

The Effect of Gamma Irradiation on Ethanolic Extract of *Temulawak (Curcuma xanthorrhiza Roxb.)* Against Human Cancer Cell Lines

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Received: 25-05-2021

Received in revised form: 16-09-2021

Accepted: 23-09-2021

DOI : 10.17146/jstni.2021.22.1.6221

Keywords: Temulawak, *Curcuma xanthorrhiza*, gamma irradiation, antioxidant, cancer, HUT78, A549, THP1, HeLa

Abstract: One of the medicinal plants that are widely used by the people of Indonesia is temulawak (*Curcuma xanthorrhiza* Roxb.) with the active ingredient is curcuminoid and xanthorrhizol. The curcuminoid is a yellow compound that is believed to have anti-cancer properties, therefore its properties must be maintained in the post-harvest process. This research aimed to study the effect of gamma irradiation on the *Curcuma* Rhizome in inhibiting the proliferative activity against human cancer cell lines. The dry powder of *C. xanthorrhiza* rhizome was irradiated with gamma at a dose of 10 kGy in 2 replications, then extracted using ethanol solvent, evaporated, to obtain an ethanol extract. As a control, an un-irradiated sample was prepared and extracted in the same manner. Each ethanol extract from irradiated and control samples was then tested for their antioxidant activity by DPPH method and antiproliferative activity against human cancer cell lines (HUT78, A549, HeLa, and THP1). The results showed that the ethanol extract of irradiated *C. xanthorrhiza* rhizome with a dose of 10 kGy still had antioxidant activity and anticancer properties based on the bioassay against four those human cancer cell lines, although the antiproliferative activity decreased between 8-30% compared to the control sample. The highest antiproliferative activity was shown by the ethanol extract against HUT78 cancer cell line with IC_{50} values of 5.4 and 10.7 $\mu\text{g/ml}$ for control and irradiated samples respectively. Based on the silica gel TLC plate, the ethanolic extracts of *C. xanthorrhiza* rhizome both unirradiated- and irradiated samples contained curcumin ($R_f = 0.58$) and demethoxycurcumin ($R_f = 0.38$).

INTRODUCTION

Indonesia has a wealth of natural plants that are very abundant and almost 75% of plants in the world are in Indonesia, about 90% of the total medicinal plants in Asia grow in Indonesia (1). Accordingly, Indonesian people already accustomed to using medicinal plants for curing various disease so called herbal medicine ("Jamu" in Indonesian) and commonly contains several components (2).

One of the medicinal plants that are widely used by Indonesian people is ginger rhizome (*Curcuma xanthorrhiza* Roxb.) (Temulawak in Indonesian), which has many benefits with the main active ingredient being curcuminoid and xanthorrhizol (3). Curcumin is a polyphenol compound isolated from the curcuma rhizome which has anti-oxidant, anti-inflammatory, and anti-cancer properties that can affect human cancer cells apoptosis in various of the body organs (4). Therefore, the curcuminoid component must be maintained in the order it is not damaged in its

handling so that its properties are still good. According to H.P. Kusumaningrum *et al.* (5), the handling and preparation of simplicia must follow the good manufacturing practices (GMP), because the wrong processing and handling will cause damage and will reduce the quality and efficacy, moreover, it can even produce mycotoxins which are toxins produced by microbes.

To prevent the product from being easily damaged, *Curcuma sp.* post-harvest processing is still done conventionally such as drying with sunlight or by oven until the moisture content less than 10% (3). Those preservation methods are used for some agricultural products in various countries, and drying by sunlight especially for *Curcuma*-based products can take a long time, 10-15 days (6).

Postharvest technology is needed by industrialists in supporting the scale of production and distribution of fresh raw materials to companies processing raw materials

so that the content and nutrients in the product are maintained (7). In various countries, damage occurs around 10-50% of agricultural products including raw materials for herbal medicines caused by microorganisms (fungi and bacteria), mice, insects, and other environmental factors (8).

