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Research Article

Nymphaea pubescens Induces Apoptosis, Suppresses Cellular Oxidants-Related Cell Invasion in B16 Melanoma Cells

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ABSTRACT

Background: *Nymphaea spp.*, Thai water lilies are aquatic plants. They contain phenolic pigments that play a major role in free radical scavenging. Melanoma is strong aggressive skin cancer-associated with oxidative stress. This study, to determine the effect of *Nymphaea spp.* extracts on cell apoptosis, cellular migration and invasion through the role of cellular oxidants in B16 melanoma cells.

Methods: Free radical scavenging activity and total phenolic were investigated by 1, 1-diphenyl-2 picrylhydrazyl (DPPH) and Ferric reducing anti-oxidant power (FRAP) methods and Folin-Denis test, respectively. Cytotoxic were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cell apoptosis was confirmed by flow cytometry. Cellular oxidants, cellular migration and invasion were determined with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), wound healing and Boyden chamber assay, respectively.

Results: *Nymphaea pubescens* showed higher capacity of scavenging free radical activity than *Nymphaea stellate* and also related phenolic content. *Nymphaea pubescens* extract was toxic to B16 melanoma cells. High concentrations cell apoptosis was induced. Contrastingly, low concentrations showed a decrease in cellular oxidants associated with the suppression of cancer cell progression. In B16 melanoma cell, *Nymphaea pubescens* extract was able to inhibit B16 melanoma cell migration and invasion through the low doses. Interestingly, the high doses of extract showed a potential of cytotoxicity to induce melanoma cell death. At the low doses, *Nymphaea pubescens* extract might suppress melanoma cells progression by interfering with both cellular migration and invasion capacity.

Conclusion: Hence, *Nymphaea pubescens* extract induced cellular apoptosis and it also suppressed cancer cell progression by reducing oxidative stress in B16 melanoma cells.

Introduction

Melanoma is the second most common invasive cancer.[1] It shows the evidence of strongly aggressive progression including invasion and metastasis that is associated with melanoma-related death.[2] Previous studies claimed that oxidative stress is not only involved in cancer initiation and promotion but also promotes cancer progression by inducing cancer angiogenesis, cellular migration, invasion and metastasis.[3] The anti-oxidant function from chemicals and natural products is generally used to control cancer cell progression.[4,5] Phytochemicals play an important role in anti-oxidant activity. They also inhibit cancer development and progression due to the function of tumor cell proliferation inhibition and cancer cell apoptosis induction.[6,7] Most of the colored pigments of natural products contain chemicals like anthocyanins and carotenoids. These phytochemicals play a crucial role through their anti-carcinogenic properties.[8,9] Anthocyanins, the natural pigments in the category of flavonoids give the intense colors of red, purple and blue.[10] They provide anti-proliferative potential in B16 melanoma cells.[11] Carotenoids, non-nitrogenous yellow, orange, or red pigments also show an association with the reduction of melanoma risk in humans.[12] *Nymphaea spp.*, commonly known as the water lily, contains various intense pigments, and is also accepted to be a major source of phenolic compounds.[13,14] The previous study has claimed that the effect of *Nymphaea stellate* which shown intense of purple color can suppress B16 melanoma cell growth and invasion through the role of cellular oxidant.[15] Moreover, the red-colored pigments of anthocyanins show a high stability and highly soluble in water.[16] Thus, this study aims to investigate the effects of *Nymphaea pubescens* extract, red-colored phytopigment on B16 melanoma cell apoptosis, cellular migration and invasion through the role of cellular oxidative stress. This information may be applied as a pharmaceutical product for melanoma treatment.

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Materials and Methods

Nymphaea spp. extraction

Nymphaea pubescens and *Nymphaea stellate* that presented with red and purple colors, respectively were obtained from Ladda Garden, Ayutthaya province, Thailand. The petal samples were harvested in January to February 2016. One hundred grams of samples were blended and mixed with 200 ml of 95% analytical grade ethanol for 3 h and the soluble part dried in a rotary vacuum evaporator (Buchi rotavapor R-200, Switzerland) and freeze dried (Supermodulyo-230, Singapore). The extract samples were dissolved in 40% ethanol analytical grade and filtered with 0.45 µm polytetrafluroethylene (PTFE) filter nylon before use in the experiment.

