

Triolein from *Coix lacryma-jobi* Induces Cell Cycle Arrest Through p53/p21 Signaling Pathway

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ABSTRACT

p53, a tumor suppressor protein, has important roles in DNA repair, cell cycle and apoptosis, is a one of the key events in cancer development. *Coix lacryma-jobi* seed has been used as a food and traditional medicine plant with anti-oxidant, anti-cancer and anti-diabetic effects. In currently research, we identified the most potent p53-increasing compound among 4 compounds (1 – 4) found in *Coix lacryma-jobi* and demonstrated its molecular mechanism in MCF-7 cells. Among the four isolated compounds (1 – 4), triolein most increased p53. Triolein treatment induced p53, p21, p27 and Bax in MCF-7 cells. Moreover, triolein caused S phase arrest through suppression of CDK1, phospho-Rb and E2F1 in dose-dependent manner. We also observed the decreasing of DNA synthesis by triolein. These data suggest that triolein may induced cell cycle restart involve DNA synthesis and apoptosis pathway in MCF-7 cells.

Key words: Triolein, *Coix lacryma-jobi*, MCF-7 cells, apoptosis, DNA synthesis.

INTRODUCTION

Cancer is a complex disease with multiple genetic and epigenetic changes. Cancer cells proliferate without stopping and spread into surrounding tissues. Chemotherapy is the most common types of cancer treatment. p53 plays an important role in cancer treatment mainly by increasing cell cycle arrest, senescence, DNA damage and apoptosis and interconnecting with many cellular functions¹⁻². Targeting p53 provides many opportunities to fight against cancer.

p53 regulates various responses to genotoxic stress by controlling the transcription of numerous target genes involved in cell cycle and

DNA repair (p21, GADD45), apoptosis (Bax, Bcl2), and senescence (Sirt1)³⁻⁴. One promising cancer treatment strategy is the development natural products that induce p53 transcription activity and its target genes to induce growth arrest and apoptosis in p53 wild-type cancer cells.

Coix seed (*Coix lacryma-jobi*) is used in Vietnam and Chinese as a nutritious food and traditional medicine. Previous studies demonstrated that Coix seed extract exhibits anti-inflammatory, protects against oxidative stress, and anti-cancer activities⁵⁻⁶. Coix extract constituents inhibited NF- κ B and protein kinase C signalling in cancer cells to suppress growth MDA-MB-231 xenograft in athymic nude mice,⁷ inhibited COX-2 expression of

human lung cancer cells⁵, activated PI3K/Akt to upregulate the Nrf2, HO-1 expression to protect against oxidative stress-induced injury⁸.

Triolein, one active component in Coix seed extract, exhibits anti-tumor activity. The anti-cancer activities of triolein are limited due to its use as a cell fuel. Triolein is degraded by lipoprotein and fatty acids released are used to provide energy via beta-oxidation⁹. However, mechanism investigations on impacts of triolein on cancer cells are necessary before drug development studies. Therefore, this study was performed to investigate impact of triolein on p53 and its target genes that involves in cell cycle arrest in breast cancer cells. We found that triolein from Coix seed extract induces p53 protein level in concentration-dependent manner in MCF-7 cells. Triolein up-regulated the expression of p53-dependent target genes that related to cell cycle arrest such as p21 and cyclin D1, reducing DNA content. This study provided scientific evidences for the use of Coix seed.

EXPERIMENTAL

Chemicals and cell culture

Most chemicals were purchased from Sigma (St. Louis, MO). p53, p21, Bax, p27, Cyclin D1, phospho-Rb, total-Rb, E2F1, anti-mouse and anti-rabbit were obtained from Cell Signaling. MCF-7 cells were obtained from the American type culture collection (Bethesda, MD) and incubated at 37 °C in a 5% CO₂/95% air atmosphere in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Western blot analysis

After protein determination using BCA protein assay kit (Pierce, Rockford, IL), aliquots of the lysates (10-30 µg of protein) were boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins in the gels were transferred onto nitrocellulose membranes and then incubated with primary antibodies specified (p53, p21, Bax, p27, Cyclin D1, phospho-Rb, total-Rb, E2F1, β -actin). Bands were visualized using ECL (GE Healthcare Life Sciences) and images were developed using the LAS 3000.

