PROTEINS FROM *MIRABILIS JALAPA* POSSESS ANTICANCER ACTIVITY *VIA* AN APOPTOTIC PATHWAY

Rith Watthanachaiyingcharoen[1],* Parkpimol Utthasin[1], Tulaya Potaros[1] and Prapan Suppakpatana[2]

[1]Faculty of Pharmacy, Srinakharinwirot University, Ongkharuk, Nakorn Nayok 26120, Thailand
[2]Department of Anatomy, Faculty of Dentistry, Mahidol University, Bangkok 10140, Thailand

ABSTRACT: *Mirabilis jalapa* is a flowering plant cultivated in many areas of Thailand. The proteins purified from this plant species show several biological activities including anti-bacterial, antiviral and immune stimulating properties. In this study, the proteins which were partially purified by ammonium sulfate precipitation, were investigated to determine the mechanisms of anticancer and cytotoxic effects. SDS-PAGE showed two major proteins at 27 kDa and 62.5 kDa in size. The cytotoxicity test on brine shrimp exhibited an LD_{50} of 95.50-489.78 µg/ml at 24 and 48 hours. In addition, the experiment testing these proteins on vero cells indicated its potent anticancer activity through apoptotic pathway. Its activity was confirmed by DNA fragmentation and cell morphology change methods.

Keywords: *Mirabilis jalapa*, plant proteins, cytotoxicity, apoptosis, anticancer

INTRODUCTION: The four-o'clock flower (*Mirabilis jalapa* L.), which is a native of tropical South America, has been naturalized as an ornamental garden plant in many parts of the world, including Thailand. The flower is open from late afternoon[1)]. The medicinal properties of *M. jalapa* have been well characterized in several studies. A protein purified from the root tubers of *M. jalapa* was confirmed to have an antiviral activity[2)]. It has an inhibitory effect on Cell-free proteins synthesis, an antiproliferative effect on tumor cells, and also inhibits the mechanical transmission of plant viruses and the in vitro protein synthesis of prokaryotes and eukaryotes[3,4)]. The major protein, which is named Mirabilis antiviral protein (MAP), is a type I Ribosome-inactivity proteins (RIPs)[5)]. The purified MAP is a lysine rich protein and is basic (pH 9.8), with a molecular weight close to 24.2 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[6)].

The objective of this study was to purify proteins from the *Mirabilis jalapa* seeds by ammonium sulfate precipitation. The obtained proteins were used to determine the mechanism of anticancer activity and the cytotoxic effect on Vero cells. The apoptotic studies were determined using cell morphology and DNA fragmentation methods. The apoptosis process caused some changes in normal living cells. First, the structure of the cells changed and organelles shrank and lost their normal intercellular contacts with neighboring cells. Subsequent processes included cytoplasmic vacuoles, plasma and nuclear membrane blebbing, chromatin condensation and cellular fragmentation into small apoptotic bodies. The morphological study used microscopic observation of the cell structure with and without stain. DNA fragmentation was the product of apoptosis.

MATERIALS AND METHODS:

Plant materials

Seeds of *M. jalapa* were bought at the local market in Nakorn Nayok Province, Thailand between January and August 2007.

Chemicals

The chemicals for protein extraction, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyltetrazolium bromide), Hoechst 33342 dye were purchased from Pacific Science Co., Ltd. Culture medium, DMEM (Dulbecco's Modified Eagle Medium) and fetal bovine serum from GibThai Co., Ltd.

Cell culture

The Vero cells were obtained from the Department of Anatomy, Faculty of Dentistry, Mahidol University, Bangkok, Thailand.

