Pharmacogn. Mag.
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ORIGINAL ARTICLE

Antiproliferative and Apoptosis Inducing Effect of Delphinidin against Human Bladder Cancer Cell Line

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Submitted: 17-Dec-2019 Revised: 27-Feb-2020 Accepted: 18-Dec-2020 Published: 15-Apr-2021

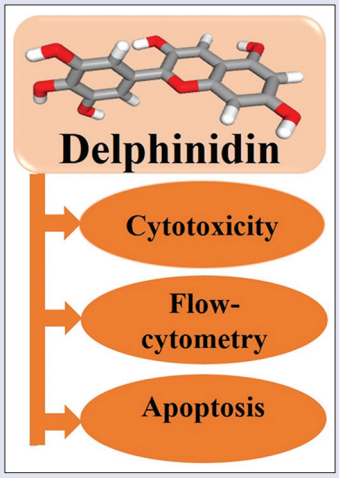
ABSTRACT

Background: Urinary bladder cancer is one of the leading causes of death worldwide. This study was conducted to evaluate the effect of delphinidin on proliferation, apoptosis, and cell cycle arrest in the urinary bladder cancer cell line (T24). **Materials and Methods:** We performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay to analyze the cytotoxic effect of delphinidin on T24 cell proliferation. We tested different concentrations of delphinidin. Furthermore, the effect of delphinidin on cell cycle arrest was studied using flow cytometric assays and the percentage of apoptotic cell death was estimated via annexin V/propidium iodide. **Results:** According to our results, delphinidin (10–60 μg/mL) demonstrated a significant cytotoxic effect against T24 cells. Morphological analysis and analysis of the oxidative stress revealed a significant number of dead cells in comparison to the control group. The results of flow cytometry revealed that delphinidin caused dose-dependent apoptosis of T24 cells. **Conclusion:** In summary, delphinidin can be a promising option for the treatment of urinary bladder cancer in the future.

Key words: Apoptosis, bladder cancer, cytotoxicity, delphinidin, flavonoids

SUMMARY

- Delphinidin is one of the bioactive molecules from the flavonoids group. In this study, delphinidin was investigated to study its effect on T24 cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used to analyze its cytotoxic effect on T24 cells. It was observed that delphinidin treatment enhances reactive oxygen specise generation in T24 cells. Furthermore, cell cycle distribution upon delphinidin treatment revealed that delphinidin induces apoptosis in T24 cells. The results were found to be significant in comparison to the control group. Further investigation is needed to study the detailed mechanisms of action of delphinidin. However, delphinidin is a promising anticancer agent that can be used in the treatment of T24 bladder cancer.



Abbreviations used: CCTCC: China Center for Type Culture Collection; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO: Dimethyl sulfoxide; LPO: Lipid peroxidase; ROS: Reactive oxygen specise; GSH: Glutathione; DCFH-DA: Dichloro-dihydro-fluorescein diacetate; FACS: Fluorescence-activated cell sorting; NAC: N-acetyl-L-cysteine; PI: Propidium iodide, PS: Phosphatidylserine.

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DOI: 10.4103/pm.pm_548_19

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INTRODUCTION

Nowadays, cancer-associated deaths are increasing at an alarming rate. Urinary bladder cancer is one of the other several types of cancers which is responsible for death worldwide.[1,2] In 2012, 430,000 new cases and 165,000 deaths were reported due to bladder cancer.[3] Although cancer chemotherapy has advanced significantly in recent times, there is no satisfactory cure available for this disease. However, cancer cells become resistant to the chemotherapy being administered.[4] Furthermore, the side effects of chemotherapy is also an important concern to consider while planning research in oncology.[5] So far, the most commonly used combination medicine for the treatment of bladder cancer is gemcitabine and cisplatin. However, this combination is found to be effective in radiosensitized cells.[6] Therefore, it is highly warranted to investigate the anticancer potential of various existing molecules. Recently, plant-based flavonoids are being tested as potential anti-cancer agents.[2] Delphinidin belongs to the class of anthocyanidins which possesses significant biological activity. It is one of the major pigmented anthocyanidins that is present in a diverse range of fruits and vegetables (e.g., berries, grapes, sweet potatoes, and pigmented cabbages).[7] Evidence suggests that delphinidin has potent anti-inflammatory and antioxidant activity due to its basic polyphenolic structure and therefore can be a promising anticancer agent.[8] Studies have shown that moderate oxidative stress

