CYTOTOXIC EFFECTS OF PROTEIN FRACTION ISOLATED FROM *Curcuma mangga* VAL RHIZOMES AND CONTAINING RIBOSOME-INACTIVATING PROTEINS ON CANCER CELL-LINES AND NORMAL CELL

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ABSTRACT

C. mangga Val. has been used as an alternative remedy for cancer in Yogyakarta. The protein fraction of C. mangga was identified to contain Ribosome-inactivating Protein which cleave supercoiled double stranded DNA in vitro. In this experiment, the protein fraction isolated from fresh, 40^0C dried and freeze dried C. mangga Val. rhizome was screened against HeLa, Raji cell-lines and normal mononuklear cells for cytotoxic effects. This would enable us to describe the sensitivity of the protein extract on different cell types. The level of cytotoxicity was determined on the level of LC_{50} which was based on the percentage of the cell death following the 24 hours incubation with the extract.

The protein isolated from C. mangga Val. was able to cleave supercoiled double stranded DNA to nick circular form. This result suggested that protein contained RIPs. The highest activity was identified in the protein isolated from fresh C. mangga Val, and this was followed respectively by freeze drying and 40^0C drying C. mangga Val. The comparison of the cytotoxic effect showed that protein of fresh C. mangga Val produced the largest number of death cells and the most toxic was on the HeLa cell line. Moreover, the LC_{50} indicated that the highest cytotoxic effect was shown by protein isolated from fresh C. mangga Val. followed respectively by freeze drying and 40^0C drying C. mangga Val. Based on LC_{50}, the highest cytotoxic effect of C. mangga Val was found on HeLa cell line, while similar cytotoxic effect was appear on Raji cell line and normal mononuclear cells

Keywords: *C. mangga Val., RIP, cytotoxic, cancer cell lines, normal cell*

INTRODUCTION

Many plants tissue produce substances which are toxic to other organism, and irreversibly inactivate eukaryotic ribosome by cleaving the N-glycosidic bond in the A_{4324} position of 28S RNA fraction so that they are no longer function in protein synthesis. These plant proteins are known as ribosome-inactivating protein (RIPs). According to their structure, RIPs can be classified into two major types. Type 1 consists of a single chain with a molecular weight around 30 kDa, while type 2, with a molecular weight around 60 kDa, usually consists of two chain (A and B) connected by disulfide bond. The A chain is homologous to type 1 RIP and is responsible for the toxicity of the molecule. The B chain is a lectin which binds the toxic to the cell surface and facilitates the entry of A chain into the cell [1].

Besides the activities of RIPs on ribosomal RNA, several RIPs demonstrate to exhibit a unique enzymatic activity on cleaving supercoiled double stranded DNA into the nicked circular or linear form. RIPs only act on supercoiled and nick-circular DNA and seldom cleave the linear form of the same molecule [2]. This fenomenon was first reported with trichosantin, an abortifacient, immunosupressive and anti tumor protein purified from the traditional Chinese herb medicine Tian Hua Fen [3].

Interest in RIPs is growing due to several discoveries, such as the anti viral activity of mirabilis antiviral protein (MAP), a type 1 RIP which has successfully focused attention on its use as potential anti-HIV [4]. The potent cytotoxicity also makes them an excellent candidates as the toxic part of immunotoxin for cancer therapy [5].

"Kunir Putih", Javanese name of *C. mangga*, has been used as an alternative remedy of cancer in Yogyakarta. Research has been done by [6] showed

that the "Kunir Putih" powder sold in Yogyakarta expressed enzymatic activity to cleave supercoiled double stranded DNA into a nick circular conformation. In order to confirm the results, identification of RNA N-glycosidase activity from the powder extraxt were carried out. Assay for RNA N-glycosidase activity on 26S rRNA *Saccharomyces cerevicease* ribosome indicated that this extracts showing the activity. Besides, the extracts have cytotoxic activity to B-Lymphoblastoid Cell Line (B-LCL) that grown from B cells of Nashoparingeal Carcinoma (NPC) Patient [7].