1, 1-diphenyl-2 picrylhydrazyl (DPPH) radical scavenging test

The total anti-oxidant property was investigated by DPPH with a modified method of Tammasakchai A., et al.[17] The working solution of DPPH was freshly prepared by dissolving and diluting with 95% analytical grade ethanol to obtain an absorbance of 0.85±0.05 units at 530 nm. The sample, 0.2 ml was mixed with 1.8 ml of DPPH solution. The mixture was immediately measured at 530 nm against a blank by a spectrophotometer (UV-2650, Labomed, USA). The radical scavenging activity of each sample was measured as a decrease in the absorbance of DPPH solution. Vitamin C was used as the standard. Vitamin C concentration required for 50% reduction (RC_{50}) against DPPH radicals was calculated. The total anti-oxidant activity in the DPPH test was expressed as vitamin C equivalent/g sample. The assay was carried out in triplicate.

Ferric reducing anti-oxidant power (FRAP) assay

The evaluation of the total anti-oxidant activity of the sample was performed by using a modified method.[18] The working FRAP reagent was prepared by mixing 25 ml acetate buffer, pH 3.6, 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 2.5 ml ferric chloride hexahydrate ($FeCl_3.6H_2O$). In the dark condition, 20 µl of the sample at various concentrations was allowed to react with 180 µl of FRAP reagent in 96 well plates for 15 min. The blue colored product (ferrous tripyridyltriazine complex) was measured at 595 nm by using an automated microplate reader (1420 Victor 2, Wallac, USA). Iron (III) sulfate heptahydrate ($FeSO_4.7H_2O$) was used as a standard. FRAP values for all samples were achieved by standard calibration curve obtained by using different concentrations of $FeSO_4.7H_2O$. Results were expressed as M $FeSO_4$/g sample. The assay was carried out in triplicate.

Total phenolic content

Total phenolic content was determined by using a modified method.[19] The sample, 20 µl was mixed with 1580 µl of distilled water and then mixed with 100 µl of Folin–Denis reagent. The mixture was incubated with 300 µl of 7.5% w/v sodium bicarbonate for 30 min at room temperature in the dark condition and its absorbance was measured at 765 nm by using a spectrophotometer (UV-2650, Labomed, USA). Gallic acid was used as a standard. The equivalent phenolic content was expressed as Gallic acid equivalent/g sample. The assay was carried out in triplicate.

B16 mouse melanoma cells (B16 cells)

B16 melanoma cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 3.17 mg/ml of $NaHCO_3$ at 37 °C and 5% CO_2.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay was used to evaluate the cellular toxicity of the sample when treated with B16 melanoma cells.[20] Cells were seeded at a density of 1.5×10^4 cells in the 96-well plates and maintained for 24 h. Old media were removed and 200 µl conditioned media were added to the sample at various concentrations. After 24 h of incubation, the media were removed and the cells were washed with phosphate buffer saline (PBS), pH 7.4. Then 100 µl of media containing 10 µl of 12 mM MTT stock solution were added to each well. After 2 h of incubation, media containing MTT were removed and 100 µl dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan. The soluble formazan crystals were obtained by using an automatic microplate reader (1420 Victor 2, Wallac, USA) at a wavelength of 530 nm. DMSO was used as a blank. The result was expressed as the percentage of viable cells (% cell viability). To determine IC_{50} values, data were presented in EXCEL graphs with the dose value (µg/mL) on the X-axis and the % viability compared to control on the Y-axis. The IC_{50} values are determined from the trend line of the data points. The cytotoxicity index was calculated as following formula:

Cytotoxicity Index= 100- [(Sample's OD value / mean OD value controls) ×100] Eq. (1)

while the cytotoxicity index would be 0 if the sample OD value/controls mean OD value was larger than 0.7, suggesting no cytotoxic influence.

