RESEARCH ARTICLE

Editorial Process: Submission:09/04/2017 Acceptance:12/03/2017

Thai Water Lily Extract Induces B16 Melanoma Cell Apoptosis and Inhibits Cellular Invasion Through the Role of Cellular Oxidants

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Abstract

Melanoma is a cancer that is associated with a high capacity of invasion. Oxidative stress is recognized as cancer growth and progression. The phytochemical pigments of natural products show either anti-oxidant or pro-oxidant activity from the redox system. In addition, the phytophenolics also prevent cancer cell proliferation and progression. Objective: This study aims to investigate the effects of Thai water lily on cell apoptosis and cellular invasion through the role of cellular oxidants in B16 melanoma cells. Methods: The cytotoxicity and cell apoptosis of Thai water lily extract treating B16 cells were performed by using the MTT and Annexin V/PI-flow cytometry methods, respectively. In addition, cellular oxidants and cancer cell invasion were also obtained by using DCFH-DA and Boyden chamber assays, respectively. Results: Thai water lily, *Nymphaea stellate* extract was shown to be markedly toxic to B16 melanoma cells with IC50 = 814 µg/ml. The extract at 800 and 1,000 µg/ml demonstrated pro-oxidant activity relating to the cell apoptosis. The low concentrations of the extract at 200 and 400 µg/ml showed the anti-oxidant function associated with the inhibitory effect of melanoma cell invasion. Conclusion: Thai water lily extract may play an important role in bioactive work as a chemo preventive agent on the modulation of cellular oxidative stress-induced apoptosis and suppressed cancer cell invasion.

Keywords: Thai water lily- melanoma cells- apoptosis- invasion- oxidative stress

Asian Pac J Cancer Prev, **19 (1)**, 149-153

Introduction

Melanoma is the second most common invasive cancer, and it has shown an increasing incidence in young adults (Reed et al., 2012; Venza et al., 2015). Melanoma shows strong aggressive cancer progression including invasion and metastasis (Fane et al., 2017). Oxidative stress is involved in cancer development, and it also promotes cancer migration, invasion and metastasis (Reuter et al., 2010). Phytochemicals from natural sources play important roles in anti-oxidants, anti-cancer cell proliferation and induced cancer cell apoptosis (Sun and Hai Liu, 2006; Wang et al., 2017b). The red and purple pigments from natural products are recognized as phytophenolics. These phytochemicals play a crucial role as an anti-carcinogenic property. (Hou, 2003; Diaconeasa et al., 2015). *Nymphaea spp*., containing the red and purple pigments, has also been accepted to be a main source of phenolic compounds (King and Young, 1999; Pal et al., 2016). The phytophenolic pigments play an important role in suppressing oxidative damage and inducing cancer cell apoptosis (Khan and Sultana, 2005; Hu et al., 2016). Thus, this study aims to investigate the effects of Thai water lily, *Nymphaea stellate*, extract which contains the purple pigment on B16 melanoma cell apoptosis and invasion through the role of cellular oxidative stress. This knowledge may be applied as a pharmaceutical product for melanoma treatment.

Materials and Methods

Nymphaea stellate extraction

Nymphaea stellate was provided from the Ladda farm Ayutthaya province, Thailand. *Nymphaea stellate*, 100 g was blended and mixed with 200 ml of 95% ethanol analytical grade. The soluble part was dried in a rotary vacuum evaporator (Buchi rotavapor R-200, Switzerland) and freeze dried (Supermodulyo-230, Singapore), respectively. The extract sample was dissolved with 40% ethanol analytical grade and filtered with 0.45 µm polytetrafluroethylene (PTFE) filter nylon before using in the experiments.

