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

RESEARCH ARTICLE

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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 phytochemical 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 polytetrafluoroethylene (PTFE) filter nylon before using in the experiments.

B16 mouse melanoma cells (B16 cells)

B16 melanoma cells (RIKEN Cell Bank, Tsukuba, Japan)
Japan) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) that were supplemented with 10% heat inactivated 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 °C, 5% of CO$_2$ for 24 h. The cells were treated with various doses of the sample for 24 h and then the treated sample was removed. DCFH-DA was added and incubated at 37 °C, 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 Annexin V/Propidium Iodine (PI) apoptosis detection kit (Sigma, Missouri, USA) and incubated at 37 °C 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≤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
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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 (Turrimi et al., 2015; Suwannalert et al., 2016). Moreover, the phenolics also altered the melanoma cell redox to suppress the melanoma cell progression (Dziale 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 of interest
No conflict of interest.

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