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CYTOTOXICITY AGAINST TUMOR CELL LINES OF A RIBOSOME-INACTIVATING PROTEIN (RIP)-LIKE PROTEIN ISOLATED FROM LEAVES OF *MIRABILIS JALAPA* L.

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The 30 kD protein fraction with properties like ribosome-inactivating protein (RIP) was isolated from the leaves of Mirabilis jalapa L. and named MJ-30. This study investigated the cytotoxic effect of MJ-30 on normal and malignant cells. MJ-30 was isolated from the leave extract of M. jalapa L. using cation-exchange chromatography with CM Sepharose CL-6B column to obtain MJ-30. The fraction contain DNA cleaving ability were pooled and checked for cytotoxicity against breast cancer T47D cell line, cervical cancer SiHa cell line, and human mononuclear cells derived from peripheral blood of healthy volunteers. Results showed that the MJ-30 produced cytotoxic effect against T47D and SiHa cell line to different extent. The LC_{50} of the MJ-30 on T47D cell line and SiHa cell line were 0.36 μg/mL and 5.6 μg/mL, respectively. While in normal cells, represented by human mononuclear cells, MJ-30 was considerably less toxic, with LC_{50} of 21.04 μg/mL. The results suggest that MJ-30 produced more cytotoxic activity toward breast and cervical cancer cells (58-fold and 4-fold, respectively) as compared to normal mononuclear cells.

Keywords: *Ribosome-inactivating protein, Mirabilis jalapa L., Cytotoxicity, T47D and SiHa cell line, Human mononuclear cells*

INTRODUCTION

Ribosome-inactivating proteins (RIPs) are a group of plant enzymes that possess capability to inactivate ribosomes by modifying the 28S rRNA through their N-glycosidase activity, which is manifested by cleavage of the N-glycosidic bond at a specific adenine. The adenine at position 4324 of rat liver 28S rRNA and corresponding adenine on other eukaryotic or prokaryotic models are the target sites (Stirpe *et al*. 1992). Through this mechanism, the binding of elongation factor 2 is prevented, with the consequent arrest of protein synthesis leading to autonomous cell death (Osborn and Hartley 1990). Such properties might contribute to its cytotoxic effect, either through necrosis or apoptosis. RIPs also have capability to cleave supercoiled double stranded DNA to acquire nick-circular and linear forms (Ling, Liu and Wang 1994). This property of

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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 are currently under study as therapeutic agents against cancer (Kreitman and Pastan 1998). The present study is a part of our continuing research to isolate active compounds from plant for anti-cancer activity. We have previously reported that crude protein fraction extracted from the leaves of *Mirabilis jalapa* L. displayed potential to cleave supercoiled DNA like RIP, and was able to induce cell death through the induction apoptosis and necrosis in different cancer cell-line models (Ikawati *et al*. 2003). However, this being in crude form, we carried out further purification of the protein extract using cation-exchange chromatography. In the present study, a protein fraction identified to have RIP-like activity has a molecular weight of 30 kD, and, therefore, was designated as MJ-30 (MJ for *Mirabilis jalapa*, and 30 for 30 kD). Cytotoxicity of the MJ-30 was tested against two cancer cell lines, namely human breast cancer T47D and cervical cancer SiHa, and human peripheral blood mononuclear cells as normal cells.

METHODS

Plant Material

Fresh leaves of *M. jalapa* L. (red flower cultivar) were collected from Yogyakarta, Indonesia. A voucher specimen was deposited in the Laboratory of Life Science Gadjah Mada University, Yogyakarta, Indonesia.

Cell Culture

The T47D and SiHa cells lines were obtained from the stock of Life Science Laboratory Gadjah Mada University. The 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).

The peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers. Briefly, each 5 mL blood sample was diluted with 5 mL of pyrogen-free normal saline and underlayered with 10 mL of Ficoll-Hypaque. The tube was centrifuged at 2000 g for 20 min. The buffy

coat layer containing mononuclear cells was then removed to a fresh tube, washed three times using RPMI 1640 medium, counted and adjusted to a final concentration of 5×10^4 cells/mL.