The use of irradiation techniques as preservation of fruits and vegetables including herbal medicines has been widely used, in average doses of 0.3 - 0.7 kGy, furthermore the irradiation at medium doses of 1-10 kGy can kill bacteria, fungi, and insects (7). Previously, the study on the effect of gamma irradiation on *Solanum nigrum* L showed that irradiation dose of 7.5 kGy was the recommended maximum dose in which at this dose the microbes could be eliminated without reducing its anti-proliferative activity (9). The previous study on the acute toxicity of ethanolic extract of irradiated *C. xanthorrhiza* Roxb. at a dose of 10 kGy showed that the sample did not appear the acute toxic symptoms in mice as a test animal (10). Another study on ethyl acetate extract of *C. xanthorrhiza* Roxb. showed that irradiation doses up to 10 kGy did not appear the significant decrease in antiproliferative activity. Further fractionation of ethyl acetate extract using column chromatography showed that the irradiation dose at 10 kGy reduced the antiproliferative activity, however, the reduction did not eliminate its antiproliferative activities (11).

A study on anti-cancer activities of *Curcuma sp.* is very useful in developing the potential of Indonesian medicinal plants for overcoming the side effects and the high cost of synthetic cancer drugs. Moreover, post-harvest preservation of medicinal plant products using gamma irradiation is also an alternative that currently becomes a trend to maintain the quality of medicinal plants. According to the previous study, the optimum irradiation dose for the preservation of *C. xanthorrhiza* rhizome was 10 kGy (12). Furthermore, the study acute toxicity of ethanolic extract from *C. xanthorrhiza* rhizome at a dose of 10 kGy has been done, nevertheless its anti-cancer properties have never been done. For this reason, it is necessary to study the anti-cancer efficacy of ethanol extract from irradiated *C. xanthorrhiza* rhizome at a dose of 10 kGy against human cancer cell lines, namely: HeLa cervical cancer, THP1 leukemia, A549 human lung carcinoma, and HUT78 lymphoma cancer cells.

EXPERIMENTAL SECTION

Material and Equipment

Curcuma xanthorrhiza Roxb. rhizome, was obtained from PT. Sido Muncul (Herbal Medicine Industry), ethanol p.a., RPMI 1640 (Gibco), bovine calf serum (Gibco), cancer cells line (Hela cervical cancer, THP 1 leukemia, A549 human lung carcinoma, and HUT78 lymphoma), trypan blue.

In this research, the Natural Rubber Gamma Irradiator at CIRA with Co-60 was used as a gamma irradiator source, CO₂ incubator, vacuum rotary evaporator (Buchi), analytic balance, microscope (Nikon), glassware, multi-well tissue culture plates, and haemocytometer.

Water Content

Determination of water content was performed by gravimetric method using oven at temperature of 105°C. A total of 2 g of *C. xanthorrhiza* powder was put into the oven for 3 h then the sample was weighed, subsequently the heating was continued and weighing for every hour until the sample weight did not change significantly.

Extract Preparation

The dried powder of *C. xanthorrhiza* rhizome was weighed 4 packs with a weight of 200 g each pack, then 2 packs were irradiated with gamma from Co-60 at a radiation dose of 10 kGy, other two packs were un-irradiated as a control. After radiation, each sample was macerated using 3 L of ethanol. Maceration was carried out for 1 day at room temperature and followed by filtration. The filtrates obtained were then evaporated using rotary evaporator to obtain the ethanolic extract.

Curcuminoid contents.

The main curcuminoid content in the extract was determined by thin layer chromatograph (TLC) compared to curcuminoid standards, namely: curcumin, demethoxycurcumin, and bisdemethoxycurcumin. The silica gel TLC plate was used with the mobile phase of chloroform:methanol (20:1). The spots were observed under UV light at $\lambda = 366$ nm and visualized by spraying with cerium sulfate (11).

Antioxidant activity.

Antioxidant activity was determined by DPPH (1,1-diphenyl-2-picrylhydrazil) method. To the samples with the concentrations of 20, 40, 60, 80, and 100 ppm was added 40 ppm of DPPH

solution, the mixture was then homogenized by vortex and incubated at room temperature for 30 min. The absorbance of the samples was measured using spectrophotometer UV-Visible at $\lambda = 517$ nm. The antioxidant activity was expressed based on the half maximum inhibition concentration (IC_{50}) value (13).

