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Original Article

Polydatin Induces Apoptosis in Thyroid Cancer TPC-I Cells by Regulating Apoptotic Proteins and Downregulating JAK/STAT Pathway

Xiao Zhao[1], Kai Gai[2], Bin Lin[3,*]

[1]Department of Medical Care, No.971, Hospital of the People's Liberation Army Navy, Qingdao, CHINA.
[2]Departments of Oncology, Qingdao Municipal Hospital, Qingdao, CHINA.
[3]Department of Thyroid Disease, Qingdao Municipal Hospital, Qingdao, CHINA.

ABSTRACT

Background: Thyroid cancer is relatively uncommon but its incidence has been steadily increasing over the past years. The etiology of thyroid cancer is multifaceted, with both genetic and environmental causes playing major roles. **Objectives:** This work was devoted to explore the anticancer properties of the polydatin against papillary thyroid carcinoma TPC-I cells. **Materials and Methods:** The cytotoxicity of polydatin (at doses of 5-160 µM/mL) against thyroid cancer TPC-I cells were tested using the MTT assay. The endogenous ROS production and incidences of apoptosis was assessed using fluorescence staining assays. The concentrations of oxidative stress indicators were assessed utilizing assay kits. The concentrations of apoptotic and cell cycle proteins, including Bax, Bcl-2, cyclin D1, JAK-1, and STAT3 were assessed in the control and treated cells with appropriate assay kits. **Results:** The growth of thyroid cancer TPC-I cells was markedly diminished following treatment with polydatin at increasing concentrations. The polydatin treatment markedly increased ROS generation and induced apoptotic cell death in TPC-I cells. Furthermore, the polydatin treatment considerably increased TBARS levels and decreased antioxidant levels in TPC-I cells. In addition, polydatin treatment elevated the Bax protein level while diminishing the levels of cyclin D1, Bcl-2, JAK-1, and STAT3 proteins in TPC-I cells. **Conclusion:** The results of this work demonstrate that polydatin treatment considerably inhibits cell growth and promotes apoptosis in thyroid cancer TPC-I cells. Consequently, it possesses the potential to serve as an anticancer candidate to treat thyroid cancer.

Keywords: Cyclin D1, JAK/STAT pathway, Polydatin, Apoptosis, TPC-I cells.

Correspondence:
Dr. Bin Lin
Department of Thyroid Disease, Qingdao Municipal Hospital, Qingdao-266000, CHINA.
Email: qdslyyjzx@outlook.com

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INTRODUCTION

Thyroid cancer is a malignant tumor growth that develops in the thyroid gland. This type of cancer is relatively uncommon, accounting for only 3.1% of all cancers globally, but its incidence has been rapidly increasing over the past years.[1] The etiology of thyroid cancer are multifaceted, with both genetic and environmental causes playing major roles. Certain genetic mutations, radiation exposure, and iodine deficiency have all been associated with higher risk of progressing the disease. The incidence of thyroid cancer has risen dramatically in recent years, with increased new cases worldwide.[2] While the mortality rate for thyroid cancer is relatively low compared to other cancers, certain subtypes of the disease can be more destructive and require more intensive therapies. Surgery and radioiodine therapy are the primary suggested modalities for the treatment of thyroid cancer, with thyroidectomy being advised for individuals with suspicious thyroid nodules. Despite the relatively favorable prognosis for most thyroid cancer patients, the disease can still pose a significant burden on individuals and healthcare systems.[3] About 10% of differentiated thyroid cancers metastasize distantly, and one-third of these metastatic instances demonstrate a failure of thyroid cells to absorb iodine, resulting in insensitivity to radioiodine therapy and the emergence of iodine-refractory differentiated thyroid cancer.[4] Existing therapeutic modalities for this variant of thyroid carcinoma, such as surgical resection, radioiodine therapy, and thyroid-stimulating hormone suppression, exhibit low or negligible efficacy, underscoring the necessity for new potent therapies.[5]

Apoptosis plays a pivotal role in the treatment of cancer. Tumor cells often demonstrate evasion of apoptosis, enabling their uncontrolled growth and proliferation. Accordingly, understanding the molecular mechanisms governing apoptosis and how resistant forms of cancer evade this process can open

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new avenues for cancer drug development.[6] The loss of balance between cell division and death is a hallmark of tumor. This imbalance can arise from defects in the apoptotic pathways, allowing the survival and progression of cancer cells. Targeting apoptosis is therefore a highly effective non-surgical approach to cancer therapy, as it can terminate the unrestrained proliferation of tumor cells by coupling the cell's own death mechanism.[7] Various signaling pathways regulate the apoptotic mechanisms. Cancer cells often exhibit defects in these pathways, contributing to their resistance to apoptosis-inducing stimuli. Therefore, a comprehensive understanding of the molecular processes underlying apoptosis induction in tumor cells is indispensable for the development of potential cancer therapies. By targeting the specific defects in the apoptotic pathways, cancer treatments can be designed to reestablish the proper balance between cell division and cell death, eventually resulting in the elimination of tumor cells.[8]

