of IC ₅₀	
	Non irradiated sample (control)	Irradiated sample (7.5 kGy)	(µg/mL)	(%)
HeLa	7.7	11.9	4.2	35
THP-1	9.8	10.7	0.9	8
HUT-78	8.0	9.4	1.4	15
A-549	9.0	12.0	3.0	25

Table 4. Bioactivity of Fraction 6 of non irradiated (control) and irradiated *mahkota dewa* bark against human cancer cell lines (HeLa, THP-1, HUT-78, and A-549)

human cancer cell lines	IC ₅₀ (µg/mL)		Increasing of IC ₅₀	
	Non irradiated sample (control)	Irradiated sample (7.5 kGy)	(µg/mL)	(%)
HeLa	3.7	8.3	4.6	124
THP-1	5.6	7.0	1.4	25
HUT-78	3.6	5.0	1.4	39
A-549	4.1	5.6	1.5	37

irradiated sample did not change the antiproliferation activity IC₅₀ value < 30 µg/mL, it was still in active category a potent anticancer [18]. The yield were not changed on Table 1, but on the previous study reported that the peak areas of major and minor components in the HPLC profiles were decrease due to gamma irradiation at doses of ≥ 10 kGy [16]. These suggested that there were degradation of the components which had efficacious as cancer agent, thus disturbing the synergy between these components as demonstrated by the impact on the decline their bioactivities against cancer cells. Nevertheless, the bioactivities were still in the active category of a potent active as anticancer. In the results of previous studies had been reported that microbial contamination on the mahkota dewa bark before irradiated found 2.14 x 10⁸ colonies of bacteria/g and 5.8 x 10⁵ colonies of mold yeasts/g, after irradiated at a dose of 5 kGy to 15 kGy was not found bacteria and molds yeasts anymore. The preservation of herbal ingredients using gamma irradiation technique refers to Codex Commission Alimentarius and the regulation of Health Minister of Republic of Indonesia. The Codex Commission Alimentarius recommends that gamma irradiation doses to 10 kGy absorbed by the irradiated material is a safe dose [20-21]. The regulation of Health Minister of No. 701/MENKES/Per/VIII/2009 about food irradiation also recommends that gamma irradiation dose to 10 kGy is used to reduce the amount of certain pathogenic microorganisms in herbs [22]. But there is no scientific evidence that supports that their bioactivities do not change as a state before the radiation. Therefore, this scientific data is needed as a data base on the preparation of an appropriate dose of gamma irradiation for preservation of Indonesia herbals medicine in

Indonesia Pharmacopoeia. Now in Indonesia Pharmacopoeia only mentioned gamma irradiation technique for preservation of herbal medicines, there is no amount of irradiation dose for a particular herbal medicines or ingredients. The dose 5 kGy to 10 kGy were optimum dose for preservation of *mahkota dewa* flesh fruit without damaging their cytotoxic activities against leukemia L1210 cells [23]. Based on this research that it was concluded the dose of 7.5 kGy were the maximum dose that did not damage the anticancer substances in mahkota dewa bark.

CONCLUSION

Based on the bioactivity results of ethyl acetate extract and fraction 6 included in the *mahkota dewa* bark against human cancer cell lines, namely HeLa, leukemia THP-1, lymphoma HUT-78, and carcinoma A-549, it can be concluded that irradiation dose up to 7.5 kGy does not eliminate the bioactivity of *mahkota dewa* bark as an anticancer, so that doses up to 7.5 kGy can be used to eliminate pathogenic microbes and to extend the shelf life for improving the quality of *mahkota dewa* bark without damaging the efficacy of *mahkota dewa* bark as an anticancer.

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