Based on the introduction, the cytotoxic effect of *C. Mangga* Val rhizomes's protein having Ribosome-inactivating Proteins (RIPs) on cancer cell line and normal cell have to be evaluated. Because of C. *mangga* was sold in Yogyakarta in the powder form, so effect of 40^0C drying and by freeze drying on protein activity was carried out too.

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EXPERIMENTAL SECTION

Materials

Protein extract containing Ribosome-inactivating proteins was isolated from fresh, 40^0C dried and freeze dried *C. mangga* Val rhizome using 5 mM sodium phosphate pH 7.2 containing 0.14M sodium chloride [8], followed by 100% ammonium sulphate precipitation [9]

Procedure

Cleavage of supercoiled DNA by the protein extract

One μg of plasmid DNA (pUC18) was incubated with various amounts of extract to volume of 20 μl containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, pH 8.0, at 30^oC for 1 hour. At the end of the reaction, 5 μl of loading buffer (30% glycerol, 200 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF) were added. Electrophoresis was carried out in 0.5xTBE buffer in a 1% agarose gel. DNA bands were visualized by staining with ethidium bromide [10].

Preparation of cancer cells

HeLa (was developed from human cervix carcinoma) and Raji (was developed from lymphoblast of burkitt lymphoma cancer patient) cell-lines were obtained from the laboratory stock collection at the Life Science Laboratory, Gajah Mada University, Jogyakarta. All cancer cells were grown in RPMI 1640 (Sigma) medium containing 5% v/v fetal calf serum (FCS) (Sigma) and 1% v/v fungison (Sigma) in the presence of 1% w/v of penicillin-streptomycin (Sigma) and incubated at 37^oC incubator in a 5% CO_2 controlled atmosphere. Cells were counted using a Neubauer Haematocytometer and resuspended in medium at the final concentration of $5x10^5$cells/mL.

Preparation of normal mononuclear cells

Normal mononuclear cells were prepared by the addition of an equal volume of Ficoll-Hypaque [11] to fresh blood obtained from a healthy individual. The mixture was then centrifuged at 2000g for 20 mins. The buffy coat layer containing mononuclear cells was then removed to a fresh tube, washed 3 times using RPMI 1640 medium, counted and adjusted to a final concentration of $5x10^4$cells/mL.

Determination of Cytotoxic effect

Cytotoxicity assays were conducted in 96 well flat bottom ELISA plates (Nunc). Protein extract was tested on a 10-fold dilution series of concentrations in culture media, from undiluted 250 ; 100 ; 50 ; 25 ; 10 ; 5 ; 2,5 ; 1,0 ; 0,5 ; 0,25 μg/mL. Each well received 100 μL of cell suspension and 100 μL of the appropriate dilution of extract. Then, plates were incubated in a 37^oC humidified incubator in 5% CO_2 for 24 h. Counting by MTT method for the cells death was used to calculate % inhibition [12, 13]. Untreated cells were used as a control (0% cytotoxicity). Mean percent cytotoxicity then calculated following treatment relative to controls. There were 3 replicate wells for each treatment.

RESULTS AND DISCUSSION

The cleavage activity seemed to be concentration-dependent, as shown on the agarose gel, where in the increasing amount of protein, supercoiled DNA became gradually fainted, whilst the nicked circular bands began to appear (Fig. 1, 2 and 3). Cleavage activity of protein extract of fresh C. mangga Val. was indicated in Fig 1. When 1 μg pUC18 was incubated with 0,1μg of protein extract of fresh C. mangga Val, it was shown that supercoiled DNA (1) began disappeared and cleaved to give a nicked circular form which moved significantly slower than the supercoiled DNA (3), and a linear form (2) which moved in between supercoiled DNA and nicked. All of supercoiled DNA was cleaved to nick circular by adding 5,0 μg of the protein. A linear DNA gradually appeared that caused by nick circular cleaving after 7,5 μg protein added.