Plants have long been recognized as an invaluable source of bioactive compounds with immense potential for various therapeutic applications, including the treatment of cancers. These phytochemicals, or plant-derived bioactive substances, have gained more interest in the area of tumor research because of their capacity to target several signaling cascades participated in the onset of tumor growth.[9] Polydatin is a bioactive stilbenoid polyphenol compound found extensively in the *Polygonum cuspidatum* plant. It has been already highlighted that polydatin has effectively inhibited cell growth and triggered apoptosis in several cancer cells, which includes colon cancer,[10] breast cancer,[11] lung cancer,[12] liver cancer,[13] osteosarcoma,[14] and oral cancer.[15] Furthermore, it has demonstrated the neuroprotective,[16] anti-inflammatory and antioxidant,[17] anti-atherosclerosis,[18] and anti-arthritic[19] activities. Nevertheless, there are no studies conducted on the anticancer properties of polydatin against thyroid cancer. Hence, this work was devoted to explore the anticancer properties of the polydatin against thyroid cancer TPC-I cells.

MATERIALS AND METHODS

Chemicals and reagents

Polydatin, Dimethyl Sulfoxide (DMSO), Fetal Bovine Serum (FBS), antibiotics, etc., were acquired from Sigma-Aldrich, USA. The diagnostic kits for quantifying the biochemical markers were acquired from MyBioSource and Abcam, USA, respectively.

Collection and maintenance of cell line

The human papillary thyroid carcinoma TPC-I cells was obtained from ATCC, USA, and cultivated in DMEM with 10% FBS and 1% antibiotics in a 5% CO_2 atmosphere. Cells were harvested upon reaching 80% confluency and utilized for subsequent fluorescence staining and biochemical evaluations.

MTT cytotoxicity assay

The impact of polydatin on the growth of TPC-I cells were studied using MTT test. Cells were cultivated on a 96-well plate for 24 hr. Consequently, cells were exposed to polydatin for an additional 24 hr at varying dosages of 5, 10, 20, 40, 80, and 160 µM/mL. Subsequent to the polydatin treatment, MTT (20 µL) combined with DMEM (100 µL) was introduced to the wells and incubated for 4 hr. The absorbance was taken at 570 nm after dissolving the formazan deposits with DMSO (100 µL) and analyzed using microplate reader.

DCFH-DA staining

The increase of ROS accumulation in TPC-I cells exposed to polydatin was analyzed by DCFH-DA staining technique. The cells were cultured in a 6-well plate and subsequently treated polydatin at dosages of IC_{50} of polydatin and/or Doxorubicin (DOX) for 24 hr. Subsequently, 10 µL of DCFH-DA was introduced to the wells and incubated for 10 min. The fluorescence microscope was utilized to assess the intensity of developed fluorescence, which correlates directly with the amounts of ROS production.

Dual staining

The dual staining assay was conducted on the control and polydatin-treated TPC-I cells to examine apoptosis. The TPC-I cells were cultured for 24 hr and subsequently exposed to IC_{50} of polydatin and/or DOX for an additional 24 hr. A solution of 100 µg/mL AO/EB dyes was subsequently added to the wells for 5 min in darkness. The occurrence of apoptotic cells were evaluated using a fluorescent microscope.

Analysis of oxidative stress markers

The TPC-I cells from both control and treatment groups were harvested, and their cell lysate was generated using cell lysis buffer to quantify the oxidative stress marker concentrations. The concentrations of TBARS, SOD, CAT, and GSH were quantified in the cell lysates of TPC-I cells, utilizing the appropriate test kits by following the manufacturer's guidelines (Abcam, USA).

Analysis of Bax/Bcl-2, cyclin D1, and JAK/STAT protein levels

To assess the concentrations of apoptotic and cell cycle biomarker proteins, the cell lysate was extracted from control and treated TPC-I cells with a cell lysis buffer. The concentrations of Bax, Bcl-2, cyclin D1, JAK-1, and STAT3 were analyzed in experimental TPC-I cell lysates. The assays were conducted utilizing assay kits received from the manufacturer (MyBioSource, USA) in accordance with the provided instructions.

Statistical analysis

The statistical studies were conducted using GraphPad software, and the data were illustrated as Mean±SD of triplicates. The

results were evaluated using one way ANOVA and Tukey's *post hoc* assays, with significance threshold of $p<0.05$.

RESULTS

Effect of polydatin on thyroid cancer TPC-I cell viability

Figure 1 illustrates the findings of MTT cytotoxicity assay, demonstrating the effect of polydatin on the proliferation of thyroid cancer TPC-I cells. Polydatin treatment markedly diminished the viability of TPC-I cells at the increasing concentrations (5-160 µM/mL). The elevated concentrations of polydatin exhibited a notable reduction in the proliferation of TPC-I cells, as illustrated in Figure 1. The IC_{50} concentration of polydatin was determined to be 40 µM/mL against TPC-I cells, and this dosage was selected for following investigations.

