

HMT Inhibits Prostate Cancer Progression by Targeting PAK-1.

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Abstract

Background: Heamatang (HMT) is a classic medicinal formula used in traditional Chinese and Korean medicine; it contains seven distinct components, mainly of herbal origin. HMT is used as an anti-aging remedy, in the treatment of urinary disorders, and to increase energy and vitality. However, the therapeutic applications of this formula have not been evaluated with evidence-based science.

Methods: Therefore, we assessed HMT through various *in vitro* methods, including cell viability assay, fluorescence-activated cell sorting assay (FACS), western blotting, migration assay, three-dimensional (3D) cell culture, siRNA-mediated PAK-1 knockdown, and crystal violet assays.

Results: HMT decreased PAK-1 expression in PC-3 cells and inhibited cell viability, growth, and motility. The inhibition of cell motility by HMT was correlated with PAK-1-mediated inhibition of Lim domain kinase (LIMK) and cofilin. HMT induced G1 arrest and apoptosis through the transcriptional regulation of cell cycle regulatory proteins (inhibition of cyclin-dependent kinase (CDK) 4, 6, and cyclin D1) and apoptosis-related proteins (increase in cleaved caspase-3 (c-cas3) and inhibition of poly ADP-ribose polymerase (PARP) and B-cell lymphoma 2 (BCL-2)). Moreover, HMT suppressed P21-activated kinase (PAK-1) expression, leading to the inhibition of AKT activities. Finally, we showed that Decursin was the active ingredient involved in the inhibitory effect of HMT on PAK-1.

Conclusion: Our findings demonstrated that HMT exerts its anticancer influence through the inhibition of PAK-1 expression. The HMT formula could be applied in various fields, including functional health food and pharmaceutical development.

Background

Prostate cancer is the second most frequent malignancy in men worldwide. The incidence and mortality rate of prostate cancer worldwide are strongly correlated with aging (over 65 years of age) [1]. Prostate cancer manifests symptoms such as frequent urination, nocturia, hematuria, erectile dysfunction, and dysuria. Prostate cancer can spread to nearby organs, such as the bladder, or travel through the bloodstream or lymphatic system to bones and other organs (www.mayoclinic.org/diseases-conditions/prostatecancer). Most prostate cancers require androgens for growth and are sensitive to androgen deprivation therapy (ADT). However, ADT leads to castration-resistant prostate cancer (CRPC). CRPC is advanced prostate cancer that has been known to evolve into either hormone-resistant prostate cancer (HRPC) or androgen-insensitive prostate cancer (AIPC). In this type of tumors, the intracrine/paracrine androgen production plays a significant role in the insurgence of prostate cancer cells resistant to testosterone suppression therapy [2].

P21-activated kinase 1 (PAK-1), a member of the serine/threonine kinase family, plays a crucial role in tumor progression and contributes to tumor invasion and metastasis in various types of human cancer. PAK-1 overexpression occurs in several types of human cancer, including prostate cancer [3, 4]. Moreover,

PAK-1 is a major downstream effector of the Rho-family GTPase Cdc42 and Rac1 and is involved in the regulation of cell morphology and motility [5].

Cancer cell migration is a fundamental process in solid tumors formation and is required for metastasis formation [6]. The formation of cancer metastasis, which includes cancer cell migration and invasion, involves changes in cytoskeletal signaling pathways, increased motility, and enhanced cell survival. Thus, the actin cytoskeleton is an important factor in tumor cell migration and invasion [7]. It has been reported that PAKs regulate the actin cytoskeleton during cell motility and invasion [8]. The phosphorylation of both LIMK and cofilin is greatly enhanced in the presence of active PAK [9–12]. Furthermore, the expression and activity of LIMK are higher in invasive breast and prostate cancer cell lines than in less invasive lines [13]. In addition, PAK-1 plays an essential role in cell growth, adhesion, migration, and survival in colorectal cancer [14] and lung adenocarcinoma [15], through the activation of AKT, ERK, and β -catenin.

The use of Complementary and alternative medicine (CAM) treatment methods, including traditional Korean medicine, has shown a steady rise among cancer patients. Many patients seek CAM therapeutic options to mitigate the side effects of chemotherapy and radiation therapy [16, 17]. However, further studies are required to examine the efficacy and safety of CAM in anticancer therapies. Heamatang (HMT) is a traditional medicine that was described in the Compendium of Materia Medica (Bancao Gamgmu), a Chinese herbarological and pharmaceutical textbook written by Li Shizhen during the Ming dynasty reign [18]. According to Bancao Gamgmu, HMT disperses hard masses caused by accumulation and assemblage of blood clot or waste discharge under deficient and excessive conditions over a long time. Thus, HMT could be applied to cancer treatment. However, there is a lack of scientific evidence regarding its clinical efficacy, as the therapeutic effects of this classic prescription have not been evaluated using conventional scientific methods. HMT is constituted by combining seahorse, and six herbal components, including *Rheum palmatum*, *Pharbitidis Nill Choisy*, *Citri unshius Markovich*, *Inula helnium*, *Croton tiglium Linne*, and *Angelica gigas Nakai*. HMT has not been employed in modern medicine because the use of Hippocampus species was prohibited on account of the originating species being endangered. However, we were able to produce HMT in-house thanks to the farming of *Hippocampus abdominalis* together with its approval for the use as food material in Korea. Therefore, in this study we evaluated the impact of HMT in prostate cancer therapy, using conventional scientific methods.

Materials And Methods

Preparation of HMT

The seven ingredients and their proportion (w/w) were as follows: *Hippocampus abdominalis* (Seahorse Australia, Beauty Point, TAS, Australia), 16.6 %; *Rheum palmatum* (Donhi Herb, Seoul, Korea),16.6 %; *Pharbitidis Nill Choisy* (Donhi Herb, Seoul, Korea), 16.6 %; *Citri unshius Markovich* (Donhi Herb, Seoul, Korea), 16.6 %; *Angelica gigas Nakai* (Donhi Herb, Seoul, Korea), 16.6 %; *Inula helnium* (Donhi Herb,

Seoul, Korea), 8.3 %; *Croton tiglium* Linne (Donhi Herb, Seoul, Korea), 8.3 %. Prof. Min-Ho Lee (Department of food technology and services, Eulji University) performed botanical identification and prepared the ethanol extract. The plant name was checked with Royal botanic gardens, Kewscience (<http://mpns.science.kew.org>). The preparation method is the following: dried and pulverized medicinal herbs were mixed and soaked in 50 % ethanol (1L x 3 changes) at room temperature for three days. The extract was filtered using Whatman filter paper, evaporated (rotary evaporator, model NE-1, Japan) and lyophilized (freeze dryer, Lioalfa-6, Telstar, Terrassa, Spain) to produce 15 g of powder.

