Apoptotic induction of ribosome-inactivating proteins (RIPs) isolated from C*arica papaya* L. leaves on cancer cell-lines

Induksi apoptosis dari *Ribosome-inactivating proteins* (RIP) yang diisolasi dari daun *Carica papaya* L. terhadap kultur sel kanker

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Abstrak

Carica papaya L mempunyai potensi untuk dikembangkan sebagai antikanker, karena mengandung Ribosome-inactivating proteins (RIPs) yang diketahui mempunyai efek sitotoksik terhadap kultur sel kanker.

Fraksi protein diisolasi dari daun *Carica papaya* L. cara mengendapkan menggunakan ammonium sulfat dan dianalisis aktivitasnya dalam memotong DNA superkoil, untuk meyakinkan keberadaan RIP pada protein tersebut. Protein aktif yang kemudian disebut sebagai 'protein CP' selanjutnya dianalisis kemampuannya dalam menginduksi apoptosis menggunakan metode pengecatan ganda dengan campuran *acrydin orange-ethidium bromide* dan kemudian di lakukan analisis ekspresi p53 dan bcl2 dengan metode immunositokimia.

Hasil menunjukkan bahwa protein CP bersifat sitotoksik terhadap kultur sel myeloma dan Raji dengan IC_{50} berturut-turut sebesar 1,80 mg/mL and 2,49 mg/mL. Apoptosos akhir dapat dideteksi pada kultur sel myeloma, tidak pada kultur sel Raji, yang diinkubasikan dengan protein CP sebesar 0,9 mg/mL and 0.6 mg/mL. Disamping itu, protein CP mampu meningkatkan ekspresi protein regulator apoptosis, p53, pada kultur sel myeloma, dan hampir tidak terdeteksi pada kultur sel Raji.

Kata Kunci : Ribosome-inactivating proteins (RIPs), *Carica papaya* L. , expression of p53 and Bcl-2, myeloma cell-line, Raji cell-line.

Abstract

Carica papaya L. leaves are potential for cancer treatment, this is due to the presence of *Ribosome-inactivating proteins* (RIPs), which demonstrated a cytotoxic effect on cancer cell lines. Therefore, this research was done to find out the effect of protein fraction containing RIPs isolated from *C. papaya* in the induction of apoptosis on cancer cell-lines (Raji and myeloma cell-lines).

The protein fraction was isolated from *Carica papaya* L. leaves using ammonium sulphate precipitation and analyzed the activity on supercoiled double stranded DNA cleavage, in order to identify the presence of RIP. The active fraction called 'CP protein', was then analyzed on the induction of apoptosis by double staining method using acrydin orange-ethidium bromide, followed by analyzing the expressions of p53 and bcl2 using immunocytochemistry.

The results demonstrated that the CP protein possessed cytotoxic activities on myeloma and Raji cell-lines with the IC_{50} of 1.80 mg/mL and 2.49 mg/mL respectively. Late apoptosis was detected on myeloma but not on Raji cell lines which was incubated with 0.9 mg/mL and 0.6 mg/mL of CP

proteins respectively. In addition, the protein was able to increase the expression of apoptotic regulatory protein, p53, on myeloma cell-line and almost undetected on Raji cell-line.

Key Words : Ribosome-inactivating proteins (RIPs), *Carica papaya* L. , expression of p53 and Bcl-2, myeloma cell-line, Raji cell-line.

Introduction

Ribosome-inactivating proteins (RIPs) are toxic proteins widely distributed throughout the plant kingdom, which are able to inhibit protein synthesis to other organism by inactivating ribosomes through the cleaving of the N-glycosidic bond at the A_{4324} position of 28S RNA (Barbieri *et al*., 1993). In addition of the activity on ribosomal RNA, several RIPs demonstrate to cleave supercoiled double stranded DNA into the nicked circular and linear form (Ling *et al*., 1994). The property of nucleic acid cleavage of RIP has been used to identify the presence of this activity of extract preparation of plant origin (Sismindari and Lord 2000).

RIPs also have cytotoxic activity that makes them an excellent candidate as the toxic part of immunotoxin for cancer therapy (Goldmacher *et al*., 1994). The rRNA depurination activity of RIPs has role in its cytotoxic activity (Bagga *et al*., 2002).