Cite this article as: Kang Y, Li J, Jing L, Zhang Y, Wang X. Antiproliferative and apoptosis inducing effect of delphinidin against human bladder cancer cell line. Phcog Mag 2021;17:101-5.

and prolonged inflammation in the tumor microenvironment are associated with cancer progression. For instance, pro-inflammatory cytokines upregulate the expression of nuclear factor-κB and activate its downstream signaling molecules that maintain tumor survival and proliferation.[9] Delphinidin demonstrated anticancer effects in ovarian cancer[10] and non-small cell lung cancer[11] by downregulating the expression of PI3K/AKT and ERK 1/2 MAPK and EGFR/VEGFR2. In this study, we aimed to evaluate the antiproliferative and apoptotic effects of delphinidin against the human bladder cancer cell line.

MATERIALS AND METHODS

Materials

The following chemicals were procured from Sigma Aldrich and E-Merck: RPMI-1640; amphotericin B; penstrep (penicillin-streptomycin); MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye; 2′,7′-dichlorofluorescin diacetate (DCFH-DA); N-acetyl-cysteine (NAC); propidium iodide (PI); and annexin. All of the chemicals were of research grade. T24 cells, human urinary bladder cancer cells, were procured from the China Center for Type Culture Collection, Shanghai, China.

Cell culture and cytotoxicity assay

T24 cells were grown in RPMI-1640 media. The medium was supplemented with fetal bovine serum, 1% penstrep, and amphotericin B. Cells were incubated in a 95% humidified atmosphere with 5% CO_2 at 37°C. Cytotoxicity of delphinidin was initially evaluated using MTT assay.[12] T24 cells were treated with different concentrations of delphinidin (10, 20, 30, 40, 50, and 60 µg/mL) for 24 h. After incubation, MTT was loaded into each well which resulted in the formation of formazan crystal. To dissolve formazan crystals, 100 µL of dimethyl sulfoxide (DMSO) was added followed by 15 min incubation and the absorbance at 570 nm was recorded using a spectrophotometer (Bio-Rad Laboratories-USA).

Oxidative stress analysis

Lipid peroxides (LPOs) and glutathione (GSH) levels in delphinidin-treated and untreated cancer cells were determined to evaluate cellular damage caused due to oxidative stress. Briefly, the supernatants obtained from the cell homogenate were used to determine the levels of LPO and GSH.[13] All the samples were stored at 4°C until further use. To estimate the level of reactive oxygen species (ROS) production, T24 cells were cultured and treated with delphinidin. After 24 h, the cells were washed twice with phosphate-buffered saline (PBS) and stained with 20 µM DCFH-DA. Cells were washed again with PBS and were analyzed via flow cytometry (BD Biosciences, San Jose, CA, USA). Furthermore, one set of drug-treated experiment was conducted in the presence of NAC (inhibitor of ROS) at a concentration of 25 µg/mL. The untreated cells were chosen as a control group and experiments were carried out in triplicates for reproducibility.

Cell cycle analysis

T24 cells (50×10^4 cells/well) were grown in 24-well culture plates for overnight. Cultured cells were treated with different concentrations (30, 40, 50, and 60 µg/mL in 0.1% DMSO) of delphinidin for 24 h. The control (untreated) cells were treated with a vehicle. The treated and control cells were washed with PBS and incubated overnight. The next day, the cells were fixed using 70% chilled ethanol. Next, PBS (400 µL), PI (50 µL, 1 mg/mL), and RNase solution (50 µL, 1 mg/mL) were added. The prepared cell suspension was incubated for half an hour and was analyzed using a flow cytometer (Cyflogic, Cyflo Ltd., Finland) to evaluate cell cycle arrest.

Detection of apoptosis using annexin

As discussed in the previous study,[12] 5×10^4 cells in 24 well plates were grown for 24 h. Next, T24 cells were exposed to delphinidin at different concentrations (30, 40, 50, and 60 µg/mL). After this, the cells were suspended in annexin buffer and stained with 5 µL of anti-annexin V dye. The stained cells were analyzed using the flow cytometer.

Statistics

All the values of the performed assays were obtained in triplicate. Differential expression of the cells in response to the various treatments specified was analyzed by taking mean values and subjecting to two-tailed unpaired Student's *t* test ($P < 0.05$).