Cellular oxidants

Intracellular oxidants were detected with fluorescent 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) in a modified method.[21] DCFH-DA is cleaved in intracellular cells by nonspecific esterase and turns to highly fluorescent 2,7-dichlorofluorescein (DCF) upon oxidation by free radicals. 7.5×10^4 cells/ml of B16 cells were seeded in 96 well plates and incubated at 37 ºC, 5% CO_2 for 24 h. Cells were treated with various doses of the sample for 1 h. Cultured cells were removed from the conditioned medium. 10 µM of DCFH-DA medium was added and incubated at 37 ºC, in 5% CO_2 for 1 h. 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). 5 mM of N-acetyl cysteine (NAC) was used as positive anti-oxidants with 100% control for untreated cells. The result was expressed as the percentage of cellular oxidants (% cellular oxidants).

Apoptotic cell population

The apoptotic cell population was determined by flow cytometry using a modified method.[22] B16 melanoma cells were cultured in 6-well plates at 37˚C, 5% CO_2. Cells were treated with various doses of the sample and incubated for 12 and 24 h. Then, cells were detached by 0.25% trypsin in Hank's balanced salt solution (HBSS) (Gibco1, Paisley, UK) and washed twice with cold PBS, pH 7.4. The cells were suspended in 100 µl of 1X binding buffer from the AnnexinV/Propidium Iodine (PI) apoptosis detection kit (Sigma, Missouri, USA). Subsequently, 4 µl of 1:1 AnnexinV:PI was added and incubated for 15 min in the dark condition. After incubation, the cells were washed with cold PBS, pH 7.4 and fixed with paraformaldehyde. Apoptotic cells were measured by flow cytometer (FAC Scan, Becton dickinson). The result was expressed as the percentage of cell apoptosis (% cell apoptosis) compared with untreated cells. The experiment was performed in triplicate.

Wound healing assay

Cell migration was performed by using wound healing assay with a modified method.[23] B16 melanoma cells, 2×10^5cells/well were seeded in 6 well plates and incubated at 37°C, 5% CO_2 until 100% confluent. The confluent monolayer was wounded by scratching lines with 10 µl of pipette tip and washed with PBS. The cells were treated with the various doses of the sample (200, 400 µg/ml) and incubated at 37°C and 5% CO_2. The migration area was analyzed by using the ImageJ program. The results were obtained in 0, 24 and 48 h under the microscope by comparing the measured area containing migrated cells with the measured area of the wounded region lacking cells. In order to assure the result, the experiment was performed in triplicate under the same conditions. The result was expressed as the percentage of cell migration (% cell migration) compared with untreated cells.

Cell invasion assay

Cell invasion assay or the Boyden chamber assay is used to study the ability of malignant cells to invade normal surrounding tissue by using a modified method.[24] In this experiment, 24-well cell culture plates that are inserted in the chamber were coated with 8.0 µm membrane pore site of the extracellular matrix (ECM) gel (Sigma-Aldrich, USA). B16 melanoma cells, 1.0×10^5 were seeded with serum free DMEM to the upper chamber and incubated for 24 h at 37°C and 5% CO_2. After 24 h, various doses of the sample with serum free DMEM were added to the upper site which contained confluent monolayer B16 melanoma cells. DMEM with 30% fetal bovine serum was added in the lower part. During the incubation period, culture media in the lower part were removed two times every 72 h. The invaded cells were analyzed by removing non-migrated cells from the upper surface and washing with PBS. At the lower surface, the invaded cells were stained with methylene blue and obtained under a microscope. The result was expressed as the number of invaded cells in 10 high power fields (HPF, 400x magnification) compared with untreated cells.

Statistical analysis

All results were presented as mean±standard deviation (mean±SD). The analysis of variance (ANOVA) was used to compare the significances between the treated- and untreated-cells. Data correlation was obtained by Pearson correlation. Statistical significance was considered at $p\leq0.05$ with SPSS version 16 computer software.

Results

Antioxidant activity and phenolic content of Nymphaea spp.

Total radical scavenging activities by DPPH radical scavenging test equivalence of *Nymphaea pubescens* demonstrated higher activity than that of *Nymphaea stellate*. In addition, *Nymphaea pubescens* also showed higher phenolic content than that of *Nymphaea stellate* (Table 1). The total phenolic contents were strongly correlated with total anti-oxidant activities by r=0.996 and r=0.982 at $p<0.001$, respectively in the DPPH method and r=0.996 ($p<0.001$) and r=0.953 ($p<0.001$), respectively in the FRAP assay. So, anti-oxidants of *Nymphaea spp*. might be drawn mainly from phenolic compounds.

