

Induction of apoptosis by protein fraction isolated from the leaves of *Mirabilis jalapa* L on HeLa and Raji cell-line

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SUMMARY

The leaves of *Mirabilis jalapa* L contains protein fraction presumed ribosome-inactivating protein (RIP). RIP is a group of protein that has RNA N-glycosidase activity that is capable to inhibit protein synthesis. Protein fraction of the plant was shown to be cytotoxic on HeLa cell-line, however, the mechanism by which the protein kill the cells is not identified yet, whether through apoptosis, necrosis, or other mechanism. This research aim to study the mechanism of cell death caused by the protein fraction isolated from the leaves of this plant on HeLa and Raji cell-line, as representative of different kind of cancer cells. Results showed that protein fraction isolated from the leaves of *Mirabilis jalapa* L was more cytotoxic to HeLa cell-line (LC50: 0.65 mg/ml) than to Raji cell-line (1.815 mg/ml) on 48 hours incubation time. Moreover, it was demonstrated that the death of HeLa cells caused by the protein fraction was due to induction of apoptosis, while on Raji cell-line was due to non-apoptosis way, presumably via necrosis.

Key words: Ribosome-inactivating protein (RIP); Leaves of *Mirabilis jalapa* L; Cytotoxicity; Apoptosis; HeLa and Raji cell-line

INTRODUCTION

Cancer is the second largest cause of death in both men and women in many countries, after cardiovascular disease (Anonymous, 1998). To date, many anticancer drugs have been developed. However, resistance to anticancer drug was discovered. Therefore, the development of new and safe drugs have become necessary by the pharmaceutical industry. Natural products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from natural products (Clark, 1996). This is especially obvious in the case of antitumor drugs, as exemplified by paclitaxel, vincristine, vinorelbin, teniposide and various water-soluble analogs of camptothecin (Pezzuto, 1997). Therefore it is promising to discover new potential anticancer drug from natural product.

Indonesia is rich of natural biodiversity, and thousands plants are used in folk medicine. One of them is *Mirabilis jalapa* L., which is known as four o'clock plant. This plant has been utilized for traditional medicine in many countries for years including Indonesia. Leaves and roots of the plant were reported to contain an antiviral protein, which was active against the mechanical transmission of certain plant viruses (Kubo *et al.*, 1990). This protein belongs to ribosome-inactivating proteins (RIPs) that are widely distributed in higher plants, and hold promise for agricultural and pharmaceutical application.

RIP is a group of protein that capable to inactivate ribosome by modifying the 28S rRNA through its 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,

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with the consequent arrest of protein synthesis. RIPs have many biological activities, one of them is the capability to cleave supercoiled double stranded DNA become nick-circular and linear form. This property may be used to identify the presence of RIP in a protein mixture from a plant (Ling *et al.*, 1994).

In addition to its antiviral property, protein fraction isolated from *Mirabilis jalapa* L was found to have cytotoxic activity on HeLa cell line (Sismindari *et al.*, 2001). However, the mechanism by which the protein kills the cells was not defined yet, whether through apoptosis or necrosis or other mechanism. Apoptosis, a kind of cellular death characterized by the early activation of endogenous proteases, cell shrinkage, membrane blebbing, and DNA fragmentation, is commonly observed following treatments with various anticancer agent *in vitro* and *in vivo* (Saikumar *et al.*, 1999), and it is the death cells expected after the treatment of anticancer drugs. In this study, we investigated the cytotoxic effect of protein fraction isolated from *Mirabilis jalapa* L in HeLa and Raji cells, which represent different kind of cancer cells, and observed whether the protein fraction could induce apoptosis. We also confirmed whether the protein belongs to RIP. We found that the protein fraction could kill the cells through different mechanism.

MATERIALS AND METHODS

Plant material

The fresh leaves of *Mirabilis jalapa* L (Nyctagynaceae) were collected from Yogyakarta area in January 2002. A voucher specimen is deposited in the Laboratory of Life Science Gadjah Mada University.

