Myristica fragrans Houtt. Methanolic Extract induces Apoptosis in a Human Leukemia Cell Line through SIRT1 mRNA Downregulation

Chintana Chirathaworn PhD*, Wisatre Kongcharoensuntorn PhD**,
Thitiporn Dechdoungchan BSc**, Alisa Lowanitchapat BSc***,
Pattaratida Sa-nguanmoo MS***, Yong Poovorawan MD****

** Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok*
*** Department of Biology, Burapha University, Chonburi*
**** Program in Biomedical Sciences, Graduate School, Chulalongkorn University, Bangkok*
***** Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok*

Background: *Myristica fragrans Houtt. (nutmeg) contains antibacterial, antiviral and anti-cancer activities. However, the mechanisms underlying those activities have not been clearly explained.*
Objective: *To study the effect of Myristica fragrans Houtt. methanolic extract on Jurkat human leukemia T cell line.*
Material and Method: *Methanol extract of Myristica fragrans Houtt. (Myristicaceae) was used to study the effect on Jurkat cell metabolic activity using an MTT assay and on apoptosis using annexin V staining. Expression of SIRT1 gene was determined by RT-PCR.*
Results: *At the concentrations 50 and 100 µg/mL, the methanol extract of Myristica fragrans Houtt significantly inhibited Jurkat cell proliferation and induced apoptosis as detected by annexin V staining. Downregulation of SIRT1 mRNA expression in Jurkat cells was observed even when the amount of methanol extract was 10 µg/mL.*
Conclusion: *Methanol extract of Myristica fragrans Houtt induced apoptosis of Jurkat leukemia T cell line in a mechanisms involving SIRT1 mRNA downregulation.*

Keywords: *Myristica fragrans, Apoptosis, SIRT1, Jurkat*

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Medicinal plants have been used for disease relief and health maintenance for a long period of time. Recently, the search for bioactive compounds in those plants has intensified. Phenolic compounds have been demonstrated to have anticancer, antioxidant, antimicrobial activity and promote the immunological functions[1-4]. Therefore, phenolic compounds are routinely investigated in the development of new drugs from medicinal plants.

Myristica fragrans Houtt (nutmeg) is one of the plants commonly found in Asian medicinal ingredients[5-7]. It contains many bioactive compounds including camphene, elemicin, eugenol, isoelemicin, isoeuglenol, methoxyeugenol, pinene, sabinene, safrol, myristic acid, myristicin, elimicin and lignan compounds[8-10]. M. fragrans extract has been shown to contain antibacterial activity against different genera of bacteria and antiviral activity against rotavirus[11,12]. Administering of this extract into hyperlipidaemia rabbits reduced the level of blood lipoprotein lipids[13]. In addition, its effect on increasing the mounting behavior and mating performance of male Swiss mice has been reported[14]. For its role as an anti-cancer agent, myristicin, found in Myristica fragrans Houtt has cytotoxic and apoptotic effects in human neuroblastoma SK-N-SH cells with an accumulation of cytochrome and activation of caspase 3 in the cytosol[15].

Correspondence to : Chirathaworn C, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. Phone: 0-2256-4000 ext. 3676, Fax: 0-2252-5952, E-mail: chintana_chula@yahoo.co.th

In the present study, the authors investigated the effect of Myristica fragrans Houtt methanol extract as anti-cancer agent using the Jurkat. leukemic T cell line. The authors further investigated the effect of this plant extract on SIRT1 (silent information regulator two ortholog 1) gene expression as SIRT1 is one of the regulators of cell life span and has been shown to be involved in aging-associated processes[16].

Material and Method

Myristica fragrans Houtt methanol extract

The bark of M. fragrans Houtt was ground to a fine powder and dried to constant weight in a desiccant at room temperature. Then, 100 g of the powdered sample was extracted using three volumes of 80% methanol for 48 hours at room temperature and stored at 4ºC for 24 hours. The extract was then filtered through Whatman paper No. 4 and evaporated in a rotary evaporator at 55ºC[9]. The yield of extract was 3.34% w/w. The extract was dissolved in dimethylsulfoxide (DMSO), filter through 0.2 m Nylon filtered paper and stored in the dark at 4ºC.