Cell Culture

Human prostate cancer cell lines (PC-3, DU-145, and LNCaP) were purchased from American Type Culture Collection (ATCC). The cells were maintained in RPMI-1640 medium with 10 % fetal bovine serum (FBS), 2 μ M l-glutamine, and penicillin/streptomycin (WelGene, Deagu, South Korea) in a humidified atmosphere with 5 % CO₂ at 37 °C.

Cell Viability Assay

We measured cell viability following HMT treatment in several prostate cancer cell lines using the CELLOMAX™ viability kit (Precaregene, Hanam, Kyungido, Korea) and the tetrazolium-salt-WST-8(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). Cells (1×10^4) were treated with different concentrations of HMT (0, 31.3, 62.5, 125, and 500 μ g/ml) in a 96-well plate for 24 h. Then, 10 μ l of CELLOMAX™ reagent was added and incubated 2 h at 37 °C in the dark. The optical density (O.D.) was measured with a microplate reader (Tecan, Sunrise, Switzerland) at 450 nm. Cell viability was calculated using the following equation: Cell viability (%) = $[\text{O.D. (FFE)} - \text{O.D. (blank)}] / [\text{O.D. (control)} - \text{O.D. (blank)}] \times 100$.

Crystal Violet Staining Assay, Cell Growth Assay

The crystal violet staining assay was used to determine the anti-proliferative effect of HMT. PC-3 cells were seeded in a 6 well plate at a concentration of 1×10^5 cells/ml/well and treated with different concentrations of HMT (0, 50, 100, and 200 μ g/ml) for five days, with daily addition of fresh media and HMT. Then, cells were fixed with 2 ml of 1 % glutaraldehyde solution (JUNSEI, Tokyo, Japan) in phosphate-buffered saline (PBS) for 15 min at 37 °C. After washing with PBS, 2 ml of 0.05 % crystal violet (Sigma Aldrich) was added for 30 min to stain cells. Cells were finally washed gently with deionized water. The plates were dried at room temperature overnight. A 70 % ethanol solution was added to each well (2 ml/well) to release the crystal violet, using a rotary shaker for 2 h at room temperature. The O.D. was measured by a microplate reader (Tecan, Sunrise, Switzerland) at 570 nm, with a reference filter at 405 nm.

Western Blot Analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % NP-40, 0.25 % sodium deoxycholate, 1 M EDTA, 1 mM Na₃VO₄, 1 mM NaF, and protease inhibitor cocktail). Protein samples were quantified using the Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA, USA), separated by electrophoresis on 8 % to 10 % SDS-PAGE gels, and transferred onto a Hybond ECL transfer membrane (Amersham Pharmacia, Piscataway, NJ, USA). The membranes were blocked in 5 % non-fat skim milk and probed with primary antibodies for PAK-1 (Abcam, Cambridge, UK), phospho-AKT, AKT, LIMK1/2, phospho-LIMK1/2, cofilin, phospho-cofilin, cyclinD1, PARP, CDK4, CDK6, and cleaved caspase-3 (Cell Signaling, Beverly, MA, USA), and β-actin (Sigma Aldrich Co., St. Louis, MO, USA). Membranes were exposed to horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit secondary antibodies. Protein expression was examined using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia, Piscataway, NJ, USA).

Wound Healing Assay

PC-3 cells (1×10^6 cells/ml) were seeded in a six-well plate and incubated at 37 °C. When the cells reached 50% confluence, they were scratched with a 200 µl pipette tip, followed by washing with PBS. The cells were then treated with 50 µg/ml of HMT in complete medium for 24 h. After incubation, cells were fixed and stained with Diff-Quick. Randomly chosen fields were photographed under a microscope (Nikon, Tokyo, Japan). The number of cells that migrated into the scratched area was calculated.

Invasion Assay Using the Boyden Chamber

PC-3 cells were treated with HMT in RPMI-1640 without FBS and were seeded in the upper chamber, containing a Matrigel-coated filter. Following this, RPMI-1640 supplemented with 10 % FBS was added to the lower wells. After incubation for 24 h, the cells on the top of the upper Matrigel-coated filter were scraped, then the invaded cells on the bottom of the inserts were fixed and stained with Diff-Quick (Sysmax). The cells on the lower surface of the filter were photographed (DFC420C, Leica, Germany) and randomly chosen fields were counted.

Fluorescence-activated Cell Sorting (FACS) Analysis

PC-3 cells were treated with HMT. Then, cells were washed and fixed in 70 % ethanol overnight at -20 °C. The following day, cells were treated with RNase A (10 mg/ml) for 1 h at 37 °C. Then, cells were stained by adding 1 ml propidium iodide (PI) (50 µg/ml). After filtering with a nylon mesh (40 µm), the DNA content of stained cells was analyzed using Cell Quest Software (BD Biosciences, San Jose, CA, USA) with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

siRNA Transfection

The PAK-1 siRNA and control siRNA were purchased from Bioneer. To transfect the siRNAs, PC-3 cells were seeded at a density of 5×10^4 cells per well in a 6-well plate. Cells were transfected using 100 nM of either the PAK-1 siRNA or the control siRNA with a siRNA transfection reagent (Polyplus) for 48 h. After treatment, cells were stimulated with HMT and then analyzed with western blotting assay and stained with crystal violet. The O.D. was measured by a microplate reader (Tecan, Sunrise, Switzerland) at 570 nm, with a reference filter at 405 nm.

3D Culture Tumor Organoids

For the generation of the PC-3 tumor organoids, cells were seeded into 96-well round-bottom ultra-low attachment plates (Corning) at 2000 viable cells per well. The PC-3 spheroids were grown in RPMI medium with 10 % FBS. The plates were incubated for 5 days at 37 °C, during which they formed spheroids. Then, the spheroids were treated with 200 µg/ml HMT for 48 h. For the apoptosis analysis, 2 µM CELL Event (Invitrogen, USA) was added to each well for 1 h. Pictures were obtained using a fluorescence microscope (Nikon, Tokyo, Japan). Fluorescence intensity of caspase-3/7 was evaluated using the ImageJ software. For the calculation of fluorescence intensity with ImageJ, two images of each well were analyzed and their average intensity was used for the statistical analysis.