All other reagents were of analytical or molecular biology grade and, when possible, RNase-free. For experiment using human volunteer to isolate the peripheral blood mononuclear cells, the experimental protocols were approved by the Ethical Committee of Health and Medical Research, Gadjah Mada University.

Isolation and Purification of MJ-30 from *Mirabilis jalapa* L.

Extraction of protein fraction from the leaves of *M. jalapa* L. was carried out as described previously (Ikawati *et al*. 2003). The fraction are referred as basic protein fractions and contained most of the RIP activity present in the starting material. To purify the protein, the protein extract was adjusted to 5 mM phosphate buffer (pH 6.5) and applied to a CM Sepharose CL-6B column (1 × 20 cm, Pharmacia LKB Biotech), and equilibrated with the same buffer at room temperature. The elution was carried out with the equilibration buffer until all peaks of the unbound protein were obtained and the absorbance at 280 nm was lowered to a base line. This was followed by elution of bound proteins with 1 liter of a linear (0–500 mM) gradient of NaCl in the same buffer. The eluted fractions were collected every 2 min in eppendorf tubes and were screened for ability to cleave supercoiled DNA as an indicator for RIP-like activity. Protein fractions with high activity of DNA cleavage were pooled and tested for cytotoxicity against cells. The protein fraction was also analysed by SDS-PAGE.

Cleavage of Supercoiled DNA by the Protein Extracts

In order to confirm the presence of RIP activity in the eluted protein fractions, their capability to cleave supercoiled double stranded DNA was determined, as previously described (Ikawati *et al*. 2003). Briefly, 1 μg of pUC18 (obtained from stock of laboratory of Life Science, Gadjah Mada University) was incubated with various volume of protein fractions (1, 2, 4, and 8 μL) to final volume of 10 μL containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, pH 8.0, at 30°C for one hour. At the end of the reaction, 4 μL of loading buffer (xylene cyanol FF 0.25% (v/v) (SIGMA), 0.25% (v/v) bromphenol blue, 30% (v/v) glycerol in 200 mM EDTA) were added. Electrophoresis was carried out on a 1% (w/v) agarose gel in 0.5 ×

TBE (Tris Borat EDTA) buffer. DNA bands were visualized by staining with ethidium bromide (Sismindari and Lord 2000). Ricin with amount of 2 µg was used for positive control. Ricin was kindly obtained from Professor JM Lord, Warwick University.

Cytotoxicity Assay

Cytotoxicity assay was carried out according to the method of Mosmann (1983) 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 in a volume of 100 µL. The media without cells was used as control media and treated with the same serial dilution of protein extract, while the phosphate buffer pH 6.5 was used as control for the protein fraction. After 24 h incubation, the number of viable cells was ascertained with MTT reaction (3-(4,5-dimethyltiazol-2-il)-2,5-diphenyl tetrazolium bromide). LC_{50} value was calculated using Reed-Muench method (1938). Percentage of dead cells was calculated as follow:

$$\% \text{dead cells} = \frac{A_{\text{untreated cells}} - A_{\text{treated cells}}}{A_{\text{untreated cells}}} \times 100\%$$

Statistical Analysis

The results were expressed as the mean ± SEM. Statistical analysis was carried out by using one way analysis of variance followed by student's paired t-test. p values less than 0.05 were considered to indicate significant differences.

