Original Article

Effect of 1,4-Napthoquinone Derivatives on Anti-proliferation and Apoptosis Induction in Skin and Lung Cancer Cells

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Background: Cancer is a major public health concern around the world. Nowadays, standard therapy in combination with targeted therapy are being used for cancer treatment, but these therapeutics show side effects or unwanted symptoms and high cost. 1,4-Napthoquinone and their derivatives were used in cancer research for a long time with high efficiency than some cancer drug. In the present study, the effect of a novel 1,4-napthoquinone, DL667 and DL666 on apoptosis induction in malignant melanoma, A375 cells and lung cancer, A549 cells, respectively were studied.

Objective: To investigate the effects of 1,4-napthoquinone derivatives, DL667 and DL66, on apoptosis induction in malignant melanoma, A375 and lung cancer, A549 cells, respectively.

Materials and Methods: Cell growth inhibition was determined by MTT assay. Apoptosis induction was via chromatin condensation or apoptotic bodies determined by Hoechst staining. In addition, changes in mitochondrial membrane potential ($\Delta\Psi m$) were done by JC-1 staining.

Results: The results showed that DL667 inhibited cell growth in A375 with an IC_{50} at 11.2±0.419 µM in a concentration-dependent manner and DL666 inhibited cell growth of A549 cells at 200 µM. Hoechst staining assay revealed that both DL667 and DL666 could induce chromatin condensation and apoptotic bodies in A375 and A549 cells. In addition, the JC-1 staining showed that DL667 and DL666 decreased the $\Delta\Psi m$, a marker event of early apoptosis via mitochondria, in A375 and A549 treated cells.

Conclusion: DL667 and DL666 showed anti-proliferation and apoptosis induction in A375 and A549 cells, respectively. Moreover, DL667 and DL666 induced chromatin condensation, apoptotic bodies and decreased the $\Delta\Psi m$, resulting in apoptosis cell death induction. Our results suggested that the decrease of $\Delta\Psi m$ may be further developed as a model for study in other cancer cell lines. However, the underlying mechanisms of apoptosis induction associated with signaling should be further studied.

Keywords: 1,4-Napthoquinone; Apoptosis; Chromatin condensation; Apoptotic bodies; Mitochondrial membrane potential

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Nowadays, cancer is a major public health concern in many countries, including Thailand. In 2020, the GLOBOCAN estimates Worldwide cancer incidence and mortality at 19.3 million new cancer cases and almost 10.0 million cancer deaths. Moreover, breast cancer is the most commonly diagnosed cancer with an estimated 2.3 million new cases (11.7%) followed by lung (11.4%), colorectal (10.0%), prostate (7.3%), and colon (6.0%) cancers, respectively. While lung cancer is the most commonly cause of cancer death with an estimate 1.8 million deaths (18%) followed by colon (9.4%) cancer[(1)]. Cancer is a group of diseases that starts from abnormal cells growing without control and spreading to other parts of the body to grow and form new tumors that replace normal tissues. Recently, there have been many types of cancer therapy, including standard methods, surgery, radiation and chemotherapy. Furthermore, targeted therapy is a target specific gene/protein or drug that works against specific cancer. However, these therapies are quite limited due to the high cost and side effects in some cases of cancer patients. In this study we used A459 cell line as a model of lung cancer and A375 cell line as a model of malignant melanoma (skin cancer). Even though skin cancer

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is not most commonly diagnosed, but melanoma is the most related death in patients with this type of skin cancer.