Protein extraction[7)]

The seeds were cleaned, dried and stored in a refrigerator at 4°C. Then the seeds were mixed with phosphate buffer (10 mM sodium phosphate

*To whom correspondence should be addressed.
Email: rith@swu.ac.th,
Tel. +66 2664 1000 ext. 1539, Fax. +66 3739 5096

buffer pH 6.3 and 50 mM NaCl) with a ratio of 2 ml buffer per gram of seeds and blended with a homogenizer for 5 minutes to make a fine emulsion at low temperature (4°C). The emulsion was stirred at 4°C for 1 hour, filtered through double cheesecloth and then centrifuged at 18,000 rpm, at 4°C for 10 minutes. The supernatant containing the crude (total) protein was stirred and dialyzed through a dialysis membrane (molecular weight cut off 1000, Cell-Sep™, USA) in phosphate buffer without NaCl at 4°C and then centrifuged at 18,000 rpm for 10 minutes. This supernatant is referred to as the dialyzed crude fraction (CR). The crude protein suspension was precipitated with 0-30%, 30-60% and 60-90% saturation of ammonium sulfate in stages and centrifuged at 18,000 rpm, 4°C for 10 minutes. The three supernatants were retained as 0-30%, 30-60%, 60-90% saturated fractions. The protein concentration was determined by Bradford assay with Coomassie® Plus Protein Assay Reagent (PIERCE®, USA).

Determination of the molecular weight of proteins

SDS-PAGE is a method for separating proteins based on the molecular weight of each protein. The SDS-PAGE mini gel system was used according to the manufacture's protocol (Whatman®, USA). The SDS-PAGE gel was prepared in discontinuous concentration (15% separating gel and 5% stacking gel) and applied at 100 constant voltages. The log 10 of the standard molecular weight proteins was plotted in a semi-logarithmic graph. The molecular weight of target proteins was calculated from the standard curve based on their R_f values.

Brine shrimp lethality assay[8,9]

Brine shrimp (*Artemia salina*) is a preliminary screening method for determining the cytotoxicity of active plant constituents. Brine shrimp eggs were laid into vessels; A half of vessel was covered with aluminium foil to protect light while other was exposed to light. Brine shrimp eggs were hatched in artificial seawater and oxygenated with an aquarium pump at room temperature. After 36-48 hours incubation, the nauplii were collected with a Pasteur pipette. A suspension of nauplii containing 10-15 organisms (50 µl) was added to each of 96 -well plates. Serial dilutions of test compounds were made into the wells and 100 µl of artificial seawater was added to each well. The vessel was covered with a plate and further incubated at room temperature for 6-48 hours. Absolute ethanol (97% w/v) was added to each well. In order to determine the cytotoxicity of proteins, plates were examined under a binocular stereomicroscope and the numbers of dead nauplii in each well were counted.

Cytotoxicity assay

The cytotoxicity assays were performed according to the micro-culture MTT method[10,11]. The cells were harvested at $5.0X10^4$ cells/well (150 µl) and inoculated in 96-well plates. The cell cultures were incubated under 5% CO_2 at 37°C for 24 hours. The cells were washed with phosphate buffer solution and culture medium. The cultured cells were then inoculated with 150 µl new culture medium either with or without the tested protein. The tested proteins were diluted in serial dilution. Next, each protein dilution was tested for at least 3 replications. Untreated cells were used as controls. After a 24 hour incubation, the medium was aspirated. 10 µl of MTT solution (5 mg/ml in phosphate buffer solution, pH 7.2) was added to each well and the plates were incubated for 4 hours at 37°C. After incubation, 100 µl of DMSO was added to the wells and gently shaken for 15 minutes to solubilize the formazan dye. Absorbency was read at 540 nm and the surviving cell fraction was calculated. The inhibition of cell viability was calculated by means of the formula: % inhibition = (1 - absorbency of treated cells/absorbency of untreated cells) x 100.

Apoptotic assay

Morphological study with fluorescence dyes[12-14]

Cells ($5x10^5$ cells/well) were seeded in 24-well plates with 0.5 ml DMEM and incubated under 5% CO_2 at 37°C for 18-24 hours. The culture medium was discarded and washed with normal saline to remove residues. The culture was replaced with new medium and serial protein solutions were added. The positive and negative controls were a culture medium containing 5-fluorouracil and the medium, respectively. The cells were incubated under 5% CO_2 at 37°C for 18-24 hours. The staining cells were flooded with Hoechst

33342 dye (1 μg/ml of bisbenzimide dye in Hank's balanced salt solution) and incubated for 15 minutes under 5% CO_2 at 37°C. The cultures were washed with Hank's balanced salt, analyzed and photographed using a fluorescence microscope.