Cytotoxic effects of Nymphaea spp. extract on B16 melanoma cells growth

The treatment of B16 melanoma cells with both strains of *Nymphaea spp.* at various concentrations (200, 400, 600, 800 and 1000 µg/ml) showed strong cytotoxicity in a dose-dependent manner with MTT assay (Figure 1).

Table 1. Anti-Oxidant activities and total phenolic content of *Nymphaea spp.*

	Anti-oxidant activities			Total phenolic content
Nymphaea spp.	**DPPH**		**FRAP**	**Folin-Denis**
	RC_{50}(µg/ml)	G Vit C/g sample	M $FeSO_4.7H_2O$/g sample	G Galic acid/g sample
Nymphaea pubescens	96.811	0.331±0.068	3.640±0.112	0.164±0.028
Nymphaea stellata	109.833	0.292±0.108	3.367±0.133	0.159±0.033
Vitamin C [(a)]	32.049	1	-	-
$FeSO_4.7H_2O$ [(b)]	-	-	1	-
Gallic acid [(c)]	-	-	-	1

a) Standard compound of DPPH assay
b) Standard compound of FRAP assay
c) Standard compound of Total phenolic content

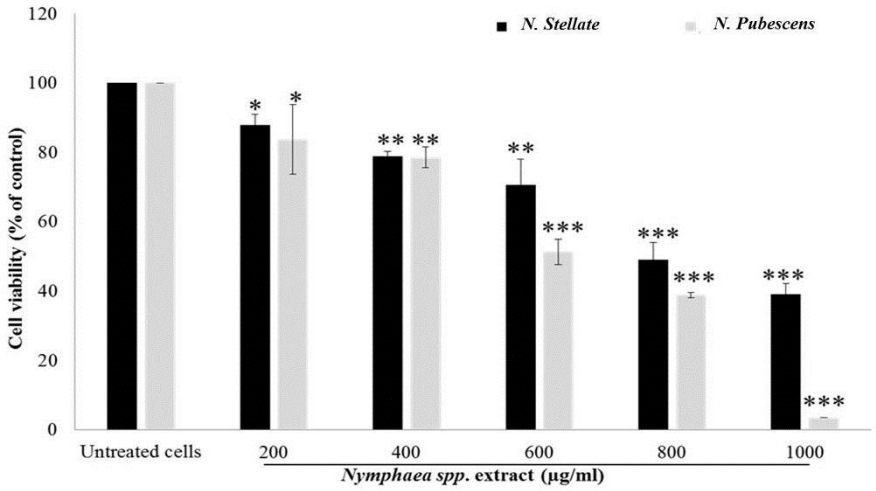

Figure 1. The effects of *Nymphaea spp.* extract at various concentrations on cell viability by the MTT method. Cell viability (% of control) of B16 treated with *Nymphaea stellata* extract (black) and *Nymphaea pubescens* extract (gray). The untreated group was used as 100% of the control. The results were expressed as mean±SD. *, ** and *** statistical significances at $p<0.05$, $p<0.005$ and $p<0.001$ when compared with untreated cells.

The cytotoxicity of *Nymphaea pubescen* on B16 melanoma cells was expressed as IC_{50} at 652 µg/ml. It was shown more effectively than *Nymphaea stellate* (IC_{50}=816 µg/ml). Thus, the high toxicity doses at 800 and 1000 µg/ml of *Nymphaea pubescens* extract which is red color were used in order to study cellular apoptosis by using the AnnexinV/PI assay. After treatment with *Nymphaea pubescens* extract at 800 and 1000 µg/ml which shown cytotoxicity index at 60.29 and 96.38, respectively in B16 melanoma cells for 12 and 24 h, the cells showed cellular apoptosis in a dose-and time-dependent manner. At 12 h, the percentages of apoptotic B16 cells in untreated, 800 and 1000 µg/ml were 0.62±0.049%, 12.41±1.272% and 18.15±2.184%, respectively. The percentages of cellular apoptosis showed high apoptotic populations at 24 h with 30.87±7.538% and 73.24±7.764% for the treated cells at 800 and 1000 µg/ml, respectively when compared with untreated cells (1.39±0.255%) (Figures 2A and 2B). *Nymphaea pubescens* extract induced B16 melanoma cell apoptosis at 12 h and effectively induced apoptosis after treated at 24 h.