RESULTS

Cytotoxicity of delphinidin

The T24 cells were exposed to different concentrations of delphinidin dissolved in RPMI-1640 media for 24 h, whereas control cells were exposed to vehicle (RPMI-1640 medium). A dose-dependent cytotoxic effect of delphinidin was observed with an IC_{50} of 34 µg/mL. The maximum growth inhibitory effect of delphinidin was at 60 µg/mL concentration [Figure 1].

Measurement of oxidative stress

According to the results of MTT assay, lower concentrations (10, 15, 20, and 25 µg/mL) were selected to analyze the oxidative potential of delphinidin. We analyzed the level of membrane LPOs [Figure 2a] and intracellular levels of GSH [Figure 2b] in T24 cells treated with delphinidin for 24 h. Lipid peroxidation was determined by estimating the formation of malondialdehyde which was significantly higher in T24 cells after delphinidin treatment. However, GSH levels were significantly decreased after delphinidin treatment in dose-dependent manner. To further validate the cytotoxic effect of delphinidin, T24 cells were exposed to NAC. According to our results, delphinidin completely inhibited the oxidative effect of NAC (data not shown). Higher concentrations of ROS were recorded in T24 cells after delphinidin treatment at a concentration of 25 µg/mL [Figure 2c and d].

Flow cytometry analysis

Figure 3a and b show the cell cycle profile of T24 cells after treatment with delphinidin. DNA histograms have shown the presence of typical sub-G1 (apoptotic) single peak at a concentration of 40 µg/mL. There

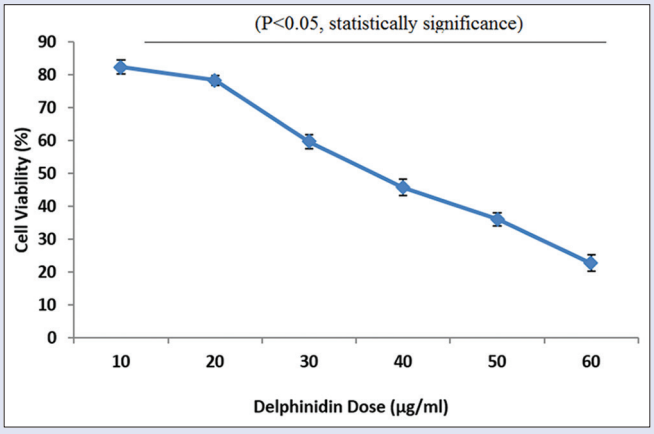

Figure 1: Cell viability assay showing dose-dependent growth inhibitory effect of delphinidin against T24 cells ($P < 0.05$ statistical significance)

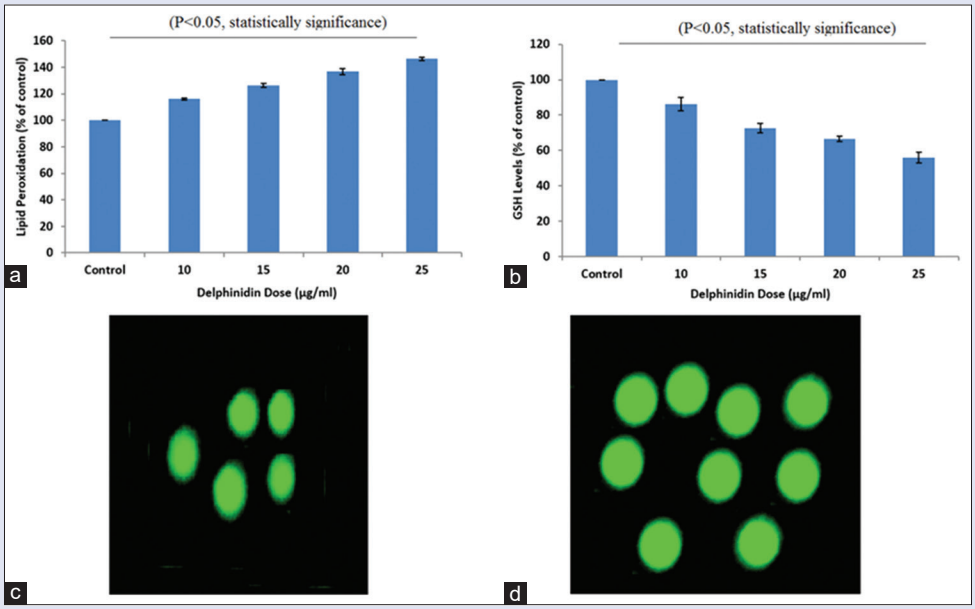

Figure 2: (a) Lipid peroxidation and (b) glutathione percentage levels have been shown at various concentrations of delphinidin in comparison to the control group ($P < 0.05$ statistical significance). (c) ROS level in without and with (d) delphinidin-treated T24 cells