1,4-Naphthoquinones are active quinone derivatives that possess many biological functions, including anticancer[2-5], ROS scavenging[4,6], antifungal[7,8] and antiviral[9]. 1,4-naphthoquinones can be synthesized by organisms such as filamentous fungi, insects[10], tenebrionid beetles[11], sea urchin[12] and some bacteria[13]. In addition, 1,4-naphthoquinones were found amongst the specialized natural products synthesized by plants, such as medicinal plants (*Lawsonia inermis*, *Tabebuia impetiginosa*, *Rubia tinctorum*, *Alkanna tinctoria*), ornamental plants (*Impatiens balsamina*, *Impatiens glandulifera*, *Dionaea muscipula*) as well as nuts and seeds (*Juglans nigra*, *Carya illinoensis*, *Sesamum indicum*)[14-17]. The report shows that quinones and their analogs, daunorubicin, doxorubicin, and mitoxantrone are important sources of cytotoxic compounds for clinical treatment of cancer[18,19]. In the present study, we investigated the effect of novel 1,4-naphthoquinones, DL667 and DL666 on apoptosis induction in A375 and A549 cells, a model for malignant melanoma and lung cancer cell lines, respectively.

Apoptosis or programmed cell death is an essential physiological process that occurs in multiple cellular with important function as cancer prevention. The marker of morphological characteristics changes in early process of apoptosis are chromatin condensation and DNA fragmentation called "apoptotic bodies" and decrease of mitochondrial membrane potential. These events can be determined by Hoechst and JC-1 staining, respectively. As described above, the effects of novel 1,4-naphthoquinones, DL667 and DL666 on apoptosis induction in A375 and A549 cells have not yet been studied. Therefore, we aimed to verify the hypothesis that DL667 and DL666 could inhibit the growth of A375 and A549 cells associate with apoptosis induction.

Materials and Methods

Cell culture

Human non small lung cancer cell line A549 and malignant melanoma cancer cells line A375 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). A549 and A375 cells were maintained as a monolayer in DMEM and RPMI1640 medium (HiMedia, Mumbai, India) supplemented with 10% FBS (HiMedia, Mumbai, India), 100 U/mL penicillin and 100 µg/mL streptomycin (HiMedia, Mumbai, India), respectively. These cells were cultured in 5% CO_2 at 37°C, and after reaching ~90% confluences, cells were subcultured and the medium was replaced 2-3 times/week.

1,4-Napthoquinone derivatives

1,4-Napthoquinone derivatives, DL666 and DL667 were obtained from Associate Professor Ratchanok Pingaew, Faculty of Science, Srinakharinwirot University, in purified powder form. They were dissolved and diluted in DMSO at the desired concentrations for the assays.

Cell proliferation and cell viability assays

The cytotoxicity of 1,4-napthoquinone derivatives, DL666 and DL667 was determined by MTT assay, a colorimetric assay for assessing cell viability, measuring mitochondrial reductase enzyme activity. In living cells, the enzyme can reduce yellow MTT to purple formazan. A375 and A549 cells were seeded in a 96-well plate ($5x10^3$ cells/well) and allowed to grow for 24 h. Then, A375 and A549 cells were treated with DL667 and DL666 at various concentrations, whereas the control group was treated with DMSO, respectively. After incubation for 24 h, 100 µL of 0.5 mg/mL MTT solution (USB corporation; Cleveland, Ohio, USA) was added to each well, and the plate was further incubated for 2 h at 37°C. The supernatant was removed, and DMSO was added to solubilize the water insoluble purple formazan crystals. The absorbance was measured using a microplate reader at 570 nm (Multiskan Sky; Thermo Fisher Scientific, Massachusetts, USA), and the IC_{50} value was calculated using the GraphPad Prism 3.03 (GraphPad Software, Inc., San Diego, CA, USA).

Nuclear morphological staining with Hoechst 33342

A375 and A549 cells were seeded at $5x10^4$ cells/well in 12-well plate for 24 h. A375 cell were treated with DL667, while A549 cells were treated with DL666 at 3, 5, 10, 30 and 50 µM for 24 h. As control, cells were treated with DMSO for 24 h and subsequently stained with 10 µM Hoechst 33342 for 30 min at 37°C and examined under a fluorescence microscope (C-P20CC; OPTIKA S.r.l., Ponteranica (BG), Italy).