BrdU assay

BrdU assay was performed as described¹⁰.

Plant materials and extraction

The seeds of *Coix lachryma-jobi* var. *mayuen* were collected in Yenbai province, Vietnam, in December 2014. The specimen of materials has been identified by one of authors Dr. Do Thi Ha (D.T.H). The specimen (HTV-211214) have been deposited with the Department of Phytochemistry, National Institute of Medicinal Materials, Viet Nam.

Sample preparation for bioassays

Each sample (adlay aril (CL1), adlay brain (CL2) and adlay seed kernel (CL3), 50 g) was extracted with 96% EtOH (3 × 0.5 L) and then evaporated to dryness under vacuum at 40 °C to obtain the 96 % CL1 (2.81 %; wt/wt), CL2 (11.64 %; wt/wt), CL3 (11.46 %; wt/wt). Adlay aril (the CL2 extract (5,32 g) was suspended in 100 mL H₂O, followed by sequential partitioning with hexane, ethyl acetate, and water, to give CL2H, 2.44 g), ethyl-acetate-soluble (CL2E, 0.1 g), water-soluble (CL2W, 2.78 g) extract. The extracts were stored at -20 °C until tested.

Extraction and isolation of compounds from the significant bioactive fraction

Adlay aril powder (5,85 kg) was extracted three times with 96% ethanol at boiling temperature (20 l × 3 h × 3 times). The combined filtrates were evaporated to dryness in vacuo at 40 °C. The residue (681,1 g) was stirred in 1 L MeOH and then suspended in 2 l H₂O, followed by sequential partitioning with hexane (Hx), ethyl acetate (EtOAc), and water (W), to give hexane-soluble, ethyl-acetate-soluble, and water-soluble fractions. Hexan-soluble fraction (280 g) was then subjected to silica gel (230–400 mesh) column chromatography with successive elution by a Hex/EtOAc gradient (100:0, 70:1, ..., 1:10, ...) and MeOH solvent system. Subfractions with the same thin-layer chromatography (TLC) patterns were combined into one fraction; thus 9 fractions were obtained (AHH1A~AHH1I). Rechromatography of the first bioactive subfraction AHH1A (80 g) on silica gel columns using Hx-EtOAc gradient (100:0, 50:1, 30:1, 10:1) to yield compound **1** (3,00 g). Fraction T1CL1F (13,6 g) was subjected to silica gel (230–400 mesh) column chromatography, eluted with dichloromethan (DCM) to obtain five sub-fractions

T1CL1F1~T1CL1F5. The fraction T1CL1F5 (1,5 g) was purified by column chromatography with a gradient solvent system DCM-MeOH (50:1, 30:1, 20:1, 10:1) obtained compound **2** (88,8 mg). Compounds **3** (3,0 g) and **4** (190.8 mg) were obtained by further purification of the fraction T1CL1F4 (13,6 g) using normal phase chromatography with a gradient solvent system DCM-MeOH (100:1~20:1) and reverse phase chromatography YMC using a solvent system acetone-H₂O (4:1).

CLH1

oil; APCI-MS: *m/z*: 886,8 [M+H]⁺ (C₅₇H₁₀₄O₆, M = 885,45). ¹H-NMR (500 MHz, CDCl₃) δ_H: 5.34 (6H, m, H-8_x, H-9_x, H-8", H-9", H-8"_x, H-9"_x), 5.26 (1H, t, H-2), 4.29 (2H, dd, *J* = 4.0, 12.0 Hz, H-1), 4.15 (2H, dd, *J* = 6.0, 12.0 Hz, H-3), 2.31 (6H, t, H-1_x, H-1", H-1"_x), 2.0 (12H, H-7_x, H-10_x, H-7", H-10", H-7"_x, H-10"_x), 1.60 (6H, m, H-2_x, H-2", H-2"_x), 1.30 (60H, CH₂), 0.88 (9H, t, H-17_x, H-17", H-17"_x). ¹³C-NMR (500 MHz, CDCl₃) δ_C: 173.3 (C-1_x, C-1"_x), 172.8 (C-1"), 130.0 (C-8_x, C-9_x, C-8"_x, C-9"_x), 129.7 (C-8", C-9"), 68.9 (C-2), 62.1 (C-1, C-2), 14.1 (C-17_x, C-17", C-17"_x), 22.7-34.2 (CH₂). ¹H and ¹³C-NMR data were consistent with literature values of triolein¹¹.