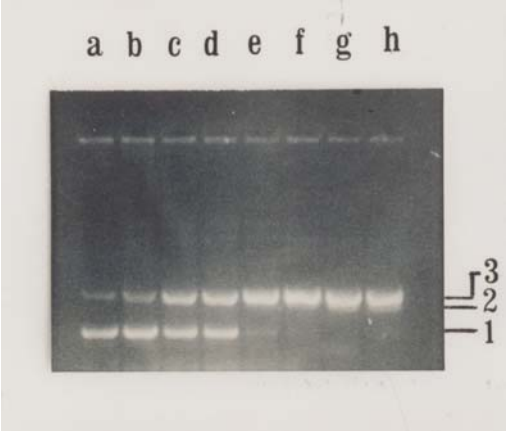

Fig. 1 Cleavage activity of protein extract of fresh C. mangga Val. (Lane a) untreated pUC18 were run as markers. (Lane b - h) treated pUC18 with protein extract at various concentrations, namely 0,1 ; 0,5 ; 1,0 ; 2,5 ; 5,0 ; 7,5 ; and 10,0 μg. Cleaving activity was shown by disappearing of (1). supercoiled form of pUC18 , (3), and appearing of open circular form of pUC18 and (2). linear form of pUC18

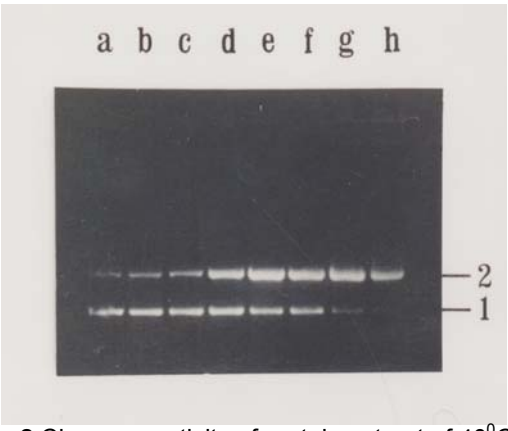

Fig. 2 Cleavage activity of protein extract of 40^0C dried C. mangga Val. (Lane a) untreated pUC18 were run as markers. (Lane b - h) treated pUC18 with protein extract at various concentrations, namely 0,1 ; 0,5 ; 1,0 ; 2,5 ; 5,0 ; 7,5 ; and 10,0 μg. Cleaving activity was shown by disappearing of (1). supercoiled form of pUC18 , (2), and appearing of open circular form of pUC18.

When pUC18 was treated with protein of C. mangga Val. dried 40^0C, similar result was also obtained. At a concentration of 0,1 μg of protein extract exhibited apparent activity on supercoiled DNA in a fashion similar to fresh extract. On the other hand, at the highest concentration (10 μg), supercoiled DNA haven't been cleaved at all to supercoiled DNA that shown by fade band of

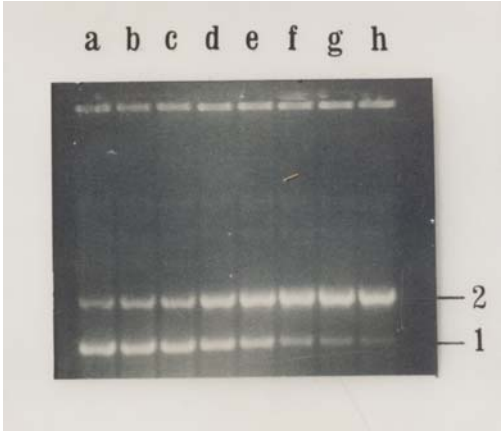

Fig. 3 Cleavage activity of protein extract of freezed dryC. mangga Val. (Lane a) untreated pUC18 were run as markers. (Lane b - h) treated pUC18 with protein extract at various concentrations, namely 0,1 ; 0,5 ; 1,0 ; 2,5 ; 5,0 ; 7,5 ; and 10,0 mg. Cleaving activity was shown by disappearing of (1). supercoiled form of pUC18 , (2), and appearing of open circular form of pUC18

supercoiled DNA (Line h). Besides, the linear DNA have not appeared yet by adding 10 μg protein

Tested protein extract of freezed dry C. mangga Val rhizomes on the ability of cleaving supercoiled double stranded DNA indicated that at the concentration of 0,1μg, the protein extract was able to cleave double stranded pUC18 into nick-circular form as indicated in Fig 3, Line b. The supercoiled band gradually became fade away and nick circular band became bolder than before, in line with increasing protein concentration. However, not all of supercoiled DNA have been cleaved to nick circular DNA at the highest concentration (10 μg) (Line h).

