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THERAPEUTIC EFFECT OF A RIBOSOME-INACTIVATING PROTEIN FROM *MIRABILIS JALAPA* L. AGAINST MOUSE SKIN CANCER INDUCED BY CO-CARCINOGEN DIMETHYLBENZANTRACENE AND UVB RADIATION

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ABSTRACT

Mirabilis jalapa L. leave has been reported to contain Ribosome-inactivating protein (RIP), which demonstrated to have *in vitro* cytotoxic effect on cancer cell lines and prevention effect against skin cancer. This research examined the therapeutic effect of protein fraction containing RIP isolated from *Mirabilis jalapa* L. (MJ protein) against skin cancer.

MJ protein was isolated using phosphate buffer pH 7.2, and then tested for its activity to cleave supercoiled DNA. To induce the skin cancer, BALB/c mice were topically exposed to single dose of 0.2 mg/0.1 ml dimethylbenzanthracene (DMBA) and UVB radiation (daily dose, 0.4 J/cm^2) for 60 days. Therapeutic effect of MJ protein was examined by observing the incidence, multiplicity, and regression of tumor and followed by analyzing the expressions of bcl2 using immunohistochemistry technique.

The results showed that MJ protein therapy (doses of 0.3/0.1ml and 1.2mg/0.1ml) was able to decrease the incidence of skin cancer (SCC) by 75.5% and tumor nodule regression (approximately 35.3%) at 10 weeks of therapy, while the dose of 1.2mg/0.1ml decreased the tumor nodule diameter by 17.36%. In addition, 0.3 mg /0.1 ml and 1.2 mg/0.1ml of MJ protein were able to significantly decrease the expressions of bcl2 protein at 82.38% and 78.40% compared to *post* UVB control sample (10 weeks of therapy). However, there was no significant difference ($p > 0.05$) among the dose groups.

Keywords: *Mirabilis jalapa* L., RIPs, skin cancer, therapeutic effect.

INTRODUCTION

Ribosome-Inactivating Proteins (RIP) are protein toxins that have N-glycosidase activity to inhibit the protein synthesis by inactivating ribosomes through the cleaving of the N-glycosidic bond at the A4324 position of 28S RNA, so that they are no longer able to function in protein synthesis[1-2]. The term RIP was used exclusively for N-glycosidase because other types of proteins that inactivate or damage ribosomes by other mechanisms (e.g., RNases or proteases) are not considered as RIP[3]. This protein receives a lot of attention in biomedical research because of their unique biological activities toward animal and human cells. Choudhary and colleagues (2008) [4] reviewed that recombinant RIPs have also been found to possess a number of enzymatic properties, such as antiviral [5], RNase activity and depurination of capped mRNAs [6].

Mirabilis jalapa L. leave has been reported to contain Ribosome-inactivating protein (RIP) cleaving single adenine residue (A4324 in rat liver 28S rRNA) [7]. This cleavage leaves the ribosomes unable to bind elongation factors, and hence incapable of performing protein synthesis. The active protein from *M. jalapa* L. was isolated, called MJ proteins. Further purification of this protein using ion exchange chromatography resulted in 2 active RIPs with the molecular weight of aproximately 30kD, they are MJ-30 protein, a basic RIP and MJ-C, an acidic RIP [8]. The MJ-30 protein was reported to have an *in vitro* cytotoxic effect on HeLa cell-line [9] and SiHa cell line [10], with the IC_{50} of 91µg/ml and 5.6 µg/ml, respectively. Whereas the MJ-C was cytotoxic on HeLa, Myeloma and T47D cell lines, with the IC_{50} of 14.3 µg/ml, 7.4 µg/ml, and 27.8 µg/ml, respectively [11]. The death of cells caused by MJ-30 protein on cell line was due to the induction of apoptosis as indicated by its ability to induce DNA fragmentation [12]. One of the apoptotic induction targets in the mitochondrial pathway is a member of the bcl2 protein family. This member can regulate the release of cytochrome c that is important to activate the executioner caspase. Chemotherapeutic agents can be indicated their ability on inducing the apoptotic program with the mechanism of modulating oncogenes that can enhance the expression of tumor suppressor genes (p53), increase expression of Bax protein, a proapoptosis members, and decrease the expression of Bcl2 [13].