CLH3

A colorless powder; M.p. 290 - 294°C; ¹H-NMR (400 MHz, Pyridin-d₅) δ: 5.35 (1H, brd, H-6); 3.53 (1H, m, H-3); 1.01 (3H, s, H-19); 0.69 (3H, s, H-20); 0.92 (3H, d, *J* = 6,8 Hz, H-21); 0.83 (3H, d, *J* = 1.6 Hz, H-26); 0.81 (3H, brs, H-27); 0.85 (3H, brs, H-29); Glu: 5.07 (1H, d, *J* = 7.2 Hz, H-1'); 4.59 (1H, dd, *J* = 2.4; 11.7 Hz, H-6a'); 4.43 (1H, dd, *J* = 5.7; 11.7 Hz, H-6b'); 4.30 (2H, m, H-3', 5'); 4.06 (1H, m, H-4'); 3.97 (1H, m, H-2'). ¹³C-NMR (100 MHz, pyridin-d₅) δ: 37.2 (C-1); 31.6 (C-2); 71.7 (C-3); 42.2 (C-4); 140.7 (C-5); 121.7 (C-6); 31.8 (C-7); 31.9 (C-8); 50.0 (C-9); 36.4 (C-10); 21.0 (C-11); 39.7 (C-12); 42.2 (C-13); 56.7 (C-14); 24.3 (C-15); 28.2 (C-16); 55.9 (C-17); 11.8 (C-18); 19.4 (C-19); 36.1 (C-20); 18.7 (C-21); 33.9 (C-22); 25.9 (C-23); 45.8 (C-24); 29.1 (C-25); 19.8 (C-26); 18.9 (C-27); 23.0 (C-28); 11.0 (C-29); Glc: 102.9 (C-1'); 79.0 (C-3'); 78.9 (C-5'); 75.7 (C-2'); 72.1 (C-4'); 63.3 (C-6'). ¹H and ¹³C-NMR data were consistent with literature values of daucosterol¹².