Antiproliferative activity test with human cancer cells.

Antiproliferative activity test of the ethanolic extract on several human cancer cell lines (HeLa, THP1, A549, and HUT78) were done by using various concentrations of 5, 10, 20, 40, and 80 $\mu\text{g/ml}$. Prior to use, each cancer cell was cultured until the concentration was 2×10^5 cells, then the ethanolic extract from each variation of the concentration was subjected to the 24-well cell culture plates. The plate containing cells and ethanolic extract of *C. xanthorrhiza* rhizome was incubated in a 5% CO_2 incubator for 72 h, then it was calculated under a microscope at 400x magnification by coloring using trypan blue.

Data analysis.

The half maximum inhibitory concentration (IC_{50}) value which is the concentration of the samples that expresses the ability to inhibit the 50% of cancer cell proliferation is calculated based on the linear regression equation as described in the previous studies (9) (14). The same calculation also applied for antioxidant activity.

The change of IC_{50} value of irradiated sample (10 kGy) was calculated by t-test of IC_{50} values of unirradiated- and irradiated samples pair using SPSS 24 with a 95% confidence level ($\alpha=0.05$).

RESULTS AND DISCUSSION

Water content, Extraction Yields, and Curcuminoid contents

The drying of the sample at room temperature (22°C) for 1 week gave the water content of 8.7%, where the value has met the quality requirements of traditional medicines, which is below 10% (15). The higher the water content, it's easy to be contaminated by fungi and microbes (16).

The yield of ethanolic extract from *C. xanthorrhiza* rhizome is shown in Table 1. There was no significant difference between unirradiated (control) and irradiated *C. xanthorrhiza* rhizome at 10 kGy. This fact clarified that gamma irradiation until a dose of 10 kGy did not damage the *C. xanthorrhiza* rhizome. The selection of

ethanol as an extraction solvent was in accordance with statement of Faraouq (2003) which cited by Riki R *et al.* (12) that ethanol is the best solvent for extracting a plant for herbal medicinal.

Table 1. The weight of ethanolic extract from *C. xanthorrhiza* rhizome

Dose (kGy)	Color of extract	Weight (g)	Yield (%)
0	Brownish yellow	13.3	6.6
10	Brownish yellow	13.2	6.5

The analysis of curcuminoid content using TLC showed that the ethanolic extract of *C. xanthorrhiza* rhizome was curcumin ($R_f = 0.58$) and demethoxycurcumin ($R_f = 0.38$). This curcuminoid content were also found in the fraction 3 of ethyl acetate extract of *C. xanthorrhiza* rhizome (11).

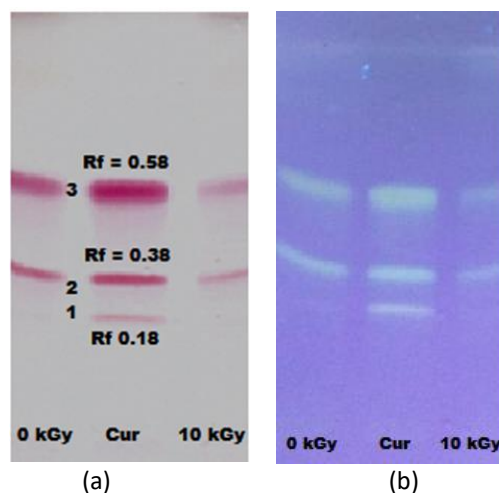


Figure 1. Thin layer chromatogram (TLC) of ethanolic extract from irradiated and unirradiated *C. xanthorrhiza* (a) spraying with reagent cerium sulfate and heating, (b) under the UV lamp at $\lambda=366$ nm

Antioxidant activity

Several studies showed that curcumin has antioxidant, antiinflammatory, and anticancer properties (17). It was clarified that the ethanolic extract of *C. xanthorrhiza* showed an antioxidant activity as presented in Table 2. The IC_{50} values of ethanolic extract of unirradiated- and irradiated samples were 52.2 ppm and 91.5 ppm. The lower of IC_{50} value is the higher its antioxidant activity due to lower samples to be needed for binding free radicals on DPPH. Although the antioxidant activity of

irradiated sample decreased caused by radiation, however the IC₅₀ value is still ≤ 100 ppm (18).