Effect of polydatin on ROS accumulation in TPC-I cells

Figure 2 demonstrates the effect of polydatin treatment on endogenous ROS generation in thyroid cancer TPC-I cells. Cells exposed to 40 µM/mL of polydatin exhibited intense green fluorescence relative to control group. The intensified green fluorescence signifies the increased endogenous buildup of ROS in the TPC-I cells exposed to polydatin. Similarly, the DOX exposure also augmented the endogenous ROS in the TPC-I cells, which supports the activity of polydatin treatment.

Effect of polydatin on apoptosis in thyroid cancer TPC-I cells

The dual staining approach was employed to investigate apoptosis in both control and treated TPC-I cells, with results presented in Figure 3. Following treatment with 40 µM/mL of polydatin, the TPC-I cells exhibited increased quantities of yellow and orange fluorescent cells, indicating the presence of both early and late-stage apoptosis. The DOX treatment results also indicated an increase in cells exhibiting bright yellow/orange fluorescence, confirming the initiation of apoptosis in the polydatin-treated TPC-I cells (Figure 3).

Effect of polydatin on oxidative stress level in TPC-I cells

Figure 4 illustrates the levels of TBARS and antioxidant levels in both control and treated TPC-I cells. The control cells demonstrated increased levels of CAT, GSH, and SOD, accompanied by a reduction in TBARS levels. Simultaneously, treatment of TPC-I cells with 40 µM/mL of polydatin led to a considerable elevation in TABRS and a reduction in antioxidant concentrations. Similarly, the treatment of DOX also resulted in an elevation in TBARS and a reduction in antioxidants in TPC-I cells. These results corroborate the hypothesis that polydatin induces oxidative stress in TPC-I cells.

Effect of polydatin on apoptosis and cell cycle-related protein levels in the TPC-I cells

The concentrations of apoptosis and cell cycle-related proteins were examined in control and treated TPC-I cells (Figure 5). In

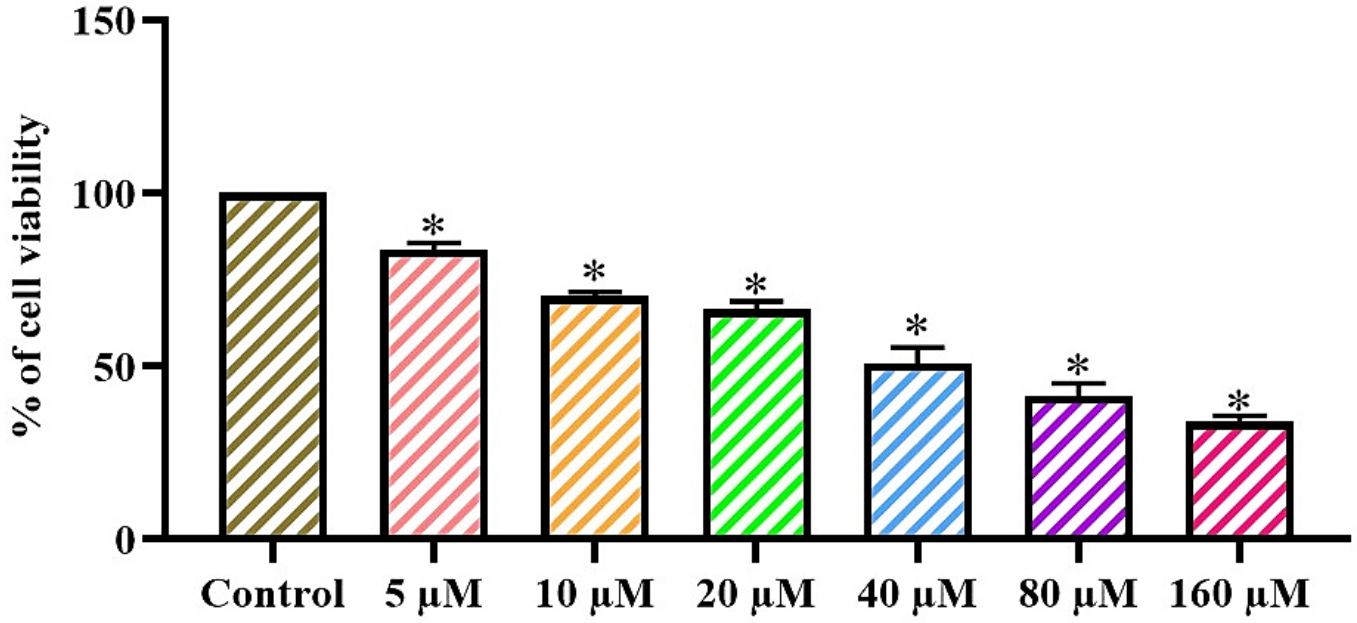

Figure 1: Effect of polydatin on the thyroid cancer TPC-I cell viability. The growth of thyroid cancer TPC-I cells was markedly diminished by the increasing concentrations (5-160 µM/mL) of polydatin. The IC_{50} concentration of polydatin against TPC-I cells was determined to be 40 µM/mL. The findings are illustrated as the Mean±SD of three individual experiments. The data are assessed using one-way ANOVA and Tukey's *post hoc* analysis using GraphPad Prism software. An asterisk '*' specifies that the values are significant at $p<0.05$ from the control group.