Cell culture

Jurkat cells, kindly provided by Professor Tada Sueblinwong, Department of Biochemistry, Chulalongkorn University, Thailand, were maintained at 37ºC under humidified air supplemented with 5% CO_2 in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 units/mL penicillin (GIBCO-BRL, USA).

MTT assay

Jurkat cells were resuspended at 5 x 10^5 cells/mL and 100 µL aliquot of cell suspension was added into a 96-well tissue culture plate. Myristica fragrans Houtt methanol extract was added to cell suspension at the final concentrations of 20, 50, and 100 µg/mL and the cell suspensions were incubated for 48 h at 37ºC under 5% CO_2 atmosphere. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) dye (10 µL of 5 mg/mL) were added into each well and cells were further incubated for 4 hours before 100 ul of DMSO was added to dissolve the formazan crystals. Absorbance at 570 nm was determined. DMSO, used for dissolving the extract, was also included as a negative control. The experiments were conducted in triplicate.

Apoptosis detection by annexin V staining

Annexin V staining for apoptosis detection was performed as previously reported[17]. In brief, Jurkat cells (1×10^6 cells/mL) were incubated with 20, 50, or 100 µg/mL of the methanol plant extract at 37ºC for 4 hours. DMSO and 10 ug/mL of camptothecin were included as negative and positive control, respectively. Treated cells were washed with cold PBS and resuspended in 100 µL of binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Cells were then incubated with annexin V-FITC (Santa Cruz Biotechnology, USA) and propidium iodide in the dark for 15 min at room temperature. Binding buffer (400 µL) was added to stained cells and cells were analyzed immediately by flow cytometry. Cells positive for annexin V-FITC staining were considered as apoptotic cells.

RT-PCR

RNA was extracted from Jurkat cells using Trizol reagent (GIBCO-BRL) according to the manufacturer's instruction. Then, 1 µg of RNA was reversed transcribed with MuMLV reverse transcriptase (Promega, USA) using oligo (dT) as a primer. Amplification of cDNA was performed using primers 5' GTA GCA CTA ATT CCA AGT TCC A and 5' GAT ACT GAT TAC CAT CAA G for SIRT1 mRNA and 5' CGC AGA AGG GGT CCT GGT GA and 5' CAG CTC CTT CTT CTG CTC CGG GGT for aldolase mRNA[18]. The thermal cycling program used was 35 cycles at 94ºC for 40 s, 57ºC for 1 min and 68ºC for 1 min for SIRT1 and 30 cycles at 94ºC for 40 s, 60ºC for 1 min and 68ºC for 1 min for aldolase. The expected product size was 586 and 176 bp for SIRT1 and aldolase respectively. The PCR products were sequenced and the sequences obtained were aligned for homology blast search in Genbank database.

SIRT1 gene expression was investigated at 1, 2 and 4 hours after treatment. The induction was observed since 1 h and there was no difference between 2 and 4 hour-treatment (data not shown). The effect of various doses of methanol plant extract on SIRT1 gene expression was determined following 2-hour treatment.

Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M.) of triplicate samples for MTT assays and of three independent experiments for propidium idodide and annexin V staining assays. Analysis of variance and student's t-test was used for statistical analysis with p value ≤ 0.05 were considered significant.

Results

Cytotoxic effect of the Myristica fragrans

Houtt methanol extract on Jurkat cells was studied using an MTT assay. As shown in Fig. 1, extracts at the concentrations 50 and 100 µg/mL significantly reduced viability of Jurkat cells. Apoptosis induction was determined by annexin V staining as described in Material and Method. Myristica fragrans Houtt methanol extract significantly induced Jurkat cell apoptosis in a dose-dependent manner in the concentration range 20-100 µg/mL (Fig. 2).

Since Myristica fragrans Houtt extract induced apoptosis of Jurkat cells, the authors further investigated whether apoptosis induction occurred through SIRT1 gene down-regulation. Plant methanol extract at 20 µg/ml was used for demonstrating the effect on SIRT1 gene expression as this was the minimum amount that could induce apoptosis. The extract at 10 µg/mL was also included in the assay. As shown in Fig. 3, the plant extract down regulated SIRT1 mRNA expression, which could be detected even when 10 µg/mL of extract was used. DMSO treatment had no effect on SIRT gene expression and aldolase gene expression was used to normalize RNA extraction.

