Original Article

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Diarylheptanoid 7-(3,4-Dihydroxyphenyl)-5- Hydroxy-1-Phenyl-(1*E*)-1-Heptene from *Curcuma Comosa* Roxb. Inhibits Nucleophosmin Localization and Induces Apoptosis in KG-1a Leukemic Stem Cells

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Abstract:

Objective: This study aimed to investigate the effect of the diarylheptanoid 7-(3,4-dihydroxyphenyl)-5- hydroxy-1-phenyl-(1*E*)-1-heptene (DDHPH) from the rhizome of *Curcuma comosa* Roxb. on nucleophosmin (NPM) protein localization followed by induced cell death in KG-1a leukemic stem cells.

Material and Methods: DDHPH was purified by column chromatography and characterized by nuclear magnetic resonance (NMR). Localization of NPM was determined by a confocal microscopy. Induction of cell death was determined by flow cytometry and Western blotting.

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Results: A previous study showed that NPM directly interacted with p53 and activates p53 function. In this study, DDHPH was found to modify the translocation of NPM and enhance both p53 and cleaved caspase-3 expression. These proteins were found to control cell cycle progression and cell apoptosis induction.

Conclusion: This study demonstrated that DDHPH is a novel compound which induces cell death through NPM localization.

Keywords: apoptosis, *Curcuma comosa*, cytotoxicity, diarylheptanoids, nucleophosmin, Zingiberaceae

Introduction

Curcuma comosa Roxb. belongs to the Zingiberaceae family. It is commonly found in southeast Asia, India, south China, Papua New Guinea, and northern Australia[1]. In Thailand, it has been used in traditional Thai medicine for postpartum uterine bleeding and is nowadays extensively used as a dietary supplement[2,3]. Active compound from *Curcuma comosa* Roxb. has already been identified and studied for its structure and biological activity. It was found that the major compound was 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene or compound 092. It showed a potential dietary supplement to prevent bone loss with low risk of breast and uterine cancers in postmenopausal women[4]. The rhizome of this plant has been traditionally used to treat estrogenic activity[5] and for its anti-inflammatory effects[6]. In this study, we purified the diarylheptanoid 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene (DDHPH) from the ethyl acetate fractional extract of *Curcuma comosa* Roxb. by column chromatography. The chemical structure was characterized by ^{1}H-NMR spectroscopic analyses as previously described[7].

DDHPH has shown cytotoxicity against KG-1a cells, leading to cell cycle arrest[7]. It also shown evidence of pro-oxidant activity (GSH and ROS determination) of the diarylheptanoid bearing a catechol moiety in the induction of apoptosis in murine P388 leukemia[8]. In leukemia treatment currently, the main problem is disease relapse, which arises from leukemic stem cells[9]. In this study, the KG-1a leukemic stem cell line was used as a model to study the effects of a diarylheptanoid on the nucleophosmin (NPM), which is a target protein involved in leukemic cell proliferation. Interestingly, DDHPH has been shown to have a non-toxic property against normal peripheral mononuclear cells (PBMCs) and red blood cells. This compound thus has the advantage of not causing chemotoxicity. However, the mechanism of how DDHPH lead to cell cycle arrest and the induction of cell apoptosis through NPM has not examined in the previous studies.

In this study, we focused on NPM protein localization associated with cell proliferation and cell death. NPM (also known as B23, numatrin, and NO38) was first identified as a non-ribosomal nucleolar phosphoprotein found at high levels in the granular regions of the nucleolus[10]. NPM has 294 amino acids with a molecular weight of 37 kDa. It contains sequences that control shuttling in and out of the nucleolus or between the nucleus and cytoplasm to participate in cellular activities[11]. The roles of NPM include ribosome biogenesis, mRNA processing, and chromatin remodeling, and it is implicated in other cellular functions[12]. NPM has diverse functions because it has multiple functional domains, including an oligomerization domain, a histone binding domain, a ribonuclease activity domain, and a DNA/RNA binding domain. These domains can interact with several proteins[13]. One study reported that overexpression of NPM enhanced proliferation of hematopoietic stem cells and progenitor cells via induction of cell cycle progression[14]. NPM was reported to be associated with drug resistance

through its escape from apoptosis by blocking BAX (a pro-apoptotic protein) through inhibition of BAX mitochondrial translocation and activation[15]. Additionally, a previous study demonstrated that NPM could stabilize and activate p53, a tumor-suppressing protein that plays a key role in the cell cycle and cell apoptosis[16]. Thus, the role of NPM in the induction of cell proliferation and promotion of cell death depends on its interactions with certain proteins. Our previous study showed that DDHPH had cytotoxicity against leukemic stem cells and induced cell cycle arrest, but the mechanism was unclear[7]. The goal of this study was to investigate the effect of DDHPH on NPM protein localization that was hypothesized to be associated with cell proliferation and cell death in KG-1a leukemic stem cells.