Carica papaya L has been demonstrated to contain ribosome-inactivating protein (RIPs) as indicates by the ability of cleaving supercoiled DNA *in vitro* and N-glycosidase on rRNA yeast (Sismindari dan Lord, 2000; Rumiyati *et al*., 2000). The protein is purified by column chromatography and has the molecular weight about 32 kDa (Rumiyati *et al*. 2000). In addition, it is found to have a cytotoxic activity on cervical cancer (HeLa) cell-lines (Rumiyati *et al*., 2003) and T47D cell-line with the IC_{50} of 2.8 mg/mL. This protein is able to induce apoptotic process, increase the expression of p53, and decrease the expression of bcl-2 on T47D cell-line (Rumiyati *et al*., 2006). Other RIPs containing protein, MJ protein, isolated from *Mirabilis jalapa* L., is also found to have cytotoxic effect on HeLa cell-line (Sudjadi *et al*., 2002)

Ricin, a RIP type 2 isolated from *Ricinus communis*, is found to induce both the apoptotic morphological changes and activation of caspase-3. It is also revealed that RIPs caused the ribotoxic stress response through their RNA-N-glycosidase activity, resulting in the activation of SAPK/JNK and p38 MAPK leading to apoptosis (Narayanan *et al*., 2005). RIPs are also able to induce apoptosis by down regulation of anti-apoptotic factors. Shiga toxin (ST), RIP from bacteria, is able to decrease in the cellular level of the bcl-2 family (Narayanan *et al*., 2005).

In this research, we found that, protein fraction of *C. papaya* was able to induce apoptotic process on Raji and myeloma cell-lines by increasing the level of p53 and decreasing of level of Bcl-2 protein.

Methodology

Fresh leaves of *C. papaya* were collected from Yogyakarta, Indonesia. A voucher specimen was deposited in the Life Science Laboratory, LPPT Gadjah Mada University (LPPT-UGM), Yogyakarta, Indonesia.

Myeloma, Raji cell-lines and pUC18 were obtained from laboratory stock of Life Science Laboratory, LPPT UGM, Yogyakarta.

Preparation of protein from C. papaya leaves

Extraction of protein from the leaves of *C. papaya* L was carried out as described previously (Sismindari *et al*. 1998). The extract was precipitated by 100 % saturated $(NH_4)_2SO_4$ followed by centrifugation at 28.000 g for 30 minutes. The precipitated protein was dialyzed overnight at 4 ^oC in sodium phosphate pH 7.2. The protein fraction was then called as 'CP protein'.

Cleavage of supercoiled DNA by RIPs

The presence of RIP activity in the CP protein, was determined by their capability to cleave supercoiled double stranded DNA, as previously described (Sismindari *et al*. 1998). Briefly, 1 μg of supercoiled double stranded plasmid DNA (pUC18) was incubated with various amounts of the CP protein containing RIPs to a final volume of 20 μL in 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, pH 8.0, at 30 °C for 1 hour. At the end of the reaction, 10 μL of loading buffer were added. Electrophoresis was carried out at 1 % agarose gel in 0.5 x TBE (tris-borat) buffer. DNA fragments were visualized by staining the gel with ethidium bromide.

Preparation of cancer cells

All cancer cells were maintained in RPMI 1640 (SIGMA) supplemented with 10 % (v/v) fetal bovine serum (Gibco), 1 % (v/v) penicillin-streptomycin (Gibco) and 0.5 % (v/v) fungizone (Gibco). The cells culture were incubated at 37° C in a humidified atmosphere of 5 % CO_2 (v/v) (Heraous-Heracell). Cells were counted using a Neubauer Haematocytometer and resuspended in medium at the final concentration of $5x10^5$ cells/mL. All other reagents were of analytical or molecular biology grade and, when possible, RNase-free (Freshney, 2000).

Cytotoxicity assay

Cytotoxicity assay was carried out according to the method of Freshney (2000) with slight modification. One hundred mL of the exponentially growing cells (5×10^4 cells/mL) were seeded in 96-well microculture plate with a serial dilution of protein extracts. The media without cells was used as a control media, while the phosphate buffer pH 6.5 was used as control for the protein fraction. After 24 hr incubation, the number of viable cells was ascertained with MTT reaction (3-(4,5-dimethyltiazol-2-il)-2,5-diphenyl tetrazolium brom-ide), and measured at λ_{550} nm followed by IC_{50} calculation.