Since the cleavage of supercoiled DNA is one of the characteristic of RIPs beside the N-glycosidase activity [2, 3], so it was strongly suggest that this protein extract contained RIPs.

The protein extract of fresh C. mangga rhizome have the highest activity on DNA supercoiled cleaving. It can be seen from Figure 1, 2 and 3 that at the same concentration, fresh C. mangga was able to cleavege supercoiled DNA more extensively than 40^0C dried dan freezed dry, indicating that fresh was more active than 40^0C dried and freezed dry C. mangga rhizome, respectivelly.

This protein extract containing RIP demonstrated cytotoxic activity in HeLa, and Raji cell lines, but gave the lowest cytotoxicity on normal mononuclear cell. The induction of the cells death were characterized by the changing of the cells morphology and the cells viability for treated and

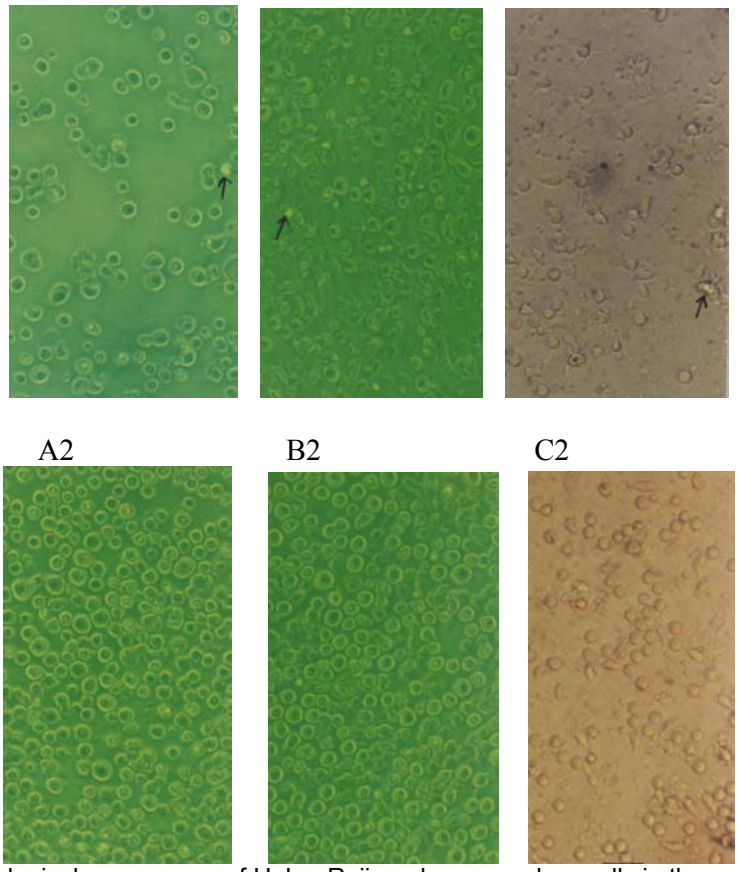

Fig 4 Morphological appearance of HeLa, Raji, and mononuclear cells in the presence of protein extract. (A1) treated HeLa cells; (A2) untreated HeLa cells; (B1) treated Raji cells (B2) untreated Raji cells; (C1) treated mononuclear cells; (C2) untreated mononuclear cells.; (→) death cell

untreated cells as shown at Fig 4. On the treated cancer cells, density of cells and percentage of cell death were higher compared to the untreated cells. The higher of the added extract the higher of the cancer cells death. For normal mononuclear cells, however, there were no differences on the cells viability between untreated and treated cells. Interestingly, this protein extract showed different level of cytotoxicity in between cancer cells.

Protein of fresh C. mangga Val gave the highest cytotoxic effect on Hela cell line, Raji cell line and normal mononuclear cells, followed by freeze drying and 40^{0}C drying respectively. It was demonstrated in Fig 5, 6, 7 and Table 1. The protein of fresh C. mangga Val still have native conformation, so it provided the highest on both cleaving of supercoiled DNA and cytotoxic activity. Similar activity was appeared on freeze dried C. mangga Val, because the rhizome was not dried by heating. The lowest activity was shown by 40^{0}C drying, since RIP underwent denaturation after heating at 40^{0}C.

