Molecular Mechanism of *Mallotus spodocarpus* Compound Induced Apoptosis on Cholangiocarcinoma Cell Lines
กลไกระดับโมเลกุลของ *Mallotus spodocarpus* ในการชักนำให้เกิด Apoptosis ของเซลล์มะเร็งท่อน้ำดี

Tapanawan Nasomyon (ฐาปนาวรรณ นาสมยนต์)* Dr. Chariya Hahnvajanawong (ดร.จริยา หาญวจนวงศ์)**
Dr. Watcharin Loilome (ดร. วัชรินทร์ ลอยลม)* Dr. Nissana Namwat (ดร.นิษณา นามวาท)*****
Dr.Wichittra Tussaneeyakul (ดร.วิจิตรา ทัศนียกุล)**** Dr. Vichai Reutrakul (ดร. วิชัย ริ้วตระกูล)*******

ABSTRACT

The growth inhibitory activity of *Mallotus spodocarpus* compound on 2 human cholangiocarcinoma (CCA) cell lines, including KKU-M156 and KKU-100 cells, was performed *in vitro* by using sulforhodamine B (SRB) assay. The *M. spodocarpus* compound exhibited strong growth inhibitory activity against KKU-M156 and KKU-100 cells with IC_{50} values of 0.07 ± 0.009 and 0.04 ± 0.01 μg/ml, respectively. The anti-proliferative effects of this compound was due to apoptosis, as seen by the appearance of membrane blebbing, chromatin condensation, and DNA fragmentation. In addition, the apoptotic process involves a decrease of Bcl-2 as well as Birc 5 gene expression levels, and an increase of Bax-alpha gene expression levels.

บทคัดย่อ

การศึกษาฤทธิ์ในการยับยั้งการเพิ่มจำนวนของเซลล์ของสารบริสุทธิ์จากต้น *Mallotus spodocarpus* ต่อเซลล์มะเร็งท่อน้ำดี 2 ชนิด คือ KKU-M156 และ KKU-100 ในหลอดทดลอง โดยใช้วิธี sulforhodamine B assay พบว่าสารบริสุทธิ์ดังกล่าว มีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ KKU-M156 และ KKU-100 โดยมีค่าความเข้มข้นของสารสกัดที่สามารถยับยั้งเซลล์มะเร็งได้ 50 % (IC50) เป็น 0.07 ± 0.009 และ 0.04 ± 0.01 μg/ml ตามลำดับ ฤทธิ์การยับยั้งการเพิ่มจำนวนของเซลล์ ของสารบริสุทธิ์นี้เกิดจากกระบวนการ apoptosis ซึ่งสังเกตจากการเกิด membrane blebbing, chromatin condensation และ DNA fragmentation นอกจากนี้พบว่ากลไกการเกิด apoptosis มีส่วนเกี่ยวข้องกับ การลดการแสดงออกของยีน Bcl-2 และ Birc 5 ในขณะที่มีการเพิ่มการแสดงของยีน Bax-alpha

Key Words : *M. spodocarpus* compound, Cholangiocarcinoma cell lines, Apoptosis process
คำสำคัญ : สารบริสุทธิ์จากต้น *M. spodocarpus* เซลล์มะเร็งท่อน้ำดี กลไกการเกิด Apoptosis

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*Student, Master of Science, Program in Medical Microbiology, Faculty of Medicine, Khon Kaen University.
**Assistant Professor, Department of Microbiology, Faculty of Medicine, Khon Kaen University.
***Doctor, Department of Biochemistry, Faculty of Medicine, Khon Kaen University.
****Assistant Professor, Department of Biochemistry, Faculty of Medicine, Khon Kaen University.
*****Associate Professor, Department of Phamacology, Faculty of Medicine, Khon Kaen University.
****** Professor, Department of Chemistry , Faculty of Science, Mahidol University.