TLC and HPLC Analysis

Decursin standard and HMT were spotted on Silica gel 60 thin-layer plates (Merck, Darmstadt, Germany). After development in isopropyl alcohol: ethyl: acetate: water (3/1/1, v/v/v), the TLC plate was dried and visualized by dipping in a solution containing 0.3 % (w/v) N-(1-naphthyl)-ethylenediamine and 5 % (v/v) H₂SO₄ in methanol and was heated at 110 °C for 10 min. We then performed a high-performance liquid chromatography (HPLC) on a Luna C18(2) 100 Å reverse phase column (250 mm×4.6 mm, 5 µm, phenomenex, Inc., Korea) connected to an Agilent Technologies 1100 series system, with a detector at 329 nm at 30 °C and using a mobile phase of 100 % ethanol: 0.1 % formic acid in water (7/3, v/v) under isocratic mode with a flow rate of 1 ml/min. The chromatography peak was identified by comparing the retention time of the sample with the reference standard.

Results

HMT Suppresses PAK-1

The PC-3 line has a high metastatic potential among prostate cancer cell lines. We thus tested whether HMT treatment could reduce PAK-1 expression in PC-3 cells by western blotting assay. Indeed, HMT inhibited the PAK-1 protein level in PC-3 cells (Fig. 1A).

HMT Inhibits Cell Growth

We then examined the cytotoxic effect of HMT in several prostate cancer cell lines. We performed a cell viability assay 24 h after HMT treatment in the PC-3, DU-145, and LNCaP lines. As shown in Fig. 1B, HMT treatment reduced cell viability in PC-3, DU-145, and LNCaP cells in a dose-dependent manner. The treatment with 31.3 $\mu\text{g/ml}$ HMT reduced prostate cancer viability by 12.2 % (PC-3), 13.5 % (DU-145), and 53.9 % (LNCaP). LNCaP cells were the most sensitive to HMT (Fig. 1B).

We then performed a cell growth assay to assess the impact of HMT treatment in the long-term (4d) growth of prostate cancer cells. As shown in Fig. 1C, HMT significantly suppressed cell growth in a dose-dependent manner. HMT suppressed cell growth, decreasing cell growth by 28 %, 46 %, and 79 % at 50, 100, and 200 $\mu\text{g/ml}$ of HMT, respectively (Fig. 1C).

HMT Inhibits Cell Migration and Invasion by Inhibiting PAK-1 Pathway

To investigate whether HMT could suppress prostate cancer cell motility without exhibiting cell toxicity, we used a wound-healing and Matrigel-coated membrane invasion assay. As shown in Fig. 2A and 2B, in the Matrigel-coated invasion assay, HMT inhibited cell invasion by 50 % compared to the untreated control. Moreover, in the wound healing assay, HMT treatment decreased serum-induced cell migration by 57 % compared to the untreated control. As shown in Fig. 1B, the inhibitory effect of cell motility by HMT was not linked to cell toxicity. Notably, 62.5 $\mu\text{g/ml}$ HMT suppressed cell viability by 15 % in the PC-3 cell lines compared to the control (Fig. 1B). To confirm that the inhibitory effect of HMT in cancer cell migration and invasion were correlated to PAK-1 regulation, we performed western blotting to measure the protein levels changes of PAK-1 and PAK-1-regulated cell motility proteins following HMT treatment in PC-3 cells. A concentration of 50 $\mu\text{g/ml}$ HMT attenuated PAK-1, AKT, LIMK, and cofilin protein levels (Fig. 2C).

HMT Induces G1 and SubG1 Arrest

To evaluate whether HMT could modulate cell cycle progression, cell cycle analysis was performed by treating PC-3 cells with high concentrations of HMT (10, 200, and 400 $\mu\text{g/ml}$) for 24 h. As shown in Fig. 3A, HMT gradually increased G1 and sub-G1 duration in a dose-dependent manner compared to those in the non-treated cells. Particularly, the increase in sub-G1 arrested cells treated with 400 $\mu\text{g/ml}$ HMT was 76 % (Fig. 3A).

To confirm the presence of HMT-mediated apoptotic cells, we stained PC-3 cells treated with 200 $\mu\text{g/ml}$ HMT for 24 h with the Cell Event Caspase-3/7 detection dye. As shown in Fig. 3C, we detected no cas3/7 fluorescent cells in the control group. On the other hand, HMT-treated cells showed green fluorescence in almost all cells.

We next assessed whether HMT-mediated G1 and sub-G1 arrest (apoptosis) were dependent on changes in G1-regulating and apoptotic-related proteins. HMT treatment decreased the levels of G1-regulated proteins (Cyclin D1 and CDK4 and 6) and increased apoptosis through the inhibition of BCL-2 and PARP and the increase of cleaved Caspase-3 (Fig. 3D).

PAK-1 Mediates HMT-Induced G1 Arrest and Apoptosis, and Suppression of Cell Proliferation and Cell Motility

Next, we assessed whether the anticancer effect of HMT is dependent on PAK-1. Upon knockdown of PAK-1 by siRNA, we observed a reduction of the expression of the G1 regulatory protein cyclin D1, and the induction of the apoptotic protein cleaved Caspase-3 (Fig. 4A). Moreover, the -1 knockdown attenuated the expression of LIMK1/2 and cofilin. The expression profile of PAK-1 knocked down cells was similar to that observed in HMT-treated cells (Fig. 4A). Moreover, PAK-1 knockdown enhanced HMT-mediated inhibition of cell growth (Fig. 4B). In addition, HMT treatment significantly suppressed PAK-1 expression in two prostate cancer cell lines, LNCaP and DU145 (Fig. 4C).

HMT Reduces Tumor Spheroid Viability

We used the PC-3 tumor spheroid model further to assess the effect of HMT treatment on tumor growth. This three-dimensional culture model mimics some aspects of the in vivo tumor organization and is better suited to study the response of cancer cells to a drug. As shown in Fig. 5A, the cell viability of the PC-3 spheroids, as measured by WST-8, was reduced by 41 % following the addition of 200 µg/ml of HMT, similarly to what was observed in 2D cultures (Fig. 4A). To measure the effect of HMT on apoptosis in the 3D PC-3 cells model, we used Cell Event, a fluorogenic Caspase-3/7 substrate: following HMT treatment, we readily detected apoptosis also in this model (Fig. 5B).

HMT Contains Decursin that Inhibits PAK-1

To identify the active compounds in HMT correlated to the anticancer activity, we determined the presence in HMT of the main herbal compounds (Naringin, Decursin, Catechin, Gallic acid, and Epicatechin) using TLC (data not shown). This preliminary TLC analysis (254 nm) revealed the presence of Decursin, as shown in Fig. 6A.

We then performed HPLC analysis to calculate the retention time of Decursin (5.014 min) and estimated its abundance in the HMT preparation (23.05 mg/g) (Fig. 6B). We then evaluated the effect of pure Decursin on -1 expression. At a concentration of 10 µM, Decursin significantly suppressed PAK-1 expression (Fig. 6C).