B16 mouse melanoma cells (B16 cells)

B16 melanoma cells (RIKEN Cell Bank, Tsukuba,

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Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) that were supplemented with 10% heat in activated fetal bovine serum, penicillin (100 µg/ml), streptomycin (100 mg/ml), and 3.7 mg/ml of $NaHCO_3$ at 37 °C and 5% of CO_2.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT assay was obtained for cellular toxicity of the sample (Stratigos and Katsambas, 2004). B16 melanoma cells were seeded in the 96-well plates and maintained for 24 h. Then, various concentrations of the sample were added on the seeded cells. After 24 h, the media were removed and 100 µl of the media containing 10 µl of 12 mM MTT stock solution were added to each well. After 2 h, the media were removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well to solubilise the formazan. An automatic microplate reader (1420 Victor 2, Wallac, USA) at a wavelength of 530 nm was used to measure the soluble formazan crystals. The DMSO was used as a blank. The result was expressed as the percentage of the viable cells (% cell viability).

Cellular oxidants

Intracellular oxidants were identified by fluorescent 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Panich et al., 2012). The B16 melanoma cells were seeded in 96 well plates and incubated at 37 ºC, 5% of CO_2 for 24 h. The cells were treated with various doses of sample for 24 h and then the treated sample was removed. DCFH-DA was added and incubated at 37 ºC, 5% of CO_2 for 1 h. The DCF fluorescence intensity was immediately assessed for cellular oxidants at excitation/emission wavelengths of 485/535 nm by using a fluorescence microplate reader (1420 Victor 2, Wallac, USA). N-acetyl cysteine (NAC) was used as positive anti-oxidants, and untreated cells were obtained at 100% of the control. The result was expressed as the percentage of the cellular oxidants (% cellular oxidants).

Apoptotic cell population

The apoptotic cell population was obtained by using flow cytometry (Komina et al., 2016). The B16 melanoma cells were cultured in 6-well plates at 37˚C, 5% of CO_2. Then, the cells with various doses of the sample were treated and incubated for 24 h. The cells were detached by 0.25% of trypsin in Hank's balanced salt solution (HBSS) (Gibco1, Paisley, UK). Then the cells were stained with an AnnexinV/Propidium Iodine (PI) apoptosis detection kit (Sigma, Missouri, USA) and incubated at 37 ºC for 15 min in a dark condition. The cells were washed with cold PBS and then the apoptotic populations by flow cytometer (FAC Scan, Becton dickinson) were measured. The results were expressed by the percentage of cell apoptosis (% cell apoptosis) compared with the untreated cells.

Boyden chamber assay

The ability of the malignant cell invasion was obtained by a Boyden chamber assay (Kramer et al., 2013). Twenty-four-well cell culture plates were inserted in the chamber and coated with 8.0 µm membrane pore site of extracellular matrix (ECM) gel (Sigma-Aldrich, USA). The B16 melanoma cells were seeded with serum free DMEM to the upper chamber. After 24 h of incubation, various doses of the sample with serum free DMEM to the upper site, which contained confluent monolayer cells, were added. The lower part was filled with DMEM with 30% of fetal bovine serum then incubated for 5 days. For the incubation period, the culture media in the lower part were removed two times every 48 h. The invaded cells were analyzed by the stained cells with methylene blue in the lower surface that measured the invasive cells obtained under a microscope. The result would be expressed as the number of invasion cells in 10 high-power fields (HPF, 40x magnification) compared with the untreated cells.

Statistical analysis

All results were presented as mean±standard deviation (mean±SD). The one-way analysis of variance (ANOVA) was used to compare the significant differences between the treated and untreated cells. Statistical significance was considered at $p \leq 0.05$ with SPSS version 16-computer software.