CLH4

A viscous oil. APCI pos *m/z* 842.3 [M+H]⁺ consistent with the chemical formula C₅₃H₉₂O₇; ESI neg 161.0 [M-C₁₈H₃₃O-C₂₉H₄₉O-H]⁻ consistent with a glucose unit C₆H₁₀O₅⁻ (162.05), *m/z* 413.3 [M-C₁₈H₃₃O-C₆H₁₀O₅]⁻ consistent with a sitosteryl unit (413.4); *m/z* 139.2 [M-C₄₃H₇₃O₇]⁺ consistent with an alkene unit (the =C-C- bond fragmentation at position 8 and 9 broken) (C₁₀H₁₉) of an oleyl unit ¹H-NMR (500 MHz, CDCl₃) δ_H: 5.36 (2H, m, -CH=CH-), 5.34 (1H, m, H-6), 4.44 (1H, dd, *J* = 5.5, 12.0 Hz, H-6'a), 4.38 (1H, d, *J* = 7.0 Hz, H-1'), 4.25 (1H, dd, *J* = 2.0, 12.0 Hz, H-6'b), 3.56 (1H, m, H-5'), 3.54 (1H, dd, 8.0, 8.5 Hz, H-5'), 3.46 (1H, m, H-6'a), 3.44 (1H, m, H-4'), 3.38 (1H, dd, *J* = 9.0, 11.5 Hz, H-2'), 3.55 (2H, dd, *J* = 6.0, 11.5 Hz, H-H3b), 2.35 (2H, m, H-23), 1.28 (30H, m, (CH₂)₁₅), 1.09 (3H, s, H-19), 0.98 (3H, d, *J* = 6.0 Hz, Me-18"), 0.95 (3H, d, *J* = 6.8 Hz, Me-21), 0.89 (3H, t, *J* = 7.0 Hz, Me-29), 0.88 (3H, d, *J* = 7.0 Hz, Me-26), 0.68 (3H, s, Me-18). ¹³C-NMR (500 MHz, CDCl₃) δ_C: 174.6 (C-1_x), olefinic carbons: 140.3 (C-5), 130.2 (C-9_x), 130.0 (C-10"), 121.1 (C-6), 73.5 (C-3), 56.8 (C-14), 56.1 (C-17), 50.2 (C-9), 45.8 (C-23), 42.4 (C-13), 39.8 (C-12), 37,3 (C-1), 36,7 (C-10), 36,1 (C-20), 34,2 (C-22), 31,9 (C-8), 31,8 (C-7), 31,5 (C-2), 29,1 (C-25), 28,3 (C-16), 25,2 (C-23), 24,3 (C-15), 23,1 (C-28), 21,0 (C-11), 19,4 (C-26), 19,3 (C-19), 19,0 (C-27), 18,8 (C-21), 11,8 (C-18), 11,0 (C-29); Glc: 100.2 (C-1'), 79.6 (C-5'), 76.0 (C-3'), 73.9 (C-2'), 70.2 (C-4'), 63.3 (C-6'), 174.6 (C-13), 33.9 (C-23), 31.8 (C-83), 31.7 (C-103), 29.2-29.7 (-CH₂-, C-43, 53, 63, 73, 123, 133, 143, 153, 163), 25.7 (C-33), 22.7 (C-173), 14.1 (C-183). ¹H and ¹³C-NMR data were consistent with literature values of sitosterol-3-O-6-oleoyl-β-D-glucopyranoside¹³.

CLH5

Colorless gum; APCI-MS pos: *m/z* 355.2 [M+H]⁺ and ESI pos: *m/z* 394.1 [M+Na]⁺ consistent with molecular formula C₂₁H₃₈O₄. ¹H-NMR (500 MHz, CDCl₃) δ_H: 5.34 (2H, m, H-9_x, H-12_x), 4.08-4.11 (2H, dd, *J* = 6.0, 11.0 Hz, H-1a, H-1b), 3.87 (1H, m, H-2), 3.63 (1H, dd, 4.5, 11.5 Hz, H-3a), 3.55 (2H, dd, *J* = 6.0, 11.5 Hz, H-H3b), 2.77 (2H, dd, *J* = 6.5, 7.0 Hz, H-11'), 2.34 (2H, t, *J* = 7.5 Hz, H-2_x), 2.00 (4H, H-8_x, H-13_x), 1.62 (2H, m, H-3_x), 1.31 (20H, 10CH₂), 0.89 (3H, t, H-18_x). ¹³C-NMR (500 MHz, CDCl₃) δ_C: 174.6 (C-1_x), olefinic carbons: 130.4 (C-9_x), 130.2 (C-13'), 129.9 (C-10_x), 128.2 (C-12'),

70.1 (C-2), 65.3 (C-1), 63.4 (C-3), 34.2 (C-11'), 32.0 (C-8', 14'), 31.7 (C-2'), 29.2-29.8 (7 CH₂), 27.3 (C-4'), 25.0 (C-3'), 14.1 (C-18x). ¹H and ¹³C-NMR data were consistent with literature values of 2,3-dihydroxypropyl octadecadienoate (1-monolinolein)¹⁴.

P-values were calculated by Student's t-test or one-way analysis of variance followed by Bonferroni post-hoc testing using GraphPad Prism 5 (GraphPad Software Inc.). *P* < 0.05 was considered statistically significant. Data are expressed as mean ± S.E.M. *, *p*<0.05; **, *p*<0.01; ***, *p*<0.001.