the control cells, the expressions of cyclin D1, Bcl-2, JAK-1, and STAT3 was increased and Bax level was reduced. Interestingly, the treatment of polydatin at a 40 µM/mL dose to TPC-I cells led to a considerable reduction in the concentrations of cyclin D1, Bcl-2, JAK, and STAT3 and increased the Bax protein levels. The outcomes of DOX treatment also corroborated these findings, as it similarly diminished the concentrations of cyclin D1, Bcl-2, JAK, and STAT3 and elevated the Bax levels. Consequently, it was evident that polydatin treatment inhibits cell growth and promotes death in thyroid cancer cells.

DISCUSSION

Cancer continues to be a primary cause of death globally, with considerable resources allocated to the advancement of new therapeutic medicines. Cytotoxicity of anticancer drugs to normal cells are major challenges in cancer therapy, as the effective dose to eliminate tumor cells can often lead to undesirable side effects. Consequently, the analysis of cell growth inhibition effects of sample drugs on cancer cells is an essential step in the drug discovery and advancement.[20] The MTT assay is a prevalent colorimetric technique for assessing cell viability and proliferation in reaction to pharmacological interventions. The MTT assay operates on the idea that mitochondrial enzymes in viable cells cleave the yellow Tetrazolium salt (MTT), resulting in the formation of a soluble blue formazan product. The quantity of formazan produced is directly related to the count of viable cells, offering a quantitative assessment of cell growth inhibition.[21] It has been already reported the utility of the MTT assay in analyzing the cytotoxicity of various drug candidates on tumor cells.[22,23] By measuring the reduction of MTT to formazan, researchers can determine the half-maximal inhibitory dosage of a drug, which represents the concentration required to inhibit 50% of cell growth.[24] This data is essential for understanding the potency and selectivity of a drug, as well as its potential therapeutic window. The present findings of MTT assay indicated that polydatin treatment markedly diminished the thyroid cancer TPC-I cell growth at the increasing concentrations. The increasing

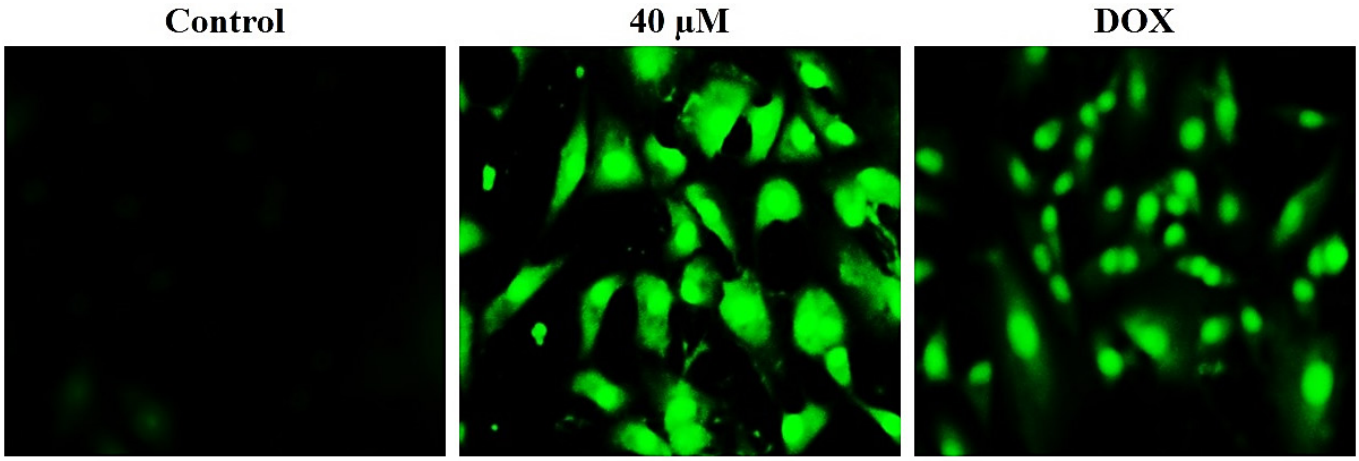

Figure 2: Effect of polydatin on the ROS generation in the TPC-I cells. The TPC-I cells demonstrated increased green fluorescence after treatment with 40 µM/mL of polydatin and/or 2 µg of DOX than that of the control cells. The augmented green fluorescence is suggests the heightened endogenous ROS production.

