Original Article:

Mirabilis Jalapa L. Protein as Inductor of Apoptosis on UVB-induced Skin Damage in Mice

Hussaana A[1], Sismindari [2], Widyarini S[3], Sudjadi [4], Ikawati Z[5]

Abstract

Background*:** *Mirabilis jalapa* L. protein (MJ-Protein) has been shown to have antioxidant and anti-inflammatory effects in vitro. Thus, it has a potential protective effect against ultraviolet B (UVB)-induced skin damage. ***Objective: To determine the protective effect and mechanism of MJ protein in UVB-radiation exposed mouse skin. ***Methods*:** In this experimentalstudy, 30 female BALB/c mice aged 6 weeks were exposed to a single dose of UVB irradiation with 3 minimal erythema doses (MEDs) and continued with the treatment of 0.6 mg MJ-Protein topically. The number of apoptotic body (sunburn cells) formed in epidermal layers of mouse dorsal skin was assessed at 1, 24, 48, 72, 96 and 120h after UVB irradiation was compared to that of the control group. The difference in the sunburn cells number between two groups were analyzed using independent T-test with the level of significance of 0.05. The apoptosis mechanism was confirmed qualitatively by caspase-3 and DNA fragmentation analysis in vitro. ***Results*:**At 24 h after the UVB exposure (peak time for sunburn cells formation), there was a significant increase in the sunburn cells number in the group treated with topical application of MJ-Protein. There was increased caspase-3 expression and DNA fragmentation in HeLa cells treated with MJ-Protein.

***Conclusions*:** MJ-Protein protects againts UVB-induced skin damage in mice trough apoptosis induction.

Keywords: *Mirabilis jalapa* L.; protein; UVB irradiation; sunburn cells; caspase-3

Bangladesh Journal of Medical Science Vol. 16 No. 03 July'17. Page : 423-427

Introduction:

MJ-Protein extracted from the leaves of *Mirabilis jalapa* L have been shown to have a typical activity of the Ribosome-inactivating proteins (RIPs), including the capability of cutting supercoiled DNA and RNA N-glycosidase activity.[1]RIP are proteins widespread in various plants. They have a function as a self-defense.[2] RIP activity is due to the inhibition of protein synthesis by ribosome inactivation. RIPs are known to have biological activity as antiviral and cytotoxic against mammalian cells,[2,3,4,5] and have been investigated as a therapeutic agent against cancer and HIV infection.[4] RIPs have alsobeen shown to cause pathogenic cell death via apoptosis pathway.[6] Previous study have demonstrated the existence of RIPs in *M. jalapa* L.[7]

The anticancer potential of MJ-Protein has been proven by their cytotoxic effects against HeLa cells (LC_{50} = 0.65-14.3 mg/ml), Raji cells (LC_{50} = 1.815 mg/ml),[8] T47D cells (LC50 0.36-27.8 ug / ml) and Siha cells (5.6 pg / ml),[7,8]and myeloma 7.4 ug / ml.[8]Cytotoxic effect of MJ-Protein was mediated by apoptosis mechanism.[8]

Apoptosis has an important physiological function in

1. Atina Hussaana, Department of Pharmacology, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang, Indonesia, 50112, atinahussaana@unissula.ac.id
2. Sismindari, Faculty of Pharmacy, Gadjah Mada University, Sekip Utara Yogyakarta, Indonesia, 55281, sismindari@ugm.ac.id
3. Sitarina Widyarini, Faculty of Veterinery, Gadjah Mada University, Jalan Fauna 2 Yogyakarta, Indonesia, 55281, sitarina@ugm.ac.id
4. Sudjadi, Faculty of Pharmacy, Gadjah Mada University, Sekip Utara Yogyakarta, Indonesia, 55281
5. Zullies Ikawati, Department of Pharmacology, Faculty of Pharmacy, Gadjah Mada University, Sekip Utara Yogyakarta, Indonesia, 55281

