

Helichrysetin Induces DNA Damage that Triggers JNK-Mediated Apoptosis in Ca Ski Cells

Ho Yen Fong, Sri Nurestri Abd Malek, Hui Shin Yee, Saiful Anuar Karsani

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

Submitted: 11-02-2017

Revised: 01-08-2017

Published: 13-11-2017

ABSTRACT

Background: Cervical cancer has become one of the most common cancers in women and currently available treatment options for cervical cancer are very limited. Naturally occurring chalcones and its derivatives have been studied extensively as a potential anticancer agent in different types of cancer and helichrysetin is naturally occurring chalcone that possess potent antiproliferative activity toward human cancer cells.

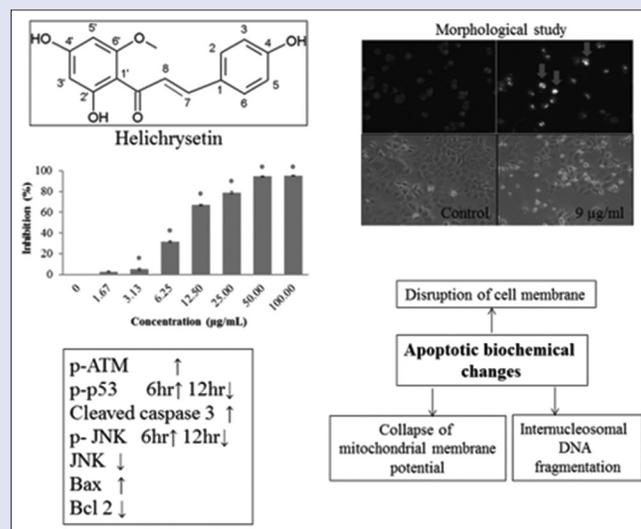
Materials and Methods: Inhibitory activity of helichrysetin was evaluated at different concentrations. Ability of helichrysetin to induce apoptosis and its relation with c-Jun N-terminal kinase (JNK)-mediated mechanism of apoptosis was assessed using flow cytometry and Western blotting.

Results: Helichrysetin inhibited Ca Ski cells at half maximal inhibitory concentration $30.62 \pm 0.38 \mu\text{M}$. This compound has the ability to induce DNA damage, mitochondrial membrane disruption, and loss of cell membrane integrity. We have shown that apoptosis was induced through the activation of JNK-mediated apoptosis by DNA damage in the cells then triggering p53-downstream apoptotic pathway with increased expression of pro-apoptotic proteins, Bax and caspase 3, and suppression of Bcl-2 anti-apoptotic protein. DNA damage in the cells also caused phosphorylation of protein ataxia-telangiectasia mutated, an activator of DNA damage response. **Conclusion:** We conclude that helichrysetin can inhibit Ca Ski cells through DNA damage-induced JNK-mediated apoptotic pathway highlighting the potential of this compound as anticancer agent for cervical cancer.

Key words: Apoptosis, Ca Ski, helichrysetin, c-Jun N-terminal kinase pathway

SUMMARY

- Helichrysetin induced DNA damage in Ca Ski cells
- DNA damage caused JNK-mediated phosphorylation of p53 resulting in p53-mediated apoptosis
- Helichrysetin is a potential DNA damage inducing agent through JNK activation to kill human cervical carcinoma cells.



Abbreviations used: ATM: Ataxia-telangiectasia mutated, DAPI: 4',6-diamidino-2-phenylindole, DMSO: Dimethyl sulfoxide, FITC: Fluorescein isothiocyanate, IC_{50} : Half maximal inhibitory concentration, JC1-5,5',6,6'-Tetrachloro: 1',3,3'-tetraethylbenzimidazolylcarbocyanine, iodide, JNK: c-Jun N-terminal kinase, MMP: Mitochondrial membrane potential, PBS: Phosphate-buffered saline, SRB: Sulforhodamine B, TUNEL: Terminal deoxynucleotidyl transferase dUTP nick labeling

Correspondence:

Dr. Saiful Anuar Karsani,
Faculty of Science, Institute of
Biological Sciences, University of Malaya,
50603 Kuala Lumpur, Malaysia.
E-mail: saiful72@um.edu.my

DOI: 10.4103/pm.pm_53_17

Access this article online

Website: www.phcog.com

Quick Response Code:



INTRODUCTION

Based on the study by GLOBOCAN 2012, cervical cancer ranked fourth most common cancer in women with an estimation of 528,000 new cases and 266,000 deaths in 2012.^[1] Different approaches have been used to treat cancer such as targeted therapy, hormone therapy, or radiation therapy.^[2,3] However, the results were not promising due to the limitation of the therapies which then brought natural products back to light in the field of drug discovery.

