Apoptosis Induction by *Rafflesia kerrii* Meijer Flower Extract *via* Caspase-Dependent and Down-Regulation of ERK Signaling Pathway in Epidermoid Carcinoma Cells

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Abstract: Plant extracts have been shown to be important sources of anti-cancer agents. *Rafflesia kerrii* Meijer (RM) is found in the rainforest of southern part of Thailand and its local name is Bua Phut. The single biggest flower has awful smell to attract flies for pollination. This study aims to investigate the activity of RM flower extract on apoptosis induction in skin cancer cells. The results showed that the RM flower extract showed the anti-proliferative activity in A431 skin cancer cells in a time- and dose-dependent manner. The morphological changes and nuclear staining with Hoechst 33342 showed evidence of apoptosis including nuclear condensation and fragmentation. RM flower extract also activated caspase-3 expression. Furthermore, phosphorylated-ERK was decreased but phosphorylated-JNK and phosphorylated-p38 were increased in A431 cells treated with RM flower extract. RM flower extract also decreased phosphorylated Akt. These results suggest that RM flower extract induced apoptosis in skin cancer cells through down-regulation of ERK and Akt signaling pathway. RM flower extract may represent an important source of novel potential antitumor agents due to its cytotoxic activity towards malignant cells.

Keywords: *Rafflesia kerrii* Meijer, cytotoxicity, apoptosis, skin cancer, A431 cells.

INTRODUCTION

Rafflesiaceae is a family of holoparasitic flowering plants most famous for being the world's largest single flower. *Rafflesia kurrii* Meijer (RM) is a second largest species in the world after *Rafflesia arnoldii* [1]. RM has been found in the tropical rain forest of southern Thailand through Malay peninsula. The Thai local name is Bua Phut. The flower has a diameter of about 70 cm with a rotting-meat-like smell to attract flies for pollination. In the south of Thailand, the decoction of flower buds is used as traditional medicine to help restore the female uterus after giving birth [2]. The phytochemistry of this plant is largely unknown. Kanchanapoom *et al*. isolated the constituents from the flower of RM and reported that this plant is rich in tannins and along with a phenylpropanoid glucoside, syringing [3]. Tannin, a plant polyphenol, has been reported to have anti-cancer [4], anti-oxidant [5] and anti-microbial activity [6].

Cancer is the major cause of human's death because of high incidence and mortality. Skin cancer is the most common form of cancer in human cancer. Ultraviolet radiation from sunlight is the main cause of skin cancer. There are three main types of skin cancer: basal cell carcinoma, squamous cell carcinoma (the nonmelanoma skin cancers), and melanoma [7-8]. The treatment of skin cancer includes surgery, radiation, chemotherapy, or a combination of radiotherapy and chemotherapy, however, the treatment is rather unsatisfactory. At present, chemoprevention and chemotherapy, which are the use of pharmacological or natural agents for inhibiting the development and for cure of cancer, are rapidly progressing. Chemoprevention can prevent a wide variety of cancers in multiple animal models [9] and naturally occurring substances are recognized to be antioxidants and cancer-preventative agents, or even cancer therapy drugs [10].

This is the first study to demonstrate the *in vitro* anti-cancer property of RM flower extract in the inhibition of skin cancer cell proliferation, focus on apoptosis induction. It was demonstrated that RM flower extract effectively inhibited A431 skin cancer cells. Additionally, RM flower extract could induce apoptosis in A431 cells *via* caspase-dependent and down-regulation of ERK signaling pathway.

MATERIALS AND METHODS

Chemical Reagent

The flower extract of *Rafflesia kerrii* Meijer (RM) included 50% hydroglycol was obtained from Dr. Malin

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Chulasiri, S&J international enterprises public company limited (Thailand). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS), penicillin/streptomycin and trypsin were purchased from PAA Laboratories (Pasching, Austria). 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 was from Invitrogen Molecular Probes (Eugene, OR). Primary rabbit polyclonal anti-caspase-3, MAPK, Akt were obtained from Cell Signaling Technology (Beverly, MA).

