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Original Article

Punicalagin Inhibits Cell Growth and Promotes Apoptosis in Bladder Cancer T24 Cells via Upregulating Oxidative Stress and Apoptotic Marker Expressions

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ABSTRACT

Background: Bladder cancer is a prevalent form of cancer worldwide and is associated with increased rates of mortality. **Objectives:** The present investigation focuses on understanding the inhibitory activities of punicalagin on the viability and promotion of apoptosis in T24 bladder cancer cells. **Materials and Methods:** The punicalagin at diverse concentrations (1-15 µM) was tested for its *in vitro* free radical scavenging effects, including DPPH, superoxide and peroxyl radicals. The effects of punicalagin against the growth of bladder cancer T24 and normal Vero cells were tested using an MTT assay. The endogenous ROS production and apoptosis were tested using fluorescent staining methods. The tryphan blue staining was done to examine the cell adhesion and viability of the cells. The oxidative stress markers and apoptotic protein expression levels were assayed using kits. **Results:** The results of the free radical scavenging assays revealed the effective antioxidant effects of the punicalagin, which reduced the DPPH, superoxide and peroxyl radicals. The treatment with diverse doses of punicalagin effectively inhibited the T24 cell growth while not disturbing the non-malignant Vero cell growth. Punicalagin effectively increased endogenous ROS accumulation and apoptosis in the T24 cells. The cell adhesion and growth was effectively reduced by the punicalagin treatment. It also increased the levels of oxidative stress markers and promoted apoptotic protein expression in the T24 bladder cancer cells. The outcomes of the punicalagin treatment were supported by the findings of the standard drug DOX treatment. **Conclusion:** Altogether, the current exploration discovered that punicalagin has anticancer properties that inhibit viability and promote apoptosis in T24 bladder cancer cells via upregulating oxidative stress and apoptotic protein expressions. Therefore, the current results suggest the punicalagin as a promising and candidate for bladder cancer treatment.

Keywords: Apoptosis, Oxidative stress, Caspases, Punicalagin, Bladder cancer.

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INTRODUCTION

Bladder cancer is a widespread cancer worldwide and is associated with increased rates of morbidity and mortality. Based on predictive characteristics, bladder cancer is categorized into two types: non-muscle-invasive and muscle-invasive bladder cancers.[1] From a clinical perspective, 70% of patients first receive a diagnosis of non-muscle invasive bladder cancer. Nevertheless, high-risk individuals who undergo transurethral resection will experience a recurrence and acquire non-muscle invasive bladder cancer.[2] Bladder cancer is a very destructive cancer type that impacts more than 500,000 people globally and causes over 200,000 deaths each year.[3] Patients with urothelial carcinoma have various treatment options, including surgery, chemotherapies and immunotherapy. However, the development of resistance and adverse effects pose challenges for long-term treatment, which may result in more severe adverse effects.[4,5] Furthermore, the requirement for continuous monitoring, high disease recurrence and therapy resistance. Bladder cancer is associated with the most expensive lifelong treatment expenses.[6] Hence, there is a requirement for novel, efficient, targeted and cost-effective therapy strategies for this type of cancer.

Apoptosis is a programmed cell death mechanism that has been shown to act as a protective barrier against the progression of cancer. Apoptosis is a fundamental mechanism in the regulation of tissue homeostasis and its stimulation is important for the effects of anticancer medicines. Thus, apoptotic resistance is an imperative stage in the onset of cancers and resistance to treatment.[7] When cancer cells undergo their metastatic transition, apoptotic signaling pathways often become dysregulated. Apoptosis is also defective in bladder cancer cells, which contributes to tumor progression and invasion.[8] In order to lessen the invasiveness

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of bladder cancer tumors, it has been suggested that inducing apoptosis in these cells could be an effective therapeutic approach.