The balance between cell proliferation and cell death is important to maintain the tissue homeostasis.^[4] Apoptosis is defined as cell suicide program involved in the elimination of unwanted cells in cell development or in respond to cellular stress.^[5] Cancer development has been related to the evasion of apoptosis in cells resulting in the resistance

of cell death.^[6] Despite being the cause of cancer development, apoptosis is also a popular target for cancer therapy. Many studies have revealed the role of flavonoid-based compounds in cancer treatment by targeting the mechanisms of apoptosis.^[7,8]

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Cite this article as: Fong HY, Abd Malek SN, Yee HS, Karsani SA. Helichrysetin induces DNA damage that triggers JNK-mediated apoptosis in Ca Ski cells. *Phcog Mag* 2017;13:607-12.

Chalcones are the bioproduct of the shikimate pathway and also the precursors of flavonoids and isoflavonoids.^[9] Chalcones are known to cause cancer cell death through the induction of apoptosis by affecting the expression of apoptotic proteins and the mitochondrial pathway.^[7] Helichrysetin, 2',4,4'-trihydroxy-6'-methoxy chalcone is a natural plant chalcone that consists of three hydroxyl groups and one methoxy group. It has been isolated from *Alpinia katsumadai* (Hua and others 2008) Hayata^[10] and *Alpinia blepharocalyx* K. Schum.,^[11] *Alpinia galanga* Willd.,^[12] and flowers of *Helichrysum foetidum* Moench and *Helichrysum odoratissimum* Sweet. Helichrysetin has also been found in the rhizomes of *Boesenbergia pandurata* (Roxb.) Schltr.^[13] Studies revealed that helichrysetin showed inhibitory activity in human breast cancer cell lines, MCF-7 and MDA-MB-435, human liver hepatocellular carcinoma, HEPG2,^[10] human cervical adenocarcinoma, HeLa,^[14] and human fibrosarcoma, HT-1080.^[11] c-Jun N-terminal kinases (JNKs) have been identified as stress-related protein kinases which have been found to be stimulated by DNA-damaging drugs,^[15] chemopreventive drugs,^[16] and cytokines.^[17] Signals given by the stimulant will be able to induce apoptosis in the cells. JNKs play very important role in the initiation of death receptor-activated extrinsic and mitochondrial-mediated intrinsic apoptosis, hence, describing the importance of JNKs in the regulation of apoptosis, cell differentiation, and cell proliferation.^[18]

Our previous study showed that helichrysetin can induce DNA damage, disruption of mitochondrial membrane, and apoptosis in A549 cells.^[19] Thus, we would like to investigate the potential of helichrysetin as an anticancer agent for cervical cancer which currently have limited treatment options. In this study, we evaluate the ability of helichrysetin to inhibit growth, induce apoptosis, and its effect on the regulation of JNK-mediated apoptotic pathways.

MATERIALS AND METHODS

Helichrysetin and cell culture

Helichrysetin was purchased from BioBioPha Co., Ltd. (China). Ca Ski cell line was obtained from American type culture collection. Ca Ski cells were cultured in RPMI-1640 medium (Nacalai Tesque, Japan), 10% fetal bovine serum, 1% amphotericin B, 2% penicillin/streptomycin (Sigma, US), and 0.5% dimethyl sulfoxide (DMSO) (for treatment only) and incubated in 5% CO₂ at 37°C. Cells were incubated overnight in culture plates for cell adherence, and treatments were performed using 10% fetal bovine serum in RPMI-1640 with 0.5% DMSO as control.

Sulforhodamine B assay

Cells were plated in 96 wells culture plate overnight. Culture media was removed and fresh media containing helichrysetin at different concentrations (1.67, 3.13, 6.25, 12.5, 25, 50, 100 µg/mL) was added. Incubation was then carried out for 72 h. Sulforhodamine B (SRB) colorimetric assay was performed for cytotoxicity screening. At the end of the incubation, protein precipitation was done using cold trichloroacetic acid at 4°C for 1 h. Precipitates were washed with distilled water five times and air dried. About 0.4% SRB solution was added to each well for 30 min for staining and then washed with 1% acetic acid to remove unbound dye. The stained proteins were extracted using Tris base and the absorbance was taken at the wavelength of 560 nm. Inhibitory activity was calculated as:

$$\text{Percentage of inhibition} = (A_{\text{control}} - A_{\text{treated}}) / A_{\text{control}} \times 100\%$$

Phase-contrast and fluorescence microscopy with 4',6-diamidino-2-phenylindole stain

Cells were cultured overnight. They were then treated with helichrysetin at 9 µg/mL for 72 h. Following treatments, the cells were observed under

phase-contrast microscope at ×20 magnification (Zeiss Axio Vert. A1). To observe the nuclear morphology, cells were harvested and washed with phosphate-buffered saline (PBS). Cells were fixed in 4% formaldehyde then stained with 0.2 µg/mL 4',6-diamidino-2-phenylindole (DAPI) fluorescent stain and 0.1% Triton X-100 for 5 min. To observe the cells under fluorescence microscope at ×40 magnification (Leica), the cells were spotted on a slide and allowed to dry.