Measurement of mitochondrial membrane potential (ΔΨm)

The ΔΨm was determined using the potential sensitive dye JC-1, which is a lipophilic cation that is incorporated into the mitochondrial membrane. Cells were seeded at $5x10^4$ cells/well in 12-well plate for 24 h. Then, A375 and A549 cells were treated with DL667 and DL666 at 3, 5, 10, 30 and 50 µM for 6 h, respectively. The control cells were treated with DMSO. The cells were then stained with 5 µg/mL of JC-1 in the dark at 37°C for 15 min and washed with PBS for 3 times before analysis by fluorescence microscopy (IX73; Olympus, Tokyo, Japan).

Statistical analysis

All data presented were obtained from at least three independent experiments and were presented as mean ± standard deviation (SD).

Results

DL667 and DL666 inhibits cell growth in A375 and A549 cells

The anti-proliferation activity of DL667 and DL666 in the A375 and A549 cells were determined by MTT assay. The IC_{50} value of DL667 and DL666 were 11.2±0.419 and 142.9±1.469 µM in A375 and A549 cells, respectively, which inhibited cell growth in a dose-dependent

manner, whereas DL667 at 25 µM reduced cell growth less than 20% comparing with the control cells upon 24 h (Figure 1). These results suggested that 1,4-napthoquinone derivatives, DL667 and DL666 showed anti-proliferative properties in A375 and A549 cells, respectively.

DL667 and DL666 induce nuclear morphological changes in A375 and A549 cells

To determine whether DL667 and DL666 induces nuclear morphological changes in A375 and A549 treated cells, Hoechst 33342 staining was carried out. The results revealed that DL667 induced chromatin condensation as well as apoptotic bodies at 5 µM and increasing number of apoptotic cells at 10, 30 and 50 µM in A375 cells (Figure 2A). In addition, DL666 induced chromatin condensation at 10, 30 and 50 µM in A549 (Figure 2B). These results suggested that 1,4-napthoquinone derivatives, DL666 and DL667 induced chromatin condensation and apoptotic bodies in A375 and A549 cells, characteristics of apoptotic cells.

DL667 and DL666 induce the loss of mitochondrial membrane potential (ΔΨm)

During apoptosis, mitochondrial membrane was disrupted by Bax leading to changes in electron transport and loss of ΔΨm. JC-1 is a lipophilic cationic fluorescent dye that used to measure ΔΨm. In healthy cells, JC-1 was accumulated in mitochondria by proton pump and forms complexes known as J-aggregates with intense red fluorescence (high ΔΨm). While, apoptotic or unhealthy cells, JC-1 remains in the monomeric form, which shows only green fluorescence (low ΔΨm). In this study, DL667 showed increasing green fluorescence at 3 µM in A375 treated cells (Figure 3A). While 10 µM DL666 showed increased green fluorescence in A549 treated cells (Figure 3B) imply early apoptosis induction. Therefore, 1,4-napthoquinone derivatives, DL667 and DL666 induced apoptosis via decreased ΔΨm in A375 and A549 treated cells, while the control cells showed only red fluorescence It is possible that DL667 and DL666 may induce apoptosis related signaling pathways in the cells.

Discussion

For anticancer study, 1,4-naphthoquinones and their derivatives show ability to inhibit cancer cell growth and induce cancer cell death in many types of cancer cells. Recently, Coulidiati et al reported that triazol-1,4-naphthoquinones induced apoptosis in leukemia cells, HL60, through Bax upregulation and increasing intracellular ROS levels via ERK pathway[20]. In breast cancer cells, MDA-MB-231, 1,4-naphthoquinone increased apoptotic markers (annexin-V positivity, caspase 3/7 activity, decreased ΔΨm)[21] same as in MCF-7 cells that 1,4-naphthoquinone inhibited cell growth and induced DNA damage via γH2AX activation[22]. In murine fibroblast cells, L929, 1,4-naphthoquinone derivatives induce apoptosis via increased levels of Annexin-V-positive staining and caspase 3 activity[23]. In this study, we used 1,4-naphthoquinones derivatives, DL667 and DL666 that were synthesized and modified by Associate Professor Ratchanok Pingaew. These compounds were published in 2015 with anticancer activity. They reported that a novel 1,4-naphthoquinones derivatives, DL667 and DL666 could inhibit cell growth in HuCCA-1 (cholangiocarcinoma), HepG2 (hepatocellular carcinoma) and MOLT-3 (lymphoblastic leukemia). Especially, DL667 and DL666 showed ability to inhibit HepG2 cells growth better than etoposide, anticancer drug, with IC_{50} at 10.32±0.58, 6.27±0.51 and 33.98±0.01 µM[24], respectively. In the present study we found that 1,4-napthoquinone, DL667 and DL666 inhibited cell growth in A375 and A549 cells, respectively, by reducing percentage of cell growth in MTT assay (Figure 1). Following Pingaew et al, in 2015, we determined whether DL667 and DL666 could induce cell