Correspondence to: Atina Hussaana, Departemen Farmakologi Fakultas Kedokteran Universitas Islam Sultan Agung, Jl. Raya Kaligawe Km 4 Semarang 50112. orcid ID: http://orcid.org/0000-0002-0244-2713, Email: atinahussaana@unissula.ac.i

treating skin cells after the exposure to the sun. DNA damaged caused by UVB radiation would be driven to undergo apoptosis as sunburn cells thereby preventing the development of cancer. RIPs have the capability to induce apoptosis through ribotoxic stress response pathway or mitokondrial pathway.[9] In addition, the mechanism of apoptosis induction by RIP can also occur by down regulating anti apoptosis factors, or by inducing DNA damage due to nuclease activity, or inducing the decrease of NAD + (Nicotinamide adenine Diphosphat) due to hyperactivation PARP (Poly ADP-ribose polymerase).[9]

Apoptosis in the skin tissue can be assessed by the occurrence of sunburn cells (SBC) in the epidermis, the degree of DNA fragmentation and the expression of caspase-3.[10] SBC sare apoptotic form of cell body, displaying a distinct morphology, a pyknotic nuclei and a shrunken glassy, eosinophilic cytoplasm.[11] The SBC shapes can be distinguished from normal cells by Haematoxylin-Eosin (HE) staining.[10] SBC is a hallmark of epidermal cells undergoing apoptosis due to sun irradiation.[10] It is necessary to study the protective effects of MJ-Protein against UVB- induced DNA damage by obtaining the SBC formation.

Materials and methods:

Selected mature leaves of *Mirabilis jalapa* L. were harvested from botanical garden at Faculty of Pharmacy Universitas Gadjah Mada (GMU).

Chemicals Chemicals and culture media used in this study were from Sigma and E.Merck. Mouse monoclonal IgG caspase-3 (Santa Cruz Biotechnology). HeLa cell lines were obtained from *Laboratorium Penelitiandan Pengujian Terpadu* (LPPT) Universitas Gadjah Mada (UGM), Yogjakarta Indonesia.

Apparatus and animals. UVB lamps (UVB tube, Philips TL40W 12/RS), were from Faculty of Medicine, Universitas Gadjah Mada (UGM), Yogjakarta Indonesia. BALB/c mice (aged 6 weeks) were obtained from *Laboratorium Penelitiandan Pengujian Terpadu* (LPPT) UGM, Yogjakarta Indonesia. Mice were housed under conventional animal house conditions in wire-topped plastic cages and were maintained at an ambient temperatureof 25°C (with a 12-h light/12-h dark photo cycle), and fed stock rodent pellet and tap water *ad libitum*. All procedures performed involving animal were approved by the UGM Animal Ethics Committee.

MJ-Protein Preparation

Fresh *M. jalapa* L. leaves was grinded in 5mM sodium phosphate buffer pH 7.2 containing 0.14mM of NaCl (10 ml/g) and filtered with sterile gauzes. The supernatant was precipitated by acetone (1:1) and then centrifuged at 1288 g for 20 minutes. The precipitated protein (referred as MJ-Protein) was diluted in 5mM sodium phosphate pH 6.5 and stored at 4°C. The molecular weight of MJ-Protein was obtained by SDS-PAGE electrophoresis.

Apoptosis induction in UVB-radiation exposed mice skin treated with MJ-Protein

Mice were divided randomly into 2 groups of 15 mice. A group as control (treated with protein solvent) and the other group was treated with 0.6 mg MJ-Protein once daily until termination. Topical DMSO was aplied first before MJ-Protein application to enhance the skin permeation. MJ-Protein was applied to the dorsum of mice induced by single dose 720 mJ/cm^2 irradiation of UVB. Three mice from each group were sacrificed at 1, 24, 48, 72 and 168 hour post UVB irradiation. Mid-dorsal skin of mice were excised, stored in a buffer-formalin solution, and paraffin embedded for sunburn cells (SBC) analysis with Haematoxylin-Eosin (HE) staining. SBC was counted on the epidermis, characterized by a pyknotic nucleus and a shrunken glassy, eosinophilic cytoplasm.[10] The SBC was observed by using light microscope with a magnification of 400 times. The average of SBC number was counted from 10 field of view. The difference of the average of SBC number between two groups were analyzed using independent T-test with $\alpha < 0.05$.