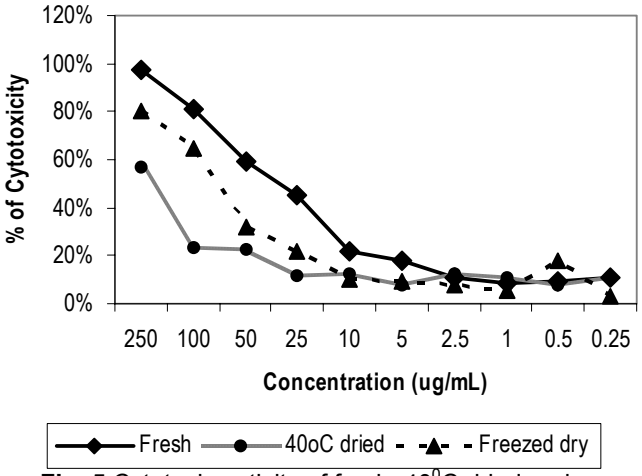

Fig. 5 Cytotoxic activity of fresh, 40^{0}C dried and freezed dry *C. mangga* Val rhizome on Raji cell line. The percent cytotoxicity was determined based on the absorbance at 550nm of the lysate of MTT-stained cells. Each value came from triplicate determinations.

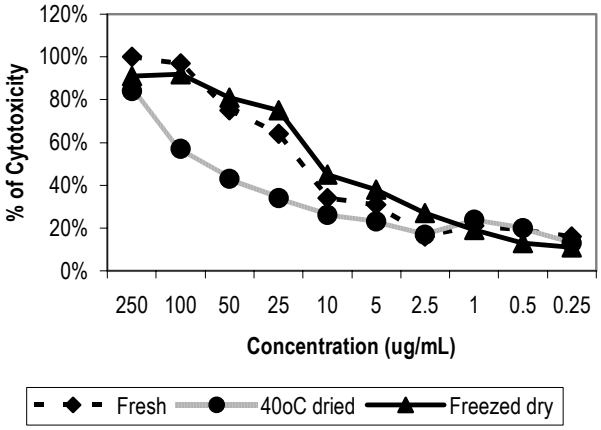

Fig. 6 Cytotoxic activity of fresh, 40^0C dried and freezed dry *C. mangga* Val rhizome on HeLa cell line. The percent cytotoxicity was determined based on the absorbance at 550nm of the lysate of MTT-stained cells. Each value came from triplicate determinations.

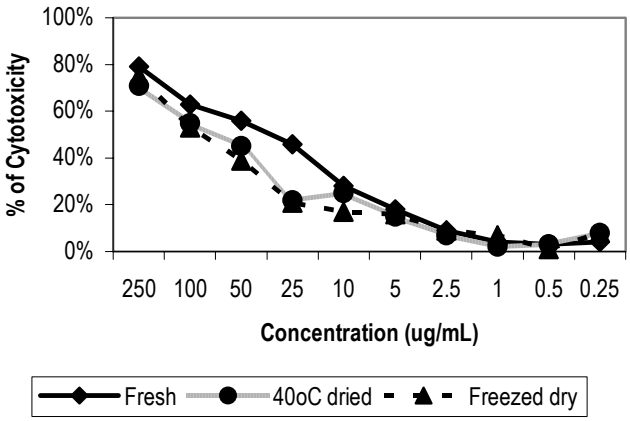

Fig. 7 Cytotoxic activity of fresh, 40^0C dried and freezed dry *C. mangga* Val rhizome on normal mononuclear cells. The percent cytotoxicity was determined based on the absorbance at 550nm of the lysate of MTT-stained cells. Each value came from triplicate determinations.

Table 1 The LC_{50} of protein extract of fresh, 40^OC dried and freeze dried *C. mangga* Val rhizome on HeLa cell line, Raji cell line and normal mononuclear cells.