Cell culture

The HeLa and Raji cell line were obtained from the stock of Life Science Laboratory Gadjah Mada University. The cells were maintained in RPMI 1640 (SIGMA) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco) and 0.5% fungizon (Gibco). The cells culture were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Isolation of RIP-containing protein from *Mirabilis jalapa* L

Leaves of *Mirabilis jalapa* L (30 gram) were ground

to a powder and stored at -20°C until use. The protein extracts were prepared by homogenizing the ground tissue with extraction buffer (5 mM sodium phosphate, pH 7.2, with 140 mM NaCl) in 4°C. The solution was subsequently centrifuged for 15 min at 1,500 g in 4°C. The supernatant was then added with saturated ammonium sulfate solution under constant stirring and followed by centrifugation for 10 min at 1,500 g. Pellet was solubilised in minimal volume of 5 mM sodium phosphate buffer pH 6.5, and were dialyzed with two changes of 5 mM NaPO₄ buffer pH 6.5 in 4°C for overnight. The dialysate was centrifuged at 8,500 g for 10 min in 4°C, and the supernatant was collected as protein sample. Protein concentration was determined using UV spectrophotometry as total protein (Layne, 1957).

Cleavage of super-coiled DNA by the protein extracts

To confirm the presence of RIP in the protein extracts, we assayed the capability of the protein to cleave a supercoiled double stranded DNA. In this experiment, pUC18 was isolated from *Escherichia coli* DH5a cultured in LB medium containing ampicillin 150 mg/ml at 37°C. After reaching the stationary growth phase, total DNA plasmid was purified by the modified alkaline lysis procedure (Sambrook *et al.*, 1989). One µg of pUC18 was incubated with various amounts of protein extracts to final volume of 10 µl containing 50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, pH 8.0, at 30°C for 1 hour. At the end of the reaction, 4 µl of loading buffer were added. Electrophoresis was carried out on a 1% agarose gel in 0.5×TBE (Tris Borat EDTA) buffer. DNA bands were visualized by staining with ethidium bromide (Sismindari *et al.*, 2000).

Cytotoxicity assay

The exponentially growing cells (5×10⁴ cells/ml) were seeded in 96-well microculture plate with a serial dilution of protein extracts in a volume of 100 µl. The blank was treated with medium containing equivalent concentration of DMSO. After 48 hr incubation, the number of viable cells was ascertained with direct observation for HeLa cells, and with Trypan Blue exclusion method for Raji cells. LC₅₀ value was calculated using Reed-Muench method.

Detection of DNA fragmentation

HeLa or Raji cells at a density of 10^6 cells/ml were treated with 2.5 mg/ml of protein extracts isolated from leaves of *Mirabilis jalapa* L for 48 hours and then collected by centrifugation at $325\times g$ for 5 min. The cell pellets were resuspended in PBS buffer and lysed in 700 μ l of TE buffer containing 0.1% SDS and proteinase K (10 μ g/ml) for overnight at 37°C. After two successive extractions with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) respectively, the aqueous layer was transferred to a fresh centrifuge tube. The DNA was ethanol precipitated and resuspended in TE buffer and containing RNase (100 μ g/ml). The DNA fragmentation was visualized by gel agarose electrophoresis using 2% agarose gel in 0.5 \times TBE buffer.

Apoptosis assay by deadend colorimetric apoptosis detection system

Apoptosis assay in HeLa cells was done with *DeadEnd Colorimetric Apoptosis Detection System* (Promega) developed from TUNEL assay. Briefly, cells (control and treatment) were incubated with biotinylated-12-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT) enzyme to label 3'-OH ends of fragmented DNA. All apoptotic cells were detected using enzyme-peroxidase staining.

RESULTS

Cleavage of supercoiled DNA by the protein extract

Protein extracts isolated from the leaves of *Mirabilis jalapa* L in the concentration of 2 μ g were found to cleave the supercoiled DNA of pUC18 to be nicked circular form (Fig. 1a lane 3). Even in the higher

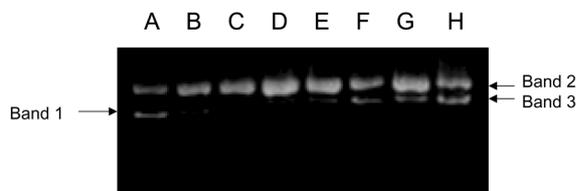


Fig. 1. Effect of protein fraction on the cleavage of DNA plasmid (pUC18). Lane A: pUC 18 control; lane B - H: pUC18 treated with protein fraction with concentration of 0.5, 1.0, 2.0, 6.0, 10.0, 12.0, and 14.0 mg/ml, respectively. Band 1: supercoiled form of DNA; band 2: nick-circular form of DNA, band 3: linear form of DNA.