Introduction

Cholangiocarcinoma (CCA) is the highest incidence primary liver cancer in the north eastern Thailand (Vatanasapt *et al.*, 1995) and still a main health problem of people in this area. All treatment approaches, including surgical treatment, chemotherapy, combined surgical and chemotherapy, and radiotherapy are considered as palliation only. Because of the less effectiveness and toxicity of conventional chemotherapeutic agents, an alternative therapy is considered. One attractive strategy for CCA therapy is the use of medicinal plants. Medicinal plants are now approved for the treatment of many diseases, including cancer, therefore, the medicinal plants may be used as the anticancer drugs directly, as a combined drug for reduce the toxic side effect of conventional drugs or as a synergistic drug.

Mallotus spodocarpus (Euphorbiaceae) is a plant indigenous to the central, northern, northeastern, and southwestern part of Thailand. It has been described in the Thai Forest Bulletin in the year 2000. Prof. Vichai Reutrakul and colleague have successed in purify novel potential medicinal compounds from *M. spodocarpus*. Among these compounds, VR-3848 (7-mer cyclic peptide) was shown to strongly inhibit cell proliferation against various human cancer cell lines such as lung (Pootrakornchai et al., 2000), breast, colon, mouth epidermoid carcinoma, and mouse lymphoid neoplasm (Sujarit *et al.*, 1998). It was also shown to induce apoptosis in the leukemic Jurkat cell line (*Uthaisang et al.*, 2004).

In this study was aimed to examine the growth inhibitory effect of compound purified from *M.spodocarpus* (VR12684) on cholangiocarcinoma (CCA) cell lines by using sulforhodamine B (SRB) assay. The molecular mechanisms of VR12684 induced cell death on CCA cell lines were also determined by using DAPI staining, DNA fragmentation, and real-time RT-PCR.

Materials and Methods

Human cancer cell lines

Two different types of CCA cell lines including KKU-M156 (moderately-differentiated adenocarcinoma) and KKU-100 (poorly-differentiated adenocarcinoma) were used in this study. These cell lines were established in the Department of Pathology, Faculty of Medicine, Khon Kaen University. The CCA cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U penicillin, 100 μg streptomycin. Both cell lines were maintained at 37°C in a 5% CO_2 humidified incubator and were subcultured weekly. The culture medium was changed twice a week.

Chemicals

VR12684 compound was kindly provided by Professor Vichai Reutrakul (Department of Chemistry, Faculty of Science, Mahidol University, Thailand). Briefly, this compound was isolated from Euphobiaceae in Thailand using bioassay-directed fractionation. Sulforhodamine B (SRB) and 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI) were purchased from Sigma (MO, USA). DNA extraction kit was purchased from Qiagen (QIAGEN GmbH, Germany). TRIzol reagent was purchase from Invitrogen (Invitrogen life technologies, Brazil). Random hexamer, dNTPs, RNase inhibitor, M-MLV reverse transcriptase enzyme were purchased

from Promega (Union Carbide Chemicals & Plastics Technology Corporation, USA). SYBR Green was purchased from Applied Biosystem (USA) Other chemicals and reagents used were analytical grade.

***In Vitro* Cytotoxicity assay**

The sulforhodamine B (SRB) assay was used in this study to estimate cell number indirectly by staining total cellular protein with the SRB. The protocol is based on that originally described by Skehan et al. (Skehan, 1990) with slight modifications. Briefly, cells (9x105 cells/well) were seeded in 96-well microtiter plates. After 24 hr incubation (Day 0), cells were treated with various concentrations of VR12684 compound in triplicate. The plates were incubated at 37 °C in a 5% CO_2 humidified incubator for 72 hr. Cell were fixed with trichloroacetic acid and stained with 0.4% SRB. The bound dye was solubilized with Tris-base buffer. The absorbance (OD) of each well was measured using ELISA plate reader (ELX-800 ; BIOTEK INSTRUMENTS, INC.) at 510 nm. Percentage of cell survival will be calculated using equation below. IC_{50} value was expressed as concentration of compound in microgram per milliliter that caused a 50% growth inhibition comparing with controls. The data are means of three independent experiments.

$$\% \text{ Survival} = \frac{\text{OD (test sample)} - \text{OD (day 0)} \times 100}{\text{OD (DMSO control)} - \text{OD (day 0)}}$$

Apoptosis assay

The mechanisms of cell death were determined by detection of nuclear morphology, DNA fragmentation, and apoptotic gene expression.

DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) staining

Apoptosis activity was determined by the detection of nuclear morphology using DAPI staining as described by Hotz *et al.* (1992) with slight modification. Cells were seeded in 12-well plate at 1-5 x 10^6 cells/well. The cells are treated with plant compound using concentration that cause 100 % cell growth inhibition and incubated for 3-5 days. The treated cells are collected at 24 hr, washed, fixed with methanol and stained with DAPI for the observation of nuclear morphology. Nuclei are considered to be the normal phenotype when glowing bright and homogenously. Apoptotic nuclei can be identified by the condensed chromatin and fragmented apoptotic nuclei.

Detection of DNA fragmentation

DNA fragmentation was analyzed by agarose gel electrophoresis as described by Sambrook and Russell (2001) with slight modification. Cells (2-3 x 10^6 cells) were treated with concentration that cause 100% cell growth inhibition and incubated for 48 hr. The treated cells were collected, washed, and used for DNA extraction by using DNA extraction kit following manufacturer instruction. Briefly, the cell pellet was lysed with lysis buffer at 56°C or 10 min. The cell lysate was treated with RNase and protease K. The DNA was precipitated by absolute ethanol and purified by column. Following elution from column, the DNA samples were run on 2% agarose gel electrophoresis at 100 volts for 40 min, stained with ethidium bromide, visualized and photographed under UV light transilluminator (Fotodyne, USA).

Detection of apoptotic gene expression by real-time RT-PCR

Primer design

Primers for PCR were designed using the GeneFisher Program reversion 1.3. (Chris Schleiermacher and Folker meyer, 1995-2001). Primers for the detection of Bcl-2, Bax-alpha, and Birc 5 were designed from Human Bcl-2 mRNA (Bcl-2), Human Bax alpha mRNA (Bax-alpha) and Homo sapiens inhibitor of Birc 5 apoptosis homolog mRNA (Birc 5). Then the primers are aligned to mRNA and genomic DNA of Bcl-2, Bax-alpha and Birc 5 since primers must locate in exon by using BioEdit.zip Program version 7.0.1. Additionally, primer sequence for the detection of GAPDH are kindly provided by miss Piyawan Amimanan, Department of Biochemistry, Faculty of Medicine, Khon Kean University. Primer for real-time PCR are summarized in table 1.

Table 1 Primers used for real-time RT-PCR.

Name	sequence (5′-3′)
Bcl-2-F	5′TGG ATG ACT GAC TAC CTG A3′
Bcl-2-R	5′TGA GCA GAC TCT TCA GAG A3′
Birc 5-F	5′AAG GCT GAG AGC CAG A3′
Birc 5-R	5′TGC CTC TTT CTC TGT CCA3′
BAX alpha-F	5′AAC CAT GAT GGG CTG GA3′
BAX alpha-R	5′CGC CAC AAA GAT CGT CA3′
GAPDH-F	5′TCA TCA GCA ATG GCT CCT GCA3′
GAPDH-R	5′TGG GTG GCA CTG ATG GCA3′

RNA extraction

Total cellular RNA was isolated from both $2x10^6$ treated and non-treated CCA cells at 48 hr according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, the cells were lysed with TRIZOL® Reagent at 15 ˚C for 5 min. Then chloroform was added and incubated at 15 ˚C for 3 min. RNA was precipitated with isopropanol. The RNA pellet was dissolved with RNase free water and stored at -70 ˚C until used.

Reverse Transcription (RT)

Reverse transcription reaction consisted of total RNA and random hexamer was mixed and heated at 70 ˚C for 10 min. After incubation, reaction buffer containing RT buffer, RNase inhibitors, dNTP mix, reverse transcriptase, and RNase free water was added. Reverse transcription was carried out using a DNA thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer Applied Biosystems). The thermal condition were 25 ˚C for 10 min, 37 ˚C for 1 hr, and 95 ˚C for 5 min.