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Based on the *in-vitro* finding, further experiment was carried out by analyzing the therapeutic effect of MJ protein *in-vivo* against skin cancer.

METHODOLOGY

Chemicals, apparatus and animals

Fresh *Mirabilis jalapa* L. leaves were harvested from garden at Faculty of Pharmacy Universitas Gadjah Mada (GMU). Deoxynucleic acid (DNA) pUC18 was obtained from laboratory stock of Life Science Laboratory, the Integrated Research and Testing Laboratory (LPPT) UGM. Chemicals and Media used in this study were from Sigma dan E.Merck. UVB lamp (UVB tube, Philips TL40W 12/RS), was from Faculty of Medical UGM. BALB/c mice (aged 6-7 weeks) were from LPPT GMU. Mice were housed in wire-topped plastic boxes and were maintained at an ambient temperature of 25°C (12 hours dark and 12 hours light), and fed stock rodent pellet and tap water *ad libitum*. All procedures using animal were approved by the GMU Animal Ethics Committee (KE/FK/212/EC).

Preparation of MJ protein

M. jalapa L. leaves extract was prepared by grinding the leaves in 5mM sodium phosphate buffer pH 7.2 containing 0.14mM of NaCl (10 ml/g). The extract was precipitated by acetone (1:1) and then centrifuged at 1288 g for 20 minutes. The precipitated protein was diluted in 5mM sodium phosphate pH 6.5 and stored at 4°C. Concentration of MJ protein was analyzed using UV spectrophotometer [7].

Cleavage of supercoiled DNA by MJ protein

Two microgram of supercoiled double stranded plasmid DNA (pUC18) was incubated with 6 µg of MJ protein to a final volume of 10 µl in mixed of 50 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl, pH 8.0, at room temperature for 1 hour. At the end of the reaction, 3 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 using a 0.8% agarose gel (containing ethidium bromide) in TBE (tris-borat) buffer. DNA bands were visualized by using UV lamp [14].

Induction of skin cancer

To induce the skin cancer, BALB/c mice were topically exposed to single dose of 0.2mg/0,1mL 9,10-dimethylbenz[α]anthracene (DMBA) and UVB radiation (daily dose, 0.4 J/cm^2) for 60 days. After the induction, two mice were randomly collected and sacrified (named *post* UVB control sample). Mid-dorsal skin of mice were excised and fixed in 10 % buffer formalin [15].

Therapy of the skin cancer and handling of the mice

Mice were divided into five groups: normal skin control group (15 mice), UVB control/untreated group (10 mice), phosphate buffer control group (20 mice), treated with 0.3mg/0.1 ml of MJ protein group (20 mice), and treated with 1.2mg/0.1 ml of MJ protein group (20 mice). MJ protein was applied to the dorsum of mice induced by DMBA co-carcinogen and UVB sixty times in 10 weeks. DMSO was used before MJ protein application to enhance the permeation of MJ protein. Tumor incidence and tumor multiplicity were observed every day. Tumor incidence is percentage of nodule-possessing mice in a group. Tumor multiplicity is total of nodules in a group per total of mice in a group.

To collect the skin samples, 2-3 mice from each treatment group were sacrified at 6 and 10 weeks of therapy. Mid-dorsal skin of mice were excised, stored in a formalin solution, and paraffin embedded. Subsequently, tissue sections were cut at 3-4 µm onto poly-L-lysine coated slides. For histological analysis, deparaffinized sections were subsequently stained with haematoxylin and eosin, and immunohistochemically stained for bcl2 [15].