Assessment of Apoptosis using Double Staining Methods

Raji cell-line (lymphoblastoid) and myeloma cell-line, myeloid cells (Bataille and Haroussearu, 1997), 1×10^6 cells/mL, were treated with the CP protein at the concentration of 0.6 mg/mL, 0,95 mg/mL for 24 h at 37 °C on plate with cover slips. Following incubation, cover slips were fixed on objective glass and treated with Etidium Bromida-Acridine Orange. Following 30 minutes incubation, morphology of the cell was analyzed under fluorescence microscope (Gasiorowski, *et al.*, 2001; Ribble *et al.*, 2005)

Assessment of the p53 and bcl2 protein Expression

The aceton-fixed cells were pretreated by microwaving in citrate buffer (10 mmol/L, pH 6.0) twice for 7 minutes at 600 W each. The sections were incubated with the monoclonal antibodies, DBA for 1 hour followed by biotinylatedhorse antimouse immunoglobulin as the secondary antibody and 3-amino-9-ethyl-carbazole (for DBA.44) as chromogens in the presence of H2O2.

Results

Cleavage of double stranded DNA by protein fraction of *Carica papaya* L.

CP protein isolated *C. papaya* was believed to contain Ribosome-inactivating proteins as demonstrated for the ability to cleave supercoiled double stranded pUC18 DNA to become nick circular and linear form (Rumiyati *et al.,2006*). The activity was gradually increased with the increased of protein concentration (Figure 1). At the concrentartion of 2mg/mL, supercoiled DNA band (band a) in the agarose gel started gradually fainted, while nicked (band c) and linear bands (band b) began to appear at the increasing concentration as indicated by slightly increasing the intensity of these bands (line 5). The supercoiled DNA completely disappeared at the concentration of 10 mg/mL (line 6). The disappearing of supercoiled DNA indicated the higher activity of the protein fraction.

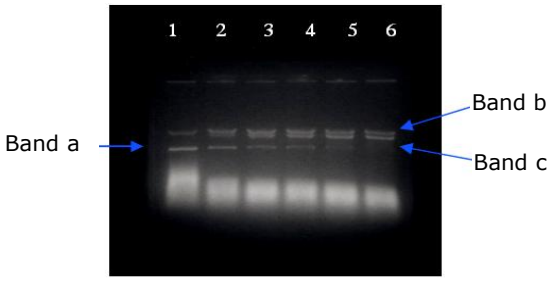

Figure 1. Cleavage activity of protein fraction isolated from *C.papaya* L on pUC19. Untreated pUC19 (1); pUC19 treated with: 2 mg/mL of protein fraction (2); 4 mg/mL of protein fraction (3); 6 mg/mL of protein fraction (4); 8 mg/mL of protein fraction (5); and 10 mg/mL of protein fraction (6). supercoiled DNA (a); linear DNA (b), nicked circular DNA (c), (Rumiyati *et al.*, 2006)

Cytotoxicity activity of protein fraction against Raji and myeloma cells-lines

Raji and myeloma cancer cell lines were treated with various concentrations (0.08 – 10 mg/mL) of the CP protein (Figure 2). At the concentration of 0.08 mg/mL, the protein

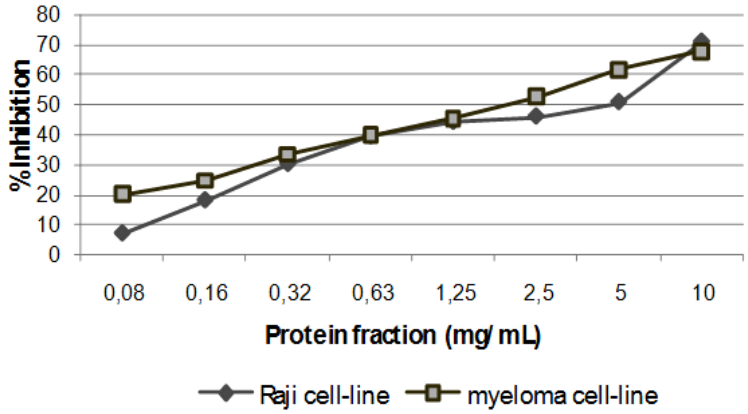

Figure 2. Inhibition of protein fraction *Carica papaya* against Raji and myeloma cells-line. Cell-lines were incubated with a series concentrations of protein fraction at 0.08 – 10 mg/mL for 24 hours and treated by MTT, resulted on producing blue colour of the viable cells, followed by detection of the colour using ELISA reader.