RESULTS AND DISCUSSION

Purification of Protein Fraction

The elution profile of bound proteins on CM-Sepharose CL6B eluted with a linear (0–500 mM) gradient of NaCl in 5 mM phosphate buffer (pH 6.5) is shown in Figure 1(a). Two major peaks were observed, which were eluted on 0.240–0.255 M NaCl and 0.33–0.37 M NaCl, respectively.
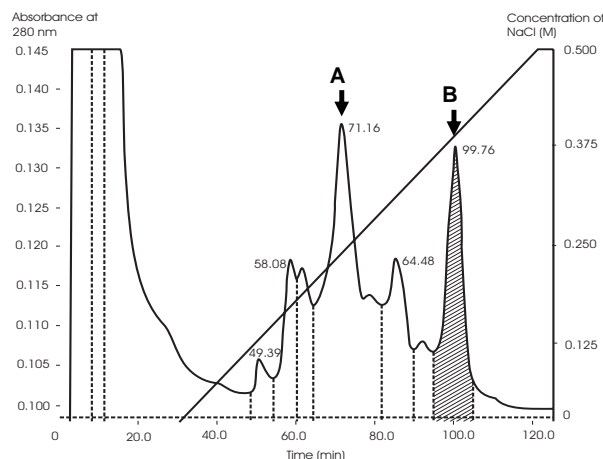
Fig. 1(a): Purification of protein from *M. jalapa* L. by column chromatography with CM Sepharose CL-6B column resulted in bound protein. Two major peak were observed, which were eluted on 0.240–0.255 M NaCl (A) and 0.33–0.37 M NaCl (B), respectively. The experimental procedures are described in material and methods. The area with hatched pattern (B) represents the fraction possessing DNA cleavage activity. The active fractions were pooled and assayed for purity, and then subjected for cytotoxicity assay as MJ-30.

Screening of Protein Fraction with Activity for Cleavage of Supercoiled DNA

All protein samples were screened for their ability to cleave supercoiled DNA as an indicator for the existence of RIP-like activity. However, only protein fractions of the bound proteins eluted on 0.33–0.37 M NaCl were found to show ability to cleave supercoiled DNA. The protein concentration was 2.88 µg/µL, and the ability to cleave supercoiled DNA was increased with increasing amount of protein used in the experiment (Fig. 2 lane B–E). The amount of protein fraction capable to cleave supercoiled DNA to be nick-circular and linear form was started from 11.52 µg (lane D). The capability of protein fraction to cleave supercoiled DNA was comparable to that of ricin, a well known RIP (Fig. 2 lane F), despite less potent, supporting that the protein from *M. jalapa* L. have RIP-like property. The active fractions are depicted with hatched areas marked in the peaks [Fig. 1(a)] in chromatogram. All the active fractions in bound protein were then pooled and analysed on SDS-PAGE and assayed for cytotoxicity against cell line. SDS-PAGE analysis revealed that

the pooled protein fraction had a protein of molecular weight 30 kD [Fig. 1(b)].
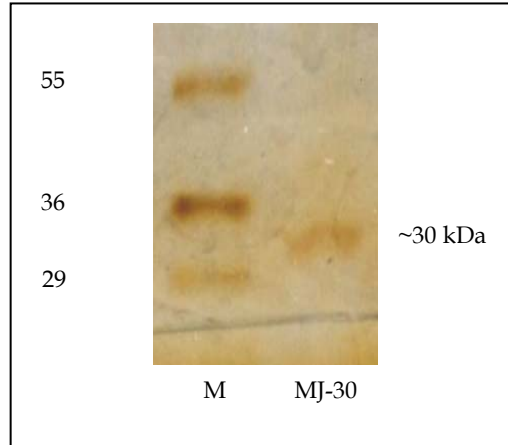
Fig. 1(b): Analysis by 12% (w/v) SDS-PAGE of the active protein fraction. The protein fraction was observed on moleculer weight of 30 kDa, therefore, it was designated as MJ-30 (MJ for *Mirabilis jalapa*, and 30 for 30 kD). M = marker protein.
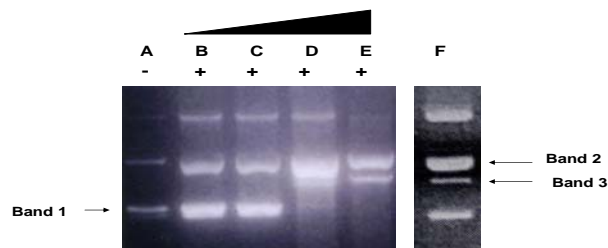