Cellular oxidant effects of Nymphaea spp. extract on B16 melanoma cells migration and invasion

Treatment of *Nymphaea pubescens* extract on B16 melanoma cells provided the pattern of cellular oxidants by the DCFH-DA method. The low-toxic doses (200 and 400 µg/ml which shown cytotoxicity index at 12.83 and 13.93, respectively) provided a lower level of cellular oxidants than untreated cells (100%) by 78±2.137% and 70±1.433%, respectively (Figure 3).

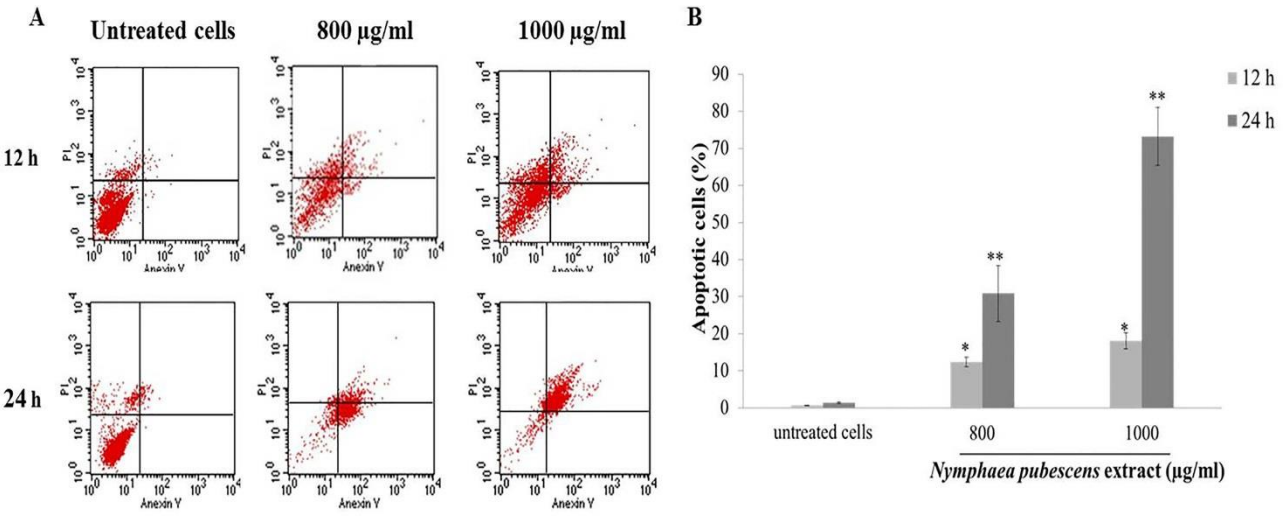

Figure 2. Apoptosis inducing effects of *Nymphaea pubescens* extract on B16 melanoma cells by Annexin V/PI assay. Flow cytometry plots show the apoptotic B16 melanoma cell population, and the dose and time course induction of apoptosis (A). The percentage of apoptotic cells was analyzed and expressed as a bar graph by mean±SD (B). * and ** statistical significances at $p<0.05$ and $p<0.01$, respectively when compared with untreated cells.