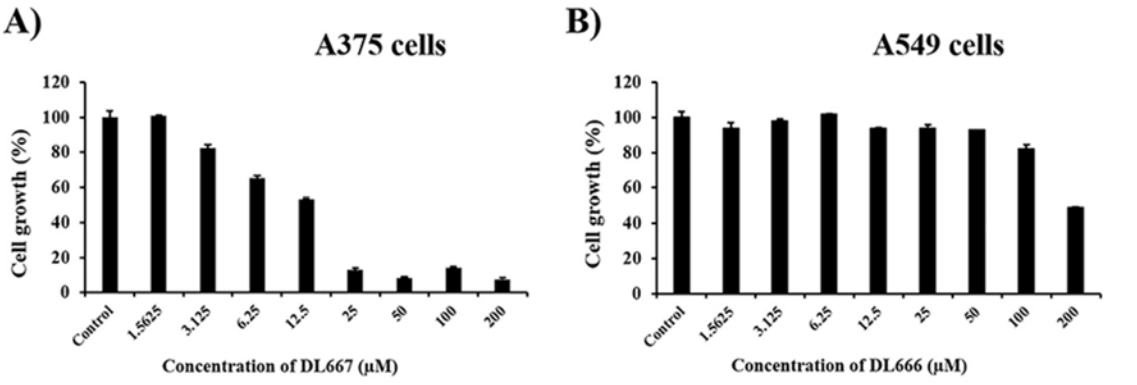

Figure 1. Effect of DL667 and DL666 on cell viability assay. A) Effect of DL667 on cell survival (%) in A375 cells following treatment with different concentrations of DL667 at 24 h. B) Effect of DL666 on cell survival (%) in A549 cells following treatment with different concentrations of DL666 at 24 h. The IC_{50} value was expressed as mean±SD from at least three independent experiments.