Because of its diffuse and infiltrative nature, bladder cancer has remarkable cell proliferation, invasion and migratory capabilities, which reduces the effectiveness of clinical treatments and the possibility of its successful removal by surgery.[9] Discovering the cellular and molecular pathways that drive bladder cancer development and invasion will lead to novel therapeutic approaches. Therefore, new compounds that can inhibit bladder cancer development are highly needed. Punicalagin is the richest and most active hydrolysable tannin compound extracted from the pomegranate. It is well known that punicalagin exhibits antioxidant,[10] antidiabetic,[11] antidiabetic nephropathy,[12] antibacterial,[13] and antiviral[14] activities. Punicalagin also exhibited antiarthritic,[15] nephroprotective,[16] and immune-suppressive[17] activities. Several previous studies already highlighted that the punicalagin also promoted apoptosis in several tumor cells, including lung cancer,[18] leukemia,[19] colon cancer,[20] cervical cancer,[21] breast cancer,[22] and glioma.[23] However, the therapeutic properties of punicalagin against bladder cancer was not reported yet. Therefore, the present investigation focuses on understanding the inhibitory properties of punicalagin on the viability and promotion of apoptosis in bladder cancer T24 cells.

MATERIALS AND METHODS

Chemicals

The following chemicals and reagents, including punicalagin, Fetal-Bovine Serum (FBS), antibiotics, etc., were attained from Sigma-Aldrich, USA. To assay the biochemical markers, the corresponding kits were purchased from Abcam, USA.

Ferric-Reducing Antioxidant Power (FRAP) assay

The FRAP analysis was executed using the earlier protocol that was previously established.[24] The diverse dosages of punicalagin (1, 2.5, 5, 7.5, 10, 12.5, and 15 µM) were added along with 1 mL of FRAP solution, acetate buffer (300 mM), TPTZ reagent (10 mM) and ferric chloride (20 mM) solution. The reaction solution (200 µL) was placed on a 96-wellplate and incubated for 10 min. Later in the incubation, the final product developed in the reaction solution was investigated at a 593 nm wavelength.

DPPH activity

The influence of punicalagin on the scavenging of DPPH radicals was investigated using the protocol established earlier.[25] The 150 µL of DPPH reagent was dissolved in 0.25 mM ethanol and added to various dosages of punicalagin (1-15 µM) for 30 min at 37°C. Later, the absorbance was measured at the 515 nm wavelength.

Chemiluminescence (CL) assay

The impacts of punicalagin on the scavenging of superoxide radicals were assessed by the protocols that were previously established.[26] The 10 µL of CL reagent was mixed to the diverse dosages (1-15 µM) of punicalagin and xanthine oxidase (80 µL) solution. The HEPES buffer was utilized to make the control solution. Later, the solution was studied using a luminometer after loading 200 µL of hypoxanthine solution (0.72 mM). The scavenging effect was studied for 10 min at 10-s intervals using a luminometer.

Analysis of Oxygen Radical Absorbance Capacity (ORAC)

The ORAC capacity of the punicalagin was investigated by assaying the peroxyl radical scavenging ability using the corresponding kit using the protocols elaborated by the kit's manufacturer (Abcam, USA).

Maintenance of cell culture

The bladder cancer T24 cells were acquired from ATCC, USA and cultivated on DMEM medium with 10% FBS in a CO_2 (5%)-provided incubator. The grown cells were obtained once they reached 80% confluency and employed for the additional treatment, fluorescent staining and biochemical examinations.

MTT assay

The control and punicalagin-treated T24 cell growth was investigated by MTT assay. On a 96-well plate, the cells were cultivated for 24 hr and then treated with the punicalagin at diverse concentrations (1, 2, 5, 7.5, 10, 12.5, and 15 µM) for 24 hr. Following the treatment, MTT (20 µL) solution and DMEM (100 µL) were mixed into the wells for 4 hr. the absorbance was measured at 570 nm after the developed formazan depositions were dissolved using DMSO (100 µL).

Dual staining

The dual staining assay was executed on the control and punicalagin-treated T24 cells to examine the apoptosis. On a 24-wellplate, the T24 cells were cultivated for 24 hr and exposed to 10 µM punicalagin and 2 µg of Doxorubicin (DOX) for 24 hr. Then, AO/EB (100 µg/mL) stains were mixed into the wells and incubated for 5 min in the dark to examine the apoptosis in T24 cells using the fluorescent microscope.