Mechanism of apoptosis inductionin vitro

Identification of caspase-3 expression using immunostaining technique. He Lacells (100 µl with a cell concentration of 5 x 10^5 mL) treated with 22.8 ng MJ-Protein and incubated for 24 h. At the end of incubation, cells were harvested and washed with PBS. Cells then dripped on poly-l-Lysine slide and immunocytochemically stained with antibodies against caspase-3. The result was observed under light microscope with a magnification of 1,000 times, compared with untreated HeLa cells. Positive caspase-3 expression was characterized by brown color in the cytoplasm.

Apoptosis detection with electrophoresis methods (modification of Saadat et al).[12] He Lacells (250 µl with a cell concentration of 5 x 10^5 mL^{-1}) treated with 22.8 ng MJ-Protein and incubated for 24 hs. At the end of incubation, the DNA of HeLa cells were isolated by suspensing in lysis buffer (10 mMTris-HCl, pH 8, 0.5 mM EDTA and 100 mMNaCl) containing proteinase-K 10 mg/ml and

0.2% Na dodecyl sulfate and incubated at 37°C over night. DNA was extracted from mixture with phenol: chloro form: isoamyl alcohol (25:24:1) continued with chloroform: isoamyl alcohol (24:1). RNase (100 mg/ml) was added and incubated at 37°C 2 hours. Mixture DNA was extracted again and precipitated with ethanol. The precipitate was dissolved in TE buffer (10 mMTris-HCl, pH 7.4 and 1 mM EDTA). DNA extract was run in 1% agarose gel electrophoresis, stained with ethidium bromide and detected by UV rays.

Ethical Clearance

All procedures performed involving animal were approved by Komisi Etik Penelitian Kedokteran dan Kesehatan (Medical and Health Research Ethics Committee) Faculty of Medicine UGM.

Results:

Protein Extracted from *Mirabilis jalapa* L Leaves (MJ- Protein)

Electropherograms of crude extract from *Mirabilis jalapa* L leaves showed a lot of protein band with molecular weight ranging from 20k Da to 200 kDa (Figure 1b). Protein precipitation using acetone produced protein extract with molecular weight ranging from 28 kDa to 30 kDa, corresponding to molecular weight of RIP. There are three predominant proteins with the molecular weight of 34.8; 30.7 and 29.6 kDa (Figure 1c). This protein extract was then referred as MJ-Protein.

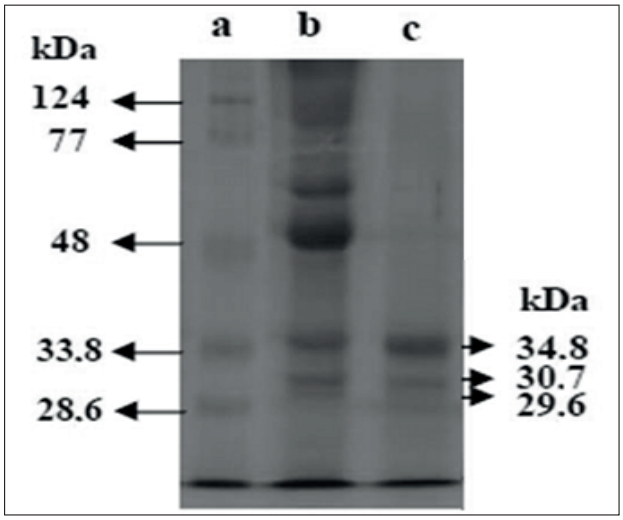

Figure 1. SDS-PAGE electropherograms of Mirabilis jalapa L. leave protein extraction (a) protein marker, (b) crude extract, (c) MJ-Protein, resulted from precipitating the crude extract using acetone. It can be seen that MJ-Protein contain proteins with the molecular weight of 34.8; 30.7 and 29.6 kDa.

This study also confirmed that MJ-Protein is capable to cleave the double stranded DNA (pUC-18) into nicked circular and linier forms. Since that capability is unique enzymatic activity of RIP (*Ribosome-inactivating proteins*), the MJ-Protein capability of of cleaving double stranded DNA into nicked circular or linier forms proving the presence of RIP in the MJ-Protein.[13]In addition, MJ-Protein has also been shown proved to have an antioxidant activity.[14]

In vitro mechanism of apoptosis induction from MJ- Protein

The results of immunocytochemistry staining of caspase-3 showed an increase in the intensity of the brown color in the cytoplasm of treated cells with 7.6 ng/ml MJ-Protein (Figure 2). Our data clearly indicate an increase in the expression of caspase-3. Figure 2 also demonstrates that MJ-Protein treatment in the both HeLa cells adding with or without caspase-3 inhibitor (Z-VAD-FMK), causing DNA fragmentation.