Identification of bcl2 protein using immuno-histochemistry technique

The tissue sections were de-paraffinized and subsequently washed with PBS (three times, each for 5 minutes). Endogenous peroxidase was quenched by incubation of tissue in 3% (v/v) of hydrogen peroxide in methanol for 20 minutes and then washed with aquadest and PBS (each of them: for 5 minutes). Citric acid buffer (pH 6.0) and microwave treatment (for 10 minutes) were used for antigen retrieval. Subsequently, the tissue sections were incubated with normal mice serum (1:50) for 5 minutes. After removing of normal mice serum (without washing), bcl2 antibody was applied onto each tissue section (1:100 dilution) at 4°C for 60 minutes. The antibody used was able to recognize wild type and bcl2 mutant. Subsequently, the tissue sections were incubated at room temperature for 5 minutes with biotinylated anti-mouse IgG and then washed with PBS for 5 minutes). Finally, the immunoreactions were visualized using Streptavidin-peroxidase complexes for 10 minutes and the peroxidase reaction was developed in 3,3'-diamino-benzidine chromogen. Tissue sections were counterstained with haematoxylin for 3-4 seconds, followed by dehydration through graded ethanol solutions and then mounted (standard procedure of Pathology and Anatomy Laboratory Sardjito Hospital).
Any statistical analysis was carried out using one way analysis of variance (ANOVA) with SPSS software. The significance level was set at 0.05.

RESULTS AND DISCUSSION

Cleavage of supercoiled DNA by MJ protein

RIP is a toxic protein that inhibits protein synthesis. This activity is believed due to an N-glycosidase activity. In additional to this activity, several RIPs also demonstrated unique enzymatic activity of cleaving double stranded DNA into nicked circular or linier forms [14]. In this experiment, activity test on cleaving supercoiled DNA (puC18) was carried out (as described in the methodology) in order to prove the presence of RIPs in the MJ protein isolated from *M. jalapa* L (Figure 1).

It was demonstrated that the supercoiled DNA band (a) and nicked circular DNA band (c1) appeared at untreated pUC18 (lane 1). At the MJ protein application (lane 2), the supercoiled DNA band (a) disappeared, while linear DNA band (b) appeared, and nicked circular

DNA band (c2) was thicker than untreated pUC18. Therefore, it was demonstrated that the MJ protein contained RIPs. This result clearly supported previous research which indicated that protein isolated from *M. jalapa* L. leaves contained RIPs [7].

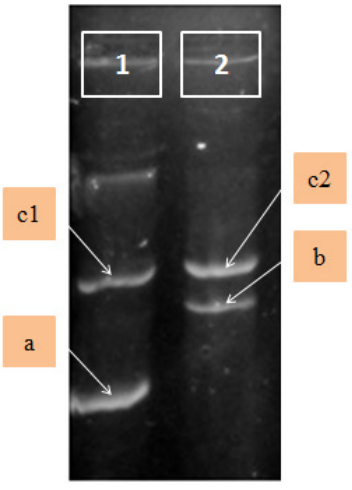

Fig. 1: *Cleavage of supercoiled DNA by MJ protein: (lane 1): untreated pUC18, (lane 2): 6 µg of MJ protein. (a): The supercoiled DNA band, (b) linear DNA band, (c1 and c2): nicked circular DNA*

Skin cancer induced by DMBA application and UV radiation to mouse skin

The results of cancer induction showed that tumor nodule started to appear after 18 times of UVB exposure, and subsequently followed by other mice at the next exposure. After 60 times of UVB exposure, the percentage of tumor incidence was 77%, while there was no mice from the control group showing tumor nodule. The differences of time needed by mice (among UVB exposure group) to show tumor nodule might be caused by genetic polymorphism, differences in immunological response and antioxidant defense system [16], and health condition. DMBA single dose and UVB radiation were used to induce the skin cancer because a previous study showed that combination of these carcinogens was more effective than UVB exposure only [15].