DCFH-DA staining

The influence of punicalagin on the accumulation of ROS in non-treated and punicalagin-exposed T24 cells was assessed using DCFH-DA staining. On a 24-wellplate, the cells were cultivated and treated with punicalagin at a 10 µM concentration and 2 µg of DOX for 24 hr. Later, the 10 µL DCFH-DA dye was added into the wells for 10 min and then the fluorescence was assessed using a fluorescence microscope.

Cell adhesion assay

The cell adhesion and viability levels of punicalagin-exposed T24 cells were examined. After culturing the T24 cells in a gelatin-coated culture plate for 24 hr, cells were treated with 10 µM of punicalagin and 2 µg of DOX for 60 min at 37°C. Then cells were rinsed with saline and stained with trypha blue to detect adhesive and viable cells, which were analyzed using an optical microscope.

Assay of oxidative stress marker levels

The control and punicalagin-exposed T24 cells were trypsinized and cell lysate was prepared using cell lysis buffer to assay the oxidative stress markers. The contents of TBARS, SOD and GSH were assayed using corresponding kits by the protocols specified by the manufacturer (Abcam, USA).

Assay of caspases and Bax/Bcl-2 levels

The caspase-3, -9, Bax and Bcl-2 expression levels were detected in the control and punicalagin-exposed T24 cells using the assay kits using the procedures specified by the manufacturer (Abcam, USA).

Statistical analysis

The statistical analyses were done using the SPSS software and the results are given as the mean±SD of triplicates. To examine the data, one-way ANOVA and DMRT tests were used, with a $p<0.05$ as a significant.

RESULTS

Effect of punicalagin on the *in vitro* scavenging of free radicals

Several free radical scavenging tests were performed to examine the antioxidant potentials of punicalagin *in vitro* and the outcomes are exhibited in Figure 1. Punicalagin at varying concentrations significantly reduced free radical levels. Punicalagin, at concentrations of 1-15 µM, significantly inhibited the production of various free radicals, including DPPH, superoxide and peroxyl radicals. Punicalagin's ability to scavenge free radicals *in vitro* was demonstrated by a decrease in the concentrations of DPPH, superoxide and peroxyl radicals in response to treatment with increased concentrations of punicalagin (Figure 1). These results provide further evidence that punicalagin has excellent antioxidant properties.

Punicalagin decreases the viability of T24 cells and not affected the normal Vero cell viability

Figure 2 displays the MTT cytotoxicity assay results for punicalagin's effect on the T24 and Vero cell growth. The viability of T24 cells was remarkably decreased by punicalagin at a range of concentrations (1-15 µM). The vitality of Vero cells was not affected by punicalagin treatment at the same concentrations (1-15 µM). The increasing pinucalagin concentration marginally suppressed T24 cell growth (Figure 2). The IC_{50} concentration of punicalagin was fixed at 10 µM against T24 cells and the same dose was selected for further investigations.