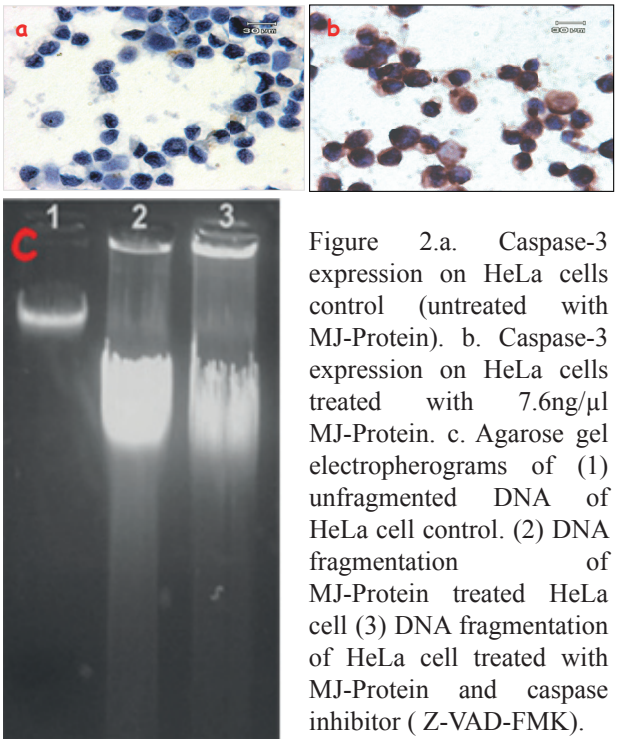

Figure 2.a. Caspase-3 expression on HeLa cells control (untreated with MJ-Protein). b. Caspase-3 expression on HeLa cells treated with 7.6ng/µl MJ-Protein. c. Agarose gel electropherograms of (1) unfragmented DNA of HeLa cell control. (2) DNA fragmentation of MJ-Protein treated HeLa cell (3) DNA fragmentation of HeLa cell treated with MJ-Protein and caspase inhibitor (Z-VAD-FMK).

Effects of MJ-Protein on UVB-induced formation of apoptotic sunburn cells in the epidermis of female BALB/c mice

In this study, the number of apoptotic cells was calculated from the number of SBC formed in the epidermis. The number of epidermal SBC after exposure to UVB radiation was shown in Figure 3. It was showed SBC formation peaked at 24hours after UVB radiation. At the peak time, the topical treatment of 0.6 mg MJ-Protein in mice skin led to a significant increase in the number of SBC ($p < 0.05$).

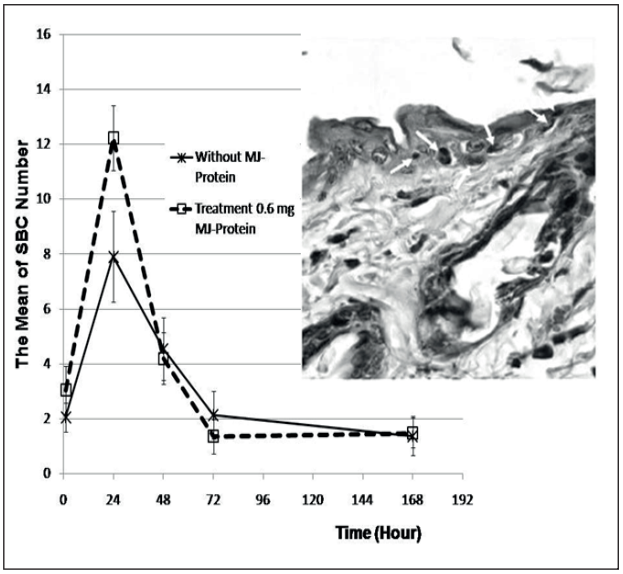

Figure 3. The profile of epidermal sunburn cells (SBCs) formation due to UVB radiation shows the peak at 24 hours. At the peak time, topical application of 0.6 mg MJ-protein significantly increases the mean of SBC number ($p < 0.05$). (Insert: the appearance of epidermal SBC 24 hours post UVB radiation, in Hematoxylin-Eosin staining, observed by using light microscope at 1,000×magnification)