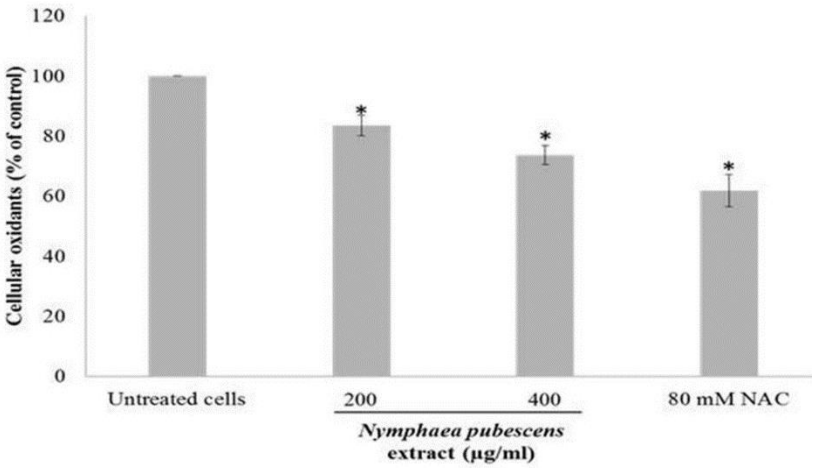

Figure 3. The effects of *Nymphaea pubescens* extract at doses of 200 and 400 µg/ml on cellular oxidants by DCFH-DA assay. N-acetyl-L-cysteine (NAC), 80 mM was used as an anti-oxidant positive control. The results are expressed by mean ± SD. * statistical significance at $p<0.001$ analyzed between treated cells and untreated cells.

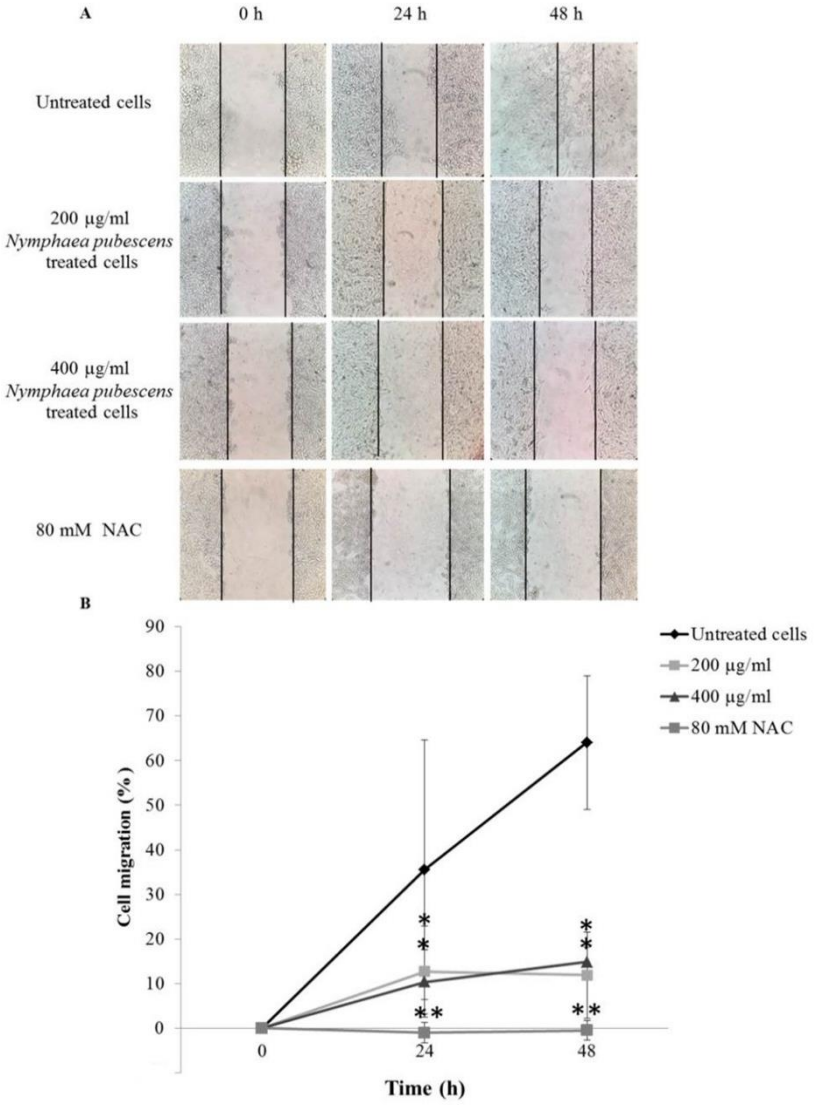

Figure 4. The effects of *Nymphaea pubescens* extract on melanoma cell migration. B16 melanoma cell migration treated with *Nymphaea pubescens* extract at 0, 24 and 48 h was obtained under a light microscope at 400X magnification (A). Eighty mM of NAC was used as an anti-oxidant positive control. The percentage of cell migration was analyzed and expressed as a bar graph by mean±SD (B). * and ** statistical significances at $p<0.05$ and $p<0.01$, respectively when compared with untreated cells.