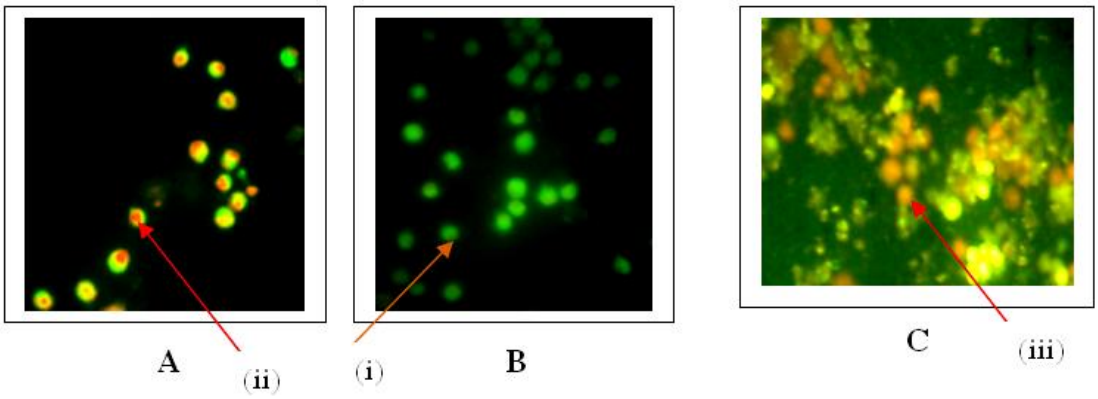

Figure 3. Image of stained cancer cells-line with mixed dye ethidium bromide-acridine orange. Treated myeloma cell line with CP protein of *C. papaya*, cells were undergo to apoptotic process (A); Untreated cell line, no apopttic process were detected (B); Treated Raji cell-line with CP protein of *C. papaya*, cells were found undergo to necrotic process. (i) viable cells (ii) cells with late apoptosis (iii) cells with necrotic process.

fraction had very low inhibition against Raji and myeloma, 7 % and 20 % respectively. At the highest concentration (10 mg/mL), it had about 70 % and 68 % inhibition. The value of IC_{50} (a concentration at 50 % inhibition) that was analyzed by probit method was approximately 2.5 mg/mL for Raji and 1.8 mg/mL for myeloma cell line.

Further analysis, induction of apoptosis and measuring the expression of p53 and Bcl2, were carried out to confirm the activity.

Effect of the protein fraction on the induction of apoptosis

Apoptotic induction of CP protein was carried out at the concentration of below IC_{50}, that were 0.6 mg/mL and 0,9 mg/mL for Raji and myeloma cell-lines respectively. The result demonstrated that the CP protein was able to induce late apoptosis on myeloma cell-line, as indicated by the appearance of apoptotic body with the orange color of the treated cell (Figure 3A), whereas, in the untreated cell, no orange