Cell Culture

Human epidermoid carcinoma cell line A431 was obtained from the American Type Culture Collection, CRL-1555 (ATCC, Manassas, VA). Cell line was maintained as a monolayer in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% Carbon dioxide (CO_2).

Detection of Nuclear Morphology by Hoechst 33342 Nuclear Staining

A431 cells were seeded at a density of 3×10^5 cells/well in a 6-well plate and allowed to grow for 24 h. The cells were then treated with 1 µg/µl of RM extract for 1, 3 and 6 h. Then cells were stained with 3 µg/ml of Hoechst 33342 for 15 min. Stained cells were washed with PBS once and the emitted light was examined by using fluorescent microscope with an ultraviolet filter.

Western Blot Analysis

A431 cells were incubated in different times at the presence or absence of 1 µg/µl of RM extract, harvested and washed once with ice cold PBS. Then, 3×10^5 cells were lysed for 30 min on ice in 50 µl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.5% Triton X-100) containing complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein was prepared by centrifugation and the protein content was determined using Bio-Rad protein assay (Bio-Rad Laboratories, USA). Proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Pall Corporation, USA) for 1 h at 100 V with the use of a Mini Trans-Blot Cell® (Bio-Rad). After blocking with TBST (10 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween 20) containing 5% nonfat milk, the blots were incubated overnight at 4°C with primary antibody (Cell Signaling Technology, Beverly, MA.) The membranes were washed in TBST and the appropriate secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Beverly, MA) was added for 1 h at room temperature. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagent (ECL, Pierce, Rockford, IL). The membranes were stripped and reprobed with β-actin antibody to assess protein loading for each lane.

RESULTS

The cytotoxic effect *of the Rafflesia kerrii Meijer flower extract* at various concentrations of 0-30 µg/µg RM extracts on A431 cells was previously studied (11). It was found that RM extract decreased cell viability of A431 cells and IC_{50} of RM was estimated to be 0.3 µg/µl. Based on time and dose dependent values apparent from growth inhibition curve of RM (11), 1 µg/µl of RM treatments for 6 h was selected for further studies.

Morphological Analysis of A431 Cell Death Mode

To determine whether the decrease in cell viability observed after treatment with RM extract is involved in apoptosis, A431 cells were stained with Hoechst 33342 dye and observed under fluorescence microscopy. The result showed that the RM extract induce the morphological characteristics of apoptotic cell death such as cell shrinkage, nuclear condensation and fragmentation (Figure **1**). These analyses confirmed that the RM extract is based on their prominent apoptotic effects.

The *Rafflesia kerrii* Meijer Flower Extract Induces Caspase-Dependent Apoptosis Pathway

A431 cells were treated with RM extract and harvested for examining the activation of caspase-3 and PARP, a substrate of caspase-3, by western blotting. Results indicate that RM extract treatment to cells caused cleavage of caspase-3 and PARP (Figure **2**). In addition, RM extract increased the expression level of Bax but no change in the expression level of Bcl-xL. These results demonstrate that RM extract is involved in apoptosis induction in A431 cells.

Effect of *Rafflesia kerrii* Meijer Flower Extract on MAPK and Akt Pathway

MAPK pathway is a transduction signaling pathway relaying on extracellular signals, including growth