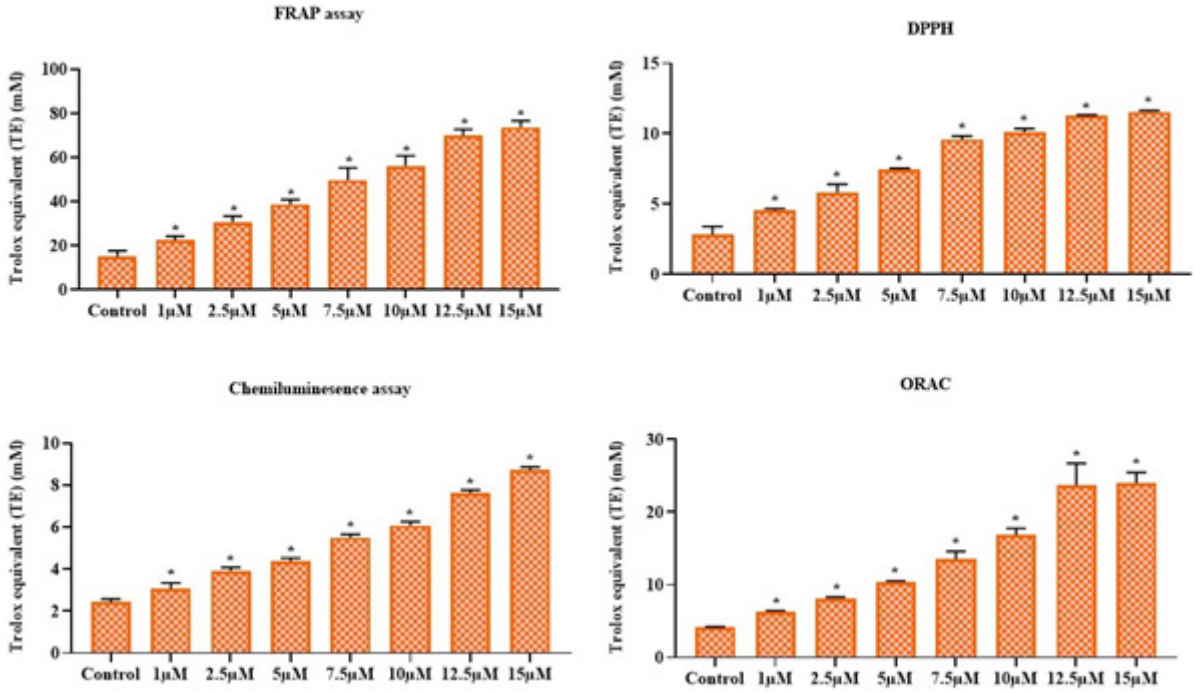

Figure 1: Effect of punicalagin on the *in vitro* scavenging of free radicals. The different concentrations of punicalagin (1-15 µM) effectively scavenged the various free radicals, including ferric radicals, DPPH radicals, superoxide radicals and peroxide radicals. The results are presented as a mean±SD of triplicate values. The values are analyzed by one-way ANOVA and Tukey's *post-hoc* test using SPSS software. '*' indicates the significance at $p<0.05$ from the control group.

Punicalagin increases the intracellular ROS production in the T24 cells

The impacts of punicalagin treatment on the ROS production was assessed in T24 cells and the results are presented in Figure 3. The T24 cells displayed a significant increase in green fluorescence following treatment with 10 µM of punicalagin when compared to control. These increased fluorescence evidence the more accumulation ROS production in the punicalagin-exposed T24 cells. The DOX treatment also exhibited higher green fluorescence, which evidences the increment in endogenous ROS accumulation.

Punicalagin increases the apoptosis in the T24 cells

The dual staining was executed to measure the apoptosis in the control and punicalagin-exposed T24 cells and the results are presented in Figure 4. The T24 cells demonstrated higher orange/yellow fluorescence following exposure to 10 µM of punicalagin, which proves the onset of both apoptosis. The DOX treatment also revealed more cells with intense orange/yellow fluorescence, which confirms the onset of apoptosis (Figure 4).

Punicalagin decreases the cell adhesion and viability of T24 cells

Trypan blue staining was used to determine how punicalagin treatment affected bladder cancer T24 cell adhesion and viability and the findings are depicted in Figure 5. A lower number of stained cells indicated that the untreated control cells contained more viable cells. A higher number of stained cells, indicative of greater cell death caused by punicalagin treatment, was seen in T24 cells, which are exposed to 10 µM of punicalagin. The DOX treatment also caused high cell death and reduced cell adhesion in T24 cells.

Punicalagin promotes the oxidative stress in the bladder cancer T24 cells

Figure 6 shows the results of measuring the TBARS, GSH and SOD levels in the control and punicalagin-exposed T24 cells. Higher levels of GSH and SOD, while reduced TBARS was noted in the control cells. Meanwhile, bladder cancer T24 cells exposed to 10 µM of punicalagin showed markedly enhanced TABRS levels and reduced GSH and SOD levels compared to control (Figure 6). The DOX also promoted the TBARS level while reducing the SOD and GSH in the T24 cells. Based on these results, it appears that punicalagin induces oxidative stress in T24 bladder cancer cells.