Cell type	LC_{50} (μg/mL)		
	fresh	40^OC dry	freezed dry
Raji *cell line*	41.3	244.2	80.4
HeLa *cell line*	18.2	105.1	15.0
Normal mononuklear cell	37.8	87.0	81.0

Interestingly, this protein extract showed different level of cytotoxicity in between cancer cells. It was more cytotoxic to HeLa cell line with LC_{50} levels 18,2 μg/mL for fresh, 15,0 μg/mL for freeze dried and 105,1 μg/mL for 40^0C dried of C. mangga Val rhizome rather than Raji cell line with with LC_{50} levels 41,3 μg/mL for fresh, 80,4 μg/mL for freeze dried and 244,2 μg/mL for 40^0C dried of C. mangga Val. The cytotoxicity of C. mangga Val's protein to normal mononuclear cells was similar to Raji cell line with LC_{50} levels 37,8 μg/mL for fresh, 81,0 μg/mL for freeze dried and 87,0 μg/mL for 40^0C dried of C. mangga Val rhizome.

These two cancer cells, HeLa, and Raji, represent different kind of cancer cell, which will enable us to describe the sensitivity of the protein extract on different cell types. HeLa cell line is an epithelial cells which are developed from human cervix carcinoma transformed by human papiloma virus 18 (HPV18) [14]. While Raji cell is lymphoblast-like cells which are developed from lymphoblast of burkitt lymphoma cancer patient. This differences on the cytotoxic level seemed to be caused by the differences on the mechanism of the cell death in these cancer cells. The mechanism by which the protein extract killed the cells was not clearly understood yet. However, it has been reported that some type 1 RIP isolated from *Aspergillus giganteus* and RIPs-like protein isolated from *Mirabilis jalapa* had cytotoxic effect and induce the cells death via apoptosis, programmed of the cell death [15-17].

In the case of HeLa cells, the mechanism of cell-death might be caused by the easier penetration of protein extract through the cell membrane of the infected cells, and inactivation either ribosomal RNA or inhibition of the E6 and E7 proteins expressed by the virus genome. The expression of E6 and E7 proteins cause the immortality of this cell since they can degrade p53 and pRb, tumor suppressor proteins, respectively. Therefore, the inhibition of these E6 and E7 proteins will cause the induction of apoptosis [14,18]. In addition, it has been reported that cleavage of the 28S ribosomal RNA is a common feature in several cases of apoptotic-mediated cell death [19]. Therefore, all these activities might cause synergistically inducing the apoptosis. The higher of the LC_{50} level on Raji cell might caused by the apoptotic resistance of the cell. This cell was reported had mutation on the *p53* gene and on the downstream of *caspase-3* gene, causing the cell became resistant to apoptosis, a program of the cell death [20]. In addition, the cells expressed

bcl-2 and c-myc which could affect synergistically on the inhibition of apoptosis [21]. Apoptotic resistance of Raji cell-line was demonstrated while this cell was treated by RIPs-like protein isolated from *Mirabilis jalapa* [16].

As indicated on Table 1, there were no different cytotoxic effects with Raji cell line when normal mononuclear were treated with the protein extract. The cytotoxic effect to normal mononuclear cells presumed due to glycosil residues of type 1 RIP could be bound to carbohydrat or by endocytosis [1]. Besides, some RIP have been founded, they have different structure with the exception of their homolog sequens. Base on the idea, some RIPs had different cytotoxic effect on normal mononuclear cells. For example, type 1 RIP of Mirabilis jalapa had cytotoxic effect to normal mononuclear cells, however, the protein of Annona squamosa had no effect [10]

CONCLUSION

1. The protein fraction of fresh *Curcuma mangga* Val. gave the highest activity on supercoiled DNA cleaving, followed by freeze drying and 40^{o}C drying, respectively.
2. The protein fraction of fresh *Curcuma mangga* Val. gave the highest cytotoxic activity to cancer cell lines and normal cell, followed by freeze drying and 40^{o}C drying, respectively
3. The protein fraction of *Curcuma mangga* Val. gave the highest cytotoxic activity to HeLa cell line, followed by Raji cell line and normal mononuclear cells with similar LC_{50} value.

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