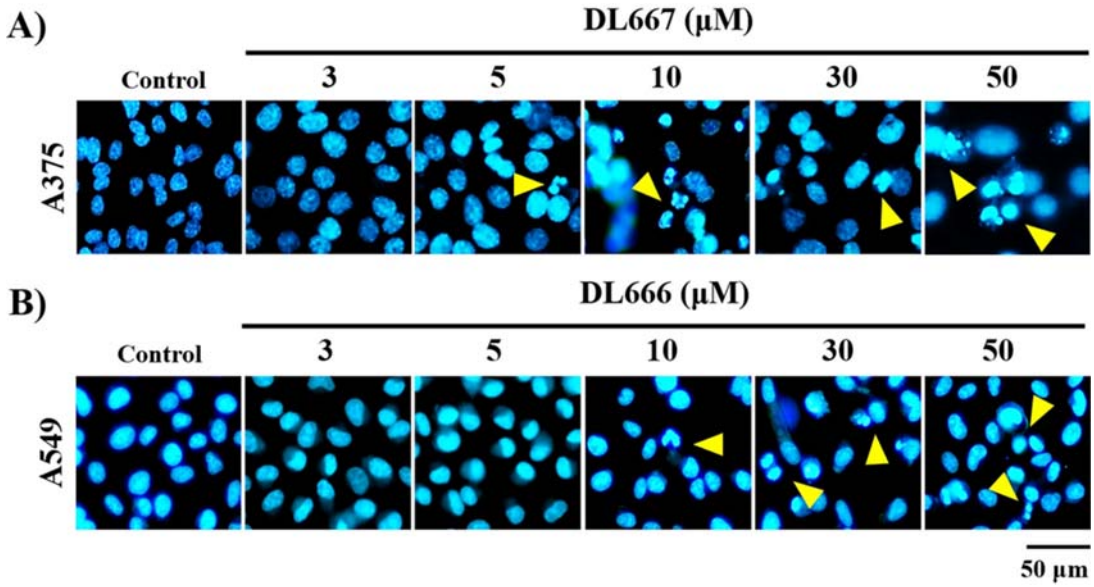

Figure 2. Effect of DL667 and DL666 on apoptosis induction. A) A375 cells were treated with DL667 at 3, 5, 10, 30 and 50 μM for 24 h. B) A549 cells were treated with DL666 at 3, 5, 10, 30 and 50 μM for 24 h. The cells were determined under a fluorescent microscope (x40). Chromatin condensation and apoptotic bodies are indicated by yellow arrow.

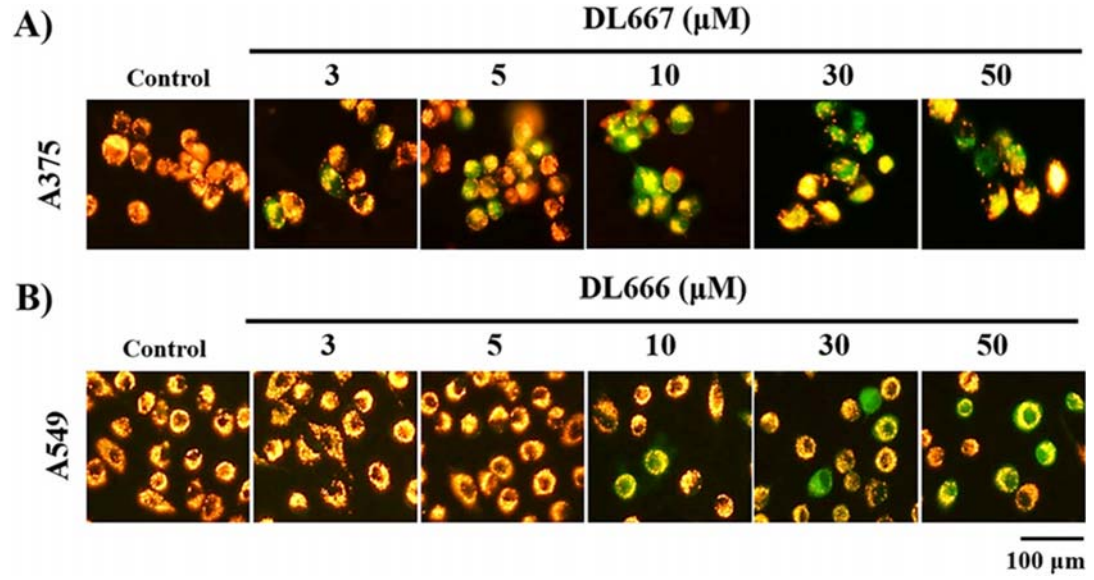

Figure 3. Effect of DL667 and DL666 on mitochondrial membrane potential. A) A375 cells were treated with DL667 at 3, 5, 10, 30 and 50 μM for 6 h. B) A549 cells were treated with DL666 at 3, 5, 10, 30 and 50 μM for 6 h. The cells were determined under a fluorescent microscope (x40). Red fluorescence in the control cells indicated high membrane potential and green fluorescence in the DL667 and DL666 treatment indicated loss of membrane potential. The DL667 and DL666 treatment showed an increased green fluorescence intensity in A375 and A549 cells, respectively.