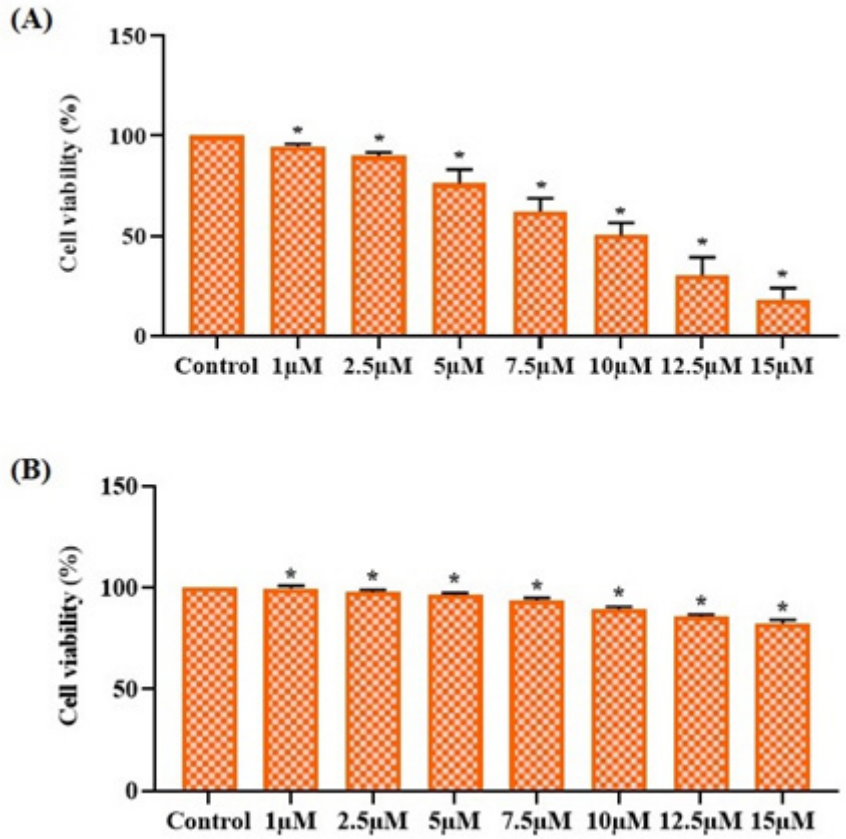

Figure 2: Effect of punicalagin on the viability of bladder cancer T24 and normal Vero cells. The results are presented as a mean±SD of triplicate values. The values are analyzed by one-way ANOVA and Tukey's *posthoc* test using SPSS software. '*' indicates the significance at $p<0.05$ from the control group. (A): T24 bladder cancer cells; (B): Non-malignant Vero cells.