DNA fragmentation[15,16]

3x10^6 Vero cells in DMEM were seeded in 6-well plates and incubated under 5% CO_2 at 37°C for 18-24 hours. The cells were exposed to the test protein solutions for 24 hours and harvested into centrifuge tubes. They were washed with phosphate buffer and centrifuged at 18,000 rpm, 4°C for 10 minutes. The residue was digested in 100 μl of lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl and 10 mM EDTA) and RNase (10 μg/ml) at 50°C for 2 hours. This was followed by digestion in proteinase K (20 μg/ml) and extraction with 600 ml of chloroform/isoamyl alcohol (24:1, v/v). The aqueous phase was precipitated and analyzed using 2% agarose gel electrophoresis.

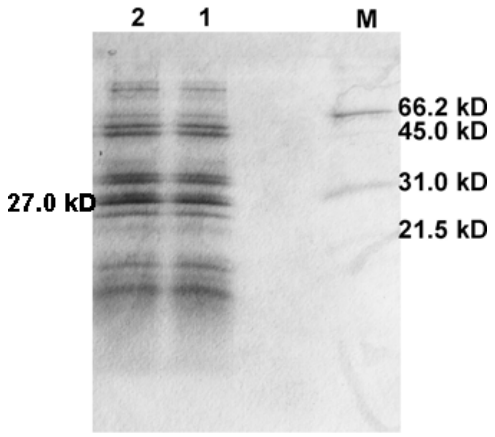

Figure 1 SDS-PAGE Analysis of the proteins extracted from *M. jalapa*. The proteins were analyzed by discontinuous gel (5% stacking gel and 15% separating gel) with coomassie blue staining: lane M = Bio-Rad marker, lane 1, 2 = dialyzed crude proteins, lane

RESULTS AND DISCUSSION: Proteins were extracted from seed of *M.jalapa* by using 10 mM sodium phosphate buffer pH 6.3 contained 5 mM EDTA. The proteins were then partially purified by precipitation with ammonium sulfate at 0-30%, 30-60% and 60-90% saturation. Proteins were characterized using SDS-PAGE, and had molecular weights of 66.7, 62.5, 55.6, 44.0 32.6, 31.5, 27.0, 24.0 and 13.5 kDa. The major protein had a molecular weight of 27.0 kDa (Figure 1). It has been reported that RIPs are widely distributed in many plant species[17]. The RIPs were separated into 3 categories: RIPs type I, II and III. The type I RIPs are a single polypeptide chain, a basic protein with pI 8-10 and a molecular mass ranging from 26-35 kDa[17,18]. Type II RIPs are highly toxic double chains that have molecular masses of about 60 kDa. Type III RIPs are synthesized as proRIPs which require proteolytic processing and form a single active chain[19]. Many researchers have reported that RIPs are proteins which have antibacterial, antiviral and anticancer properties. In addition, a RIP with a molecular weight of about 27 kDa from *M. jalapa*[4,5] and *M. expansa*[20] has previously been described.

Brine shrimp lethality test was used to test the cytotoxicy of the protein. Cytotoxicity to brine shrimp could not be used to determine the LD_{50} over a short time period (at 6 and 12 hours), but it could be determined at 24 and 48 hours. At 24 hours, the LD_{50} was 359.75, 368.13, 1406.05 and 489.78 μg/ml for dialyzed crude protein, in a solution of 0-30%, 30-60% and 60-90% ammonium sulfate precipitated proteins. At 48 hours, the LD_{50} was 186.64, 263.03, 95.50 and 197.70 μg/ml. This assay suggested this protein was only slightly toxic to a normal cell.

The IC_{50} in Vero cells was 3981.07, 6309.57, 1412.54 and 50.12 μg/ml of dialyzed crude protein for the protein solutions precipitated at 0-30%, 30-60% and 60-90% ammonium sulfate, respectively. Cytotoxic testing in brine shrimp at 24 hours with the protein solution precipitated in 60-90% ammonium sulfate exhibited the highest cytotoxicity against Vero cells. These results suggested that this protein fraction had a greater cytotoxic effect against permanent cells than normal brine shrimp cells.