Discussion

Sirtulins are proteins in a class of evolutionarily conserved molecules with protein deacetylation activity. Sir2 (silent information regulatory 2), a sirtulin in yeast, is involved in many processes such as gene silencing, regulation of p53, fatty acid metabolism, cell cycle regulation, and life span extension[16]. Overextension of Sir2 extended the lifespan of budding yeast and Caenorhabditis elegans[19,20]. SIRT1 (Silent information regulator two ortholog 1), the mammalian Sir2 homologue, is involved in life prolonging in mammal. The examples of known SIRT1 substrates are p53, peroxisome proliferators-activated receptors (PPAR), forkhead-box transcription factors (FOXO), histone H1, H3 and H4[21]. The mechanisms of SIRT1 in regulating senescence and apoptosis have been widely studied[22-25]. Deacetylation of p53 or FOXO by SIRT1 attenuates apoptosis induction, which might shift cellular response away from cell death towards cell survival[21,26]. SIRT1 has been suggested to be a determinant for cell survival and cancer development.

In the present study, the authors have demonstrated that Myristica fragrans Houtt methanol extract inhibited Jurkat cell proliferation though apoptosis induction. As this plant extract is known to contain phenolic compound and it has been shown that polyphenols stimulate deacetylase activity of SIRT1 protein[27], the authors also demonstrated that the extract inhibited a Jurkat T-leukemia cell line proliferation and induced SIRT1 mRNA downregulation.

Although apoptosis induction was observed when at least 20 µg/mL of the methanol extract was used, induction of SIRT1 gene expression could be demonstrated when only 10 µg/mL extract was used. The amount as low as 10 µg/mL can induce SIRT gene

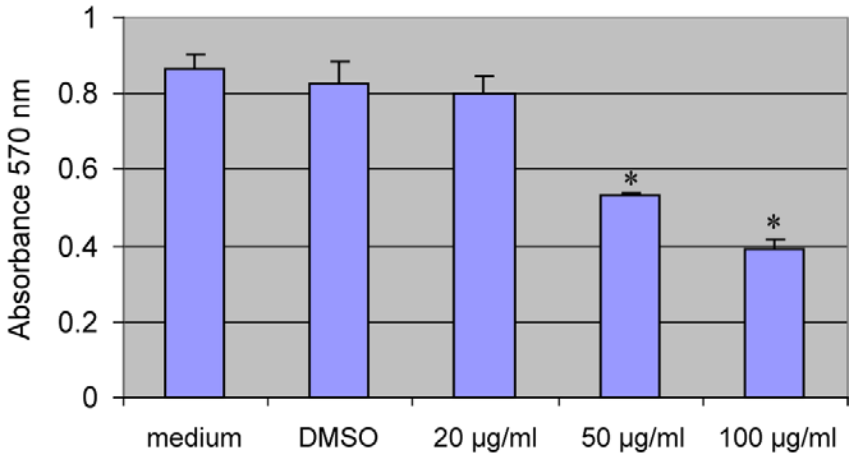

Fig. 1 Cytotoxic effect of Myristica fragrans Houtt methanol extract on Jurkat cell using MTT assay, Jurkat cells were treated with medium, DMSO, 20, 50 or 100 µg/mL plant extract for 48 h at 37°C, absorbance at 570 nm represents viability of Jurkat cells, three independent experiments were conducted in triplicate
* $p \leq 0.05$ compared to DMSO control