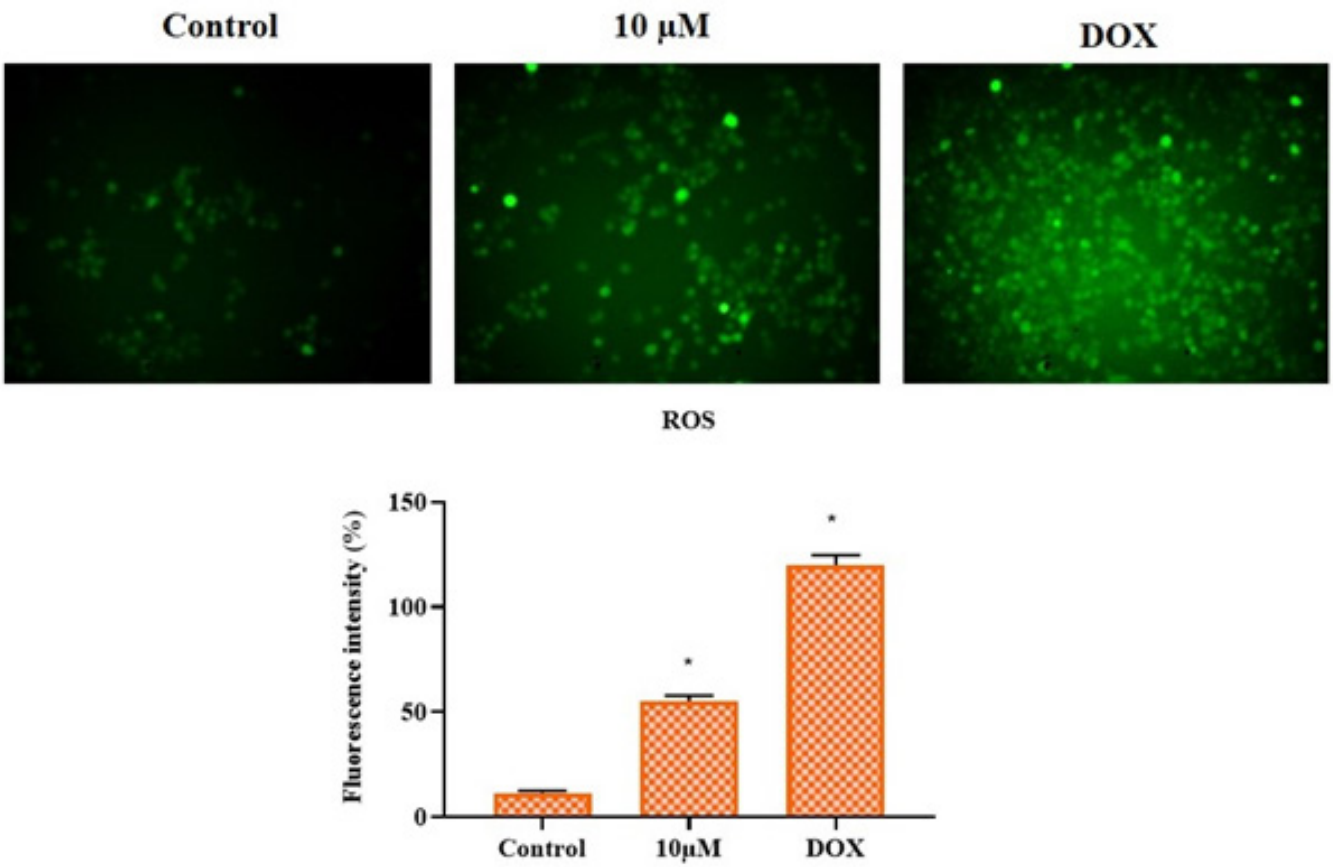

Figure 3: Effect of punicalagin on the intracellular ROS production in the T24 cells. The T24 cells treated with 10 µM of punicalagin showed increased green fluorescence compared to the control, which evidences higher endogenous ROS production.