Material and Methods

Plant extraction and DDHPH characterization

Curcuma comosa Roxb. was obtained from an herbarium, the Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand. The specimen had been obtained from Chiang Dao District, Chiang Mai Province, Thailand, in August 2018. The specimen number voucher was 023237.

The fresh rhizomes of *Curcuma comosa* Roxb. (5 kg) were cleaned, peeled, and then dried in an air-dry oven at 50 °C. The dried rhizomes were ground to a powder. Then, the powder was extracted as previously described[7]. Briefly, the rhizome powder was macerated in ethyl acetate for three days. The liquid portion was collected, and the residual marc was further macerated and collected the liquid portion for three times. The liquid portions of the extractions were pooled together and filtrated with Whatman filter paper No. 1, pore size 11 µm (Buckinghamshire, United Kingdom). The filtrate was evaporated using a rotary evaporator (N-1000, EYELA, Shanghai, China) and subsequently dried to obtain the ethyl acetate fraction. The residual powder was dried in a hot air oven at 45 °C.

Column Chromatography

Ethyl acetate fractional extract was further purified to isolate the DDHPH by column chromatography using silica gel grade 60 (Merck, Darmstadt, Germany) as previously described[7]. Silica gel 60 was packed in a glass column (3.5×50 cm) with the volume of 290 cm^3, and 1.577 g of ethyl acetate fraction was added to the top of the silica gel column. The column was eluted with Hex:EtOAc at a ratio of 1:1. The pooled fractions were observed using TLC. The most purified pooled fractions had a percentage yield of 32.4%. Fractions containing the DDHPH were characterized at the Faculty of Science, Chiang Mai University, to determine its chemical structure using nuclear magnetic resonance (NMR) spectroscopy (Bruker, Fällanden, Switzerland). The fractional extracts or DDHPH were dissolved in DMSO to obtain the working concentration (25 mg/mL) and stored at –20 °C for later use. The purity of the DDHPH was determined using ^{1}H-NMR and ^{13}C-NMR in conditions previously described[7].

Cell cultures and DDHPH preparation

Leukemic cell line KG-1a (leukemic stem cell-like cell line with a stem cell population) was cultured in IMDM medium supplemented with 20% FBS containing 1 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cell lines were incubated at 37 °C under 95% humidity and 5% CO_2. Diarylheptanoid 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene (DDHPH) was extracted from *Curcuma comosa* Roxb. by column chromatography as previously described[7]. DDHPH was dissolved in DMSO for stock preparation at a concentration of 25 mg/mL. The DDHPH was stored at –20 °C before use.

Preparation of the whole protein extract

To investigate the NPM expression after compound treatment, KG-1a cells were seeded into 6-well plates

at a concentration of 1.5×10^5 cells/mL and treated with the DDHPH at the concentration value of the inhibitory concentration associated with 20% of cell growth (IC_{20}). After 48 hr of incubation, the cells were harvested and washed with cold PBS, pH 7.4, three times. The whole protein was extracted using an RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail). The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, IL, USA). The protein extracts were stored at −20 °C until use.

Western blot analysis

Extracted protein (30–50 µg) was loaded into a 12% polyacrylamide gel and then protein was separated using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. After that the protein in the gel was transferred to the PVDF membrane. The method was as previously described[7]. The proteins were determined using primary and secondary antibodies as indicated in Table 1. The protein bands were visualized by Luminata™ Forte Western HRP Substrate (Millipore Corporation, Billerica, MA, USA). Quantity one 1-D Analysis software was used for quantitating the densitometry. The levels of the NPM, p53, and caspase-3 bands were normalized to GAPDH bands. In this study, curcumin was used as a positive control for the high expression level of p53 and cleaved caspase-3 proteins[17,18].

Confocal immunofluorescence imaging

To study the effects of *Curcuma comosa* Roxb. DDHPH on NPM protein localization, KG-1a cells were adjusted to 1.5×10^5 cells/mL in 20% FBS IMDM complete medium. The cells were treated with DDHPH at the IC_{20} value concentration for 10, 20, and 30 min and fixed with 2% formaldehyde in Hanks' balanced salt solution (HBSS) for 20 min. The fixed cells were permeabilized with 0.5% Triton X-100 in PBS, pH 7.4, for 10 min then washed with PBS, pH 7.4. After that, the fixed cells were blocked by 2% BSA in PBS, pH 7.4, for 30 min, and then washed three times with PBS, pH 7.4. After centrifugation at 1,200 rpm for 3 min, the cell pellet was resuspended and stained with mouse monoclonal anti-NPM IgG (C-19; SC-192) (Santa Cruz, CA, USA) at 1:1,000 dilution in blocking buffer for 1 hr then washed with PBS, pH 7.4, three times followed by adding Alexa Flour™ 568 (red fluorescence color)