Flow cytometry analysis

Cells were plated in 6 well culture plates and incubated overnight. Treatments with helichrysetin were performed, 4.5, 9, and 18 µg/mL and from 6 to 72 h. After treatment, cells were harvested and washed twice with PBS, and staining was then performed according to assay kit's manufacturer's instructions. Annexin-V/fluorescein isothiocyanate (FITC) assay (BD Biosciences), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Invitrogen, US) and JC-1 mitochondrial membrane potential (MMP) assay (BD Biosciences) were performed to analyze cell membrane integrity, internucleosomal DNA fragmentation, and mitochondrial membrane, respectively. For cell cycle analysis, cells were stained with propidium iodide (PI). All cells were acquired using Accuri C6 flow cytometer by capturing 10,000 events per run (BD Biosciences).

Western blotting

2 × 10⁶ cells were treated with helichrysetin at half maximal inhibitory concentration (IC₅₀) for 6 and 12 h. Cells were harvested and the proteins were extracted with RIPA buffer. About 50 µg of cell lysates were separated by 6% stacking gel and 12% separating gel in acrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membrane and blocking was done with 5% bovine serum albumin at room temperature. Incubation with primary antibody (1:1000) was performed at 4°C overnight. Next, the membrane was incubated with peroxidase-conjugated secondary antibody (1:2000) and visualized using chemiluminescence.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 17, IBM, US. Results were shown as mean ± standard error with at least three independent experiments. Statistical significance was set at *P* < 0.05. The analyses performed were the normality test (Shapiro-Wilk), Student's *t*-test, snf Levene's test for determination of variances equality and comparison of means using one-way analysis of variance with Games-Howell test.

RESULTS

Helichrysetin inhibits proliferation of Ca Ski cells

SRB assay revealed that the increase in the concentration of helichrysetin (from 1.67 to 100 µg/mL) caused a significant (*P* < 0.05) increase in the percentage of inhibition at 72 h treatment. The concentration at which helichrysetin caused 50% cell growth inhibition of Ca Ski cell lines was 8.76 ± 0.11 µg/mL (30.62 ± 0.38 µM, IC₅₀). The result is summarized in Figure 1.

Changes in cell and nuclear morphology of Ca Ski cells

Phase-contrast microscopy revealed a loss of cell volume in Ca Ski cells treated with helichrysetin at 9 µg/mL. The cells appeared to be rounded up and detached from substratum upon treatment with helichrysetin while untreated cells remain in its regular morphology and continue to proliferate and increase in number [Figure 2]. Changes in cell nuclear morphology can be observed by staining the cellular DNA with DAPI

stain [Figure 3]. Cells that were not treated with helichrysetin remain dim while the cells treated with 9 $\mu\text{g}/\text{mL}$ of helichrysetin have bright appearance and the nucleus appeared to be condensed and fragmented. Condensed chromatin becomes DAPI-dense chromatin region, hence emitting high intensity of fluorescence showed by the bright cells in Figure 3.

Detection of early and late apoptosis by annexin-V/ fluorescein isothiocyanate-propidium iodide assay

In the annexin-V/FITC-PI assay, helichrysetin caused significant increase of apoptotic cells in a time- and dose-dependent manner ($P < 0.05$). The results were summarized in Figure 4. Percentage of annexin-V-positive cells increased significantly at 9 and 18 $\mu\text{g}/\text{mL}$ to $41.96 \pm 1.42\%$ and $42.57 \pm 1.12\%$, respectively. Time-dependent study results showed that the percentage of annexin-V-positive cells increased significantly after 24, 48, and 72 h of treatment at IC_{50} $40.93 \pm 1.09\%$, $45.23 \pm 1.26\%$, and $41.96 \pm 1.42\%$, respectively.

Helichrysetin caused DNA damage and disruption of mitochondrial membrane potential

Results of TUNEL assay revealed that $34.58 \pm 1.40\%$ of the treated Ca Ski cells were apoptotic at 9 $\mu\text{g}/\text{mL}$ of helichrysetin [Figure 5]. The apoptotic region is represented by the top region (R2) of the density plot in Figure 5. This indicates that helichrysetin caused damage to DNA observed by the fragmentation of internucleosomal DNA in Ca Ski cells. Terminal deoxynucleotidyl transferase labels the nicks in the DNA by adding nucleotides to it, hence increased of labeling indicates increase of

nicks in DNA. Helichrysetin treatment showed disruption of the MMP in Ca Ski cells. The percentage of apoptotic cells increased significantly from 12 h of treatment to 72 h of treatment and also in dose-dependent study from 9 to 18 $\mu\text{g}/\text{mL}$ [Figure 6]. The apoptotic cells indicate the depolarization of the MMP where the apoptotic population presents a reduction in the red fluorescence (JC-1) signal intensity.