Results

The B16 melanoma cells were treated with Thai water lily, *Nymphaea stellate* extract at concentrations of 200, 400, 600, 800 and 1,000 µg/ml. The results demonstrated

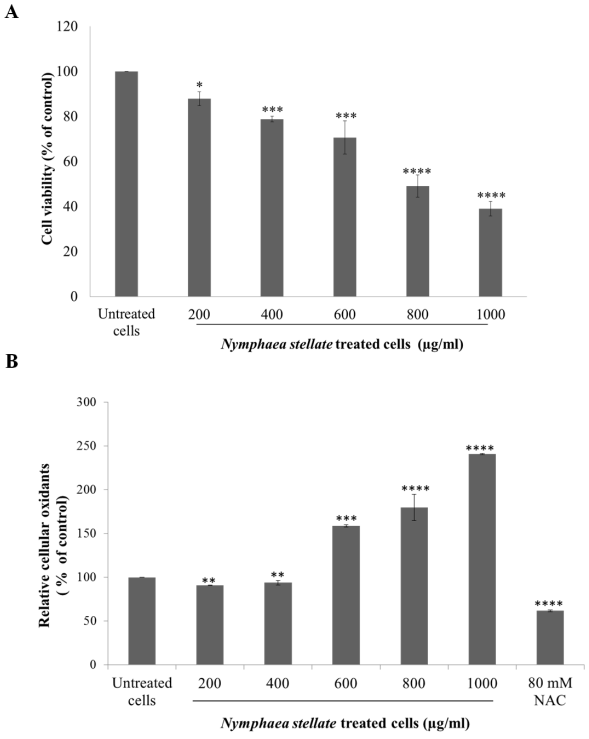

Figure 1. The Effects of the *Nymphaea Stellate* Extract at Various Concentrations on B16 Cell Vability by the MTT Method (A) and cellular oxidants by the DCFH-DA assay. N-acetyl-L-cysteine (NAC) was used as positive control (B). The results are expressed as a mean±SD (n=3). The ANOVA test was used for statistical analysis. *, **, *** and **** represented the statistical analysis between the treated cells and untreated cells at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.005$ and $p \leq 0.001$, respectively.

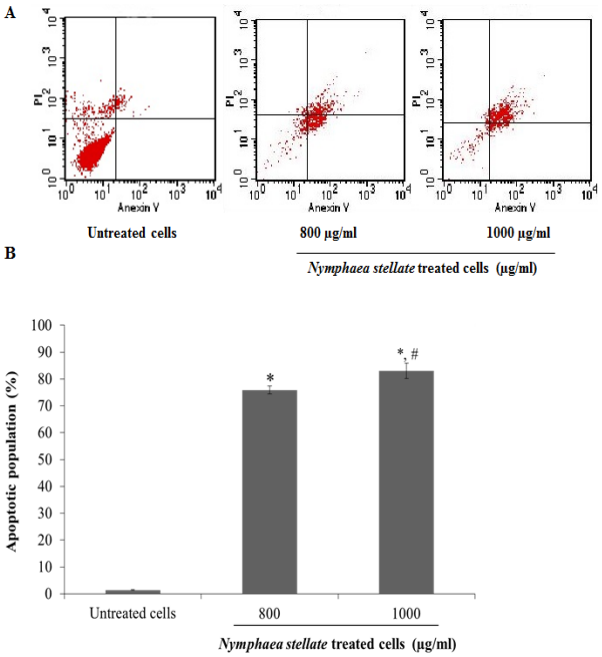

Figure 2. The Effects of the *Nymphaea Stellate* Extract on Cellular Apoptosis of the B16 Cells in 24 h. The flow cytometry analysis showed four quadrants as lower-left, upper left, lower right, and upper right that was represented with viable cells, necrotic cells, early, and late apoptotic cells, respectively (A). The percentage of the apoptotic population was calculated from the summation of the early and late apoptotic cells with a mean±SD (n=3) (B). * represented the statistical analysis between the treated cells and untreated cells at p≤0.05. # represented the 1,000 µg/ml treated cells compared with 800 µg/ml at p≤0.001.