Table 1 Details of sources and dilutions of antibodies for NPM, p53, caspase-3, and GAPDH protein level detection by Western blotting

Targeted protein	Primary antibody	Secondary antibody
Nucleophosmin (NPM)	Mouse monoclonal anti-NPM IgG (C-19; SC-192) (Santa Cruz, CA, USA) at 1:1,000 dilution	HRP conjugated goat anti-mouse IgG (Carlsbad, CA, USA) at 1:20,000 dilution
p53	Rabbit monoclonal anti-P53 IgG (Cincinnati, OH, USA) at 1:1,000 dilution	HRP conjugated goat anti-rabbit IgG (Carlsbad, CA, USA) at 1:20,000 dilution
Caspase-3	Rabbit monoclonal anti-caspase-3 IgG (Cincinnati, OH, USA) at 1:1,000 dilution	HRP conjugated goat anti-rabbit IgG (Carlsbad, CA, USA) at 1:20,000 dilution
GAPDH	Rabbit monoclonal anti-GAPDH IgG (Burlington, MA, USA) at 1:1,000 dilution	HRP conjugated goat anti-rabbit IgG (Carlsbad, CA, USA) at 1:20,000 dilution

GAPDH=glyceraldehyde 3-phosphate dehydrogenase

conjugated goat anti-mouse IgG (Sigma Aldrich, MO, USA) at 1:1,000 dilution for 1 hr. DAPI (Sigma Aldrich, MO, USA) at 2 μg/mL in PBS, pH 7.4, was used to stain the nuclei (blue fluorescence color). The cells were seeded on glass slides and mounted, then visualized under confocal immunofluorescence microscopy (Leica, NC, USA). NPM (red fluorescence color after anti-NPM and Alexa Flour™ 568 staining) was observed and compared with a vehicle control to determine NPM localization.

Apoptosis analysis by flow cytometry

Annexin V-FITC and a PI Apoptosis Detection Kit (BioLegend, CA, USA) were used in this experiment, according to the instructions of the manufacturer. KG-1a cells were adjusted to 1.5×10^5 cells/mL and treated with DDHPH at concentrations of IC_{50} values, then incubated at 37 °C for 48 hr. Vincristine was used for treatment in a similar condition as the positive control. Then, the cells were harvested and washed with cold PBS, pH 7.4. The cells were resuspended in buffer at a concentration of 1×10^6 cells/mL. The cells were transferred to a microcentrifuge tube (1×10^5 cells/mL) and Annexin V-FITC and PI were added. The cells were gently vortexed and incubated for 15 min at 25 °C in the dark. Buffer was added to each tube and the cell solutions were determined by a flow cytometer within 1 hr. The data were analyzed by FlowJo V10 program.

Statistical analysis

All data are expressed as means±standard deviations (S.D.) from triplicate samples of three independent experiments. Statistical differences between the means were determined using one-way ANOVA or student's t-test. The differences were considered significant when the probability value obtained was found to be less than 0.01 (p-value<0.01).

Results

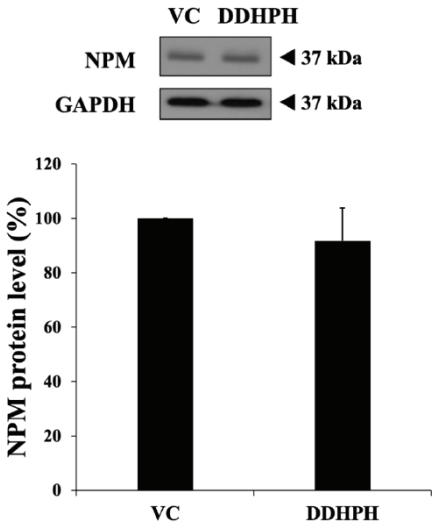
NPM protein expression level in whole cell extracts after DDHPH treatments in KG-1a cells

The IC_{20} value of DDHPH after a MTT assay in a KG-1a cell line in our previous report (2.30 μg/mL) was used to examine total NPM protein expression[7]. The level of total NPM protein in whole protein lysate after DDHPH treatment was normalized using the GAPDH protein level and then the calculated percentage of NPM protein expression was compared with the vehicle control. The NPM protein expression after treatment with DDHPH was 91.31±11.94% compared with the vehicle control (100±0%) (Figure 1). However, this difference was not statistically significant.