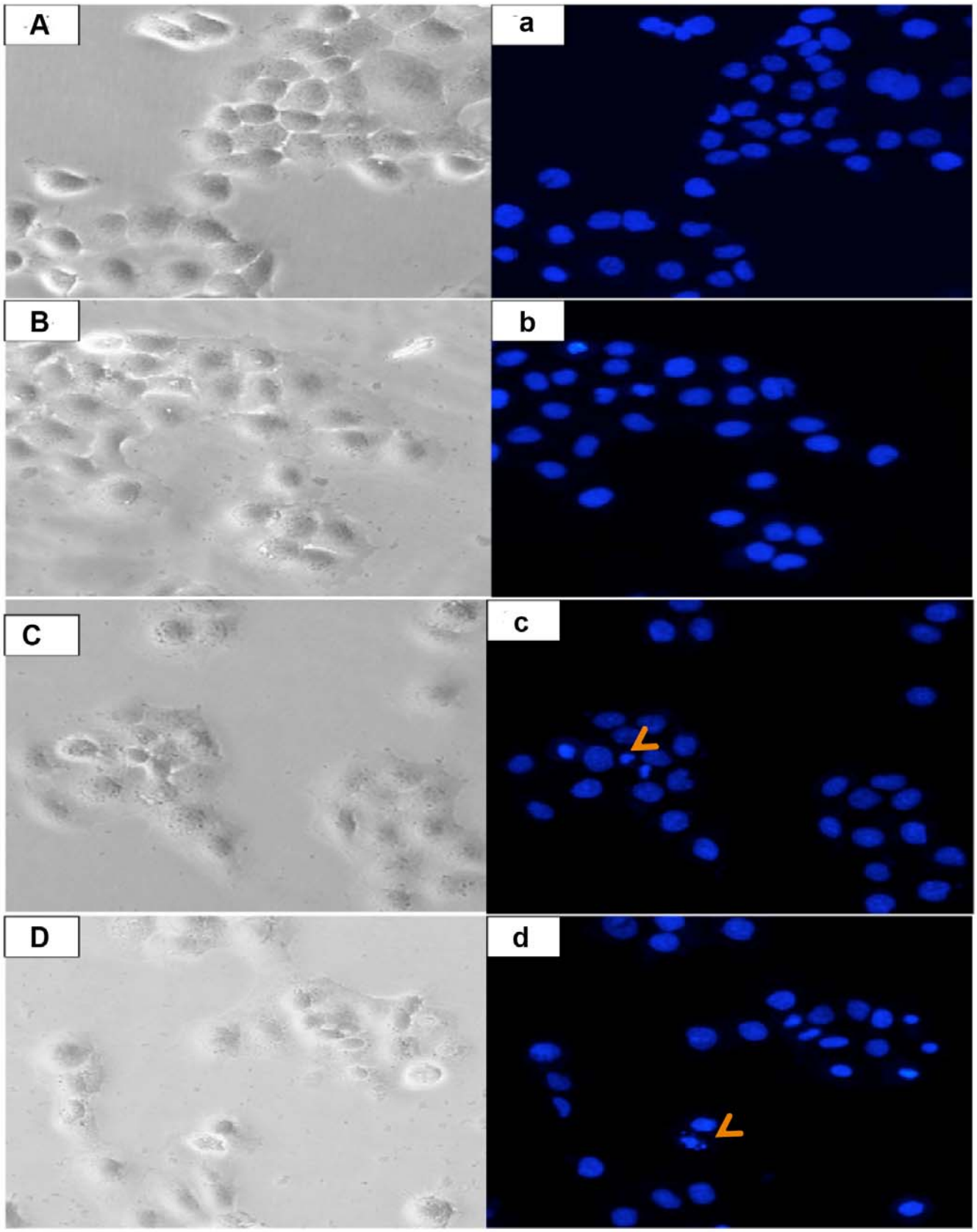

Figure 1: The morphology changes of A431 cells non-treated (**A**) and treated with 1 μg/μL of RM flower extract for 1 (**B**), 3 (**C**) and 6 h (**D**), then stained with Hoechst 33342 and examined under white light (left panel) and fluorescent light (right panel) microscope (20X magnification).