Fig. 2: Effect of protein fractions of M. *jalapa* L (MJ-30) eluted on 0.33–0.37 M NaCl with increasing amount on the cleavage of DNA plasmid (pUC18). Lane A: pUC 18 control; lane B–E: pUC18 treated with 1, 2, 4, 8 µL, respectively, of protein with concentration of 2.88 µg/µL. Lane F: pUC18 treated with 2 µg of ricin. The ability of the MJ-30 to cleave supercoiled DNA was started from the amount of 11.52 µg (lane D). Band 1: supercoiled form of DNA; band 2: nick-circular form of DNA, band 3: linear form of DNA.

Cytotoxic Activity

Protein fractions of bound protein with DNA cleaving activity demonstrated different levels of cytotoxic activity against T47D and SiHa cell lines, and very less toxic against human mononuclear cells (Fig. 3).

After 24 h of incubation, LC_{50} of the MJ-30 against T47D, SiHa, and human mononuclear cells were found to be 0.36, 5.6 and 21.04 µg/mL, respectively. Percentage of dead cells caused by the highest concentration of protein used (0.55 µg/mL) to treat T47D, SiHa, and mononuclear cells were 65.39, 18.14 and 7.61%, respectively.

The results of the present study demonstrated that the protein fractions isolated from extract of the leaves of *M. jalapa* L. contain protein with RIP-like activity, which is indicated by the capability of the protein fractions to cleave supercoiled DNA into linear and nick-circular forms. This property is one of the characteristics of RIPs beside the N-glycosidase activity. The observations suggest that the fractions may contain RIP-like activity. As known so far, RIP from plants may be classified into type 1 or 2 according to their single- or double-chain structure (Barbieri, Batteli and Stirpe 1993). Besides the classical type 1 and 2 RIPs, a 60-kDa RIP (called JIP60) has been identified in barley (*Hordeum vulgare*) that consists of an amino-terminal domain closely related to the RIP enzymatic chain linked to an unrelated carboxyl-terminal domain with unknown function, which may be classified as type 3 RIP (Reinbothe *et al*. 1994). Our protein is most likely similar to type 1 RIP as it has molecular weight in the range of 30 kD. Several isoforms of RIPs have been identified in the seeds of *M. jalapa*, designated as Mirabilis antiviral protein (MAP) with molecular weight of 27.788 kD, MAP-2 (30.412 kD), MAP-3 (29.771 kD), and MAP-4 (29.339 kD) (Bolognesi *et al*. 2002). We did not confirm yet whether our MJ-30 is similar to MAP-2, however, it is likely that RIPs may be different in different tissue.
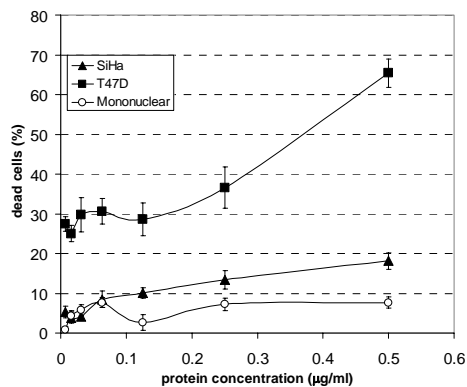
Fig. 3: Cytotoxic effect of MJ-30 on T47D and SiHa cells line compared to that on human peripheral mononuclear cells. Phosphate buffer pH 6.5 was used as negative control for protein fraction. Data are means of 4–6 experiments in triplicate.

The MJ-30 containing protein fraction demonstrated cytotoxic effect against T47D and SiHa cell lines, but human mononuclear cells were comparatively more resistant compared to T47D and SiHa cell line. Mononuclear cells are less-dividing cells that usually relatively insensitive to anti cancer therapy. This finding shows that MJ-30 relatively less cytotoxic for normal cells, and produces more cytotoxic activity against tumor cells despite in different levels.