Discussion

In conclusion, that MJ-Protein is capable to increase the caspase-3 expression in HeLa cells. It means that the signal transduction process towards by MJ-proteins induced apoptosis is through the specific activation of cystein-aspartic acid protease(caspase). However, the fact that the both MJ-Protein treated HeLa cells with or without inhibitor of caspase-3 (Z-VAD-FMK) undergoing DNA fragmentation, suggests that the induction of apoptosis in HeLa cells by MJ-Protein involves two pathways (caspase-dependent and caspase-independent apoptosis). Thus, while inducing apoptosis in one way inhibited, apoptosis is still possible to occur with the other pathway.

In this study, the increase in the number of epidermal SBC caused by UVB radiation indicate the capability of MJ-Protein to induce the apoptosis in mouse skin. Since the existence of SBC describing the potential to eliminate unrepaired DNA damage,[15] the capability to increase the number of epidermal SBC furthermore describes the potential to eliminate the possibility of skin cancer. It was found that there was increase in the number of epidermal SBC due to UVB radiation in MJ-protein treated mouse skin, indicated that MJ-Proteins can be used as therapy against UVB radiation induced skin damage.

Overall, our study is consistent with previous studies finding that the number of SBC incidence peaks at 10 to 24 hours following UVB iradiation and almost dissappears at 36 to 48 hour.[10,15] The increased number of SBC after MJ-protein treatment indicated that it could induce apoptosis of cells undergoing DNA damage due to UVB radiation. MJ-Protein involvement in the regulation of apoptosis is present in common with caffeine. Topical application of caffeine has also been shown to increase the apoptotic sunburn cells of skin,[16,17] so that cells undergoing DNA damage will be faster and more eliminated from skin.

It is well known that the regulation of apoptosis may be a decisive factor protection against UV radiation-induced carsinogenesis. MJ-Protein-containing RIP was shown to to cause the death of damaged cells not only by caspase-dependent but also by independent caspase pathway. The caspase-dependent induction of apoptosis by MJ-Protein involving extrinsic or intrinsic pathways, ends on caspase 3 as the executor of apoptosis. This apoptosis mechanism is similiar to that of in ricin, one of other RIP.[18] Activation of caspase 3 by MJ-Protein will cause DNA fragmentation.

Meanwhile, MJ-Protein induces the apoptosis in the caspase-independent pathway by inhibiting protein synthesis. Termination of protein synthesis can occur through the activity of the 28S rRNA N-glycosidases to cleave the N-glycosidic bond between the adenine No. 4324 and its ribose in the 60S subunit of ribosomes. It would inhibit the elongation factor and stop the protein synthesis.[6] The rRNA N-glycosidase activity resulting rRNAdepurination, seems involve in the capacity of MJ-protein to induce apoptosis.

In our study we did not observe the effect of MJ-Protein on DNA repair enzyme system. Thus, it could not be ascertained whether the MJ-Protein has the preventive effect against the UVB radiation induced DNA damage. Nevertheless, the results of this study provides a rational development of MJ-Protein as skin care cosmetics agent againts skin damage due to UVB radiation.

Conclusions:

It can be concluded that MJ-Protein is capable to induce the apoptosis in UVB irradiated mouse skin. The induction of apoptosis occurs through both caspase-dependent and caspase-independent mechanism in vitro.

Acknowledgement:

The author wish to thank the Directorate for Guidance of Research and Community Service *(Direktorat Pembinaan Penelitian dan Pengabdian kepada Masyarakat, Dirbinlitabmas)* of the Directorate General of Higher Education (DitjenDikti) for funding this study through a grant from the Competitive Research Grant *(Program Hibah Bersaing)* fund for the year 2012.

Conflict of interest:

The investigators declare that they have no conflictof interests.

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