Effect of DDHPH on NPM localization in the cytoplasm and nuclei of KG-1a cells under confocal immunofluorescence microscope

NPM protein localization was visualized under a Leica Laser Scanning Confocal Upright Microscope at the Microscopy and Analytical Imaging Research Resource Core Lab, University of Kansas to study the location of NPM after DDHPH treatment in KG-1a cells. DDHPH at IC_{20} values (2.30 μg/mL) was used to treat KG-1a cells at various incubation times (10–30 min). DAPI (blue fluorescence color in Figure 2) was used to identify areas of cell nuclei. NPM protein (red fluorescence color in Figure 2) was observed and the determined NPM localization was compared with the vehicle control. After 10–30 min of DDHPH treatment, NPM localization was more in the nucleus compared with the vehicle controls. When NPM localized to a nucleus, the high intensity of NPM in red and nucleus stained with DAPI in blue showed a purple color after merging (Figures 2A–C). Additionally, the vehicle control did not interfere with the distribution of NPM. Quantification of cells was done with NPM localized in the nuclei following DDHPH treatment at 10, 20, and 30 min. The results of the study indicated that

there were significant differences between vehicle control and DDHPH treatment at 20 and 30 min (Figure 2D). At 20 min, there was an increase of NPM localized in a nucleus following DDHPH treatment by 22.89%. However, after treatment for 30 min, the percentage increase was 34.30%. These images and percentages of cells containing NPM localized in nuclei suggest that DDHPH induced NPM protein localization from the cytoplasm to the nucleus when compared with the controls. Moreover, it might be possible that DDHPH blocked NPM transportation from the nucleus to the cytoplasm, leading to NPM accumulation in the nuclei of the KG-1a cells.

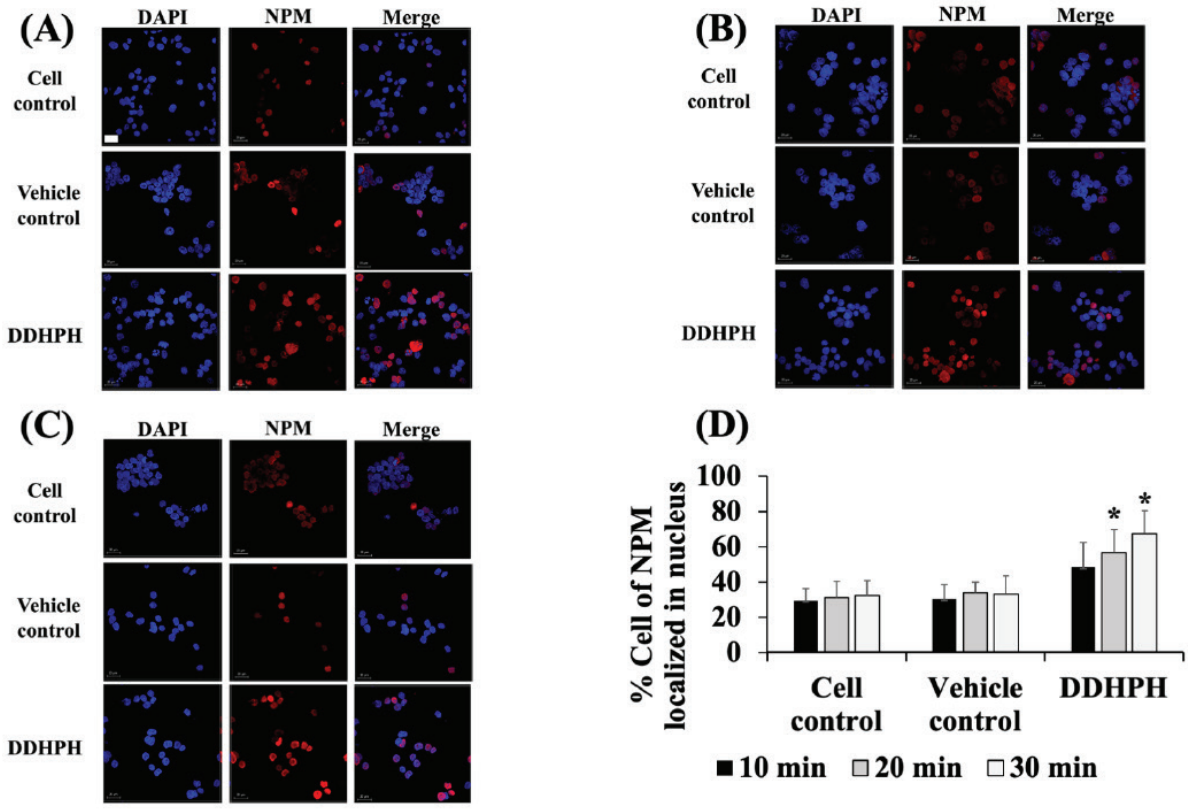


VC=vehicle control, DDHPH=diarylheptanoid 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene, NPM=nucleophosmin, GAPDH=glyceraldehyde 3-phosphate dehydrogenase