Table 2. Antioxidant activity of ethanolic extract of un-irradiated- and irradiated *C. xanthorrhiza*

Ethanolic extract of un-irradiated sample (0 kGy)						
Concentration (µg/ml)	0	20	40	60	80	100
Inhibition rate (%)*	0	22.8	44.9	58.5	72.0	78.8
Linear regression equation	Y = 0.6959 X + 13.668					
IC ₅₀ (µg/mL)	52.2					
Ethanolic extract of irradiated sample (10 kGy)						
Concentration (µg/ml)	0	20	40	60	80	100
Inhibition rate (%)*	0	8.2	26.1	36.3	46.7	50.2
Linear regression equation	Y = 0.5228 X + 2.1411					
IC ₅₀ (µg/mL)	91.5					

*result from three repetitions

Antiproliferative activity against human cancer cell lines

As seen in Fig. 2.a, 2.b, 2.c, and 2.d, the antiproliferative activity of ethanolic extract against human cancer cell lines (HeLa, THP1, A549, and HUT78) showed that those activities decreased with increasing their sample concentrations,

Subsequently, to find out more specific antiproliferative activity values, the half maximum inhibitory concentration (IC₅₀) value of the samples were calculated using linear regression curve, where the probit of the inhibitory activity percentage as Y-axis and logarithmic of concentration as X-axis as shown in Fig. 3.

From Figure 3, it can be seen that the linearity of the curve is very good with a correlation coefficient (R) = 0.99 for all curves as reported in previous study (19). Furthermore, the results of calculating the IC₅₀ value of control and irradiated ethanol extract of *C. xanthorrhiza* rhizome for all cancer cells are shown in Table 3.

This research showed that the ethanol extract of *C. xanthorrhiza* rhizome has the potential as an anti-cancer against four human cancer cell lines tested, although their antiproliferative activities were different. Among them, the highest IC₅₀ value was obtained from HUT78 cancer cells because it had the smallest IC₅₀ (5.4 µg/ml), followed by A548 (14.3 µg/ml), HeLa (15.5 µg/ml), and THP1 (17.9 µg/ml) (Table 2).

Anti-cancer properties of curcumin because it has high antioxidant properties that

can scavenge free radicals, curcumin has two hydroxy phenolic groups and β-diketone, which are two phenolic groups on one mole of curcumin can scavenge two free radicals. Free radicals scavenging consists of two stages, firstly scavenging the free radicals by hydroxy phenolic groups, follow by β-ketone groups (14).

Table 2. The IC₅₀ values of the ethanol extract of control and irradiated *C. xanthorrhiza* rhizome against human cancer cell lines

Cell lines	IC ₅₀ (µg/ml) of ethanol extract		Increase of IC ₅₀ (%)
	Dose 0 kGy	Dose 10 kGy	
HUT78	5.4	10.7	98
A549	14.3	27.5	92
HeLa	15.5	24.7	59
THP1	17.9	24.0	34

Curcumin contained in *Curcuma* can induce apoptosis, so it will weaken cancer cells by modulating proteins (20). Cancer cell apoptosis induction done by curcumin through regulation of p53 expression and initiation of apoptotic pathways in mitochondria by increasing BAX which will then bind to receptors on cancer cells (21). Apoptosis is the path of death of a cancer cell by stimulating and involving a protein (19). The ability of curcumin to kill cancer cells is not followed by attacking the normal cells, since the high antioxidant content of curcumin will protect the normal cells (22).