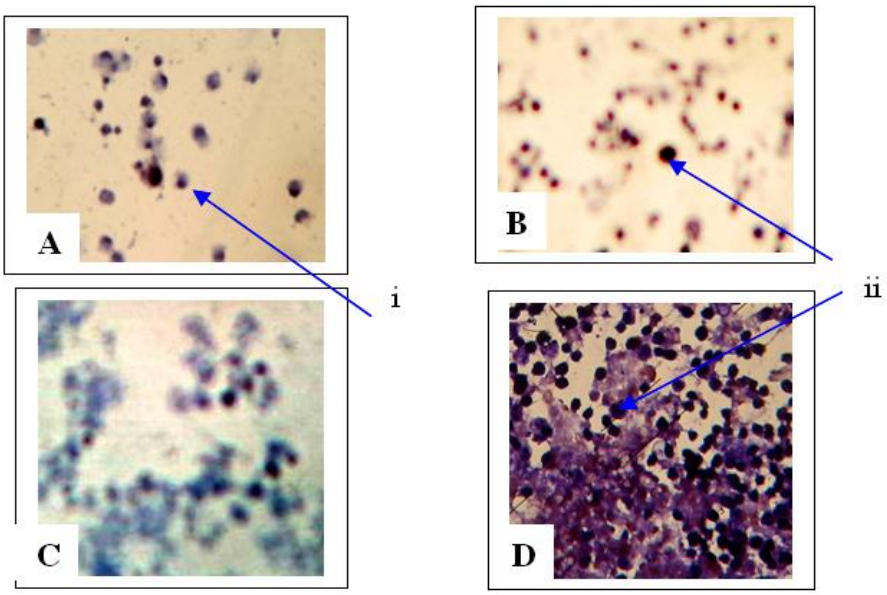

Figure 4. Effect of CP protein on the p53 expression on cancer cells-line. Treated and untreated cells were fixed and incubated with the p53 antibody.The expression of p53 was indicated by the dark brown colour of the nucleous. Untreated Raji cell-line (A) and treated Raji cell-line with 0,9 mg/mL CP protein (B), no differences on the p53 expression. Untreated myeloma cell-line (C) and treated myeloma cell line with 0.9 mg/mL CP protein (D), expression of p53 was dramatically increase on the treated cells. Low expression p53 protein (i). high expression p53 protein (ii).

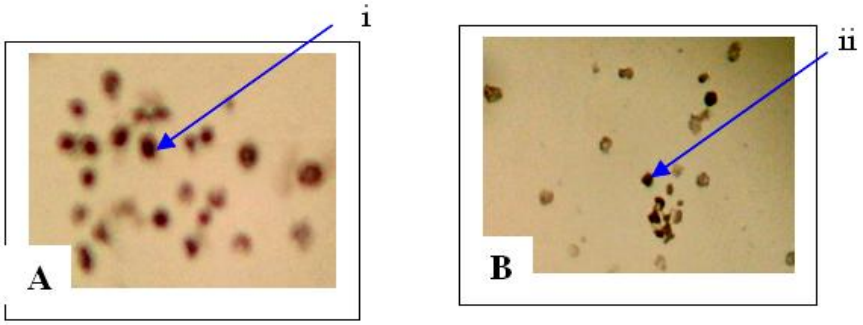

Figure 5. Effect of CP protein on the bcl2 protein expressions of Raji cell line. Treated and untreated cells were fixed and incubated with the bcl2 antibody.The expression of bcl2 was indicated by the dark brown colour of the cells. Untreated cells (A) and (B) treated cells with 0.9 mg/mL CP protein, The expression of bcl2 was decrease on the treated cells. Expressed bcl-2 protein (i). un-expressed bcl2 protein (ii).

color was observed (Figure 3B). The induction of apoptosis of this protein was also demonstrated at T47D cell line treated with CP protein (Rumiyati *et al.* 2006), HeLa cell-line treated with MJ protein, a RIPs-like protein isolated from *M. jalapa* (Ikawati *et al.,* 2002). On Raji cell-line, most of the cells was undergo into the necrotic process, as demonstrated in the appearance of broken cells with the (full orange color) Figure 3C. It seemed that Raji cell-line was resistant to apoptotic process. Similar result was found when Raji cell-line was treated with MJ-30, a basic RIP isolated from *M.jalapa leaves* (Ikawati *et al.*, 2003).

The mechanism of apoptotic induction in myeloma cell lines was proposed due to the RNA-N-glycosidase activity of the protein fraction. RNA-N-glycosidase activity led to damage of 28S rRNA resulting in an activation of SAPK/JNK thus activating a ribotoxic stress

response and apoptosis (Narayanan *et al.*, 2005). Induction of apoptosis was also proposed via mitochondrial pathway involving the regulation of tumor suppressor gene (p53) expression as well as an anti-apoptotic factor (Bcl-2 protein).