Figure 1 Effect of DDHPH on NPM protein expression in KG-1a cells and the levels of NPM protein following treatment with DDHPH at the IC_{20} value (2.30 μg/mL). Western blotting was used to evaluate protein levels. The protein levels were analyzed using a scanning densitometer. GAPDH protein levels were used to normalize the levels of NPM. Each bar represents mean±S.D. of three independent experiments performed in triplicate.

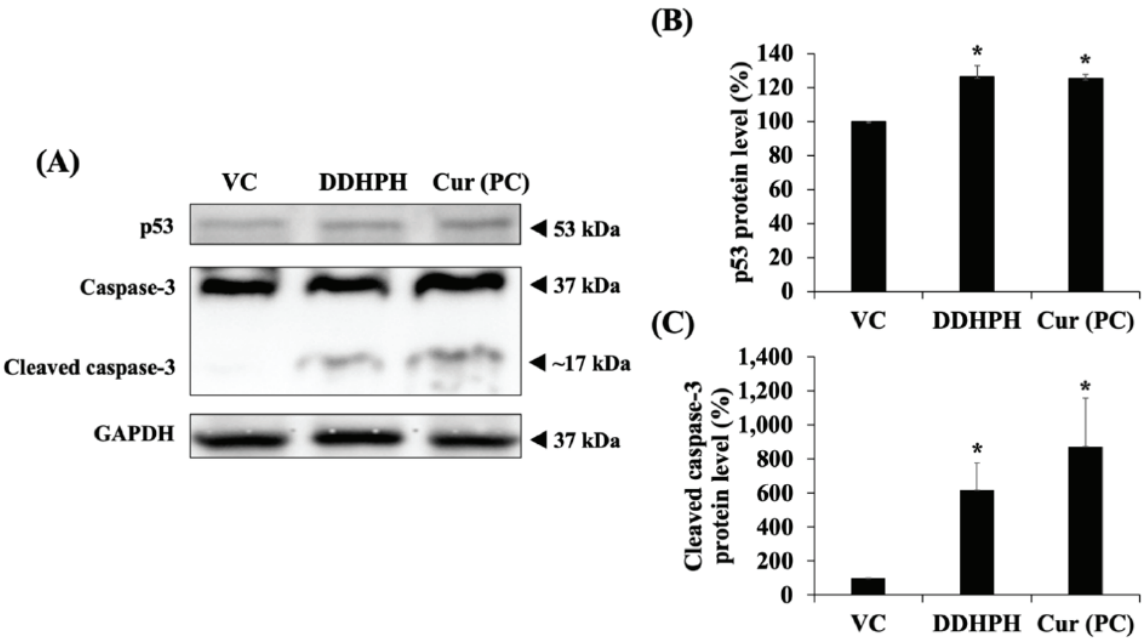
Upregulation of p53 expression and cell apoptosis induction in KG-1a cells after DDHPH treatment

In a previous study, DDHPH significantly affected cell cycle arrest at the S phase[7]. However, the mechanism of how the DDHPH did this was not studied. The p53 protein was previously reported to be involved in cell cycle progression[16,19]. In this study, DDHPH at the IC_{20} value (2.30 μg/mL) was used for further investigation. The results showed that DDHPH affected p53 protein expression. The level of p53 was normalized using the GAPDH protein, then the percentage of p53 protein expression was calculated. The expression of p53 significantly increased after DDHPH treatment for 48 hr which gave results correlating to the cell cycle arrest levels found in the previously noted study[7]. Moreover, cleaved caspase-3 expression was increased after DDHPH treatment at the IC_{20} value, suggesting induction of apoptosis (Figure 3C). Curcumin was used as a clear positive control as there are previous studies of its effect on increasing p53 and cleaved caspase-3 protein expression[17,18]. In addition, cell apoptosis after DDHPH treatment at the IC_{20} (2.30 μg/mL) and IC_{50} (5.11 μg/mL) treatments was determined by flow cytometer. PI and Annexin-V FITC were used to stain cells and indicate cell viability. PI can stain the DNA and RNA of damaged cells while Annexin V-FITC stains the phosphatidylserine of the plasma membrane of cells undergoing apoptosis, indicating apoptotic cells. Vincristine, a chemotherapeutic drug, was used as a positive control. The apoptotic cell population after DDHPH treatment was 32.9±2.2% following DDHPH treatment at the IC_{50} value. Meanwhile, at the IC_{20} value, the apoptotic cell population after DDHPH treatment was 28.6±3.2% (Figures 4 and 5). These results correlated with a previous study[8] that diarylheptanoid from *Curcuma comosa* was found to induced apoptosis in a P388 murine leukemic cell line. From these findings, it can be concluded that DDHPH could upregulate p53 and activate caspase-3, proceeding to arrest the cell cycle and induce apoptosis, which lead to cancer cell death.