SYBR Green real-time PCR assay

Real-time PCR of Bcl-2, Birc 5, Bax-alpha and GAPDH were performed using a SYBR Green assay. Each reaction contains of single stranded cDNA, 1X PCR buffer (Tris-HCl pH 8.3, KCl, $((NH_4)_2SO_4)$, $MgCl_2$, dNTP mix, forward and reverse primers, 1X SYBR Green and Hot start Taq polymerase. The PCR cycling conditions are 95 ˚C for 10 min, then 40 cycles of 95 ˚C for 1 min, 55 ˚C for 1 min, and 72?C for 1 min, followed by 72 ˚C for 10 min. At each cycle, the accumulated PCR products were detected by monitoring an increase in fluorescence of the reporter dye from dsDNA-binding SYBR Green. After PCR, a dissociation curve (melting curve) is constructed in the range of 50 ˚C to 99 ˚C. All data were analyzed using the ABI PRISM 7500 Real time PCR system (Applied Biosystem). The mRNA expression level was presented as the ratio between absolute

quantification numbers of gene of interest and GAPDH.

Statistical Analyin

Mean expression levels of each target genes of treated and untreated control cells were tested for the statistical difference using Student's t-test. P-value less than 0.05 was considered for the statistical significant difference.

Results

Effect of *M. spodocarpus* compound on cell growth

The *M. spodocarpus* compound (VR12684) showed marked inhibitory effect on KKU-M156 and KKU-100 cell proliferation (Table 1). The concentration required by this compound for 50% growth inhibition of KKU-M156 and KKU-100 cells was found to be 0.07 and 0.04 μg/ml, respectively.

Table 2. The IC_{50} value of M. spodocarpus compound (VR12684) on KKU-M156 and KKU-100 cell lines. The results are expressed as mean ± SE. (n=3)

Cell lines	IC_{50} (μg/ml)
KKU-M156	0.07 ± 0.009
KKU-100	0.04 ± 0.01

Apoptotic assay

In order to determine whether the growth inhibitory effect of the *M. spodocarpus* compound was due to the induction of apoptotic cell death, the ability of the *M. spodocarpus* compound to induce apoptotic cell death was determined by analyzing its effect on cell morphology under bright field inverted microscope and nuclear morphology using DAPI staining. The induction of apoptosis was confirmed by DNA fragmentation assay.

Effect of M. spodocarpus compound on cell morphology and nuclear morphology

After incubation with the IC_{100} concentration of *M. spodocarpus* compound for 48 h. the morphological alterations in KKU-M156 and KKU-100 cell lines were observed under bright field inverted microscope. Untreated control KKU-M156 cells were cuboid and polygonal in normal shape (Figure1A). Exposure of KKU-M156 cells to the *M. spodocarpus* compound led to retraction, rounding and some sensitive cells were detached from the surface. Membrane blebbing (Figure1C, arrowhead) and apoptotic body (Figure1C, arrow) were observed.

Untreated control KKU-100 cells were polygonal in normal shape (Figure 1B). Treatment of KKU-100 cells to the *M. spodocarpus* compound also led to retraction, rounding and detaching from the surface. Membrane blebbing (Figure 1D, arrowhead) and apoptotic body (Figure 1D, arrow) were observed.

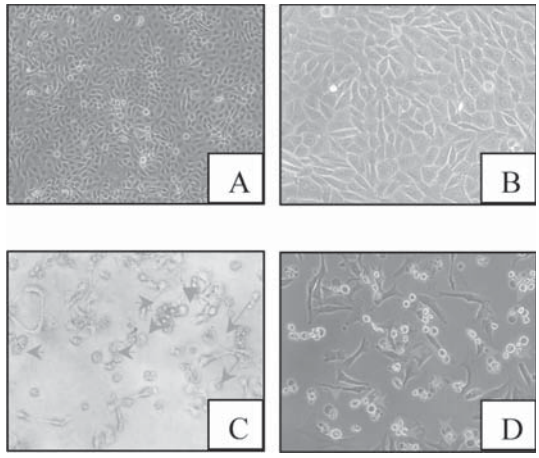

Figure 1 Morphological changes of KKU-M156 cells treated with the *M. spodocarpus* compound observed under bright field microscopy. Untreated KKU-M156 control cells were cuboid and polygonal in normal shape (A). Untreated KKU-100 cells were polygonal in normal shape (B). While treated cells showed the retraction, rounding, detached from the surface, membrane blebbing (arrow) were observed in KKU-M156 (C) and KKU-100 cells (D)