Effect of MJ protein towards tumor incidence and multiplicity

The results showed that the therapeutic effect did not show a consistent result at 4 weeks of therapy. However, there was an inclination of tumor incidence decreased on dose of 0.3 mg/0.1 ml, while dose of 1.2 mg/0.1 ml did not show the decrease of tumor incidence. The effect of MJ protein at both doses (0.3 mg/ml and 1.2 mg/0.1ml) towards the decrease of tumor incidence started to appear at 5 weeks of therapy and continued till 10 weeks of therapy (Figure 2). MJ protein-treated group showed the decrease of tumor incidence by 26 to 38% at 10 weeks of therapy. The MJ protein seemed to be more effective for a preventive effects as indicated by decrease the skin tumor incidence by 50% at the dose of 1.2 mg/0.1 ml [17].

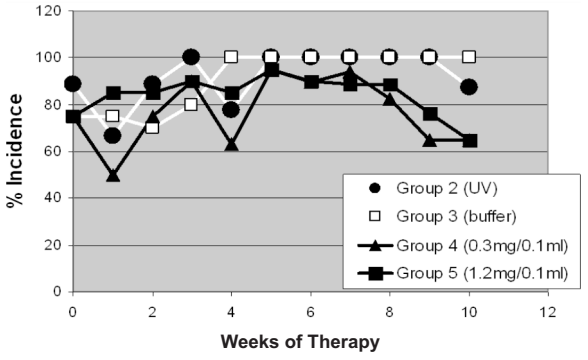

Fig. 2: *The decrease of tumor incidence caused by MJ protein*

Besides observing the tumor incidence, this study also observed the growth of tumor nodule shown in tumor multiplicity. The results showed that MJ protein (doses of 0.3 mg/0.1 ml and 1.2 mg/0.1 ml) was able to decrease the tumor multiplicity. The decrease of tumor multiplicity started to appear at 5 weeks of therapy and continued till 10 weeks of therapy (Figure 3).

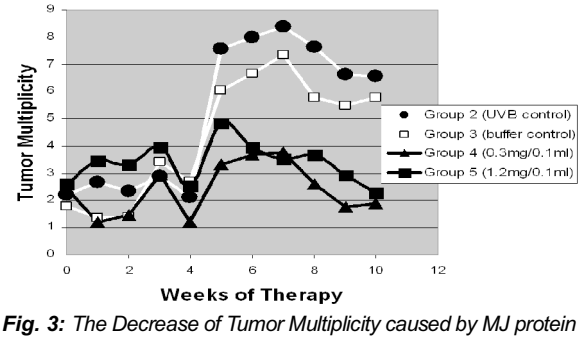

Fig. 3: *The Decrease of Tumor Multiplicity caused by MJ protein*

Tumor nodule regression was also observed with two parameters, namely (1) the rate and amount of mice which was free from nodule tumor, and (2) the diameter of tumor nodule. In the first parameter, the results showed that 12.5% of untreated group (UVB control group) was free from tumor nodule at 10 weeks of therapy, while each of treated group (0.3 mg/0.1 ml and 1.2 mg/0.1 ml) showed amount of mice which is free from tumor nodule by 35.3% at 8 weeks of therapy (Figure 4). It means that the tumor nodule regression of MJ protein-treated group was two weeks faster than untreated group. Subsequently, the percentage of the mice which is free from tumor nodule in treated group was more than untreated group.

The second parameter of tumor nodule regression was the decrease of nodule diameter. The group 5 (dose of 1.2 mg/0.1 ml) showed the mean decrease of nodule diameter by 17.36%, while the group 4 (dose of 0.3 mg/0.1 ml) showed the mean increase of nodule diameter (not shown).

The skin cancer types which appeared in this experiment were: papilloma, squamous cell carcinoma (SCC) and fibroma. Papilloma and fibroma are benign tumor at epithelial layer and fibrous connective tissue,

respectively, while SCC is malignant tumor. This experiment showed that MJ protein therapy (doses of 0.3 mg/0.1 ml and 1.2 mg/0.1 mL) was able to decrease the incidence of skin cancer (SCC) by 75.5%.