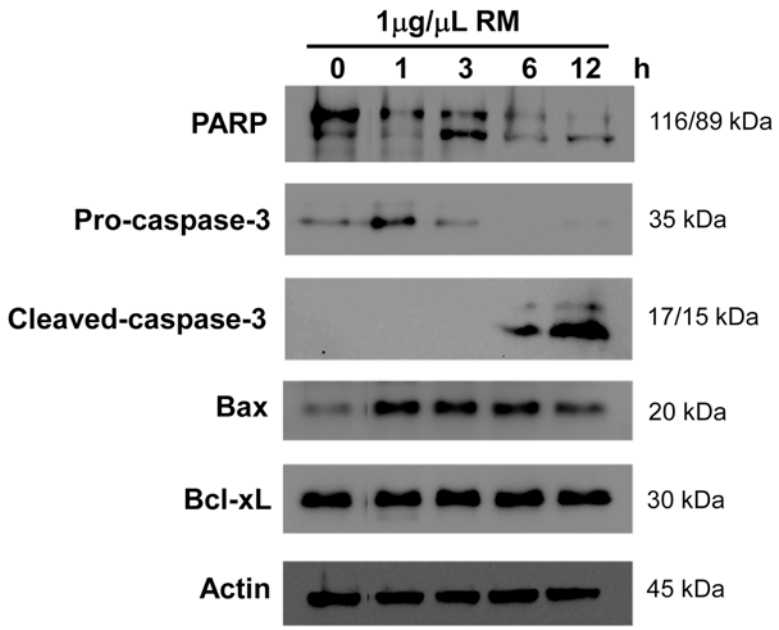

Figure 2: Effects of RM flower extraction on expression of PARP, caspase-3 and Bcl-2 family proteins in A431 cells. Cells were treated with 1 μg/μL of RM flower extract for 1, 3, 6 and 12 h and examined by Western blot analysis. Actin was used as the internal control.

factors, hormones, cytokines and stress, from plasma membrane to nucleus *via* an ordered series of consecutive phosphorylation events. There are three major pathways in mammals, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 pathways. Generally, the ERK pathway responds to growth factor signals, whereas the JNK and p38 pathways typically respond to a variety of extracellular stress signals. As shown in Figure **3**, the results revealed the RM extract decreased the level of phosphorylated and total ERK1/2. On the other hand, RM extract increased the level of phosphorylated and total JNK and p38.

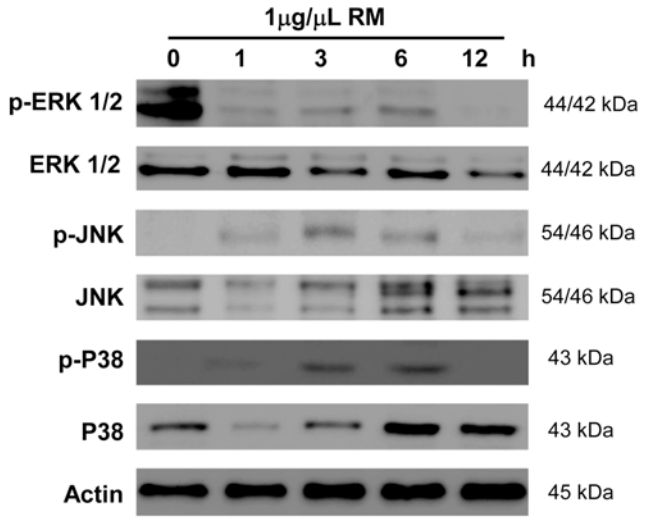

Figure 3: Effects of RM flower extraction on expression of MAPK pathway in A431 cells. Cells were treated with 1 μg/μL of RM flower extract for 1, 3, 6 and 12 h and examined by Western blot analysis. Actin was used as the internal control.

Akt plays a key role in controlling survival and apoptosis. The result showed phosphorylated Akt decreased after treatment with RM extract (Figure **4**). Our results suggest that RM flower extract induced apoptosis through down-regulation of phosphorylated ERK and Akt signaling pathway in A431 cells.

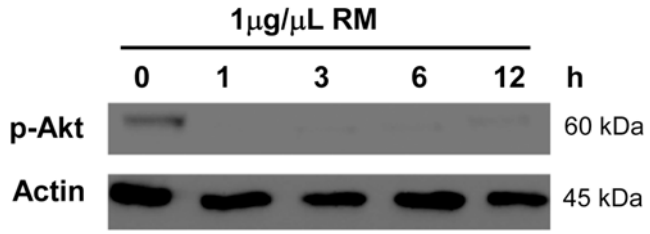

Figure 4: Effects of RM flower extraction on expression of phosphorylated Akt in A431 cells. Cells were treated with 1 μg/μL of RM flower extract for 1, 3, 6 and 12 h and examined by Western blot analysis. Actin was used as the internal control.