Cell cycle analysis

In cell cycle analysis, the percentage of apoptotic cells in the untreated sample is $6.36 \pm 0.39\%$ while at 4.5, 9, and 18 $\mu\text{g}/\text{mL}$, the percentages are $12.68 \pm 0.96\%$, $10.58 \pm 1.55\%$, and $20.70 \pm 1.43\%$, respectively. The apoptotic cells in cell cycle are represented by sub-G1 phase in the cell cycle. Result in Figure 7 showed that helichrysetin can cause disruption in the progression of the cell cycle with the accumulation of cell in the sub-G1 phase, the hypodiploid peak subpopulation containing the fragmented DNA.

Western blotting of DNA damage, c-Jun N-terminal kinase, and apoptotic-related markers

Treatment with helichrysetin at IC_{50} caused the increase of phospho-JNK at 6 h in comparison to the untreated cells and subsequently decreased after 12 h of treatment while the expression level of JNK protein decreased

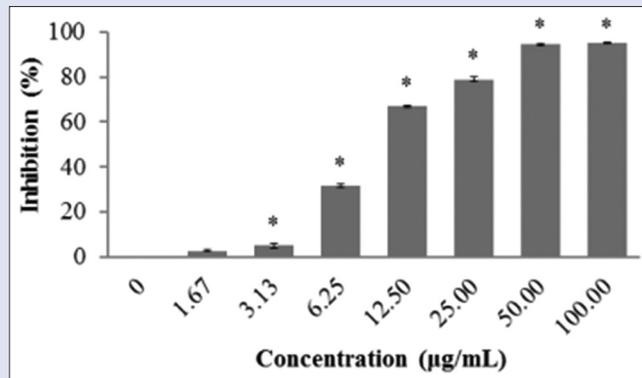


Figure 1: Percentage of Ca Ski cells inhibition by helichrysetin at different concentrations. Data shown as mean \pm standard error and asterisk as $P < 0.05$ of three independent experiments performed in triplicates (at column width)

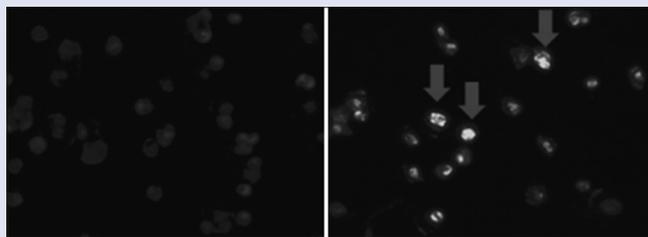


Figure 3: Fluorescence microscopy image. Control (left) and treated (right) cells. Magnification $\times 40$. Arrows indicate cells with pyknosis and karyorrhexis (at column width)

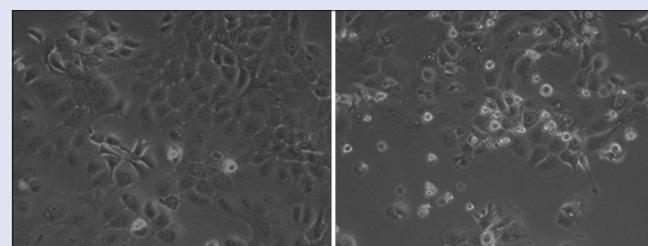


Figure 2: Phase contrast microscopy. Control (left) and cells treated at 9 $\mu\text{g}/\text{mL}$ for 72 h (right). Magnification $\times 20$ (at column width)

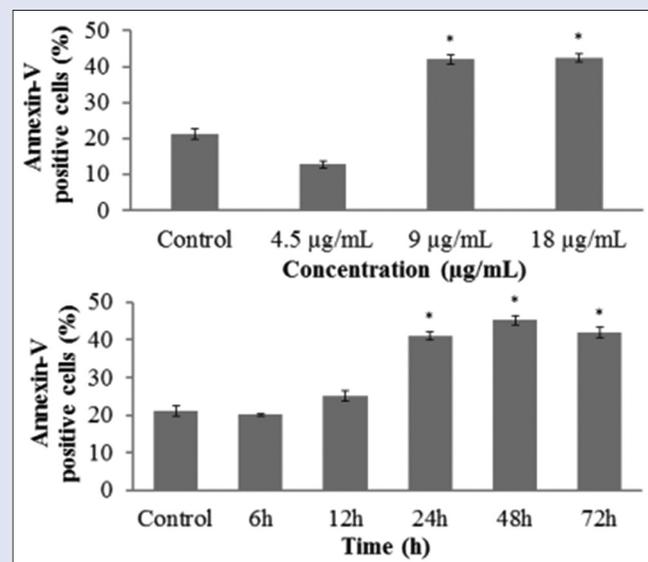


Figure 4: Bar chart corresponds to the annexin-V/fluorescein isothiocyanate assay results. Results shown as percentage mean \pm standard error and asterisk as $P < 0.05$. Top: Dose-dependent study. Bottom: Time-dependent study (at column width)