Following DAPI staining, it was found that after treatment of KKU-M156 and KKU-100 cells with *M. spodocarpus* compound, apoptotic cells could be clearly seen under fluorescent microscope as shown in figure 2. These apoptotic cells exhibited characteristic of nuclear morphological changes, including chromatin condensation, segmentation of nuclear chromatin of irregular size in treated cells (Figure 2C, D). This was clear contrast to the spherical and regular form of control nuclei (Fugure 2A, B)

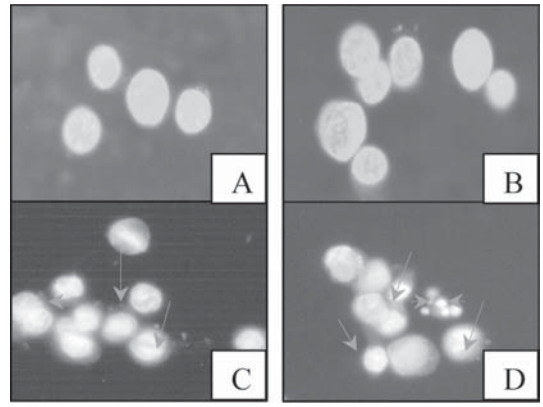

Figure 2 Effect of *M. spodocarpus* compound on nuclear morphology of KKU-M156 and KKU-100 cells for 24 hr. Cells were stained with DAPI. Nuclear morphology was observed under fluorescent microscope. Nuclei of control cells were round with a normal contour of nuclei in KKU-M156 (A), in KKU-100 (B). *M. spodocarpus* compound treated KKU-M156 (C) and KKU-100 cells (D) showed chromatin condensation (arrow head) and nuclear fragmentation (arrow)

Effect of M. *spodocarpus* compound on DNA fragmentation

After treatment of KKU-M156 and KKU-100 cells with *M. spodocarpus* compound for 24 hr, the DNA fragmentation was detected on agarose gel electrophoresis. The fragmented DNA was clearly seen in treated KKU-M156 cells (Figure 3, lane 4) and KKU-100 cells (Figure 3, lane 5). Whereas DNA control of untreated KKU-M156 cells (Figure 3, lane 2) and KKU-100 cells (Figure 3, lane 3) did not provide ladders.

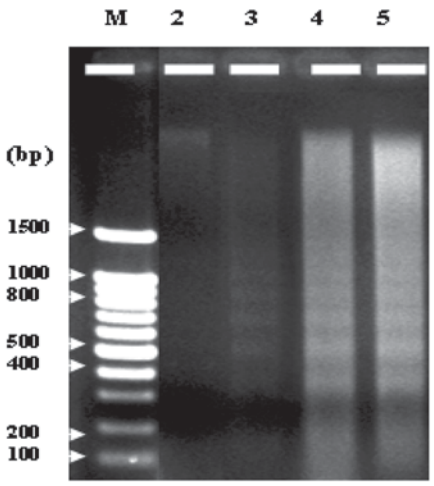

Figure 3 DNA fragmentation KKU-M156 and KKU-100 cells after 24 hr exposure to *M. spodocarpus* compound. Lane 1: 100 base-pair DNA ladder marker, lane 2 : untreated KKU-M156 cell, lane 3 : untreated KKU-100 cell lane 4 : KKU-M156 cells treated with *M. spodocarpus* and 5: KKU-100 cells treated with *M. spodocarpus* compound

Effect of M. spodocarpus compound on apoptotic gene expressions

Using real-time PCR analyses, we found that M. spodocarpus compound significantly reduced the level of Bcl-2 and Birc 5 gene expressions in both KKU-M156 (Figure 5) and KKU-100 (Figure 6) (p-value < 0.05). This compound increased the expression of Bax-alpha in both KKU-M156 (Figure 5) and KKU-100 cells (Figure 6) as compares with the controls.