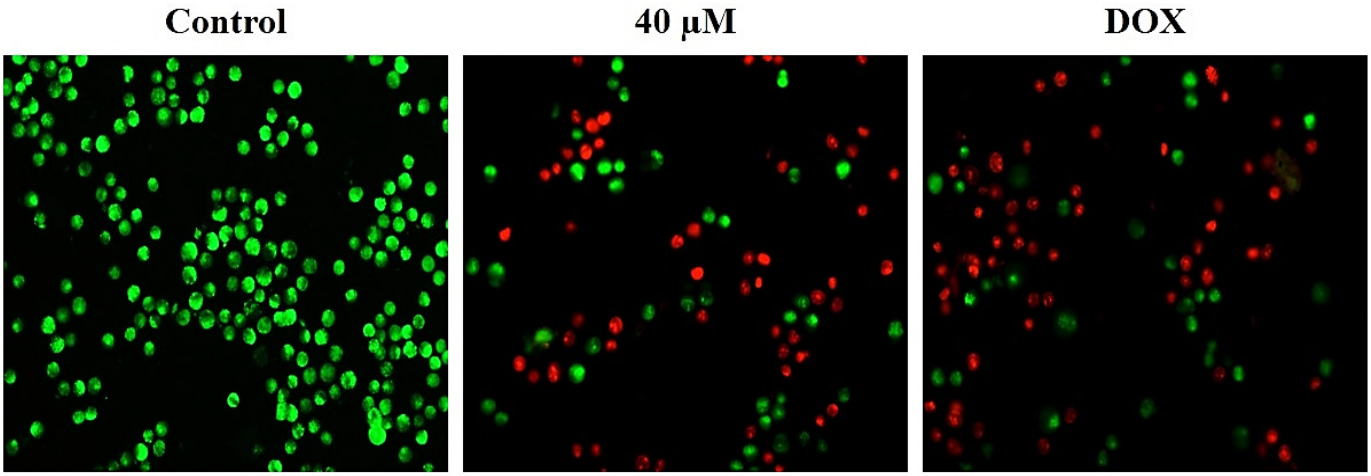

Figure 3: Effect of polydatin on the apoptosis in thyroid cancer TPC-I cells. Following treatment with 40 µM/mL of polydatin and/or 2 µg of DOX, the thyroid cancer TPC-I cells displayed increased yellow and orange fluorescence. This observation validated the presence of early and late apoptosis in the polydatin-treated TPC-I cells.

concentrations of polydatin exhibited a notable reduction in the TPC-I cell growth.

The analysis of intracellular ROS production in drug-treated cancer cells is essential for understanding the processes of action and therapeutic applications of various pharmacological agents.[25] Elevated levels of intracellular ROS can induce irreversible damage to vital cellular components, resulting in apoptosis. The fluorescent probe DCFH-DA has been extensively utilized to assess the accumulation of endogenous ROS in both normal and tumor cells.[26] Apoptosis plays a crucial role in the pathogenesis and treatment of cancer. Comprehending the mechanisms of apoptosis in tumor cells is crucial for formulating successful therapeutic options. Cancer cells often exhibit defective apoptotic pathways, resulting in unrestrained cell growth and resistance to various anticancer treatments.[27] One approach to studying apoptosis in tumor cells is the utilization of the dual staining assay. This technique, which combines the use of AO and EB, allows to detect and quantify the apoptotic and necrotic cells. The ease of this method and its ability to provide both qualitative and quantitative data on cell death make it a valuable tool in cancer research.[28] The dual staining assay has been widely utilized to analyze the activity of several anticancer drugs on the induction of apoptosis in cancer cell lines. By understanding the balance between cell death and proliferation, researchers can advance insights into the progression of malignant disease and its responsiveness to therapy. Additionally, research has explored the use of targeting apoptotic pathways as a strategy for cancer treatment.[29,30] By comprehending the molecular mechanisms underlying apoptosis in cancer cells, researchers can develop novel therapeutic targets and advance more potential anticancer