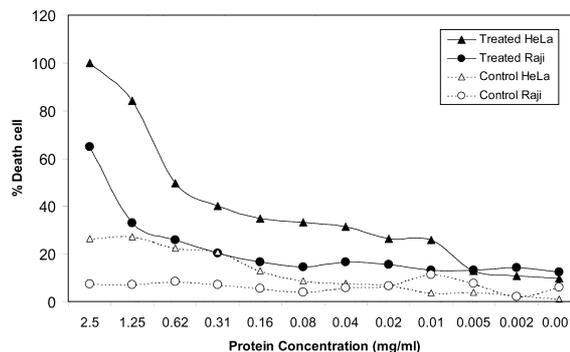


Fig. 2. Cytotoxic effect of protein fractions from *Mirabilis jalapa* on HeLa (\blacktriangle) and Raji (\circ) cells. Data are mean of 4-6 experiments.

concentration, i.e. 3 μ g, the nicked circular was cleaved to be linear form (Fig. 1), which moved between supercoiled DNA and nicked circular form. The cleavage of supercoiled DNA activity seemed to be concentration-dependent, as shown by gradual fainting of supercoiled DNA and gradual appearing of nicked and linear DNA band in the agarose gel, parallel with the increasing amount of protein (Fig. 1). As the cleavage of supercoiled DNA is one property of RIP (Ling *et al.*, 1994), these results indicate that the protein extracts might contain RIP.

Cytotoxic activity

The presumed RIP-containing proteins demonstrated cytotoxic activity in HeLa and Raji cell line with different level, respectively, as shown in Fig. 2. After 48 hours incubation, LC50 of the protein fraction on HeLa cell line was 0.65 mg/ml, while on Raji cell-line was 1.815 mg/ml.

Induction of apoptosis

In order to confirm the mechanism of death cells caused by the extracts, apoptosis assay was done. Apoptosis assay was carried out with two methods, i.e. agarose gel electrophoreses to detect DNA fragmentation and staining method developed from TUNEL assay. As indicated on Fig. 3, it was found that protein extracts broke-down the DNA of HeLa cells into fragments (see Fig. 3a). The phenomenon, however, was not found in Raji cells (see Fig. 3b). The apoptotic death of HeLa cells was also shown by staining method using *DeadEnd Colorimetric Apoptosis Detection System* (Fig. 4a and b).

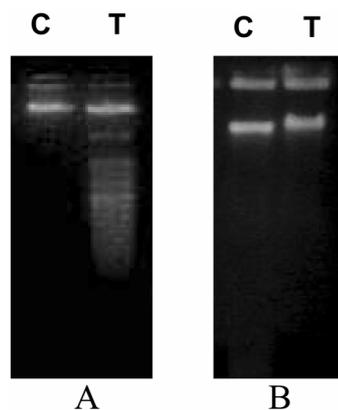


Fig. 3. Induction of DNA fragmentation in HeLa cells (A) but not in Raji cells(B). The cells were treated with 2.5 mg/ml protein fraction isolated from *Mirabilis jalapa* for 24 hours at 37°C and were then harvested for DNA isolation. Characteristic apoptotic DNA ladder were observed in HeLa cells but not in Raji cells.

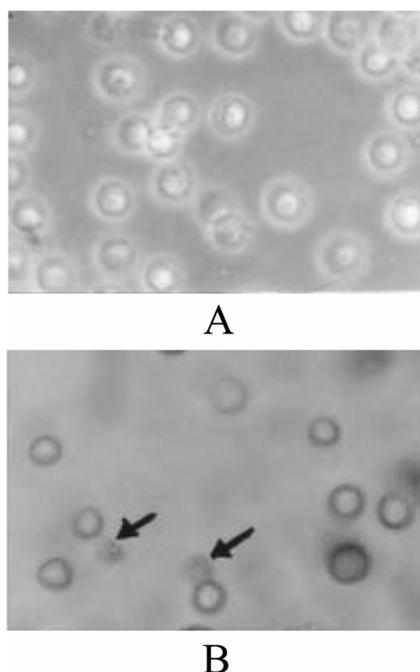


Fig. 4. HeLa cells stained with *DeadEnd Colorimetric Apoptosis Detection System* after incubation with medium for control (A) and protein fraction isolated form *Mirabilis jalapa* (B). The cells was shrunked and stained dark brown indicating that the cells undergo apoptosis process (arrow).