The cytotoxicity of MJ-30 seems to be varied on the type of target cell line. The RIP showed markedly higher cytotoxicity on T47D cell line, but only slightly cytotoxic against SiHa cell lines. T47D cell line is originally derived from pleural effusion of 54 years old human female with breast carcinoma, while SiHa cells are derived from a surgically removed cervical carcinoma, which carry one or two copies of human papillomaviruse (HPV) 16 DNA as an integrated form in chromosome (Friedl *et al.* 1970). The difference in cell type may account for the difference cytotoxic response against anti cancer agents.

In the case of RIP, variation of cytotoxicity against different cells was also observed in another RIP, trichosantin (Chan *et al*. 2002). Chan *et al.* (2002) employed three different cell types IC21, JAR and Vero cell lines, which were shown to be high, medium and low sensitivity to trichosantin. A good relationship was demonstrated between intracellular trichosantin concentration and toxicity. It seemed that variation of cytotoxicity of RIPs in different cells may be dependent upon the mechanisms affecting its internalization.

This phenomenon may also be applied generally for other RIP, including MJ-30. It has been proposed that type 1 RIPs enter cells through passive mechanisms such as fluid-phase pinocytosis. However, some observations, such as the difference in sensitivity of type 1 RIPs among different cell types, and the organ-specific toxicity of type 1 RIPs, indicate a specific mechanism for the entry of these proteins into target cells (Cavarallo *et al.* 1995). A receptor responsible for the binding and endocytosis of RIP is alpha 2-macroglobulin receptor (alpha 2MR). A study with saporin and trichosantin, a potent type 1 RIPs, indicated that general mechanism of complex interactions between RIPs and cellular membranes is mediated by alpha 2-macroglobulin receptor (Cavarallo *et al*. 1995; Chan *et al*. 2000). The alpha 2-macroglobulin receptors are expressed in cancer cell line, including breast cancer cell line, T47D and BT-20 cell line (Li *et al*. 1998). Despite the lack of data concerning the expression of alpha 2-macroglobulin receptors on various type of cells, it

is assumed that there may be differential expression of RIP receptor type on the different cells used in the present study, where T47D cells shows the highest expression.

Other possibilities that may explain the different cytotoxicity of the RIP toward different kind of cells is the difference in the downstream death pathways. Indeed, the inhibition of protein synthesis by cleavage of the N-glycosidic bond of a specific adenine of 28S rRNA has been accepted as the mechanism by which plant RIPs cause cytotoxicity. However, beside inhibition of protein synthesis, RIPs have been shown to induce apoptosis or programmed cell death (Olmo *et al*. 2001). Apoptosis is not a general direct consequence of protein biosynthesis inhibition, as deduced from the comparative analysis of the effects of alpha-sarcin and cycloheximide (Olmo *et al*. 2001). Olmo and colleagues reported that cycloheximide does not induce apoptosis even at concentrations far beyond its IC_{50}, where protein biosynthesis is null. It indicates that there may be several mechanisms of cytotoxicity induced by RIPs. Consequently, more mechanisms may contribute to more cytotoxic activities of RIPs against tumor cells. In SiHa cancer cells, for example, p53 are disrupted by HPVs E6 and E7, respectively (Hausen 1996), while T47D cells still express normal level of p53 (Hurd *et al*. 1995), this may cause the SiHa cells become more resistant to apoptosis than T47D cells. This may explain the different sensitivity between SiHa and T47D cells against RIPs.

The variation of sensitivity of two tumor cells employed in our study against RIPs is not fully understood yet. There are still some contradictory reports whether the cytotoxic effect of RIPs is dependent or independent of a surface receptor-mediated pathway. These hypothesis need further elucidation, however, these results may give insight to develop anticancer agent derived from RIPs for certain type of cancer.

CONCLUSION

The protein with RIP-like activity isolated from the leaves of *M. jalapa* showed high cytotoxicity against T47D and SiHa cell lines with different extent (LC_{50}: 0.36 µg/mL and 5.6 µg/mL, respectively), and relatively less cytotoxic to mononuclear cells (LC_{50}: 21.04 µg/mL).

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