death via apoptosis induction. We observed the nuclear morphological changes by chromatin condensation and apoptotic bodies detection. A375 and A549 treated cells were stained with DNA and nuclei staining fluorescent dye. The result showed that DL667 induced both chromatin condensation and apoptotic bodies in A375 treated cells (Figure 2A), whereas DL666 only induced chromatin condensation in A549 treated cells (Figure 2B). Moreover, the result from JC-1 staining found that DL667 decreased ΔΨm in A375 treated cells, the same as A549 cells treated with DL666. The decrease of ΔΨm occurs in early apoptosis process following chromatin condensation and apoptotic bodies that resulting from caspases activation. These results correlated with Li et al, reported that plumbagin, plant-derived naphthoquinone induced apoptotic bodies in breast cancer, MDA-MB-231 cells[25]. Furthermore, a novel 1,4-naphthoquinone derivative induced apoptotic bodies and decreased ΔΨm in MDA-MB-231 cells[21]. Additionally, plumbagin inhibited cell growth via STAT3, survivin and Mcl-1 with apoptosis induction via Annexin-V positive and STAT1 expression[25].

In the present study, we suggested that a novel 1,4-napthoquinone, DL667 and DL666 showed cell growth inhibition associated with apoptosis induction in A375 and A549 cells through chromatin condensation, apoptotic body formation and the loss of ΔΨm. However, the mechanism of apoptosis induction in both A375 and A549 cells by DL667 and DL666 need to be further determined.

Conclusion

A novel 1,4-napthoquinone, DL667 and DL666 showed anti-proliferation and apoptosis induction in A375 and A549 cells, respectively, through growth inhibition, nuclear morphological changes including chromatin condensation and decreased ΔΨm. Therefore, DL667 and DL666 may be further studied for the underlying mechanism of apoptosis cell death associated with signaling pathways.

What is already known on this topic?

1,4-Napthoquinone and their derivatives have been reported to have several activities including anticancer, antimalarial, antifungal, antiviral as well as ROS scavenging. Moreover, 1,4-napthoquinone was used in cancer research for developing a new anticancer drug.

What this study adds?

A novel 1,4-napthoquinone, DL667 and DL666 were synthesized and modified by Associate Professor Ratchanok Pingaew showed growth inhibition associated with apoptosis induction in A375 and A549 cells, respectively.

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Potential conflicts of interest

The authors declare no conflict of interest.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71:209-49.
2. Verma RP. Anti-cancer activities of 1,4-naphthoquinones: a QSAR study. Anticancer Agents Med Chem 2006;6:489-99.
3. Schepetkin IA, Karpenko AS, Khlebnikov AI, Shibinska MO, Levandovskiy IA, Kirpotina LN, et al. Synthesis, anticancer activity, and molecular modeling of 1,4-naphthoquinones that inhibit MKK7 and Cdc25. Eur J Med Chem 2019;183:111719.
4. Majiene D, Kuseliauskyte J, Stimbirys A, Jekabsone A. Comparison of the effect of native 1,4-naphthoquinones plumbagin, menadione, and lawsone on viability, redox status, and mitochondrial functions of C6 glioblastoma cells. Nutrients 2019;11:1294.
5. Liu YM, HuangFu WC, Huang HL, Wu WC, Chen YL, Yen Y, et al. 1,4-Naphthoquinones as inhibitors of Itch, a HECT domain-E3 ligase, and tumor growth suppressors in multiple myeloma. Eur J Med Chem 2017;140:84-91.
6. Liu H, Yan C, Li C, You T, She Z. Naphthoquinone derivatives with anti-inflammatory activity from mangrove-derived endophytic fungus talaromyces sp. SK-S009. Molecules 2020;25:576.
7. Halicki PCB, Ferreira LA, De Moura KCG, Carneiro PF, Del Rio KP, Carvalho T, et al. Naphthoquinone derivatives as scaffold to develop new drugs for tuberculosis treatment. Front Microbiol 2018;9:673.
8. Ibis C, Tuyun AF, Ozsoy-Gunes Z, Bahar H, Stasevych MV, Musyanovych RY, et al. Synthesis and biological evaluation of novel nitrogen- and sulfur-containing hetero-1,4-naphthoquinones as potent antifungal and antibacterial agents. Eur J Med Chem 2011;46:5861-7.
9. Tandon VK, Singh RV, Yadav DB. Synthesis and evaluation of novel 1,4-naphthoquinone derivatives as antiviral, antifungal and anticancer agents. Bioorg Med Chem Lett 2004;14:2901-4.
10. Pollard DR, Gill P, Day A. The group-specific protein marker: a possible indicator of syphilis, not human immunodeficiency virus infection. CMAJ 1988;138: 1013-5.
11. Pankewitz F, Hilker M. Polyketides in insects: ecological role of these widespread chemicals and evolutionary aspects of their biogenesis. Biol Rev Camb Philos Soc 2008;83:209-26.
12. Calestani C, Rast JP, Davidson EH. Isolation of pigment cell specific genes in the sea urchin embryo by differential macroarray screening. Development 2003;130:4587-96.
13. Babula P, Adam V, Havel L, Kizek R. Noteworthy secondary metabolites naphthoquinones-their