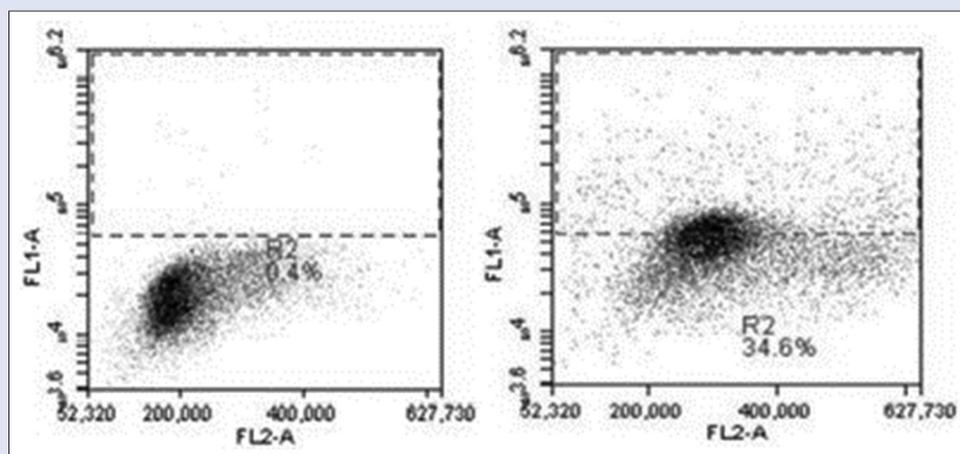


Figure 5: Density plot of terminal deoxynucleotidyl transferase dUTP nick labeling assay. Control (left) and treated at 9 µg/mL (right) (at column width)

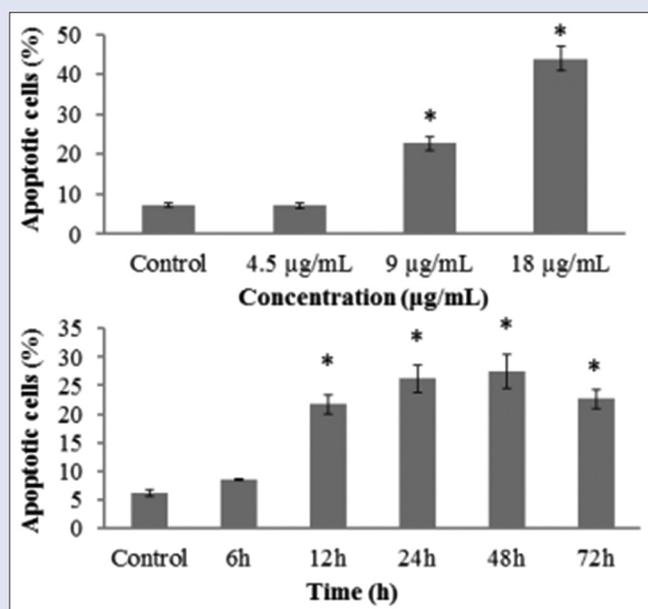


Figure 6: Bar chart corresponded JC-1 mitochondrial membrane potential assay. Results shown as percentage mean ± standard error and asterisk as $P < 0.05$. Top: Dose-dependent study. Bottom: Time-dependent study (at column width)

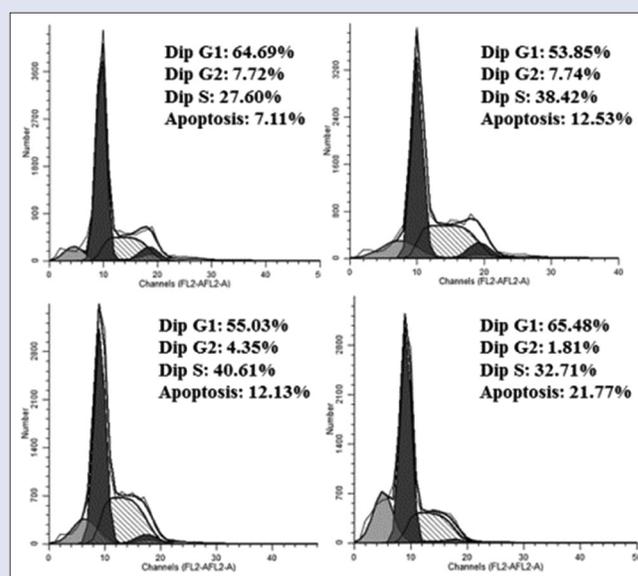


Figure 7: Histogram of cell cycle analysis. Control (top left), 4.5 µg/mL (top right), 9 µg/mL (bottom left), 18 µg/mL (bottom right) (at column width)

upon treatment of helichrysetin at 6 and 12 h. Then, we explored the protein expression of phospho-p53 in helichrysetin-treated Ca Ski cells. At 6 h, phospho-p53 is highly expressed in Ca Ski then decreased at 12 h. This observation suggests that phosphorylation of p53 occurred at the early stage of the treatment. Our result revealed that helichrysetin upregulated phospho-ataxia-telangiectasia mutated (ATM) at 6 and 12 h in comparison to untreated cells. Phosphorylation of ATM is the indication of activation of ATM protein in the cells.