RESULTS AND DISCUSSION

p53 tumour suppressor protein has been demonstrated to induce apoptosis and cell cycle arrest in cancer cells [1]. To screen natural drug candidates against cancer, we first determined the effect of ethanol 96% extractions from *Coix lacryma-jobi* includes: adlay aril, adlay brain and adlay seed kernel (1 mg/ml) on p53 protein expression using western blot analysis. Fig 1A indicated that adlay aril extraction most potential increased p53 in MCF-7 cells. From adlay aril extraction, 3 fractions include: n-hexan, ethyl acetate and water were prepared and further examine the effects of these fraction on p53 expression. As shown as in Fig 1B, n-hexan fraction most activated p53 protein expression in MCF-7 cells. From this fraction, we isolated 4 compounds: CLH1 (triolein), CLH3, CLH4 and CLH5. Using western blot analysis, we found triolein (100 iM) compound most significant increase p53 protein expression level in MCF-7 cells (Fig 1C).

p53 expression up-regulated p21-cell cycle arrest inducer to induce cell cycle arrest and then repair the damaged DNA due to increase apoptosis in cancer cells [15]. Thus, we examine the effect of triolein on p21, p27 and Bax protein expression. As expect, p53, p21, p27 and Bax protein level were significant increased by triolein in dose-dependent (Fig. 2A).

Cyclin D1 is an important regulator in G1/S phase of cell cycle, cell proliferation and

apoptosis. E2F1 was reported to induce apoptosis result from the inhibition of Rb. By using western blot analysis, Fig. 2B presented Cyclin D, phospho-Rb and E2F1 were significant suppressed by triolein in dose-dependent manner (10 iM-300 iM). Moreover, we observed the cell cytotoxicity by triolein at 300 iM. p53/p21 plays the important key event in the control of cell cycle, apoptosis and cellular stability (ref). During DNA damage process, wild-type p53 is expressed to increase cell cycle arrest at several checkpoints to repair the damaged DNA. Our study demonstrated that triolein inhibited DNA synthesis in dose-dependent manner. These data suggest triolein isolated from *Coix lacryma-jobi* induces cell cycle arrest through p53/p21 signaling pathway

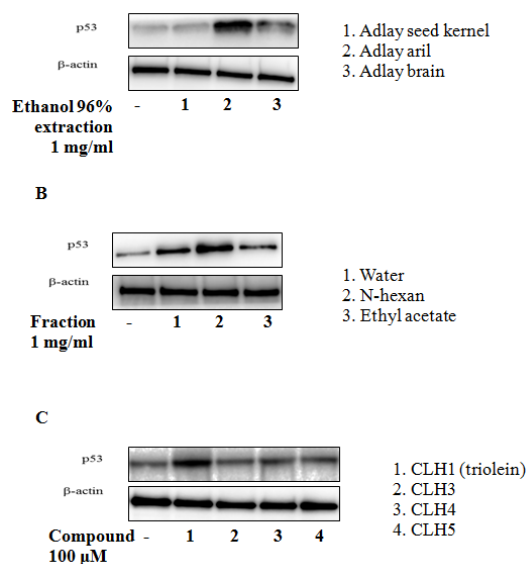


Fig. 1: Triolein activates p53 in MCF-7 cells (A) Effect of p53 by 96% ethanol extraction from *Coix lacryma-jobi* (adlay aril, adlay brain and adlay seed kernel) at 1mg/ml. Cells were incubated with different extractions for 24h. Cells were then harvested and analysis by western blot analysis. (B) Effect of three fraction: n-hexan, ethyl acetate and water from adlay aril extraction on p53 protein expression. (C) Effect of four compounds isolated from n-hexan fraction on p53 protein expression. (n=3)