Discussion

Since PAKs were discovered in 1994, several studies demonstrated their crucial roles in numerous cellular processes, such as cell cycle progression and cell survival [19]. PAK-1 belongs to the Group I of the PAK family and was identified for the first time in a screening for proteins that interacted with GTP-bound Rac [20]. PAK-1 is overexpressed in a variety of cancers, including prostate cancer [21]. PAK-1 was recently shown to be a valid therapeutic target for cancer treatment [22]. Since then, several PAK-1 inhibitors have been developed for use as biological markers and therapeutic agents [23].

Recent studies investigating the antitumor effect of natural products suggested that they may lead to promising alternative therapy for the treatment of cancer. Traditional Chinese or Korean medicine is widely accepted as an alternative treatment for cancer [24–26]. Furthermore, research on traditional Chinese and Korean medicinal and herbal formulations has been recognized internationally by medical researchers [27], and several studies elucidated the molecular and cellular mechanisms of herbal medicine-derived phytochemicals in the context of cancer research [28–30]. In this study, we investigated the effect of oriental medicinal herbs on PAK-1 signaling. Previously, we have reported the effect of PAK-1 inhibitor EOPK (Essential oil of *Pinus koraiensis*) in HCT 116 cells [31]. Now, we identified a second PAK-1 inhibitor isolated from HMT.

Recent studies have shown that there are several possible links between PAK and PI3K and Raf–MAPK pathways [4, 31–33]. To confirm the involvement of PAK-1 in the HMT-mediated anticancer effect, we evaluated expression changes of proteins involved in the PAK-1 pathway by knocking down PAK-1 with siRNA. This knockdown enhanced the HMT-mediated anticancer effects by inhibiting the PAK-1/AKT pathway. Consistently with our data, PAK-1 inhibition using shRNA or siRNA strategies induced apoptosis and CDK4/6 inhibition [34–36].

HMT is mainly prepared using extracts from *Hippocampus abdominalis*, *Angelica gigas* Nakai, *Rhem palmatum*, and *Critri unshius* Markovich. The *Hippocampus* extract contains steroids and fatty acids and possesses various pharmacological properties including anti-tumor effects [37, 38] and anti-prostatic hyperplasia activity [39, 40]. *Rhem palmatum* contains emodin, that has been reported to target the AR directly and induce apoptosis [41, 42]. Moreover, emodin has been reported that inhibits tumor cell migration through suppression of PI3-K-cdc42/Rac1 pathway in MDA-MB-231 cells [43]. *Rhem palmatum* contains catechin and epicatechin. Green tea catechins have been reported to possess anticancer effects by many researchers [44–47]. The therapeutic mechanisms of catechins, especially in prostate cancer, include direct action on cancer cells and indirect action on tumor-associated inflammation [48]. *Critri unshius* Markovich contains hesperidin and naringin. According to a recent research, naringin inhibits cell viability and induces apoptosis in PC-3, LNCaP, and DU145 cells. Furthermore, naringin synergistically increases the effect of paclitaxel in the treatment of prostate cancer cells [49]. Korean *Angelica gigas* Nakai is a major medicinal herb. Traditionally, its dried root has been used to treat anemia, pain, infection, and articular rheumatism in Korea, most often by boiling the roots in water. TLC and HPLC data show that HMT contains Decursin (Fig. 6A and B). Decursin is a major chemical component of *Angelica gigas* extract and has been previously been associated with antitumor effects in prostate cancer [50–53]. Moreover, Decursin decreases cell proliferation and angiogenesis and increases apoptosis in vitro and in

PC-3 and DU-145 xenograft models [52, 54]. By using TLC, we did not find the presence of other compounds (Naringin, Emodin, Catechin, Gallic acid, and Epi-catechin). However, further analyses are necessary to identify the presence of other compounds, since the anticancer activity of these components may contribute to the total HMT anti-tumor activity.

Three-dimensional (3D) growth of immortalized cell lines or primary cells is regarded as a more physiological model to perform in vitro screening, as 3D cell cultures possess several in vivo features of tumor organization [55, 56]. We evaluated the anticancer-effect of HMT in a 3D culture growth model. As shown in Fig. 5B, the PC-3 tumor organoids were bumpy, not showing the typically spheroid and uniform morphology. Thus, the PC-3 line has high metastatic potential among prostate cancer cell lines. PC-3 cells lack cell-cell adhesion molecules that are related to cell motility, migration, and invasion, making it difficult to form spheroid tumor formation in the absence of a synthetic matrix, such as ECM (extracellular matrix) components. Also, previous studies reported that PC-3 cells have no or low levels of E-cadherin and alpha-catenin [57]. As shown in Fig. 5A, the organoids of the control group were brighter than those of the HMT-treated group in bright field pictures. On the other hand, the spheroids of the HMT-treated group were darker than those of the control group in the whole area. Consistently, the Cell Event-positive spheroids showed a dark section, consistent with apoptosis induction [58–60].

Conclusions

In summary, we demonstrated that, in PC-3 cells, a non-toxic dose of HMT decreased cell migration, cell invasion, and the expression of PAK-1, AKT, LIMK1/2, and Cofilin. HMT reduces cell proliferation by arresting cells in the G1 phase, through the reduction of CDK4/6 and cyclin D1 expression levels. HMT also induces apoptosis through sub-G1 arrest by decreasing PARP and BCL-2 expression levels and inducing the expression of cleaved Caspase-3. Interestingly, HMT treatment and PAK-1 knockdown by siRNA showed a similar phenotype in PC-3 cells. Moreover, we identified that the inhibition of PAK-1 by HMT treatment is dependent on Decursin. Our findings demonstrated that HMT inhibits cell motility and growth and induces apoptosis by inhibiting the PAK-1/AKT and the PAK-1/LIMK1/2/Cofilin signaling pathway. Collectively, these data suggest that HMT could be used as a starting point for the development of functional health foods and medicine.

Abbreviations

HMT: Heamatang; FACS: Fluorescence-activated cell sorting; PAK1: P21-activated kinase 1; siRNA: Small interfering RNA; ADP: Adenosine diphosphate; ADT: Androgen deprivation therapy; CRPC: Castration-resistant prostate cancer; HRPC: Hormone-resistant prostate cancer; AIPC: Androgen-insensitive prostate cancer; CAM: Complementary and alternative medicine; O.D.: Optical density; ECL: Enhanced chemiluminescence

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

HJL conceived and designed experiments and revised the manuscript; SOL and XYH performed the experiments. YKL analyzed the data. LMH prepared and provided the HMT. YSI performed TLC and HPLC analysis. All authors read and approved the final manuscript.