In additions, the apoptotic processes were established in protein-treated vero cells by using microscopic and DNA fragmentation techniques. After incubation with the protein solutions, Vero cells were altered due to shrinking of organelles, blebbing in cell membranes and chromatin condensations. Hoechst dye solution penetrated into the nucleus and chelated with the chromatin

and visualized as fluorescence-labeled chromatin. After staining, we observed chromatin condensation and strong fluorescence. The change in the genetic material was evidence that they were going through an apoptotic process (Figure 2). The saturated proteins of 60-90% ammonium sulfate exhibited high levels of cytotoxic and apoptotic activity (Figure 2F).

Using agarose gel electrophoresis technique, the DNA fragments of treated-vero cells were separated as small pieces of DNA as shown in figure 3. Our result was correlated with previous study. Their results also showed anticancer activity of crude protein extracted from *M. jalapa* against the permanent cell lines L929[7]. These results suggested that the proteins from seeds of *Mirabilis jalapa* had potential as anticancer compounds and for use in clinical research laboratories.

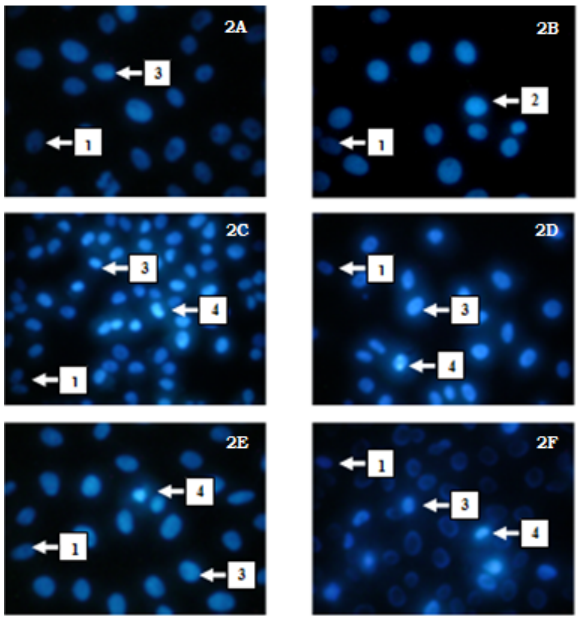

Figure 2 The Vero cells after exposure to the proteins and Hoechst 33342 staining

2A = Vero cells exposed to 5-fluorouracil (positive control)
2B = Vero cells exposed to absolute ethanol (necrosis)
2C = Vero cells exposed to the crude proteins from M. jalapa
2D = Vero cells exposed to 0-30% ammonium sulfate precipitated proteins from M. jalapa
2E = Vero cells exposed to 30-60% ammonium sulfate precipitated proteins from M. jalapa
2F = Vero cells exposed to 60-90% ammonium sulfate precipitated proteins from M. jalapa
(1 = normal cell, 2 = necrosis cell, 3 = early apoptotic cell, 4 = late apoptotic cell)

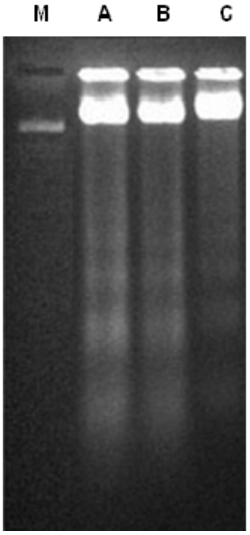

Figure 3 The DNA fragmentation of Vero cells using agarose gel electrophoresis

M = Vero cells exposed to absolute ethanol (necrosis)
A = Vero cells exposed to 0-30% ammonium sulfate precipitated proteins from *M. jalapa*
B = Vero cells exposed to 30-60% ammonium sulfate precipitated proteins from *M. jalapa*
C = Vero cells exposed to 60-90% ammonium sulfate precipitated proteins from *M. jalapa*

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