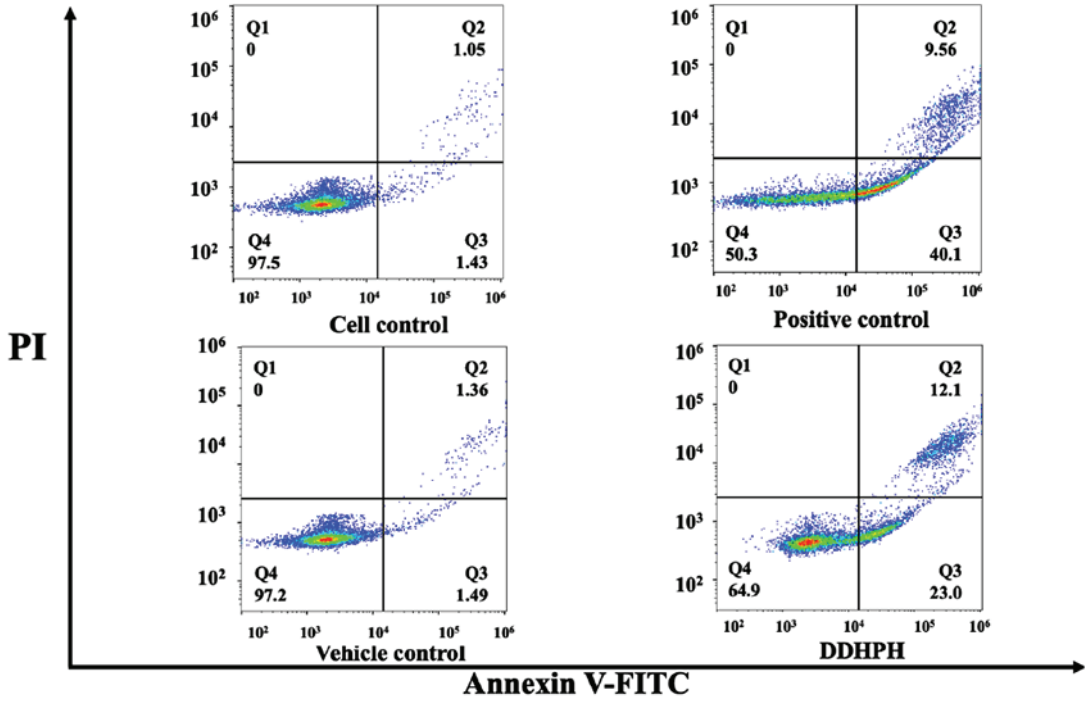
DAPI=4', 6-diamidino-2-phenylindole, NPM=nucleophosmin, DDHPH=diarylheptanoid 7-(3,4-dihydroxyphenyl)-5- hydroxy-1-phenyl-(1*E*)-1-heptene

Figure 2 Effect of DDHPH at the IC_{20} value (2.30 μg/mL) on NPM localization in the cytoplasm and nuclei of KG-1a cells under confocal microscope at various incubation times (scale bar, 20 μm). KG-1a cells were stained with mouse monoclonal anti-NPM IgG. Alexa Flour™ 568 conjugated goat anti-mouse IgG was used as a secondary antibody. The nuclei were stained with DAPI. (A) 10 min after DDHPH treatment (B) 20 min after DDHPH treatment (C) 30 min after DDHPH treatment. NPM was observed to be localized in the nucleus at 10, 20, and 30 min after DDHPD treatment. (D) Percentage of cells with NPM localized in the nuclei is demonstrated as a bar graph (n=300 in each control and DDHPH treatment). Each bar represents mean±S.D. of three independent experiments performed in triplicate. Asterisk (*) denotes significant differences at p-value<0.01.