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Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of Interest

We declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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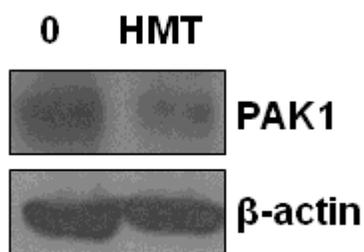
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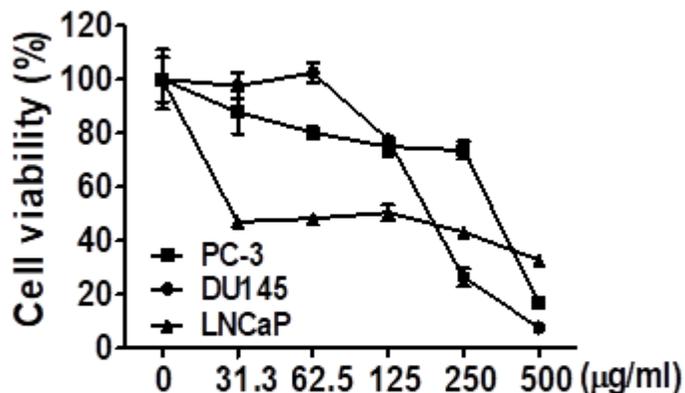
Figures

Figure. 1

A.



B.



C.

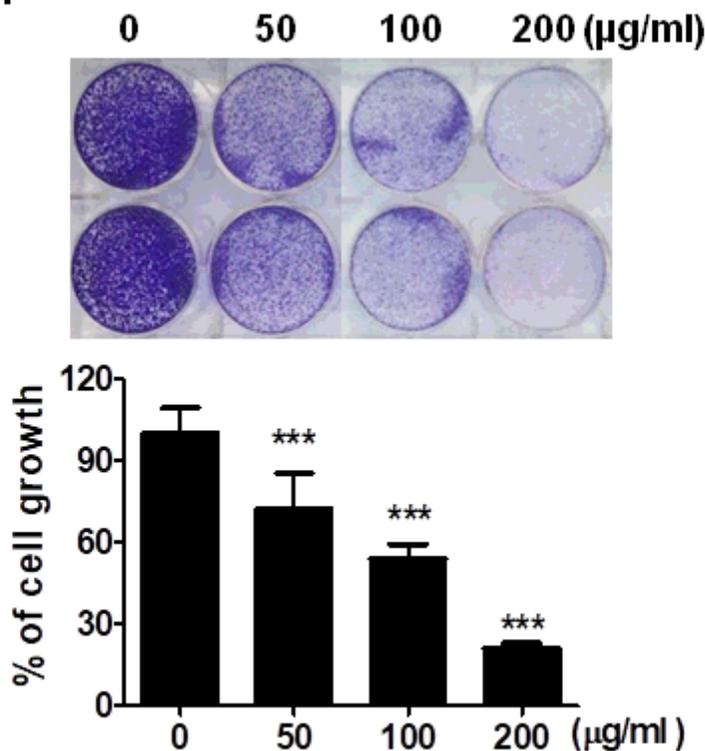


Figure 1

The inhibitory effect of HMT on PAK-1 expression and cell growth in PC-3 cells. (A) HMT-treated PC-3 cell lysates were prepared and subjected to western blotting for PAK-1 expression. (B) Cells (LNCaP, DU-145, and PC-3 cells) were treated with various concentrations of HMT for 24 h, and cell viability was measured by using CELLOMAX™ viability kit. Data represent mean \pm SD. (C) The anti-proliferation activity, cell growth, for long term treatment of HMT was carried out by cell growth-crystal violet assay. HMT (50, 100, and 200 μ g/ml) treated to PC-3 cells for 4 days. The cells were stained with crystal violet and were photographed and resolved in 70% EtOH, and the absorbance was measured using a microplate reader. Data represent mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control.