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DISCUSSION

The results of our research show that the leaves of

Mirabilis jalapa L contain protein fraction that presumed to belongs to RIP. The presence of RIP is indicated by the capability of the protein extracts to cleave supercoiled DNA to be linear and nick-circular form. This property is one of the characteristics of RIP beside the N-glycosidase activity.

Several proteins have been found in genus *Mirabilis*. One of them is *Mirabilis* Antiviral Protein (MAP) isolated form the root of *Mirabilis jalapa* (Kataoka et al., 1990). MAP, a type I RIP, was purified to homogeneity and was revealed to be lysine rich and basic (pI 9.8), with molecular weight close to 24.2 kDa as determined by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) (Takanami et al., 1990). MAP was shown to inhibit protein synthesis in *Escherichia coli* as well as in eukaryotes (Habuka et al., 1991) and to possess repellent properties against aphid and white flies (Verma and Kymar, 1979). Others that are isolated from the roots of *Mirabilis expansa* are ME1 (27.5 kDa) and ME2 (27 kDa), and shows high similarity to the MAP (Vivanco et al., 1999). The protein fraction in this study has not been investigated and identified further, however, from the purification we found a single protein with the molecular weight close to 30 kDa (Sudjadi et al., 2003). Other kind of RIP is expected to be found in *Mirabilis jalapa* L planted in Indonesia as we are investigating now. A study concerning this matter is now being carried out in our laboratory.

Our protein fraction showed cytotoxic effect on HeLa and Raji cell line with different level of potency. The protein was found to be more cytotoxic to HeLa than to Raji cell line. After 48 hours incubation, the LC₅₀ of the protein was 0.65 mg/ml for HeLa and 1.815 mg/ml for Raji cell. On the same concentration (the highest concentration tested: 2.50 mg/ml), the protein caused 100% cell death in HeLa cells, but only less than 65% on Raji cells. HeLa and Raji cells represent different kind of cancer cell, which enable us to have description of the effect of the protein fraction on different cell system. HeLa cells are the classic example of an immortalized cell line. These are human epithelial cells from a fatal cervical carcinoma transformed by human papillomavirus 18 (HPV18). While Raji cells are lymphoblast-like cells which was established by R.J.V. Pulvertaft in 1963 from a Burkitt lymphoma

of the left maxilla of an 11-year-old Negro male. It seems that the mechanism of cell death in both cells is different each other, as consequence of different level of cytotoxicity of the protein fraction.

To address this question, we study the effect of the protein on induction of apoptosis, one of the mechanism of cell death. From this study, we found that the protein fraction caused DNA fragmentation in HeLa cells, indicating that the cells undergo apoptosis process (Fig. 3A). While in Raji cells, there was no evidence of DNA fragmentation after 48 hour incubation with the protein, indicating that there was no apoptosis. The resistance of Raji cells against induction of apoptosis by the protein fraction may correlate with the lower cytotoxic effect of the protein on those cells compared to HeLa cells. These results are in line with Kawabata (1999) reporting that Raji cells were resistant to apoptosis induction, 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 Tripan Blue exclusion. The necrotic cells were recognized by swelling and nuclear flocculation, and have no ability to exclude the dyes.

The mechanisms by which the protein fraction, presumed RIP, are able to induce apoptosis in HeLa cells are still not well understood. Apoptotic cell death is initiated by a cascade of events mainly through two different mechanisms involving distinct initiator caspases. One of these mechanisms is receptor-dependent and is directly related to the activation of caspase-8 and possibly caspase-10. The other mechanism, in response to genotoxic agents, anti-cancer drugs, etc., provokes alterations in the mitochondria, leading to the induction of a pathway that activates caspase-9. We did not yet investigate whether the protein fraction initiates the activation of any caspase. But some authors have suggested that any toxin that produces approximately 90% inhibition of protein biosynthesis may induce apoptosis (Kochi *et al.*, 1993). In fact, it has also been reported that cleavage of the large ribosomal subunit 28S rRNA is a common feature in several cases of apoptosis-mediated cell death (Houge *et al.*, 1995). As mentioned before, the capability to cleave of the N-glycosidic bond of the 28S rRNA is a property of RIP. Therefore, if the

protein fraction is really RIP, it is possible that the protein fraction from *Mirabilis jalapa* induces apoptosis in HeLa cells by its N-glycosidase activity. However, the molecular mechanism by which the RIP induce apoptosis through its N-glycosidase activity, whether dependent or independently from caspase activation, specially caspase-3, is still need to be elucidated.

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