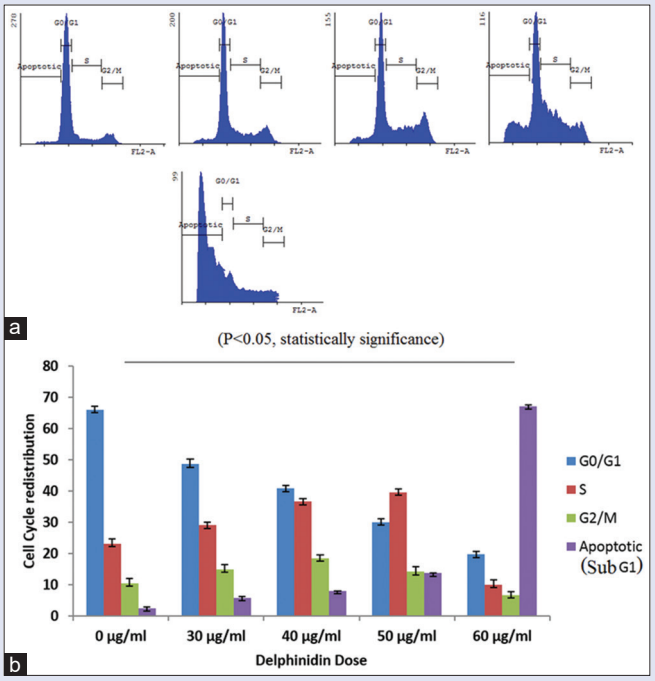

Figure 3: Flow cytometry analysis. (a) Cell cycle profiles of T24 cells are showing maximum proportion at G1 phase. Cell cycle arrest was found at G1 phase which is increasing with delphinidin dosage. The significant apoptotic activity has shown at 60 µg/ml concentration. (b) The graph represents the redistribution of cells in the delphinidin-treated group in comparison to control ($P < 0.05$ statistical significance)

was a significant increase in sub-G1 proportion of cells (13.68% and 67.16%) with 50 and 60 µg/mL delphinidin.

Apoptotic percentage of delphinidin

A graph between annexin V- and PI-gated cells using flow cytometer represents the percentage of viable (annexin V− PI⁻), non-apoptotic (PI⁻), early apoptotic (annexin V+ PI⁻), and late apoptotic (annexin V+ PI+) cells [Figure 4]. There was a dose-dependent apoptotic effect of delphinidin on T24 cells. Delphinidin demonstrated apoptotic effect against T24 cells at 0, 30, 40, and 50 µg/mL concentrations. The percentage of apoptotic cells was higher in the treated group than that of the control group.

DISCUSSION

In this study, we analyzed the anticancer activities of delphinidin using T24 urinary bladder cancer cells. Multiple therapeutic effects of flavonoids have already been reported previously. To the best of our knowledge, this is the first report on cytotoxicity and apoptotic effect of delphinidin on human urinary bladder cancer cells. Our results have confirmed that the incidence of urinary bladder cancer is increasing and there is an urgent requirement for new drugs to reduce the mortality rate. Delphinidin demonstrated a significant growth inhibitory effect on T24 cells at 30–60 µg/mL. Previous studies on other cancer cells have revealed that delphinidin significantly inhibits the proliferation of cancer cells at 20–30 µg/mL.[9,10] In this study, we observed that delphinidin induced the oxidative stress-mediated apoptotic cell death in T24 cells. Intracellular ROS content after incubation with 25 µg/mL delphinidin was analyzed using DCFH-DA staining method. DCFH-DA dye is passively transported into the cells where it combines with ROS and forms dichlorofluorescein.[14] Elevated levels of ROS have been correlated with inflammation, DNA fragmentation, and apoptosis activation.[15-17] Earlier reports, using cancer cell lines (human colon cancer cells [HCT116] and human breast cancer cells [MCF7]), have demonstrated that higher toxicity of delphinidin might be due to its ability to induce oxidative stress.[18,19] According to the results of MTT assay, delphinidin shows broad-spectrum cytotoxic activity against T24 cells. Our results were consistent with previously published data.[8,20-22] More interestingly, recent findings in cancer research have revealed that the generation of ROSs via oxidative stress results in the induction of apoptotic cell death.[23] Apoptosis activation in cancer cells is an important cellular event against tumor growth.[12,24] Apoptotic induction by chemotherapeutic agents is considered a crucial therapeutic