Figure. 2

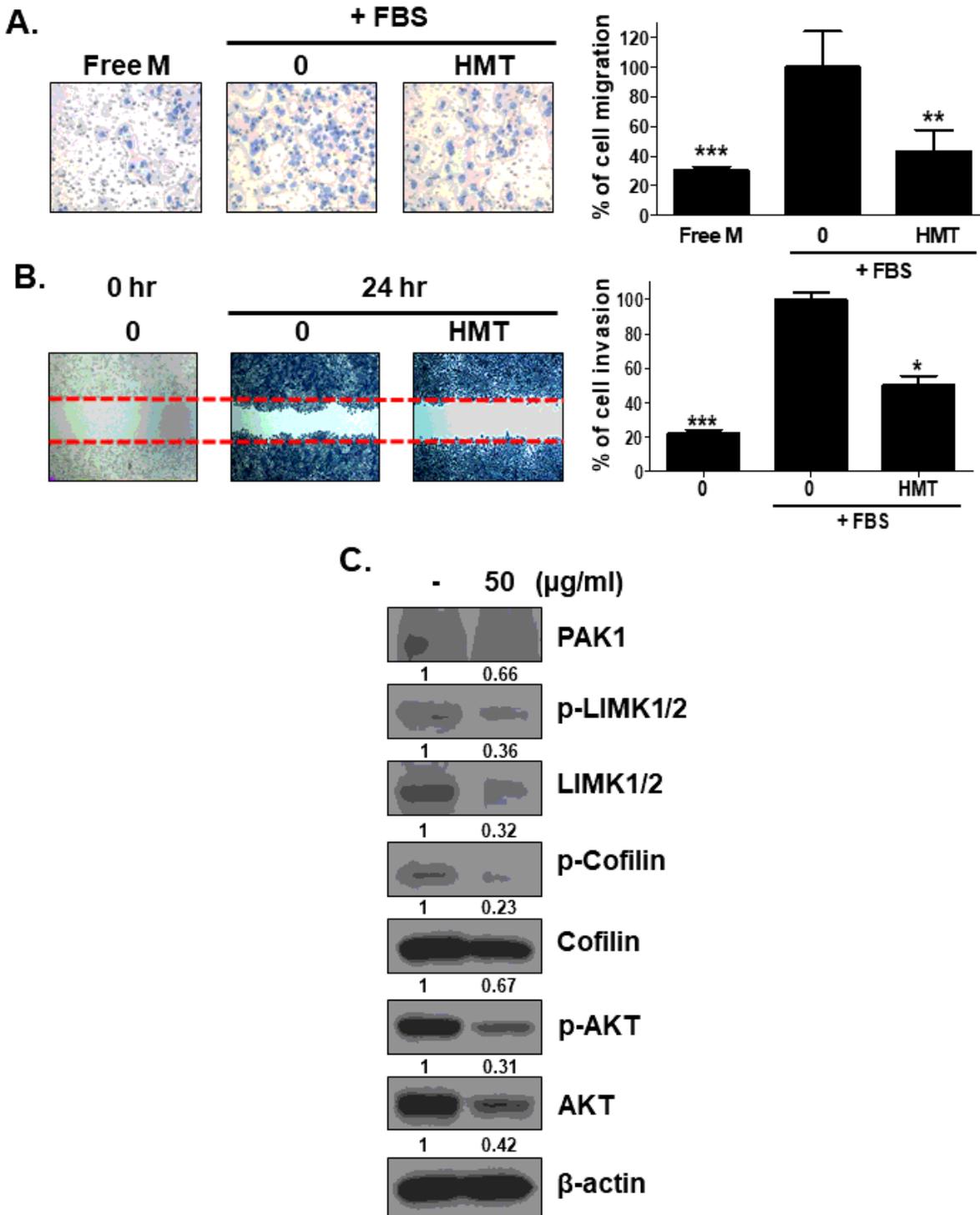


Figure 2

Inhibitory effect of HMT on cell migration and invasion in PC-3 cells. Cells were treated with HMT (50 µg/ml) for 24 h. (A) The number of invasive cells into the matrigel coated membrane was photographed (X100) and calculated as a percentage of invasion. (B) The number of cells migrating into the scratched area was photographed (X100) and calculated as a percentage of migration. (C) HMT-treated PC-3 cell

lysates were prepared and subjected to western blotting for cell motility related-proteins in PAK-1 signaling pathway (PAK-1, LIMK1/2, p-LIMK1/2, Cofilin, p-Cofilin, AKT, p-AKT, and β -actin).

Figure. 3

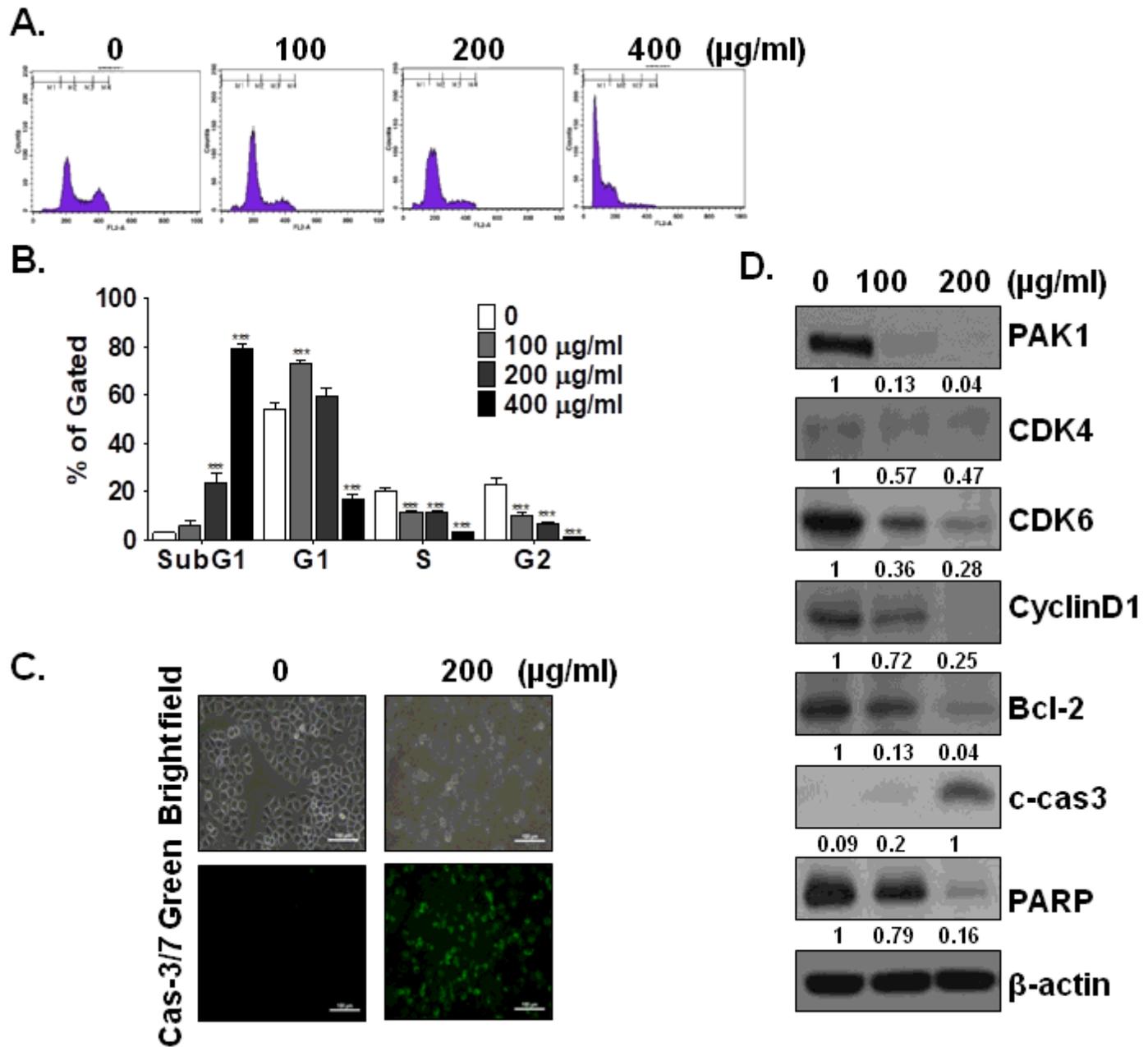


Figure 3

Effect of HMT on cell cycle arrest and apoptosis in PC-3 cells. HMT (100, 200, and 400 $\mu\text{g/ml}$) treated to PC-3 cells for 24 h. (A) The treated cells were stained with propidium iodide (PI) and analyzed by flow cytometry. (B) Bar graphs showed the quantification of the cell cycle population (%). Data represent mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control. (C) PC-3 cells were treated with HMT (200 $\mu\text{g/ml}$) for 24 h and stained with 2 μM CELLEvent for apoptosis analysis and imaged using a fluorescence microscope (X 200) (Nikon, Tokyo, Japan). (D) HMT (100 and 200 $\mu\text{g/ml}$) -treated PC-3 cell lysates were prepared and subjected to western blotting for cell cycle-and apoptosis-related proteins

(PAK-1, CDK4,6, CyclinD1, BCL-2, cleaved caspase-3, PARP, and β -actin). Band density of proteins was quantified using the Gelpro analyzer (Media Cybernetic, Bethesda, MD, USA).