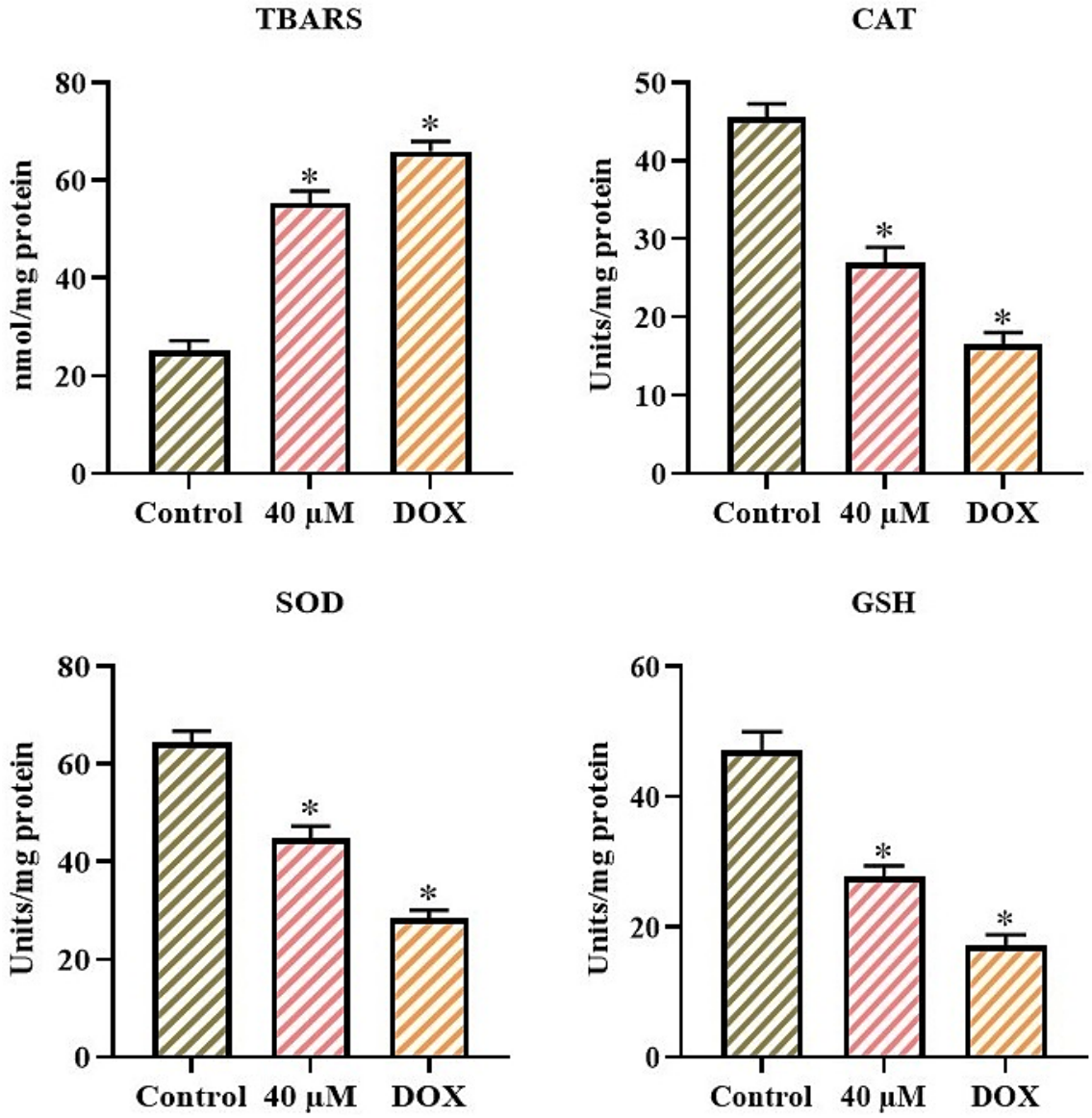

Figure 4: Effect of polydatin on the oxidative stress level in the TPC-I cells. The findings are illustrated as the mean±SD of three individual experiments. The data are assessed using one-way ANOVA and Tukey's *post hoc* analysis using GraphPad Prism software. An asterisk '*' specifies that the values are significant at $p<0.05$ from the control group.