occurrence, pharmacological properties and analysis. Curr Pharmaceut Anal 2009;5:47-68.
14. Seigler DS. Benzoquinones, naphthoquinones, and anthraquinones. In: Seigler DS, editor. Plant secondary metabolism. Boston: Springer; 1998. p. 76-93.
15. Thomson RH. Naphthoquinones. In: Thomson RH, editor. Naturally occurring quinones IV: Recent advances. 4th ed. Dordrecht: Springer Netherlands; 1997. p. 112-308.
16. Hook I, Mills C, Sheridan H. Bioactive naphthoquinones from higher plants. In: Atta-ur-Rahman, editor. Studies in natural products chemistry, vol. 41. Amsterdum, Netherlands: Elsevier; 2014. p. 119-60.
17. Egan PA, van der Kooy F. Phytochemistry of the carnivorous sundew genus Drosera (Droseraceae) - future perspectives and ethnopharmacological relevance. Chem Biodivers 2013;10:1774-90.
18. Wolkenberg SE, Boger DL. Mechanisms of in situ activation for DNA-targeting antitumor agents. Chem Rev 2002;102:2477-95.
19. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 1999;57:727-41.
20. Coulidiati TH, Dantas BB, Faheina-Martins GV, de Morais Gomes ER, Goncalves JCR, de Araujo DAM. Proapoptotic effects of triazol-1,4-naphthoquinones involve intracellular ROS production and MAPK/ERK pathway in human leukemia cells. Anticancer Agents Med Chem 2020;20:2089-98.
21. Karakas D, Akar RO, Gokmen Z, Deniz NG, Ulukaya E. A novel 1,4-naphthoquinone-derived compound induces apoptotic cell death in breast cancer cells. Turk J Biol 2019;43:256-63.
22. Ourique F, Kviecinski MR, Felipe KB, Correia JF, Farias MS, Castro LS, et al. DNA damage and inhibition of akt pathway in mcf-7 cells and ehrlich tumor in mice treated with 1,4-naphthoquinones in combination with ascorbate. Oxid Med Cell Longev 2015;2015:495305.
23. Ramirez O, Motta-Mena LB, Cordova A, Estrada A, Li Q, Martinez L, et al. A small library of synthetic di-substituted 1, 4-naphthoquinones induces ROS-mediated cell death in murine fibroblasts. PLoS One 2014;9:e106828.
24. Pingaew R, Prachayasittikul V, Worachartcheewan A, Nantasenamat C, Prachayasittikul S, Ruchirawat S, et al. Novel 1,4-naphthoquinone-based sulfonamides: Synthesis, QSAR, anticancer and antimalarial studies. Eur J Med Chem 2015;103:446-59.
25. Li N, Ou J, Bao N, Chen C, Shi Z, Chen L, et al. Design, synthesis and biological evaluation of novel plumbagin derivatives as potent antitumor agents with STAT3 inhibition. Bioorg Chem 2020;104:104208.