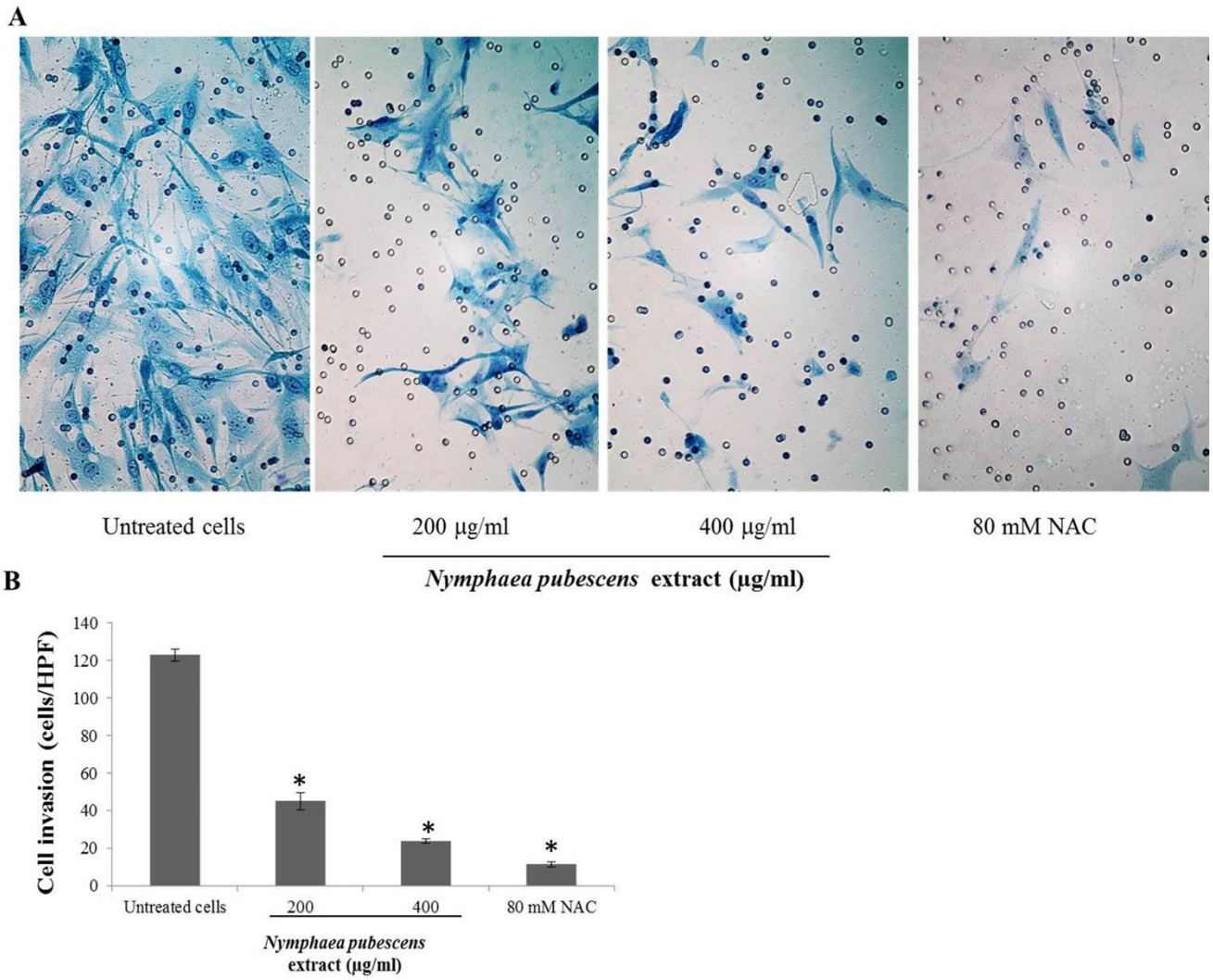

Figure 5. The effects of *Nymphaea pubescens* extract on melanoma cell invasion. B16 melanoma cell invasion treated with *Nymphaea pubescens* extract for 72 h was observed under a light microscope at 400X magnification (A). Eighty mM of NAC was used as an anti-oxidant positive control. The invasion of melanoma cells was counted for 10 high power field (HPF) and expressed as a bar graph by mean±SD (B). * statistical significance at $p<0.001$ when compared with untreated cells.