DDHPH=diarylheptanoid 7-(3,4-dihydroxyphenyl)-5- hydroxy-1-phenyl-(1*E*)-1-heptene

Figure 3 Effect of DDHPH on p53, caspase-3, and cleaved caspase-3 protein expressions in KG-1a cells. (A) The levels of p53, caspase-3 and cleaved caspase-3 protein following treatment with DDHPH at the IC_{20} value (2.30 μg/mL) (VC: vehicle control; Cur: curcumin; PC: positive control). Western blotting was used to detect proteins and then the proteins were measured for their levels using a scanning densitometer. GAPDH protein levels were used to normalize the levels of p53, caspase-3, and cleaved caspase-3. (B) The protein level of p53 following treatment with DDHPH at IC_{20} value was upregulated compared with the vehicle control. (C) The cleaved caspase-3 protein level following treatment with DDHPH at IC_{20} value was upregulated compared with the vehicle control. Each bar represents the mean±S.D. of three independent experiments performed in triplicate. Asterisks (*) denote significant differences at p-value<0.01.



PI=propidium iodide, Annexin V-FITC = Annexin V conjugated with fluorescein isothiocyanate, DDHPH=diarylheptanoid 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene

Figure 4 The induction of apoptosis after DDHPH treatment in KG-1a cells. KG-1a cells (1.5×10^5 cells/mL) were cultured with DDHPH at IC_{50} values (5.11 μg/mL). PI and Annexin V-FITC were used to stain cells then apoptotic cells were determined. The representative flow cytometry dot plot indicates cell population (Q1: cells were stained with PI, necrotic cells; Q2: cells were stained with PI and Annexin V-FITC, apoptotic cells; Q3: cells were stained with Annexin V-FITC, early apoptotic cells; Q4: cells were not stained, live cells). Following DDHPH treatment, apoptotic cells (Q2 and Q3 cell populations) were found to be increased when compared with vehicle control.

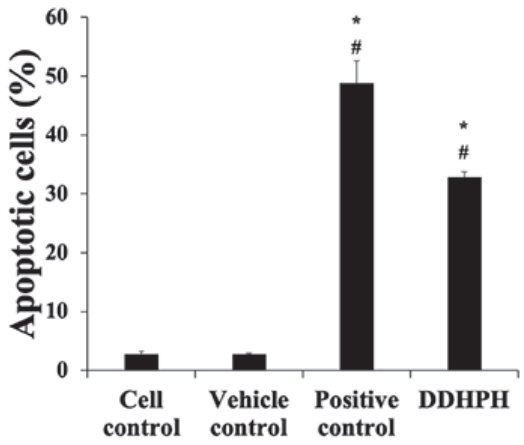


Figure 5 The induction of apoptosis after DDHPH treatment in KG-1a cells. KG-1a cells (1.5×10^5 cells/mL) were cultured with DDHPH at IC_{50} values (5.11 µg/mL). PI and Annexin V-FITC were used to stain the cells then the apoptotic cells were determined. The percentages of apoptotic cells were compared between treatments. Each bar represents the mean±S.D. of three independent experiments performed in triplicate. An asterisk (*) denotes significant differences at p-value<0.01 as compared with the cell control. A hash mark (#) denotes significant differences at p-value<0.01 as compared with the vehicle control.

Discussion

Curcuma comosa Roxb. is well known in Thailand and used to treat unpleasant symptoms in women's urogenital systems for its estrogen-like activity[5]. In a recent study, diarylheptanoids were found to be the dominant active compounds from this plant[4]. They have been found to possess various properties, including estrogenic activity as well as antioxidant, anti-inflammatory, and anti-cancer effects[5-8]. In another previous report, a diarylheptanoid named trans-1,7-diphenyl-5-hydroxy-1-heptene, showed drug modulation in the K562/ADR drug-resistant cell line by inducing drug accumulation in drug-resistant cells[20]. In this study, we focused on an active compound from an ethyl acetate fractional extract, diarylheptanoid 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene, or DDHPH. DDHPH was previously studied for its antioxidant, anti-inflammatory, and anti-cancer abilities. It was found to have cytotoxic activity and to decrease the expression of the WT1 protein that promotes cell proliferation in leukemic cells. Moreover, it could induce cell cycle arrest at the S phase in a leukemic stem cell line[7,8]. In addition, in a study mentioned earlier, DDHPH neither induced red blood cell hemolysis nor had cytotoxicity against peripheral blood mononuclear cells in normal healthy volunteers[7]. This current study aimed to fully examine the mechanism of cell cycle arrest and cell death after treatment with DDHPH.

Leukemia is a disease of abnormal proliferation of white blood cells. There are two main types of leukemia, namely lymphoblastic and myeloblastic leukemia. However, the cause of the disease remains unknown. The factors proposed to be involved in leukemia development are genetics, age, race, radiation effects, prior immunosuppression and chemotherapy, parental and residential factors, and infection[21]. To treat leukemia, chemotherapy is a popular treatment option. Unfortunately, chemotherapy-related toxicity can occur after a patient receives chemotherapeutic agents, especially in elderly patients, 75.4% of whom in one study had potential drug interactions after receiving chemotherapy[22]. In addition, chemotherapy-induced central neurotoxicity has been reported in 10–20% acute lymphoblastic leukemia pediatric patients[23]. Moreover, the disease can relapse after the patient has recovered due to small populations of leukemic stem cells (LSCs)[9]. The new treatment approach is to destroy both cancer cells and cancer stem cells to assure the efficiency of therapy. While targeted therapy has been used for this purpose, medicinal plants are another option as some plants do not have adverse effects on normal cells, such as DDHPH.