DISCUSSION

Research into the anti-cancer potential of plant extracts to use as chemotherapy and chemoprevention have been growing and expanding [12]. Fruits and vegetables possess various pharmaceutical properties and have been shown to be rich source of phytochemicals with anti-carcinogenic potential, especially phenolic compounds [13]. Phytochemicals

have been reported to affect different intracellular signaling pathways implicated in the initiation, promotion and progression of cancer. The anticancer effects of plant extracts have been involved in cytotoxic induction, the scavenging of free radicals, anti-inflammatory activity, cell cycle arrest, the induction of apoptosis, inhibition of tumor angiogenesis and invasion [14-16].

Rafflesia kerrii Meijer (RM) is a parasitic flowering plant which found in tropical rain forest of southern Thailand. It has been reported that RM extract is rich in tannins which possess anti-cancer, anti-oxidant and anti-microbial property [3-6]. In the present study, we found that RM flower extract decreased cell proliferation of A431 epidermoid carcinoma cells. Apoptotic cells are characterized by distinct morphological features including cell shrinkage, chromatin condensation, membrane blebbing and formation of apoptotic bodies. Morphology changes of apoptotic cell death were observed in RM flower extract treated-cells. Activation of apoptotic pathways is a mechanism by caspase cascade signaling *via* two major pathways: the mitochondrial (intrinsic) and death-receptor (extrinsic) pathway. The results indicated that treatment of RM extract activated apoptosis *via* cleavage of caspase-3 and PARP.

The formation of skin tumors is divided into three stages: tumor initiation, tumor promotion and tumor progression. Both UVA and UVB can act as complete carcinogens for skin cancer by causing DNA damage. Moreover, UV radiation mediates tumor promoter effects through increases in gene transcription and through activation of MAPK pathway [17]. The MAPK signaling pathway plays key roles in a wide range of cellular processes and is often deregulated in disease states. The activation of the MAPK pathway regulates key processes such as cell proliferation, invasion, metastasis, survival and angiogenesis, which are involved in cancer development [18]. The MAPK pathway is a family of Ser/Thr kinases composed of ERK, JNK and p38. ERK has been activated in response to growth stimulation and linked to cell survival and cell proliferation. JNK is also activated in response to growth factor signaling but is also activated in response to cellular stress as in p38. It has been studied that ERK was responsible for high levels of cell proliferation and differentiation of many cancers [19]. On the other hand, down-regulation of ERK was indicated that blockage of the function of ERK might be associated with apoptosis [20]. JNK activates apoptotic signaling either through the up-regulation of pro-apoptotic genes through the transactivation of specific transcription factors such as c-Jun or by directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins through phosphorylation events [21]. p38 regulates apoptosis through transcriptional and posttranscriptional mechanisms involving the protein from Bcl-2 family protetins and death receptor pathway [22]. In this study, RM flower extract was shown to down-regulate the phosphorylated and total ERK1/2 but up-regulate the phosphorylation and total of JNK and p38. Therefore, induction of apoptosis in A431 cells by RM flower extract may be associated with the MAPK pathway.

The serine/threonine protein kinase Akt which is downstream of phosphoinositide 3-kinase (PI3 K), is an important intracellular signaling pathway downstream of many growth factor receptor. This pathway is able to activate proliferation and survival pathways in cancer [23]. Our result showed that RM flower extract could decrease the phosphorylation of Akt in A431 cells. These results correspond with previous studies that reported down-regulation of ERK/Akt induced apoptosis in cancer cells [24-26].

Together, our study demonstrates that RM flower extract has the ability to decrease cell viability of A431 skin cancer cells and induce apoptosis through caspase-dependent and down-regulation of ERK and Akt signaling pathways. This new information suggests that RM flower extract may have a role in skin cancer chemoprevention.

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