Treatment of B16 melanoma cells with *Nymphaea pubescens* extract also suppressed cell migration at 24 h by providing the percentages of B16 melanoma cell migration at 13±10.139% and 10±7.162%, at the treated doses of 200 and 400 µg/ml, respectively when compared with untreated cells (36±0.718%). This suppression still persisted to 48 h of treatment by 17±4.487% and 15±3.311% at the doses of 200 and 400 µg/ml, respectively when compared with untreated cells (72±10.557%). These results suggested that *Nymphaea pubescens* extract at the doses of 200 and 400 µg/ml demonstrated the potential to inhibit B16 melanoma cells migration. NAC was used as a positive control (Figures 4A and 4B). In addition, *Nymphaea pubescens* extract treated cells showed the dose dependent capacity to suppress B16 melanoma cell invasion (Figure 5A and 5B) at 45±5.283 ($p<0.001$) and 24±4.125 ($p<0.001$) cells/HPF at the doses of 200 and 400 µg/ml, respectively when compared with untreated cells (123±4.653 cells/HPF). These results demonstrated that *Nymphaea pubescens* extract was able to inhibit B16 melanoma cell migration and invasion. As the results indicate, *Nymphaea pubescens* extract had the potential to induce melanoma cell death at doses of 800 and 1000 µg/ml. At the low-toxic doses of 200 and 400 µg/ml, *Nymphaea pubescens* extract might suppress melanoma cells progression by interfering with both cellular migration and invasion capacity. In addition, at the low-toxic doses it also showed a tendency to decrease cellular oxidants in doses-dependent manner. Therefore, it is possible to use the extract of *Nymphaea pubescens* as a pharmaceutical product for the treatment of melanoma.

Discussion

Previously, the over production of reactive oxygen species (ROS) is known as being involved in a melanoma cell progression.[2] The ability to scavenge ROS related to phenolic structure of phytochemical in cancer cells.[25] In this study, we demonstrated that *Nymphaea pubescens* and *Nymphaea stellate* extract which show in red and

purple respectively can scavenge free radicals in *in vitro* tests by DPPH and FRAP methods. The anti-radical property was also strongly correlated with total phenolic content. Many phytochemicals, especially phenolic acid had an association with cancer cell death.[26,27] Treatment with *Nymphaea spp*. extract at the doses of 200, 400, 600, 800 and 1000 µg/ml also showed cytotoxicity in a dose-dependent manner. This result was in agreement with previous studies which showed that, *Nymphaea spp.* contained a major source of phenolic compounds which act as a chemopreventive against experimental carcinogenesis including in melanoma cells.[28,29] *Nymphaea pubescens*, the intense of red color have demonstrated better than that *Nymphaea stellate* (purple) in scavenging of free radical, total phenolic content and suppress in cell viability related with the previous support of stability in red-colored pigments which is the predominantly in the form of flavylium cations to be highly soluble in water.[30] *Nymphaea pubescens* also induced B16 melanoma cell apoptosis by treatment doses of 800 and 1000 µg/ml. These results were in agreement with previous reports, in which some phenolic compounds had pharmacological activity to suppress cancer cell promotion by induction of melanoma cell apoptosis.[31,32] In addition, the anti-oxidant property of *Nymphaea pubescens* extract at low-toxic doses (200 and 400 µg/ml) showed strong association with suppression in B16 melanoma cell migration and invasion. These results followed those of the previous studies, whereby the increase of ROS in melanoma cells was contributing to melanoma cell transformation and progression.[33,34] The modulation of melanoma cells was maintained by the anti-oxidant activity on the treated melanoma cells with some phenolic compounds.[34,35]

Conclusion

Nymphaea pubescens extract shown strongly effect on cell viability by inducing melanoma cell apoptosis. The anti-oxidant activity shown in low-toxic doses provided suppressive activity on melanoma cell progression. Hence, *Nymphaea pubescens* extract can be used and developed as a pharmaceutical product for the treatment of melanoma.

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Conflict of Interests

The authors claim that there is no conflict of interest.

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