As mentioned above, our previous study showed that DDHPH had cytotoxic activity in KG-1a leukemic stem cells. It also showed significant suppression of the WT1 protein and induction cell cycle arrest. However, the mechanism of its potential against human leukemic cells was not elucidated in that earlier study. In this current study, NPM was focused on as it plays a key role in many cellular activities related to both cell proliferation and cell death[24]. According to the study of Li et al., NPM overexpression enhanced hematopoietic cell proliferation[14]. Firstly, we aimed to study the effect of DDHPH at the IC_{20} value on the suppression of NPM expression leading to decreased cell proliferation in KG-1a cells. However, in the whole cell lysate, there was no difference in NPM expression in cells exposed to DDHPH when compared with the vehicle control. This result demonstrated that DDHPH did not affect the total NPM protein level or NPM protein expression in KG-1a cells. Thus, transportation of NPM between the cytoplasm and nucleus was further examined by confocal immunofluorescence imaging. This current study showed that NPM tended to localize in the nucleus after treatment. The alteration of NPM location can result in different functions with various proteins. It has been found that the NPM protein normally works as a shuttling protein between the nucleus and cytoplasm for different functions in various cellular activities[25]. Additionally, DDHPH might block the transportation of NPM from the nucleus to the cytoplasm. This result suggested that the localization of NPM had changed after DDHPH treatment and affected cell proliferation and cell death.

In our previous study, we found that DDHPH could induce cell cycle arrest at the S phase while the upstream regulators were not identified. In another study, the pathways of p53 and the cell cycle were intensively studied. In brief, p53 was expressed in low levels in normal conditions. When cells became damaged, p53 turned into its active form and was expressed at a high level. Activated p53 acted as a transcription factor and transactivated downstream target genes. Those target genes then induced cell cycle arrest and cell apoptosis[26]. The relationship between NPM and p53 was previously studied, although the coactivation remains unclear[2]. As Colombo and colleagues reported, NPM directly interacts with p53 by stabilizing and increasing its transcriptional activity[16]. In this current study, p53 expression was evaluated and was found to have an increased expression level after DDHPH treatment. These findings indicate that NPM localization toward the nucleus possibly causes p53 activation and results in cell cycle arrest and cell apoptosis.

In this current study, apoptosis induction after DDHPH treatment was studied along with caspase-3 expression, a well-known marker for apoptosis activation. In that study, apoptosis was induced after treatment with DDHPH at IC_{20} and IC_{50} values. Caspase-3 expression levels were evaluated after treatment at the IC_{20} value. The level of cleaved caspase-3 was increased after DDHPH treatment compared with the vehicle control. These results could support the induction of apoptosis after DDHPH treatment. Our study is in line with a prior study wherein caspase-3 activity was reported to increase after similar treatment[8]. In that study, NPM was reported to interact with various proteins in the apoptotic pathway and to regulate apoptosis depending on its location. Other previous studies found that NPM tended to suppress apoptosis but also possessed the ability to interact with apoptotic proteins as chaperones[27-29]. Our study confirms the hypothesis that an imbalance of NPM protein transportation between cytoplasm and nucleus inhibits KG-1a cell proliferation and induces apoptosis. Moreover, our findings also confirmed the interaction of NPM and p53 proteins. Our results indicate that DDHPH induces NPM localization toward nucleus. Then, NPM acts through a p53-dependent pathway to induce cell cycle arrest and activate caspase-3 in the apoptotic pathway.

Conclusion

This study suggests an explanation for the bioactivity mechanism of the diarylheptanoid 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene, or DDHPH, extracted from rhizomes of *Curcuma comosa* Roxb., on the inhibition of NPM localization, which in turn affects cell cycle arrest and cell apoptosis in a leukemic stem cell line. This study provided knowledge that DDHPH is involved in NPM function alteration leading to the upregulation of p53 and caspase-3 activity, causing cell cycle arrest and cell apoptosis. Thus, DDHPH could be used as a model for chemotherapeutic agents, particularly for patients suffering from leukemia. Moreover, as DDHPH had no toxicities in normal blood cells, it could be more advantageous for vulnerable patients. Nonetheless, further investigations *in vivo* should be done to assure the competency of this compound.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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