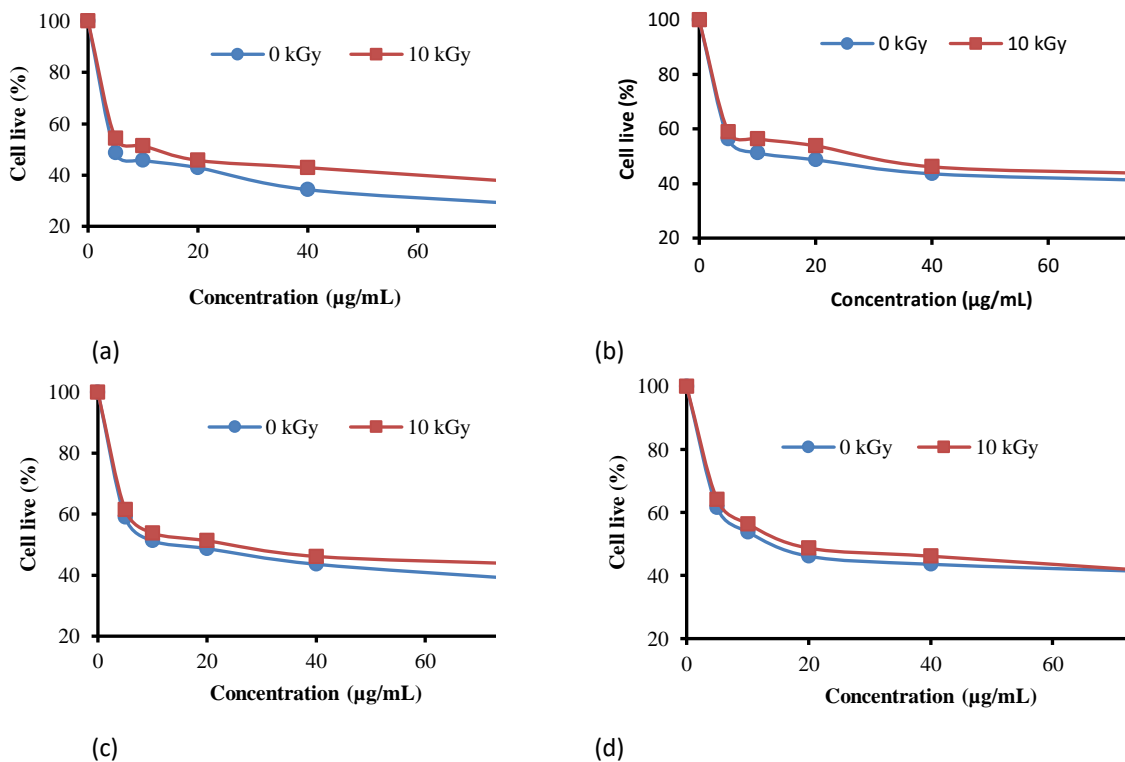


Figure 2. Relationship between cell live (%) of (a) HUT78, (b) A549, (c) HeLa, and (d) THP1 with ethanolic extract of irradiated and un-irradiated *C. xanthorrhiza* rhizome in various concentration