Upon the treatment of Ca Ski cells with helichrysetin at IC_{50} , anti-apoptotic protein, Bcl-2, was suppressed in this study. The expression of Bcl-2 was downregulated after 6 and 12 h of treatment with helichrysetin in comparison to its untreated cells. The expression of pro-apoptotic protein, Bax, increased after 6 h treatment with helichrysetin and decreased at 12 h treatment. This trend is similar to the expression pattern of p-p53 and p-JNK in Ca Ski cells upon helichrysetin treatment. Result also showed

that there was a slight decrease of cleaved caspase 3 at 6 h and after 12 h treatment, and the expression of cleaved caspase 3 was upregulated in Ca Ski cells. All results from Western blotting were summarized in Figure 8.

DISCUSSION

Helichrysetin has been reported to be biologically active toward breast cancer cells, lung cancer cells, and colorectal cancer cells by inhibiting the proliferation of these cancer cells. In the previous study, we have demonstrated that helichrysetin can induce cell death and trigger apoptosis in A549 cells. In this study, we aim to investigate the effect of this natural compound on cervical cancer cells, Ca Ski, since cervical cancer has limited treatment options.

Our result from the cell inhibition assay showed that helichrysetin inhibits the proliferation and reduces the viability of Ca Ski cells. A compound is considered active against cancer cell when the IC_{50} is $< 50 \mu M$.^[20] Hence, the IC_{50} value of $30.62 \pm 0.38 \mu M$ indicates that helichrysetin is active against Ca Ski cells.

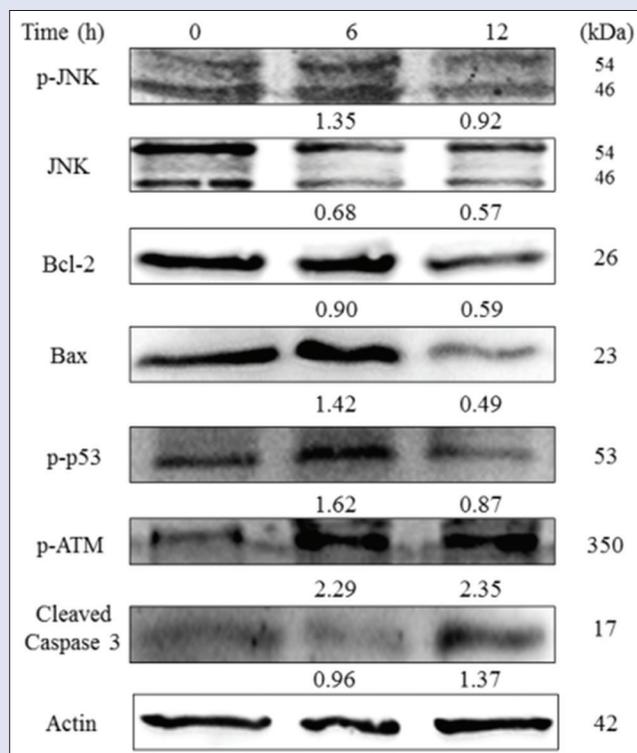


Figure 8: Western blot analysis of helichrysetin-treated Ca Ski. Cells treated with helichrysetin (9 µg/mL) at 6 and 12 h (at column width)

During the initiation of apoptosis, apoptotic features such as cell shrinkage, reduction of cell number, formation of apoptotic bodies, chromatin condensation, nuclear fragmentation, and detachment of cells can be observed.^[21] Our results from the phase-contrast microscopy and nuclear morphological study have revealed these apoptotic morphological features upon helichrysetin treatment.

Cell shrinkage is a passive process leading to the breakdown of cells to form apoptotic bodies which will be engulfed through phagocytosis by macrophages.^[22] Chromatin condensation starts at the peripheral of the nuclear membrane. Further condensation at the late stage of apoptosis will finally cause the nucleus to break up inside the cell. This phenomenon is named karyorrhexis. The occurrence of pyknosis happens when the cell chromatin becomes a solid and irregular mass through condensation. Occurrence of chromatin condensation and nuclear fragmentation is important hallmark of apoptosis.^[23]

During the early stage of apoptosis, externalization of phosphatidylserine will occur, and this phenomenon can be detected in the annexin-V/FITC assay. Our study showed that helichrysetin can cause the externalization of phosphatidylserine from the inner leaflet of plasma membrane to the outer leaflet. This event is an important hallmark of apoptosis^[24] which is also an early sign that allows the recognition of Ca Ski cells for phagocytic engulfment.^[25]

Helichrysetin caused damage to DNA observed by the fragmentation of internucleosomal DNA in Ca Ski cells which is a result of both single- and double-strand DNA breaks.^[26] The fragmentation of double-stranded DNA in the internucleosomal region shown in Figure 5 by TUNEL assay is known to be involved in the signal transduction of cell cycle activation and apoptosis.^[27] DNA damage in the cells may be regarded as a point of no return for the cells which will execute the cellular apoptotic pathway.