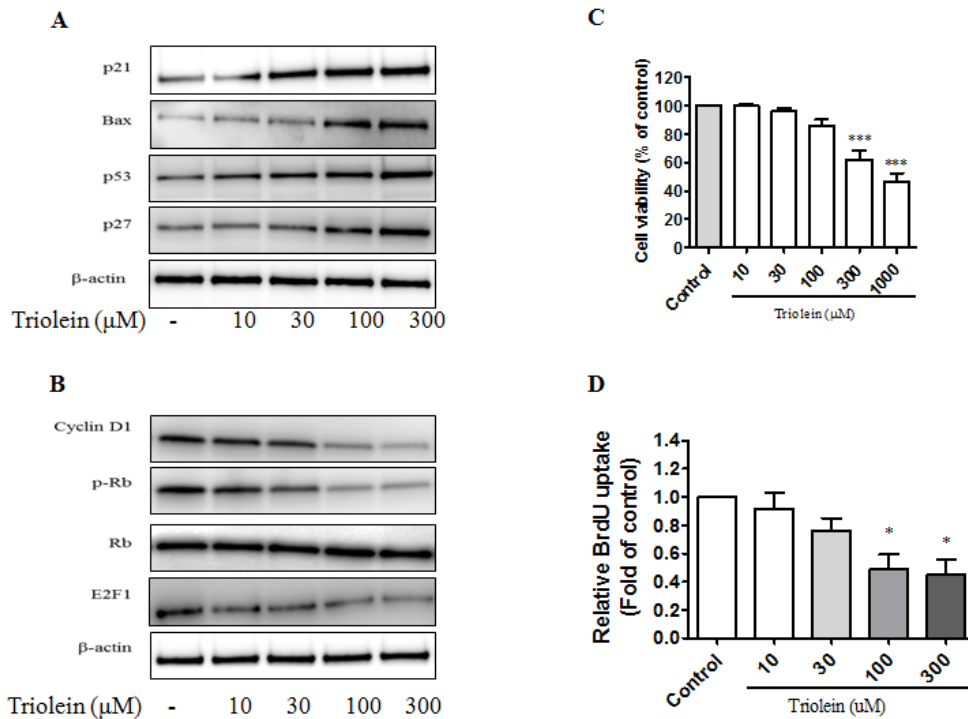


Fig. 2: (A) Effect of triolein on p53, p27, Bax and p21 protein expression in dose-dependent manner in MCF-7 cells. Cells were treated with various concentrations of triolein and then cells were harvested to subject to western blot analysis (n=3). (B) Effect of triolein in dose-dependent on Cyclin D1, phospho-Rb, Rb and E2F1 protein expression (n=3). (C) Cell viability after treatment of triolein in MCF-7 cells by MTT assay (n=4). (D) Effect of triolein on DNA synthesis by BrdU assay (n=4). *, p<0.05; **, p<0.01; ***, p<0.001 relative to control

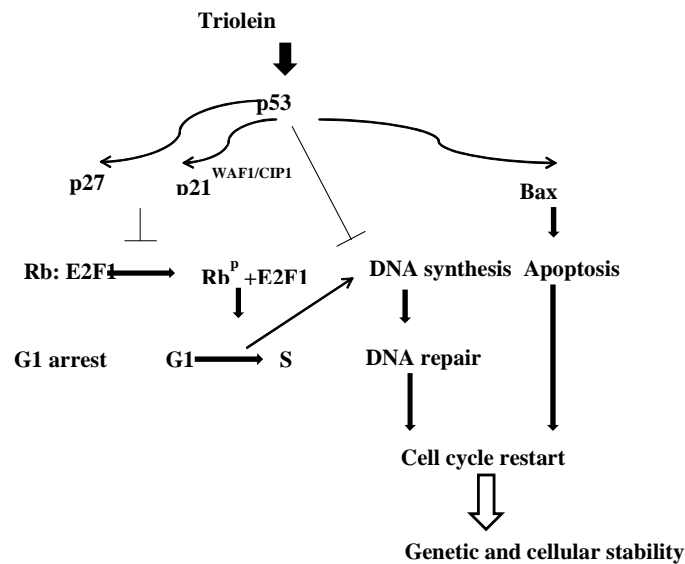


Fig. 3: Molecular mechanism of triolein in cell cycle in MCF-7 cells

Declaration of interest

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