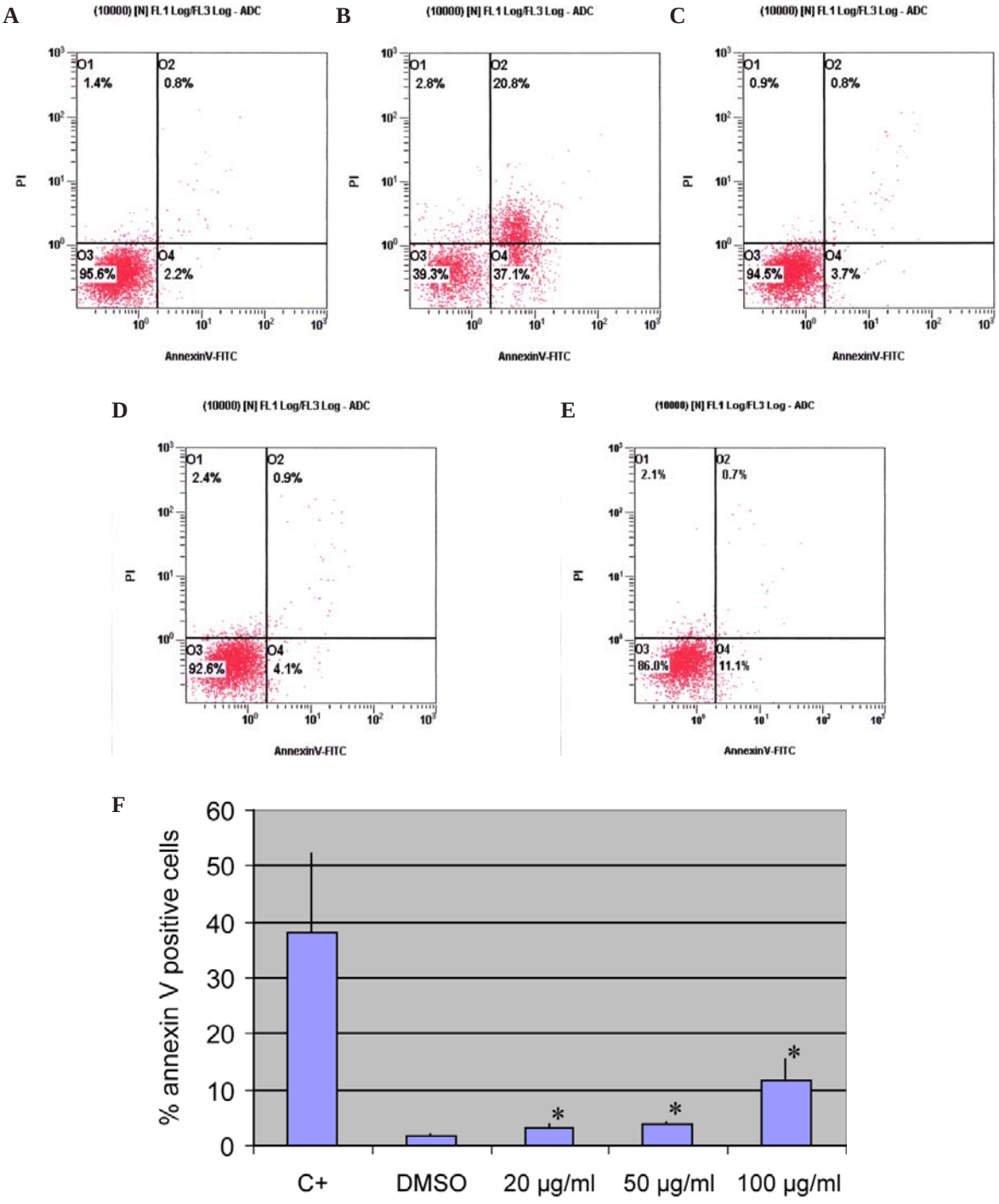

Fig. 2 Myristica fragrans Houtt methanol extract induction of apoptosis of Jurkat cells demonstrated by annexin V staining, Jurkat cells were treated with DMSO (A), campothecin (B), 20 (C), 50 (D) or 100 (E) µg/mL of plant extract for 48 h at 37ºC, apoptotic cells were demonstrated by determining the number of annexin V positive cells compared to flow cytometer, A-E are data from one experiment shown in dot plot and F presents the average of three independent experiments