Results in Figure 7 showed that helichrysetin can cause disruption in the progression of the cell cycle with the accumulation of cell in the sub-G1 phase, the hypodiploid peak subpopulation containing the fragmented DNA.^[28] Low molecular weight DNA is present in this subpopulation as a result of DNA degradation in apoptotic cells.^[29]

Mitochondrion is the metabolic and bioenergetic center of all eukaryotic cells. When apoptosis occurs, BH3-only proteins that are involved in apoptosis will be translocated to mitochondria in response to apoptosis stimuli.^[30] Upon translocation to mitochondria, apoptogenic proteins such as cytochrome c will be released. Collapse of MMP is related to the release of cytochrome c from mitochondria followed by the activation of caspase cascade resulting in mitochondrial-activated apoptosis.^[31]

Studies have shown that apoptosis can be induced by death stimuli such as DNA damage, UV radiation, and chemotherapeutic agents.^[32] Western blotting and the study of MMP have revealed that helichrysetin can cause the disruption of MMP and the increase of cleaved caspase 3 and Bax protein. This shows that helichrysetin can upregulate the BH3-only protein mediator, Bax causing the release of cytochrome c indicated by the collapse of MMP, and finally initiating the caspase cascade.^[33]

Phosphorylation of JNK has been reported to promote p53-mediated apoptosis in response to death stimuli.^[34] Previous study showed that p53, tumor suppressor protein, can be phosphorylated upon the action of DNA damage-inducing agents,^[35] and phosphorylation of p53 activates the protein to induce apoptosis.^[36] Phosphorylation of p53 is mediated by protein kinase, ATM, which stabilizes and activates p53.^[37] ATM commonly recognizes the presence of DNA double-strand breaks that trigger the downstream effects to activate cell cycle arrest, DNA damage response, and cell death mechanism.^[38] Results from Western blot study suggested that helichrysetin induced apoptosis in Ca Ski cells by DNA damage-induced JNK-mediated apoptosis through the activation of p53 by DNA damage response protein ATM.

In summary, this study uncovers the inhibitory activity of helichrysetin in Ca Ski, human cervical carcinoma cells. Helichrysetin has the ability to cause DNA damage in the cells which act as a death stimuli for the induction of programmed cell death. As a result of DNA damage in the cells, JNK-mediated phosphorylation of p53 occurred which contribute to the induction of p53-mediated apoptosis. Hence, helichrysetin can serve as a potential DNA damage inducing agent and through activation of JNK, initiate the killing of human cervical carcinoma cells.

Financial support and sponsorship

This work was supported financially by Ministry of Higher Education of Malaysia and University of Malaya through High Impact Research MoE Grant UM.C/625/1/IR/MoE/SC/02.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al.* Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359-86.
2. Basmadjian C, Zhao Q, Bentouhami E, Djehal A, Nebigil CG, Johnson RA, *et al.* Cancer wars: Natural products strike back. *Front Chem* 2014;2:20.
3. Schlom J. Therapeutic cancer vaccines: Current status and moving forward. *J Natl Cancer Inst* 2012;104:599-613.
4. Lockshin RA, Zakeri Z. Cell death in health and disease. *J Cell Mol Med* 2007;11:1214-24.
5. Fernald K, Kurokawa M. Evading apoptosis in cancer. *Trends Cell Biol* 2013;23:620-33.
6. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* 2011;144:646-74.