Figure. 4

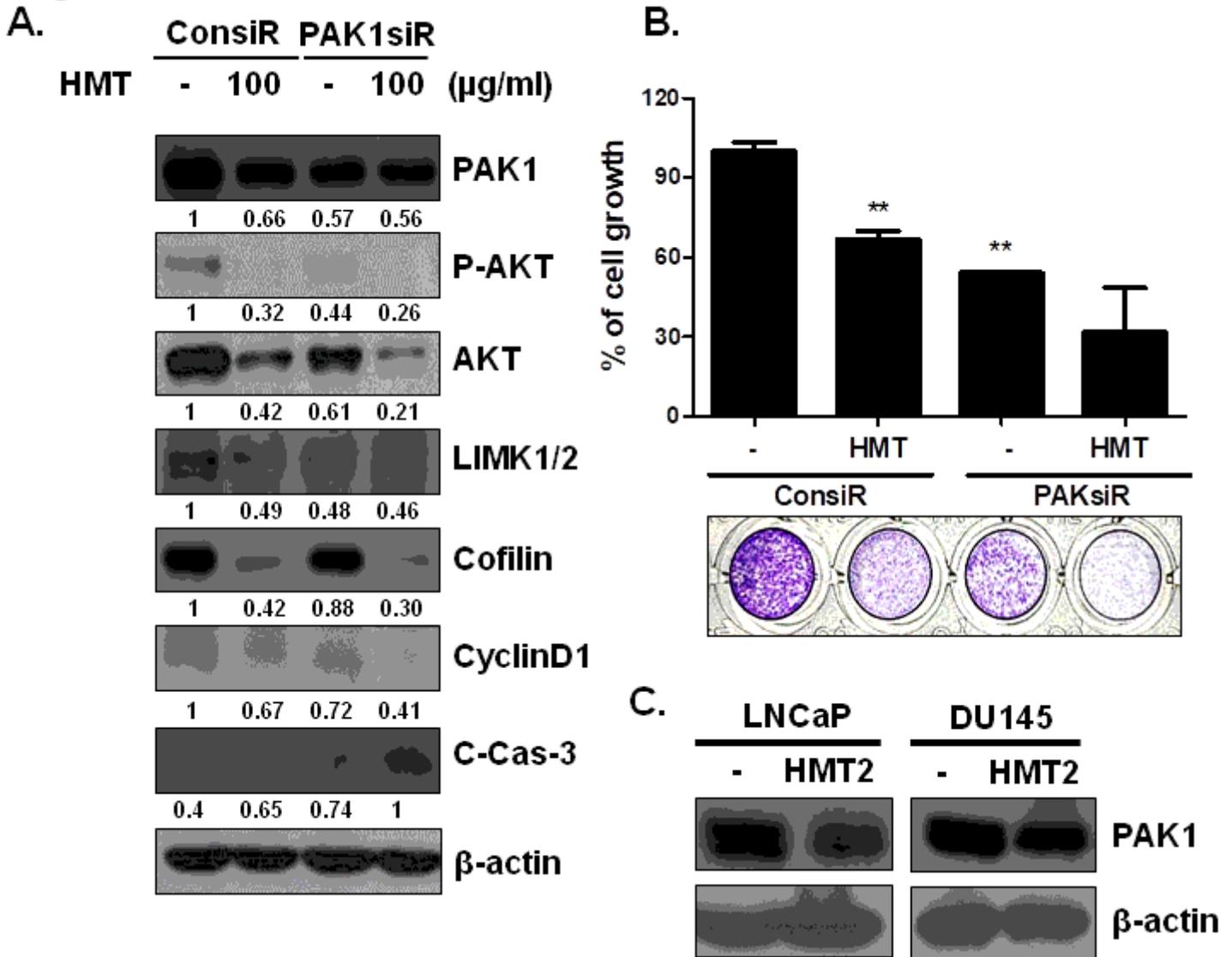


Figure 4

Effect of PAK-1 siRNA on cell migration, proliferation, and apoptosis-related markers in HMT treated PC-3 cells. PAK-1 siRNA and HMT treated PC-3 cell lysates were prepared and subjected to western blotting (A) with antibodies against PAK-1, AKT, p-AKT, LIMK1/2, Cofilin, CyclinD1, C-Cas3 and β -actin, and (B) cell growth was assayed by crystal violet staining. The stained cells were photographed and resolved in 70% EtOH, and the absorbance was measured using a microplate reader. Data represent mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control. (C) HMT-treated PAK-1 expression in LNCaP and DU145 cells by Western Blotting.