Efect of CP protein on p53 and Bcl-2 proteins expressions

The effect of CP protein on apoptotic process against Raji and myeloma cancer cell line was examined on the expression of p53 and bcl2 using immunocytochemistry methode. The results as shown in figure 4, indicated that CP protein was able to highly induce the expression of p53 on myeloma cancer cells (Figure 4D) compared to the untreated ones (figure 4C). Whereas, the induction of p53 expression was almost un-detected on the treated Raji (Figure 4B). However, CP protein was able to slighly decrease the expression of Bcl-2 protein, the anti-apoptotic protein (Figure 5 B).

Discussions

Based on the cytotoxic analysis, it was found that HeLa cell line had the highest sensitivity to the CP protein with the IC_{50} of 0.6 mg/mL (Rumiyati *et al.*, 2003), followed by myeloma, Raji and T47D cell line (IC_{50} of 2.8 mg/mL) (Rumiyati *et al.*, 2006). The selectivity efect of RIPs to cancer cell-line was also found to other isolated RIPs, a basic (MJ-30) and acidic (MJ-C) RIP-like proteins isolated from *Mirabilis jalapa*. MJ-30 was more sensitive to T47D cell-line with the IC_{50} of 0.4 mg/mL (Ikawati *et al.*, 2006) compare to myeloma and HeLa cell-line with the IC_{50} of 1,1 mg/mL and 7.1 mg/mL respectively (Sudjadi *et al.*, 2002). Therefore, MJ-C found to be more sensitive to myeloma (IC_{50} 7 μg/mL) compare to HeLa and T47D with the IC_{50} of 0.002 μg/mL and 0.03 mg/mL respectively (Sudjadi *et al.,* 2007). The differences of the cytotoxicity of these proteins might caused by the differences of cancer cell characterization which have different sensitivity to different structure of RIPs.

CP protein was able to induce apoptotic process and p53 protein expression, on myeloma cell-line. Induction of apoptotic process was also found on some cancer cell-lines treated by many RIPs (Ikawati, *et al.*, 2003, Narayanan *et al.*, 2005), this was suggested that the mechanism of apoptosis was regulated via mitochondrial pathway and passed through p53-dependent. One of the targets of apoptotic induction in the mitochondrial pathway is a member of the Bcl-2 protein family. This member can regulate the release of cytochrome-c that is important to activate cascade of caspase to active executioner caspase. It can cleave the cellular substrates that lead to characteristic biochemical and morphological changes of cells (Burlacu, 2003 ; Kuwana and Newmeyer, 2003). Some chemotherapeutic agents are indicated that they can induce of the apoptotic program with the mechanism as follows : modulate oncogenes that can enhance the expression of tumor suppressor genes (p53), increase expression of Bax protein, a pro-apoptosis members, and decrease the expression of Bcl2, an anti-apoptosis members (Xu, *et al.*, 2001).

This study demonstrated that there was no apoptotic induction on Raji cell-line. The resistance of Raji cells to apoptosis by the CP protein may correlate to the lower cytotoxic effect compared to HeLa cells. These results are in line with Kawabata (1999) which reporting that apoptotic resistant of Raji cells-lines due to a defect in the apoptotic pathway in the cytoplasm downstream of caspase-3. The death of Raji cells found on the cytotoxicity study is presumably due to necrosis as indicated by double staining and the unable to induce p53 expression as well as to decrease bcl2 expression. The apoptotic resistant of Raji cell-line was also found when this cells was treated by MJ-30 (Ikawati *et al.*, 2003). Some cancer cells have demonstrated mutation on tumor suppressor genes such as p53, beside changes of expression of proteins that regulate of apoptosis. As a result of that is destruction of apoptosis program (Antonsson, 2001).

Furthur study need to carried out to purified the active protein in order to analysed the mechism of action.

Conclusions

The CP protein possessed citotoxicity activity against Raji and myeloma cell lines with IC_{50} of 2.5 mg/mL for Raji and 1.8 mg/mL for myeloma cell. The CP protein was able to

induce apoptotic processes on myeloma cell, induce p53 protein expression on Raji and myeloma cell-lines. In addition, the CP protein could decrease the expression of the anti apoptotic protein, bcl2 on Raji cell-line.

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