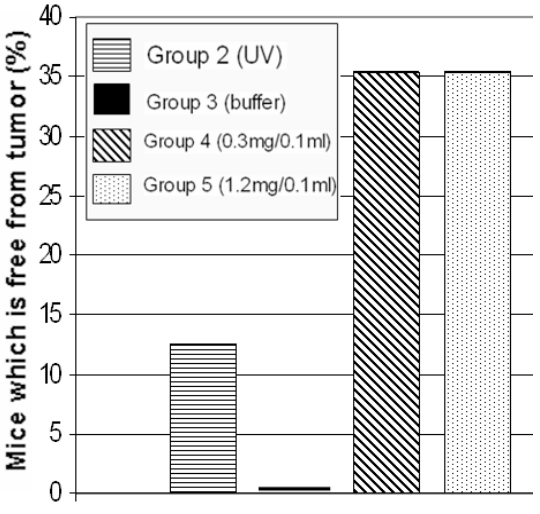

Fig. 4: *The effect of MJ protein towards tumor nodule regression (amount of mice which was free from tumor nodule).*

Effect of MJ protein towards bcl2 protein expression

As shown in Figure 5, the expression of bcl2 protein (epidermal cells expressing bcl2 proteins had brown membrane and cytoplasm), the anti-apoptotic protein, in the group treated with the dose of 0.3 mg/0.1 ml and 1.2 mg/0.1 ml MJ protein was significantly decreased at 82.38% and 78.40% compared to *post* UVB control sample (10 weeks of therapy). However, there was no significant difference ($p > 0.05$) among the dose groups. On the other hand, the expression bcl2 protein in the group treated with phosphate buffer was decreased at 15.87% compared to *post* UVB control sample. This result supported the previous finding that protein containing RIPs was able to induce the p53 expression and decrease the bcl2 expression on cancer cell lines [18].

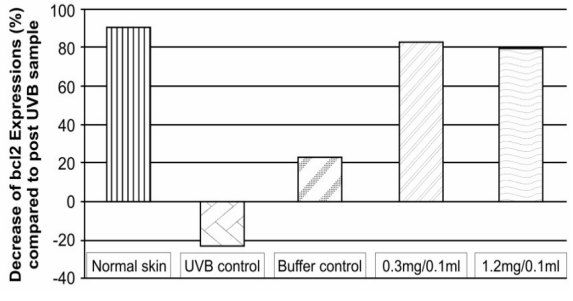

Fig. 5: *The decrease of bcl2 protein expression by MJ protein*

Bcl2 protein expression was observed at epidermis layer because most of UVB-induced skin cancer happens in this layer [19]. In addition, long-term exposure to UVB radiation significantly changes the expression of the Fas receptor and its ligand in the epidermis, so that it results in UVB-induced immunosuppression, a process promoting skin cancer outgrowth [20].

Suppression of bcl2 protein expression showed that the therapeutic effect of MJ protein may have been caused by apoptotic mechanism via mitochondrial pathway, as indicated by the apoptotic effect this protein on cancer cell lines [21]. Induction of apoptosis was also demonstrated by MJ protein on prevention effects, when it was applied prior UVB radiation [22]. This apoptotic effect is found on most protein inhibitor compounds which can decrease protein synthesis more than 90% [23], as was the case on MJ protein. In addition of all these effects, MJ protein was able to protect the inflammation process and had immunosuppressive effect on UVB induced mice [24].

Based on all these finding, MJ protein become a potent candidate for cancer therapy and prevention, especially when it could be formulated as nanoparticles, such as using chitosan. Recently, nanoparticle for drug formulation become interesting since this formula could protect the drug in the systemic circulation, so that could increase therapeutic benefit and minimizing the side-effects [25,26]. The mechanisms by which MJ protein decreased the expression of bcl2 protein on skin cancer therapy need to be investigated further.

CONCLUSION

MJ protein therapy was able to decrease the incidence of skin cancer (SCC) by 75.5% and tumor nodul regression (approximately 35.3%) at 10 weeks of therapy. It was also able to decrease the tumor nodul diameter by 17.36%. In addition, using 95% of confidence level, MJ protein was able to significantly decrease ($p > 0.05$) the expressions of bcl2 protein at 82.38% and 78.40% compared to *post* UVB control sample (10 weeks of therapy).

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