* $p \leq 0.05$ compared to DMSO control

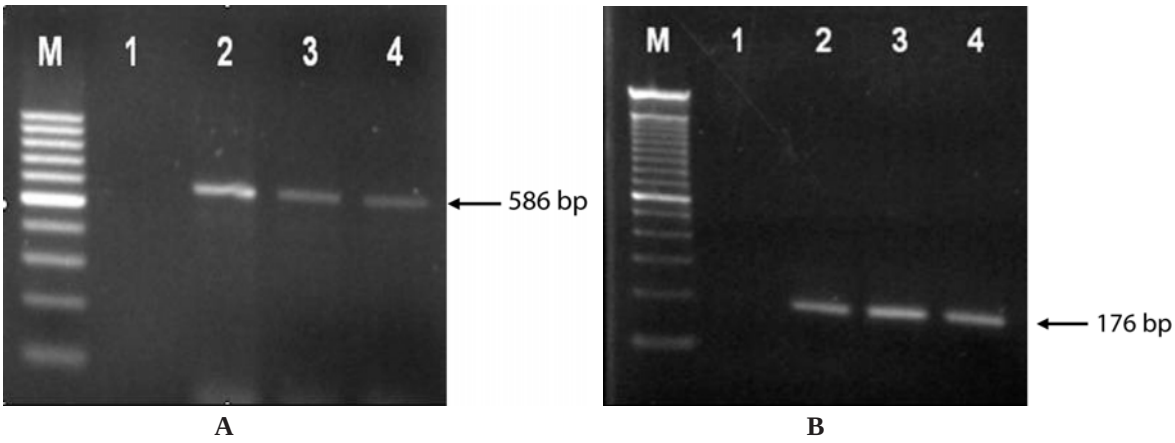

Fig. 3 Myristica fragrans Houtt methanol extract downregulation of SIRT1 gene Expression, RT-PCR was performed using RNA extracted from Jurkat cells treated with DMSO (lane 2), 10 µg/mL (lane 3) and 20 µg/mL (lane 4) plant extract for 2 hours, A and B demonstrate PCR product from SIRT1 and aldolase gene specific primers respectively, Lane M is DNA markers and lane 1 is PCR reagent control, similar results were obtained in 3 independent experiments

expression, which can be detected by RT-PCR. However, this amount may not be enough to induce detectable apoptosis.

The presented data can be useful for further study on mechanisms of cancer development and the anti-cancer effect of medicinal plant extract.

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สารสกัดจาก Myristica fragrans Houtt กระตุ้นให้เซลล์ Jurkat เกิด Apoptosis โดยลดการแสดงออกของจีน SIRT 1

จินตนา จิรถาวร, วิสาตรี คงเจริญสุนทร, ฐิติพร เดชดวงจันทร์, อลิสา โล่วณิชพัฒน์, ภัทริดา สงวนหมู่, ยง ภู่วรวรรณ

ภูมิหลัง: *Myristica fragrans Houtt (nutmeg) เป็นพืชสมุนไพรที่มีรายงานว่าสารสกัดมีฤทธิ์ในการต้านแบคทีเรีย ไวรัส และทำลายเซลล์มะเร็ง แต่กลไกการออกฤทธิ์ดังกล่าวยังไม่เป็นที่ทราบแน่ชัด*

วัตถุประสงค์: *ศึกษาผลของสารสกัดจาก Myristica fragrans Houtt ต่อเซลล์มะเร็ง*

วัสดุและวิธีการ: *ใช้เมธานอลสกัด Myristica fragrans Houtt. ศึกษาผลของสารสกัดที่ได้ต่อเซลล์มะเร็งชนิด Jurkat ในการเพิ่มจำนวนโดยใช้วิธี MTT assay ศึกษาการเกิด Apoptosis โดยย้อมเซลล์ด้วยสาร annexin V และดูการแสดงออกของจีน SIRT1 ด้วยวิธี RT-PCR*

ผลการศึกษา: *สารสกัด Myristica fragrans Houtt. ที่ความเข้มข้น 50 และ 100 ไมโครกรัม/มิลลิลิตร ยับยั้งการเพิ่มจำนวนของเซลล์ Jurkat โดยการกระตุ้นให้เกิด Apoptosis วัดด้วยวิธีการย้อมด้วย annexin V โดยพบว่า สารสกัดในปริมาณเพียง 10 ไมโครกรัม/มิลลิลิตร สามารถลดการแสดงออกของจีน SIRT1 ได้ และกลไกหนึ่งในการกระตุ้น apoptosis เกิดจากการลดการแสดงออกของจีน SIRT1*

สรุป: *สารสกัดจาก Myristica fragrans Houtt. extract กระตุ้นให้เซลล์ Jurkat ตายโดยวิธี Apoptosis โดยกลไกหนึ่งที่เกี่ยวข้องคือการลดการแสดงออกของจีน SIRT1*