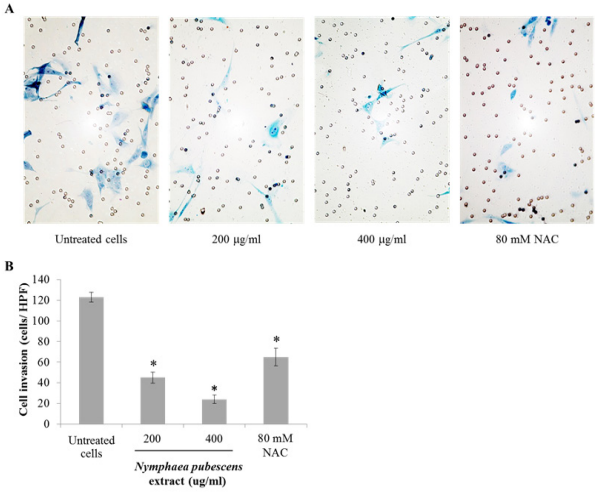

Figure 3 The Effects of the *Nymphaea Stellate* Extract on Melanoma Cell Invasion. The B16 melanoma cells penetrated the ECM gel under a light microscope at 40X magnification (A). The invasion of the melanoma cells was counted for 10 high-power fields (HPF) and expressed as a mean±SD (B). N-acetyl-L-cysteine (NAC), 80 mM was used as an anti-oxidant positive control. * represented the statistical analysis between the treated and untreated cells at p≤0.001.

the decreasing percentage of cell viability associated with the dose dependent manner by the MTT assay. The 50% inhibition (IC_{50}) of *Nymphaea stellate* extract was 814 µg/ml (Figure 1A). In addition, the treated cells were also obtained for cellular oxidants with the same concentration. At a low concentration, the 200 and 400 µg/ml treated cancer cells showed a tendency to slightly decrease the cellular oxidants at 91±0.130% (p=0.01) and 94±2.564% (p=0.01), respectively when compared with 100% untreated cells. Contrastingly, at high concentrations 600, 800 and 1,000 µg/ml treated cancer cells showed increasing cellular oxidants with a dose dependent manner at 159±1.291% (p=0.005), 180±14.842% (p=0.001) and 241±0.784% (p=0.001), respectively when compared with untreated cells (100% of the control) (Figure 1B). Cytotoxicity doses, which were related to high toxic doses of 800 and 1,000 µg/ml, were further used to study the cellular apoptosis. The results showed a high summation of early and late apoptotic cells at 75.92±1.478% (p≤0.001) and 83.02±2.772% (p≤0.001), respectively when compared with the untreated cells (1.41±0.226%) (Figure 2A and 2B). Low concentrations were used to study the cellular invasion. Treated doses at 200 and 400 µg/ml showed the dose dependent capacity to suppress the B16 melanoma cell invasion by the Boyden chamber assay (Figure 3A). The results demonstrated invasive cancer cells at 15±4.583 (p=0.001) and 6±1.155 (p=0.001) cells/HPF of 200 and 400 µg/ml treated doses, respectively when compared with the untreated cells (47±3.215 cells/HPF) (Figure 3B). The results suggested that *Nymphaea stellate* extract at low concentrations might decrease the cellular invasion through an anti-oxidant function. Contrastingly, high concentrations of treated cells showed induced cell apoptosis that was strongly associated with pro-oxidants. Therefore, it was possible to use the extract of *Nymphaea stellate* as a pharmaceutical product for the treatment of melanoma.

Discussion

An overproduction of the reactive oxygen species (ROS) is known as being involved in melanoma cell progression (Fane et al., 2017). Phytochemicals, especially phenolic compounds, can induce cancer cell death and suppress cancer cell progression due to the ability of the redox function (Lo et al., 2010; Cao et al., 2014). *Nymphaea stellate* extract at various concentrations showed cytotoxicity with a dose dependent manner. This result was in agreement with previous studies; Nymphaea spp. contained the main source of phenolic compounds, which acted as a chemo preventive against experimental carcinogenesis (Rani et al., 2012; Paudel and Panth, 2015). The intensity of the purple pigments in *Nymphaea stellate* showed high phytophenolics relating to anthocyanin, which is reported as anti-cancer cell proliferation and progression (Wang and Stoner, 2008). The increasing of ROS could contribute to melanoma cell progression including invasion (Campos et al., 2007; Wang et al., 2017a). In this study, the treatment of the *Nymphaea stellate* extract at low treated doses could suppress cellular oxidative stress and cancer cell migration. These results may be due to the function of the phenolic compounds that had been reported in the inhibition capacity of cancer