Figure. 5

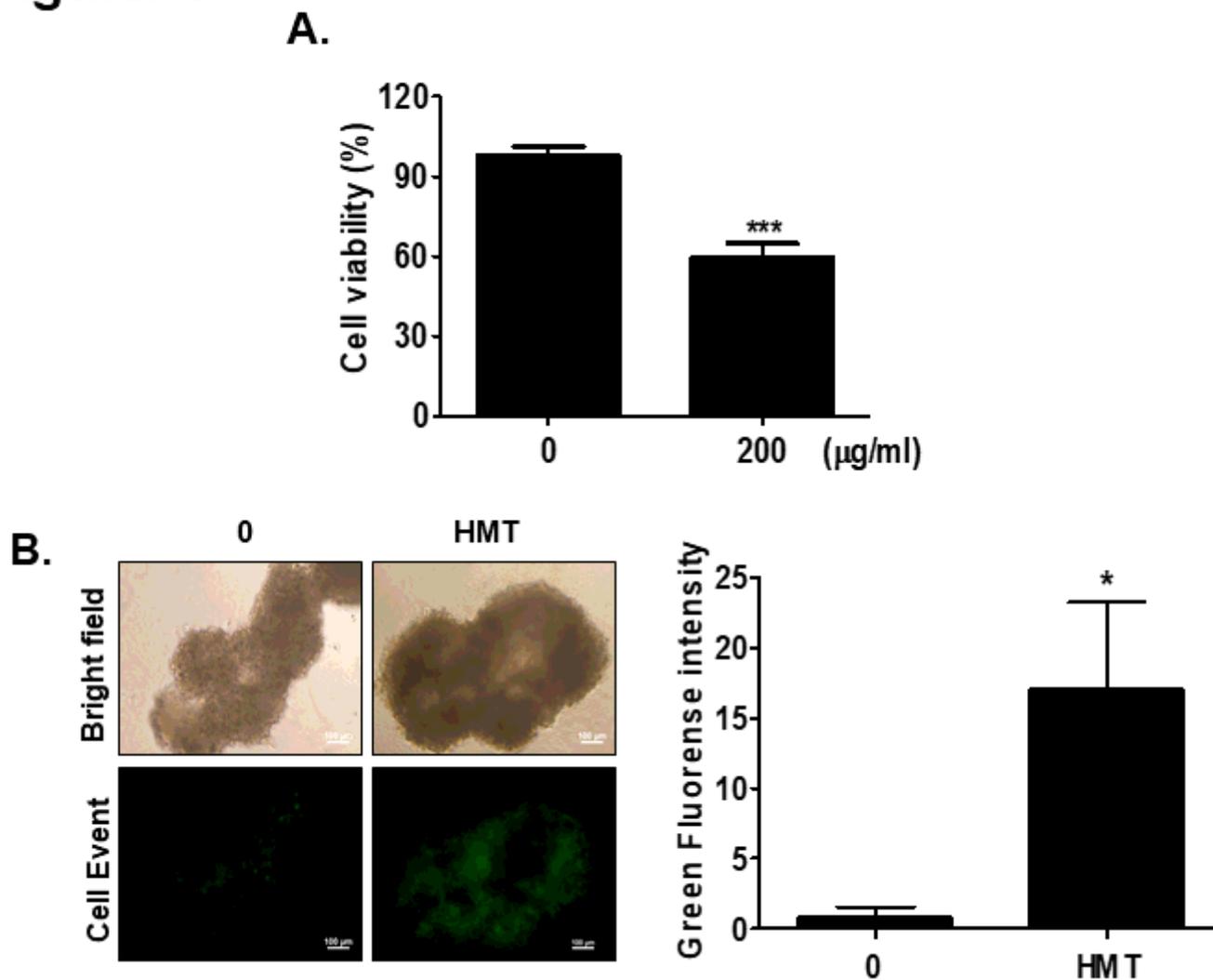


Figure 5

Inhibitory Effect of HMT on 3D culture PC-3 tumor organoids growth. 5days after formed spheroid, 200µg/ml HMT was treated to the formed spheroids for 48 h. Six organoids per group were used. (A) For organoids viability, the CELLOMAX™ viability kit was added each well and reacted for 18 h. Data represent mean ± SD. *p<0.05, **p<0.01 and ***p<0.001 compared with control. (B) For apoptosis analysis, 2 µM CELLEvent (Invitrogen, USA) was added to each well and reacted for 1 h. Pictures were obtained using a fluorescence microscope (Nikon, Tokyo, Japan).

Figure. 6

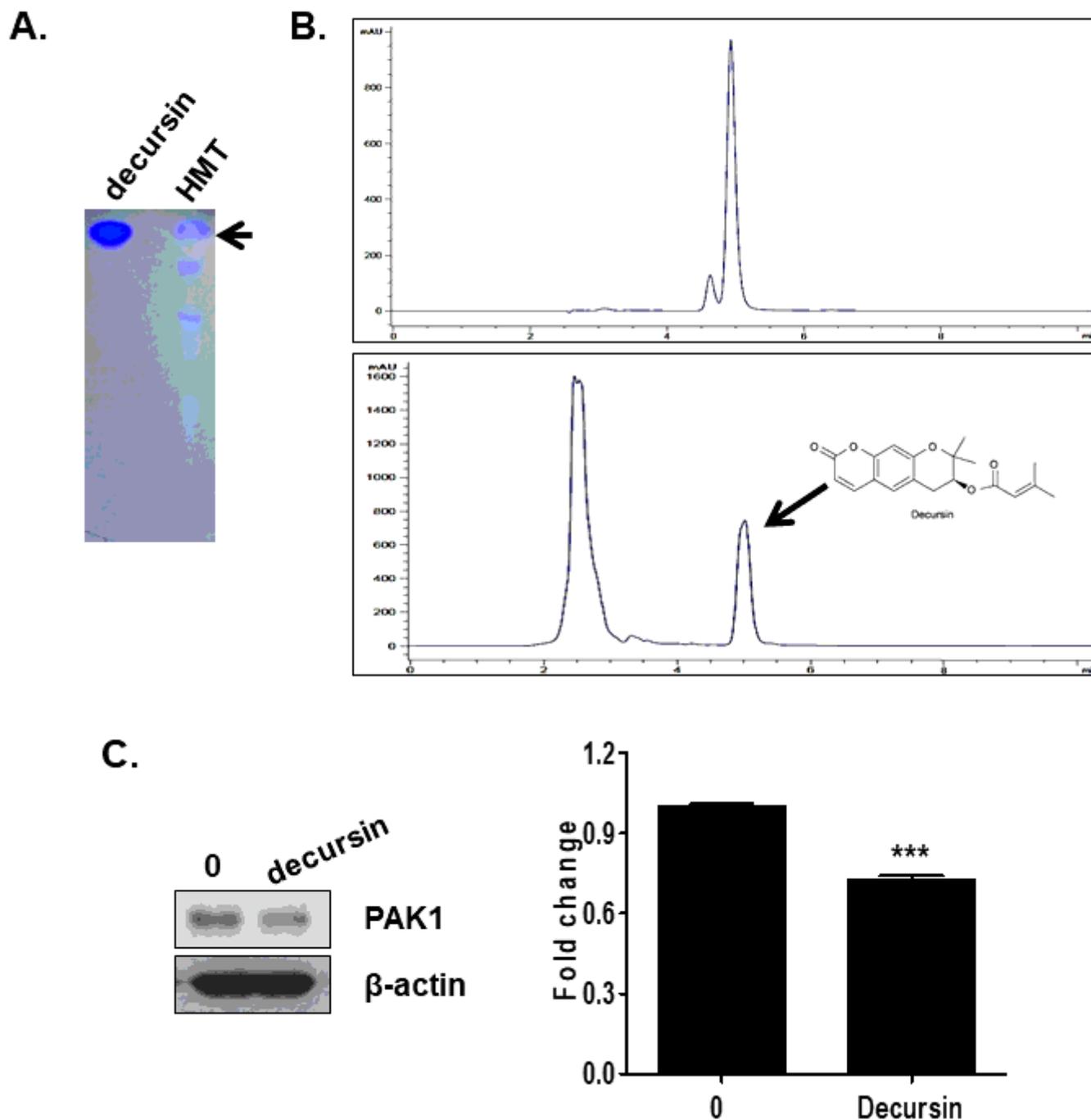


Figure 6

HMT contains decursin that inhibits PAK-1 (A) TLC analyses of HMT. For the detection of decursin, the TLC plate to char carbon-containing compounds was dipped in 10 % sulfuric acid. (B) HPLC profile. Upper section: standard of decursin, lower section; HMT (C) Changes in PAK-1 expression by decursin in PC-3 cells. Cells were treated with decursin (at 10 μ M) for 24h. Cell lysates were prepared and subjected to western blotting using PAK-1 antibodies.