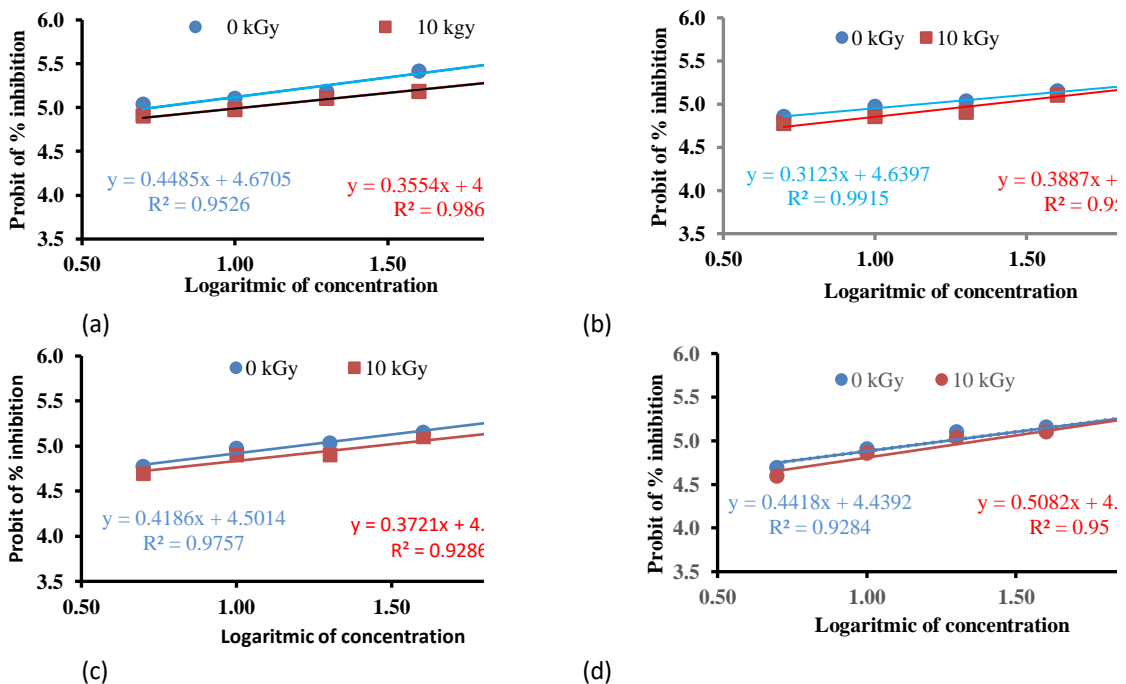


Figure 3. The linear regression curve of probit of the inhibitory activity percentage versus logarithmic of concentration of the ethanolic extract against human cancer cell lines: (a) HUT78, (b) A549, (c) HeLa, and (d) THP1

Based on Table 2, it shown that the radiation dose of 10 kGy reduce the anti-cancer properties of *C. xanthorrhiza* rhizome. Analisis by t-test using SPSS 24 at 95% confidence level

($\alpha=0.05$) showed that the cancer cell lines HUT78, A549, and HeLa had significance values <0.05 ($\alpha=0.000$), so it can be stated that the IC50 of irradiated sample (10 kGy) was significantly

different compared to the control (unirradiated sample). Whereas, the IC₅₀ of irradiated sample on THP1 was not significantly different compared to controls with a significance value > 0.005 ($\alpha=0,484$). However, the irradiated sample at 10 kGy still has potential as an anticancer, since the IC₅₀ value obtained was $\leq 30 \mu\text{g/ml}$ against HUT78, A549, HeLa and THP1 cells (23). The smaller IC₅₀ value obtained will be more potent as an anticancer drug. The increase in radiation dose in the sample of *C. xanthorrhiza* rhizome which was irradiated by gamma 10 kGy resulted in a decrease in the amount of curcumin in *C. xanthorrhiza*. A dose of 10 kGy resulted in a decrease of curcumin in *Curcuma* sp. by 38.5% and the degradation of curcumin compounds produced new compounds (11,24). Decreasing levels and changes in curcumin compounds will affect the cytotoxic properties of *C. xanthorrhiza* rhizome against cancer cells.

CONCLUSION

Irradiation dose up to 10 kGy can be used to extend the shelf life of *C. xanthorrhiza* rhizome, although at these irradiation dose reduced their anticancer properties based on the antiproliferative activity test against human cancer cell lines: HUT78, HeLa, THP1 and A549. However, these decreases did not eliminate their activities, whereas the IC₅₀ still < 30 $\mu\text{g/ml}$. The same pattern was also occurred on antioxidant activity which was its antioxidant activity of irradiated sample decreased due to irradiation. Based on the silica gel TLC plate, the ethanolic extracts of *C. xanthorrhiza* rhizome both unirradiated- and irradiated samples contained curcumin (Rf = 0.58) and demethoxycurcumin (Rf = 0.38).

ACKNOWLEDGEMENTS

The authors would like to thank CIRA-BATAN who provided research facilities and the staff of the Irradiator gamma facilities who had irradiated *C. xanthorrhiza* samples. Special thanks is also addressed to Prof. drh. Bambang Pontjo Priosoeryanto, Ph.D for providing laboratory facilities to conduct the bioassay against human cancer cell lines.

All authors (Susanto, Ermin Katrin Winarno, and Hendig Winarno) have equal contributions as the principal/main contributor.

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