cell invasion by the up regulation of tumor suppressor genes and down regulation of the cell cycle (Turrini et al., 2015; Suwannalert et al., 2016). Moreover, the phenolics also altered the melanoma cell redox to suppress the melanoma cell progression (Dzialo et al., 2016; Wang et al., 2017a). Contrastingly, the high treated doses could promote pro-oxidant activity that strongly induced B16 melanoma cell apoptosis. As a redox stage, the excessive ROS production was able to stimulate the transcription factor of APE/Ref1 leading to induced cell apoptosis. Phytochemicals also induced Bax and suppressed Bcl-2 on both melanocyte and melanoma cells (Iwashita et al., 2000; Galati and O'Brien, 2004). Pro-oxidant activity had a pharmacological activity to promote melanoma cell apoptosis through the role of cellular oxidants relating to hyper oxidation in endoplasmic reticulum (ER) (Jiang et al., 2009; Habtemariam and Dagne, 2010).

In conclusion, Thai water lily, *Nymphaea stellate* extract could induce cell apoptosis and suppress cellular invasion by both pro-oxidants and anti-oxidants due to the doses treatments. Thus, *Nymphaea stellate* extract can be used and developed as a pharmaceutical product for the treatment of melanoma.

Statement conflict of interest

No conflict of interest.

Acknowledgements

This study was supported by grants from the Science Achievement Scholarship of Thailand (SAST) and Faculty of Science, Mahidol University. The researchers are grateful to the Department of Pathobiology, Faculty of Science, Mahidol University for providing the laboratory space and facilities. The researchers also appreciate Mrs. Maliwan Emyeam from the Department of Pathobiology, Faculty of Science, Mahidol University for assisting in the B16 melanoma cell maintenance.

References

Campos AC, Molognoni F, Melo FH, et al (2007). Oxidative stress modulates DNA methylation during melanocyte anchorage blockade associated with malignant transformation. *Neoplasia*, **9**, 1111-21.

Cao HH, Tse AK, Kwan HY, et al (2014). Quercetin exerts anti-melanoma activities and inhibits STAT3 signaling. *Biochem Pharmacol*, **87**, 424-34.

Diaconeasa Z, Leopold L, Rugina D, et al (2015). Antiproliferative and antioxidant properties of anthocyanin rich extracts from blueberry and blackcurrant juice. *Int J Mol Sci*, **16**, 2352-65.

Dzialo M, Mierziak J, Korzun U, et al (2016). The potential of plant phenolics in prevention and therapy of skin disorders. *Int J Mol Sci*, **17**, 160.

Fane ME, Chhabra Y, Hollingsworth DE, et al (2017). NFIB mediates BRN2 driven melanoma cell migration and invasion through regulation of EZH2 and MITF. *E Bio Medicine*, **16,** 63-75.

Galati G, O'Brien PJ (2004). Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radic Biol Med*, **37**, 287-303.

Habtemariam S, Dagne E (2010). Comparative antioxidant, prooxidant and cytotoxic activity of sigmoidin A and eriodictyol. *Planta Med*, **76**, 589-94.

Hou DX (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr Mol Med*, **3**, 149-59.

Hu X, Chen L, Shi S, et al (2016). Antioxidant capacity and phenolic compounds of Lonicerae macranthoides by HPLC-DAD-QTOF-MS/MS. *J Pharm Biomed Anal*, **124**, 254-60.