7. Jandial DD, Blair CA, Zhang S, Krill LS, Zhang YB, Zi X. Molecular targeted approaches to cancer therapy and prevention using chalcones. *Curr Cancer Drug Targets* 2014;14:181-200.
8. Liu M, Hansen PE, Wang G, Qiu L, Dong J, Yin H, *et al.* Pharmacological profile of xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus*). *Molecules* 2015;20:754-79.
9. Rahman MA. Chalcone: A valuable insight into the recent advances and potential. *Chemical Sciences Journal* 2011;2011:1-16.
10. Hua SZ, Wang XB, Luo JG, Wang JS, Kong LY. A pair of unique sesquiterpene - Chalcone conjugates isolated from the seeds of *Alpinia katsumadai*. *Tetrahedron Lett* 2008;49:5658-61.
11. Ali MS, Tezuka Y, Awale S, Banskota AH, Kadota S. Six new diarylheptanoids from the seeds of *Alpinia blepharocalyx*. *J Nat Prod* 2001;64:289-93.
12. Morita H, Itokawa H. Cytotoxic and antifungal diterpenes from the seeds of *Alpinia galanga*. *Planta Med* 1988;54:117-20.
13. Tewtrakul S, Subhadhirasakul S, Karalai C, Ponglimanont C, Cheenpracha S. Anti-inflammatory effects of compounds from *Kaempferia parviflora* and *Boesenbergia pandurata*. *Food Chem* 2009;115:534-8.
14. Vogel S, Ohmayer S, Brunner G, Heilmann J. Natural and non-natural prenylated chalcones: Synthesis, cytotoxicity and anti-oxidative activity. *Bioorg Med Chem* 2008;16:4286-93.
15. Yu R, Shtil AA, Tan TH, Roninson IB, Kong AN. Adriamycin activates c-jun N-terminal kinase in human leukemia cells: A relevance to apoptosis. *Cancer Lett* 1996;107:73-81.
16. Yu R, Jiao JJ, Duh JL, Tan TH, Kong AN. Phenethyl isothiocyanate, a natural chemopreventive agent, activates c-Jun N-terminal kinase 1. *Cancer Res* 1996;56:2954-9.
17. Sluss HK, Barrett T, Dérijard B, Davis RJ. Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol Cell Biol* 1994;14:8376-84.
18. Liu J, Lin A. Role of JNK activation in apoptosis: A double-edged sword. *Cell Res* 2005;15:36-42.
19. Ho YF, Karsani SA, Yong WK, Abd Malek SN. Induction of apoptosis and cell cycle blockade by helichrysetin in a549 human lung adenocarcinoma cells. *Evid Based Complement Alternat Med* 2013;2013:857257.
20. Boik J. Natural compounds in cancer therapy. *J Nat Prod* 2001;64:1605-6.
21. Bortner CD, Cidlowski JA. Apoptotic volume decrease and the incredible shrinking cell. *Cell Death Differ* 2002;9:1307-10.
22. Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* 2000;45:528-37.
23. Prokhorova EA, Zamaraev AV, Kopeina GS, Zhivotovsky B, Lavrik IN. Role of the nucleus in apoptosis: Signaling and execution. *Cell Mol Life Sci* 2015;72:4593-612.
24. Bronckers AL, Goei SW, Dumont E, Lyaaruu DM, Wöltgens JH, van Heerde WL, *et al.* *In situ* detection of apoptosis in dental and periodontal tissues of the adult mouse using annexin-V-biotin. *Histochem Cell Biol* 2000;113:293-301.
25. Lee SH, Meng XW, Flatten KS, Loegering DA, Kaufmann SH. Phosphatidylserine exposure during apoptosis reflects bidirectional trafficking between plasma membrane and cytoplasm. *Cell Death Differ* 2013;20:64-76.
26. Ribeiro GF, Côte-Real M, Johansson B. Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock. *Mol Biol Cell* 2006;17:4584-91.
27. Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. *Oncogene* 2004;23:2797-808.
28. Halicka DH, Pozarowski P, Ita M, Ardelt WJ, Mikulski SM, Shogen K, *et al.* Enhancement of activation-induced apoptosis of lymphocytes by the cytotoxic ribonuclease onconase (Ranpirinase). *Int J Oncol* 2002;21:1245-50.
29. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 1997;27:1-20.
30. Wilfling F, Weber A, Potthoff S, Vögtle FN, Meisinger C, Paschen SA, *et al.* BH3-only proteins are tail-anchored in the outer mitochondrial membrane and can initiate the activation of Bax. *Cell Death Differ* 2012;19:1328-36.
31. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 2000;10:369-77.
32. Lopez J, Tait SW. Mitochondrial apoptosis: Killing cancer using the enemy within. *Br J Cancer* 2015;112:957-62.
33. Smaili SS, Hsu YT, Sanders KM, Russell JT, Youle RJ. Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential. *Cell Death Differ* 2001;8:909-20.
34. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002;4:E131-6.
35. Higashimoto Y, Saito S, Tong XH, Hong A, Sakaguchi K, Appella E, *et al.* Human p53 is phosphorylated on serines 6 and 9 in response to DNA damage-inducing agents. *J Biol Chem* 2000;275:23199-203.
36. Oleinik NV, Krupenko NI, Krupenko SA. Cooperation between JNK1 and JNK2 in activation of p53 apoptotic pathway. *Oncogene* 2007;26:7222-30.
37. Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E, *et al.* ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. *J Biol Chem* 2002;277:12491-4.
38. Oberle C, Blattner C. Regulation of the DNA damage response to DSBs by post-translational modifications. *Curr Genomics* 2010;11:184-98.