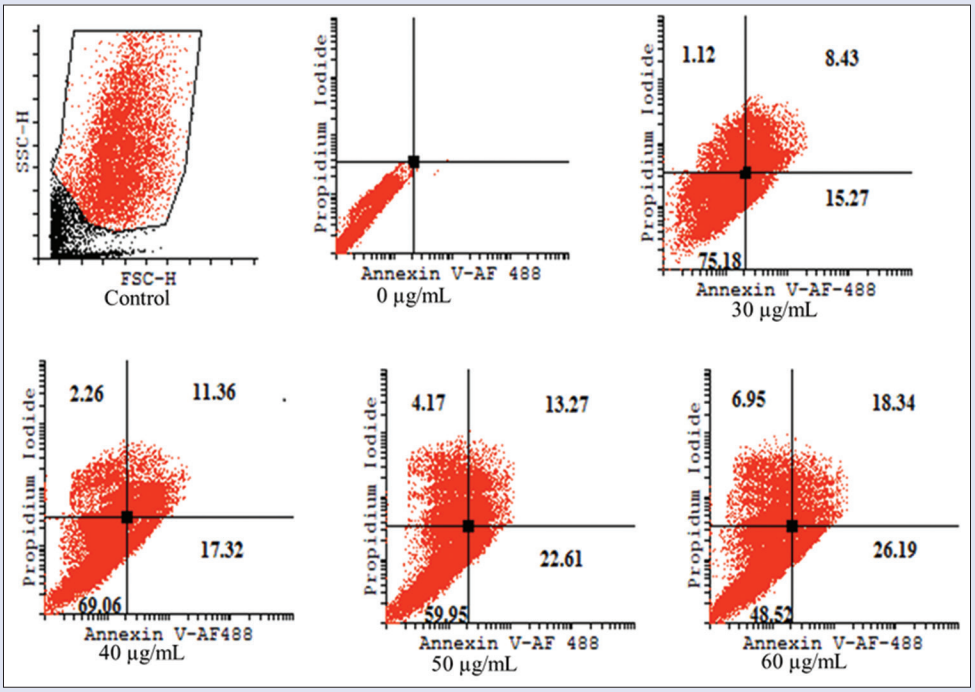

Figure 4: Urinary bladder cancer T24 cells were treated with delphinidin at various concentrations (0, 30, 40, 50, and 60 µg/mL) for 24 h and examined using annexin V/PI staining on a flow cytometer

approach.[25-27] In this study, flow cytometry was conducted to examine cell cycle redistribution and apoptotic effect of delphinidin in T24 cells. The cell cycle redistribution data show that there is an increase in the accumulation of cells in S and G2/M phase of T24 cells treated with delphinidin at 30 and 40 µg/mL [Figure 3]. Furthermore, the number of cells arrested at the S and G2/M phase was reduced when the cells were treated with higher concentrations of delphinidin (50 and 60 µg/mL). This is due to the increase in apoptotic cell death at higher concentrations. To further confirm the apoptotic effect of delphinidin, the treated cells were stained using annexin and observed using a flow cytometer. The apoptotic and necrotic cells were identified based on the principle of protrusions of phosphatidylserine receptors to the plasma membrane surface. Alexa Fluor 488 annexin V is a fluorescent dye that binds specifically to phosphatidylserine and helps in the early detection of apoptotic cancer cells. In addition, disruption of plasma membrane integrity has shown the cancer cell death due to both apoptotic (at 60 µg/mL dose, 6.95% and 18.34% cells were in early and late apoptosis, respectively) and necrotic (at 60 µg/mL, 26.19% cells were undergone in necrosis) processes.

CONCLUSION

This study demonstrates that delphinidin shows potent anticancer activity against human urinary bladder cancer cells. The growth inhibitory effect of delphinidin was due to apoptosis induction and ROS generation. Therefore, delphinidin could be considered a potent and inexpensive therapeutic agent for cancer therapy. Further research is highly warranted to confirm its use in human clinical trials.

Acknowledgements

The authors would like to acknowledge the Department of Oncological Radiotherapy, The People's Hospital Of Zhangqiu for their support.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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