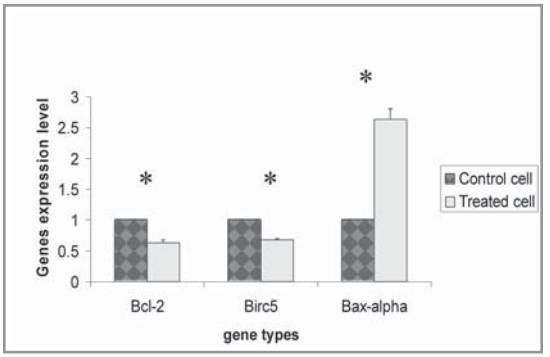

Figure 5 Expression levels of Bcl-2, Birc 5 and Bax-alpha genes in KKU-M156 cells treated with *M. spodocarpus* compound at IC100 concentration for 48 hr. The values are presented as mean ± S.D. (n = 3). Significant differences with control cells are designated as * (p value < 0.05)

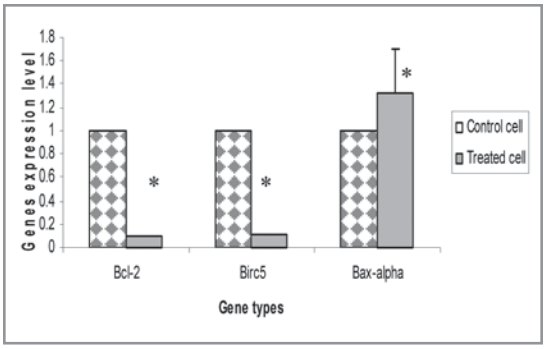

Figure 6 Expression levels of Bcl-2, Birc 5 and Bax-alpha genes in KKU-100 cells treated with *M. spodocarpus* compound at IC100 concentration for 48 hr. The values are presented as mean ± S.D. (n = 3). Significant differences with control cells are designated as * (p value < 0.05)

Discussion and Conclusion

The search for anticancer agents from natural sources has been successful worldwide. Active constituents have been isolated and are nowadays used to treat human tumors. In the present study we analyzed the *in vitro* effect on two CCA cell lines of the *M. spodocarpus* compound. Our results demonstrate that the compound has marked growth inhibitory effects on the two CCA cell lines. These results are consistent with the previous studies, which reported the growth inhibitory activity of a 7-mer cyclic peptide isolated from *M. spodocarpus* to several human cancer cell lines such as lung (Pootrakornchai R *et al.*, 2000), breast, colon, mouth epidermoid carcinoma, and mouse lymphoid neoplasm (Sujarit K et al., 1998).

Previously, a 7-mer cyclic peptide isolated from *M. spodocarpus* has been shown to induced apoptosis in the leukemia Jurkat cell line (Uthaisang *et al.*, 2004). To further characterize whether the growth inhibitory activity of *M. spodocarpus* compound in KKU-M156 and KKU-100 cells was related to the induction of apoptosis. We analyzed the cell morphology, nuclear morphology and DNA fragmentation of treated and untreated control cells. After treatment of the compound, we observed the trypical morphological characteristics of apoptosis, such as chromatin condensation, formation of apoptotic bodies, and DNA ladder formation. These results indicate that the cytotoxic effect of this compound on KKU-M156 and KKU-100 cells are associated with induction of apoptosis.

The Bcl-2 family protein plays a crucial role in apoptosis, an increase expression of Bax-alpha can induce apoptosis by suppressing the activity of Bcl-2 .The Bax-alpha : Bcl-2 ratio, rather than Bcl-2 alone is important for the survival of drug-induced apoptosis in leukemic cell lines. Using real-time PCR analyses, we found that *M. spodocarpus* compound significantly reduced the levels of Bcl-2 and Birc 5 gene expressions in both KKU-M156 and KKU-100 cells. However, this compound increased the expression of Bax-alpha in both KKU-M156 and KKU-100 cells.

Taken together, the decreased expression levels of Bcl-2 and Birc 5 genes but increased expression level of Bax-alpha gene might contribute to the induction of apoptosis in *M. spodocarpus* compound treated KKU-M156 and KKU-1000 cells. We are now in the process of studying the expressions of Bcl-2, Birc 5 and Bax-alpha proteins of *M. spodocarpus* compound treated KKU-M156 and KKU-100 cells. More research on the application of *M. spodocarpus* compound in cancer treatment in warranted.

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