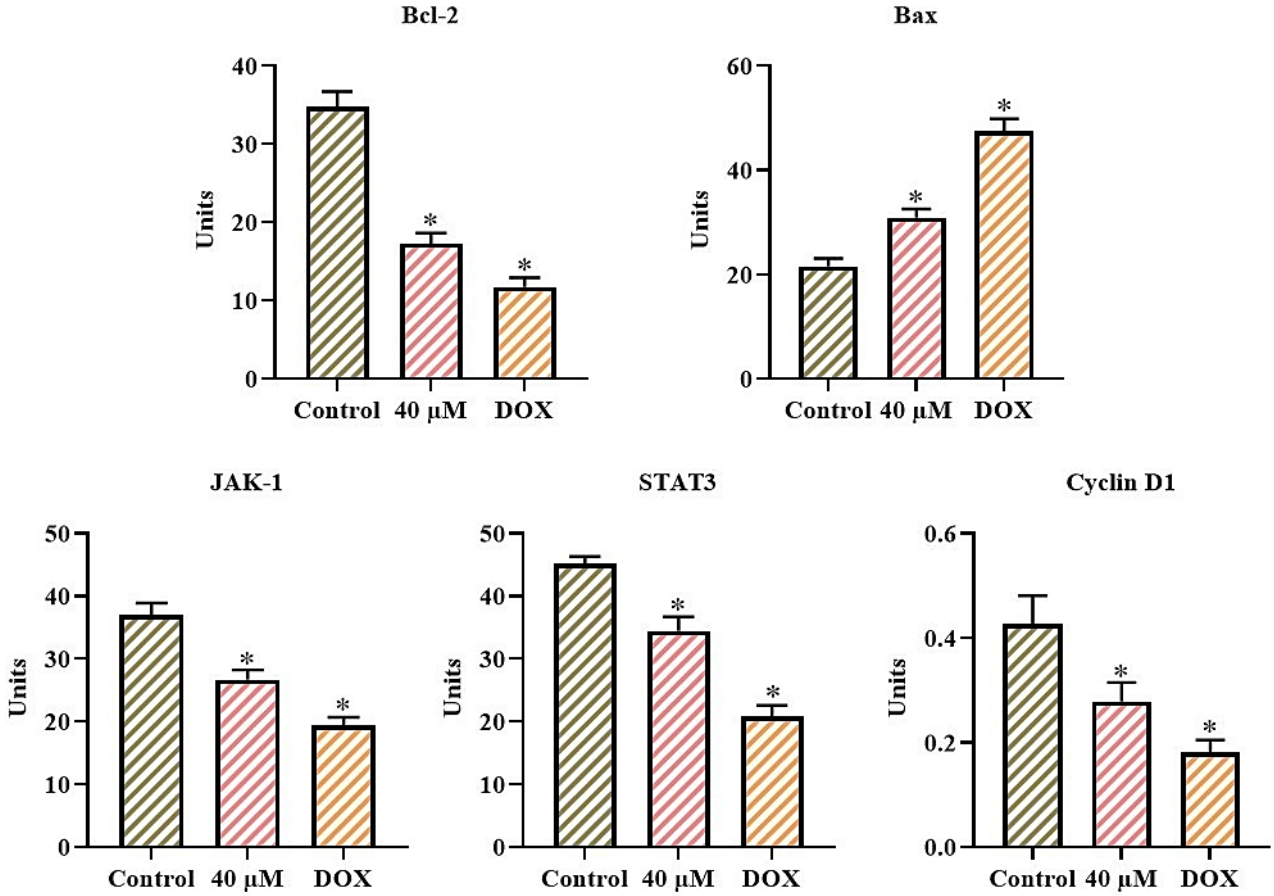

Figure 5: Effect of polydatin on apoptotic and cell cycle protein levels in the TPC-I cells. The findings are illustrated as the mean±SD of three individual experiments. The data are assessed using one-way ANOVA and Tukey's *post hoc* analysis using GraphPad Prism software. An asterisk '*' specifies that the values are significant at $p<0.05$ from the control group.

strategies. In this study, the findings of fluorescent staining assays demonstrated that polydatin treatment considerable elevated the endogenous ROS accumulation and increased apoptosis in thyroid cancer TPC-I cells. Supportively, Zheng *et al.*,[31] already mentioned that resveratrol induced apoptosis via increasing ROS accumulation and oxidative-related cellular lesions in THJ-16T cells.

Oxidative stress is a crucial cause in the advancement and progression of cancer, as it can lead to DNA damage, genetic instability, and ultimately, apoptosis in tumor cells. The equilibrium between oxidative and antioxidant markers, such as TBARS, SOD, and GSH, plays a central role in maintaining the cellular redox state and determining the fate of cancer cells.[32] Oxidative stress develops from a disproportion between the generation of ROS and the capacity of cells to neutralize them through antioxidant defense mechanisms. In cancer cells, increased levels of ROS can trigger various signaling cascades, including those participated in cell growth, survival, and metastasis. When redox equilibrium is disrupted, oxidative stress can induce abnormal cell death and facilitate disease progression.[33] The analysis of oxidative and antioxidant indicators, like TBARS, CAT, SOD, and GSH in the drug-treated cancer cells offers useful insights into the cellular metabolic state and the advancement of therapeutic interventions. The assessment of these markers in drug-treated cancer cells can help elucidate the mechanisms by which certain drugs show their anti-cancer activities, potentially through the modulation of the oxidative stress response.[34] Therefore, the investigation of oxidative stress-mediated apoptosis in cancer cells and the analysis of key oxidative and antioxidant markers, is essential to understand the mechanisms underlying the therapeutic potential of various anti-cancer agents. The current findings clearly proved that the polydatin treatment remarkably increased the TABRS levels and reduced the antioxidants SOD, CAT, and GSH levels. These findings further confirmed by the standard drug DOX treatment. Therefore, it was clear that polydatin treatment facilitates oxidative stress-mediated apoptosis in thyroid cancer TPC-I cells.

Apoptosis is an essential mechanism that preserves cellular homeostasis. The Bcl-2 protein family is pivotal in modulating this process, comprising both pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl-2) constituents. Among the pro-apoptotic proteins, Bax is a key player in triggering the mitochondria-dependent apoptotic pathway.[35] Cancer cells frequently demonstrate dysregulation of the apoptotic machinery, enabling them to escape apoptosis and grow continually. The equilibrium between the pro-apoptotic Bax and

the anti-apoptotic Bcl-2 proteins is a crucial factor influencing a cell's destiny. Bax is predominantly found in the cytoplasm of healthy cells; however, when receiving stress signals, it relocates to the mitochondria, facilitating the release of cytochrome c and initiating the apoptotic cascade.[35] In contrast, Bcl-2 localizes to the mitochondrial membrane and inhibits the pro-apoptotic activity of Bax, thus supporting cell survival.[37] Cancer cells often upregulate Bcl-2 expression or downregulate Bax, tipping the equilibrium in favor of cell survival. This mechanism is observed in a variety of cancers, including hematological malignancies. Furthermore, several stress, an inducer of apoptosis, can also lead to the translocation of Bax to the mitochondria and the inactivation of Bcl-2, eventually leading to apoptosis.[38] The importance of the Bax-Bcl-2 balance in tumor growth has led to the investigation of therapeutic methods targeting this axis. Drugs that selectively induce Bax-mediated apoptosis or inhibit Bcl-2 have demonstrated promising results in tumor treatment and are being actively explored for clinical applications.[39,40] These results highlight the importance of targeting the Bax-Bcl-2 axis as a therapeutic strategy to treat tumors. The current results clearly demonstrated that polydatin treatment considerably increased the Bax protein level subsequently reduced the Bcl-2 in the TPC-I cells. These findings suggests that polydatin treatment can induce apoptosis by targeting Bax/Bcl-2 axis in the thyroid cancer cells.