Iwashita K, Kobori M, Yamaki K, et al (2000). Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. *Biosci Biotechnol Biochem*, **64**, 1813-20.

Jiang CC, Wroblewski D, Yang F, et al (2009). Human melanoma cells under endoplasmic reticulum stress are more susceptible to apoptosis induced by the BH3 mimetic obatoclax. *Neoplasia*, **11**, 945-55.

Khan N, Sultana S (2005). Anticarcinogenic effect of Nymphaea alba against oxidative damage, hyperproliferative response and renal carcinogenesis in Wistar rats. *Mol Cell Biochem*, **271**, 1-11.

King A, Young G (1999). Characteristics and occurrence of phenolic phytochemicals. *J Am Diet Assoc*, **99**, 213-8.

Komina A, Palkina N, Aksenenko M, et al (2016). Antiproliferative and Pro-Apoptotic Effects of MiR-4286 Inhibition in Melanoma Cells. *PLoS One*, **11**, e0168229.

Kramer N, Walzl A, Unger C, et al (2013). In vitro cell migration and invasion assays. *Mutat Res*, **752**, 10-24.

Lo C, Lai TY, Yang JH, et al (2010). Gallic acid induces apoptosis in A375.S2 human melanoma cells through caspase-dependent and -independent pathways. *Int J Oncol*, **37**, 377-85.

Pal HC, Hunt KM, Diamond A, et al (2016). Phytochemicals for the management of melanoma. *Mini Rev Med Chem*, **16**, 953-79.

Panich U, Onkoksoong T, Limsaengurai S, et al (2012). UVA-induced melanogenesis and modulation of glutathione redox system in different melanoma cell lines: the protective effect of gallic acid. *J Photochem Photobiol B*, **108**, 16-22.

Paudel KR, Panth N (2015). Phytochemical profile and biological activity of nelumbo nucifera. *Evid Based Complement Alternat Med*, **2015**, 789124.

Rani DD, Kumar SA, Shuaib M, et al (2012). Nymphaea stellata: A potential herb and its medicinal important. *JDDT*, **2**, 41-4.

Reed KB, Brewer JD, Lohse CM, et al (2012). Increasing incidence of melanoma among young adults: an epidemiological study in Olmsted county, Minnesota. *Mayo Clin Proc*, **87**, 328-34.

Reuter S, Gupta SC, Chaturvedi MM, et al (2010). Oxidative stress, inflammation, and cancer: how are they linked?. *Free Radic Biol Med*, **49**, 1603-16.

Stratigos AJ, Katsambas AD (2004). Optimal management of recalcitrant disorders of hyperpigmentation in dark-skinned patients. *Am J Clin Dermatol*, **5**, 161-8.

Sun J, Hai Liu R (2006). Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Lett*, **241**, 124-34.

Suwannalert P, Payuhakrit W, Koomsang T (2016). Anti-oxidant, pro-oxidant and anti-inflammatory effects of unpolished rice relevant to colorectal cancer. *Asian Pac J Cancer Prev*, **17**, 5047-56.

Turrini E, Ferruzzi L, Fimognari C (2015). Potential effects of pomegranate polyphenols in cancer prevention and therapy. *Oxid Med Cell Longev*, **2015**, 938475.

Venza M, Visalli M, Beninati C, et al (2015). Cellular mechanisms of oxidative stress and action in melanoma. *Oxid Med Cell Longev*, **2015**, 481782.

Wang JJ, Wu CC, Lee CL, et al (2017a). Antimelanogenic, antioxidant and antiproliferative effects of antrodia

camphorata fruiting bodies on B16-F0 melanoma cells. *PLoS One*, **12**, 1-15.
Wang LS, Stoner GD (2008). Anthocyanins and their role in cancer prevention. *Cancer Lett*, **269**, 281-90.
Wang S, Shen P, Zhou J, et al (2017b). Diet phytochemicals and cutaneous carcinoma chemoprevention: A review. *Pharmacol Res*, **119**, 327-46.