Cancer is a multifaceted disease marked by the unregulated proliferation and dissemination of atypical cells. Several key proteins play essential roles in the advancement of cancer, including cyclin D1, JAK-1, and STAT3. Cyclin D1 is a cell cycle regulatory protein that is essential for the advancement of cells by G1 stage of the cell cycle. Abnormal cyclin D1 expression has been implicated in the development of various types of cancer.[41] Cyclin D1 develops a complex with cyclin-dependent kinases and facilitates the retinoblastoma protein phosphorylation, which in turn allows the cell to develop via cell cycle. In the context of thyroid cancer, cyclin D1 was shown to be deregulated in a significant proportion of cases, contributing to the unrestrained growth of tumor cells.[42] The JAK-STAT cascade is another important player in tumor development. Activation of this pathway, particularly through the JAK-1 and STAT3 proteins, has been observed in several solid tumors.[43] The JAK-STAT signaling is participated in the control of various cellular mechanisms, including cell proliferation, differentiation, and apoptosis. Constitutive stimulation of this signaling can lead to the increased expression of genes participated in cell growth and survival, ultimately participating in cancer growth.[44] Moreover, the analysis of cyclin D1, JAK-1, and STAT3 protein levels in drug-treated cancer cells is crucial for understanding the effectiveness of therapeutic interventions. Drugs that target these proteins or the pathways they are involved in can have a considerable effect on tumor cell proliferation and survival. For instance, drugs that inhibit JAK-STAT pathway have the positive effects to treat several solid tumors.[45] Therefore, the cyclin D1, JAK-1, and STAT3 proteins play essential roles in the advancement of cancer, and the analysis of their concentrations in drug-exposed cancer cells is essential for evaluating the efficacy of therapeutic interventions. In addition, the upregulation of JAK-STAT pathway was already well reported in the thyroid cancer.[46] The present results demonstrated that polydatin treatment led to a considerable diminution in the cyclin D1, JAK, and STAT3 protein concentrations in TPC-I cells. These results are also supported by the findings of standard drug DOX treatment, as it similarly decreased the cyclin D1, JAK, and STAT3 concentrations in the TPC-I cells. Therefore, it was evident that polydatin can inhibit the proliferation and trigger apoptosis in thyroid cancer cells. The previous study reported that fisetin induced apoptosis in TPC-1 cells via inducing oxidative damage and down-regulating JAK 1 and STAT3 expressions.[47] The curcumin also inhibited the viability, migration, and invasion of TPC-1 cells by inhibiting the STAT3 axis. These previous reports supported the anticancer activity of polydatin.[48]

CONCLUSION

This study's findings indicate that polydatin effectively inhibits growth and promotes apoptosis in thyroid cancer TPC-I cells. The treatment with polydatin enhanced ROS production and induced cell death by elevating apoptotic protein levels while inhibiting Bcl-2 expression. Moreover, polydatin treatment considerably decreased cyclin D1, JAK, STAT3, and antioxidant levels and enhanced the TBARS level in TPC-I cells. Consequently, it possesses the capacity to serve as an antitumor candidate to treat thyroid cancer. Further studies are essential to elucidate the exact molecular processes involved in polydatin-induced apoptosis in thyroid cancer cells.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DMEM: Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **MTT:** (3-[4,5-Dimethylthiazol-2-yl]-2,5 Diphenyl Tetrazolium Bromide); **DMSO:** Dimethyl Sulfoxide; **AO/EB:** Acridine Orange/Ethidium Bromide; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **GSH:** Glutathione.

SUMMARY

This work was devoted to explore the anticancer properties of the polydatin against thyroid cancer TPC-I cells. TPC-I cells were tested using the MTT assay, ROS production and incidences of apoptosis was assessed using fluorescence staining assays. The concentrations of oxidative stress indicators were assessed utilizing assay kits. Also, apoptotic and cell cycle proteins were assessed in the control and treated cells with appropriate assay kits. Polydatin effectively inhibits growth and promotes apoptosis in TPC-I cells. The treatment with polydatin enhanced ROS

production and induced cell death by elevating apoptotic protein levels while inhibiting Bcl-2 expression. Moreover, polydatin treatment considerably decreased cyclin D1, JAK, STAT3, and antioxidant levels and enhanced the TBARS level in TPC-I cells. Consequently, it possesses the capacity to serve as an antitumor candidate to treat thyroid cancer.

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