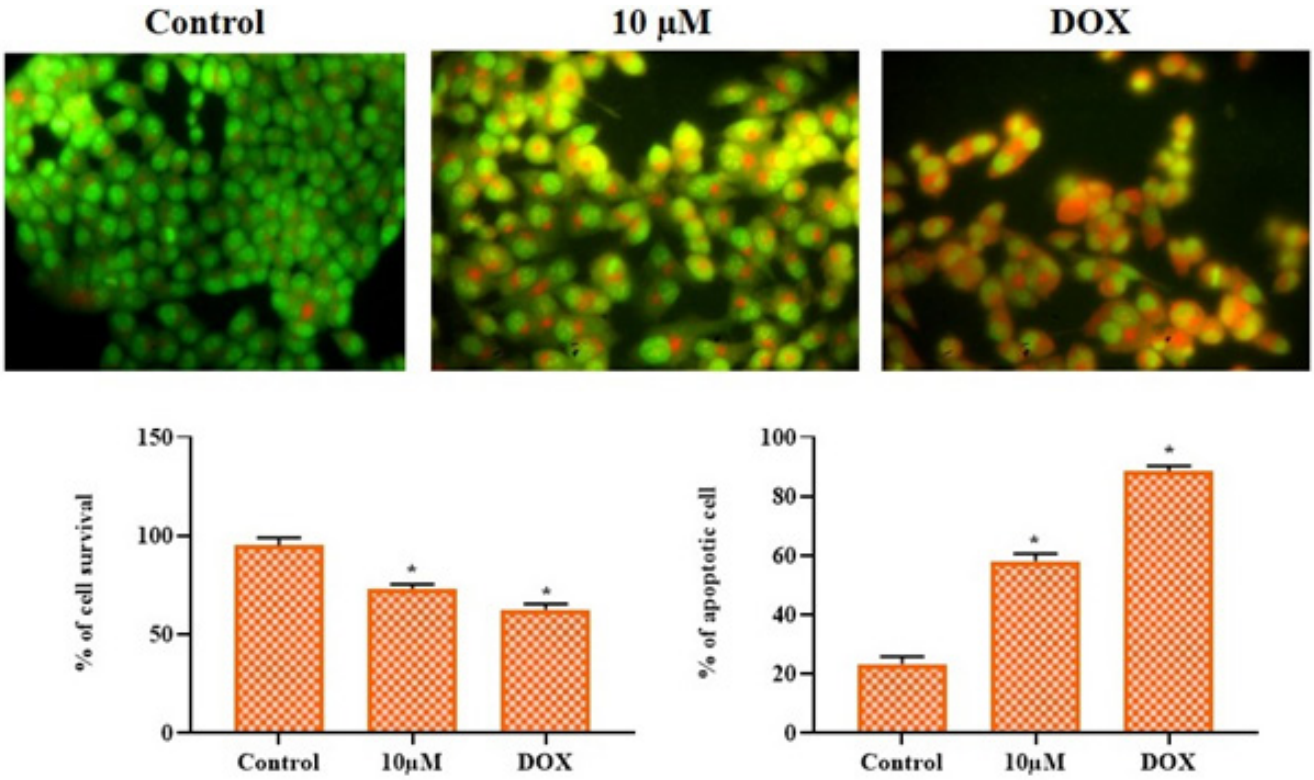

Figure 4: Effect of punicalagin on the apoptotic cell death in the bladder cancer T24 cells. The T24 bladder cancer cells produced more yellow and orange fluoresced cells after the treatment with 10 µM of punicalagin, which confirms the occurrence of early and late apoptosis in the T24 cells.

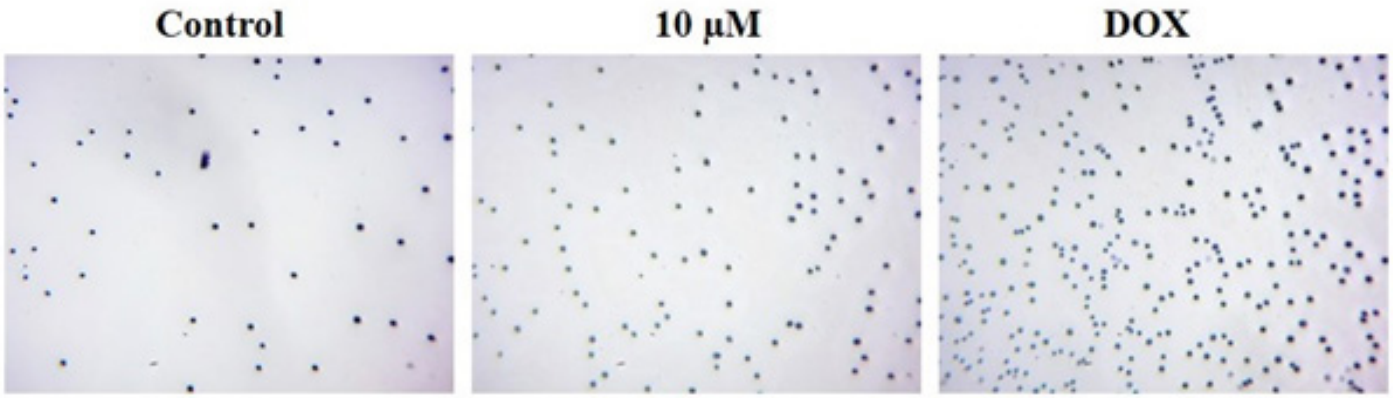

Figure 5: Effect of punicalagin on the cell adhesion and viability of T24 cells. The T24 bladder cancer cells resulted in decreased cell adhesion and viability after treatment with 10 µM of punicalagin.

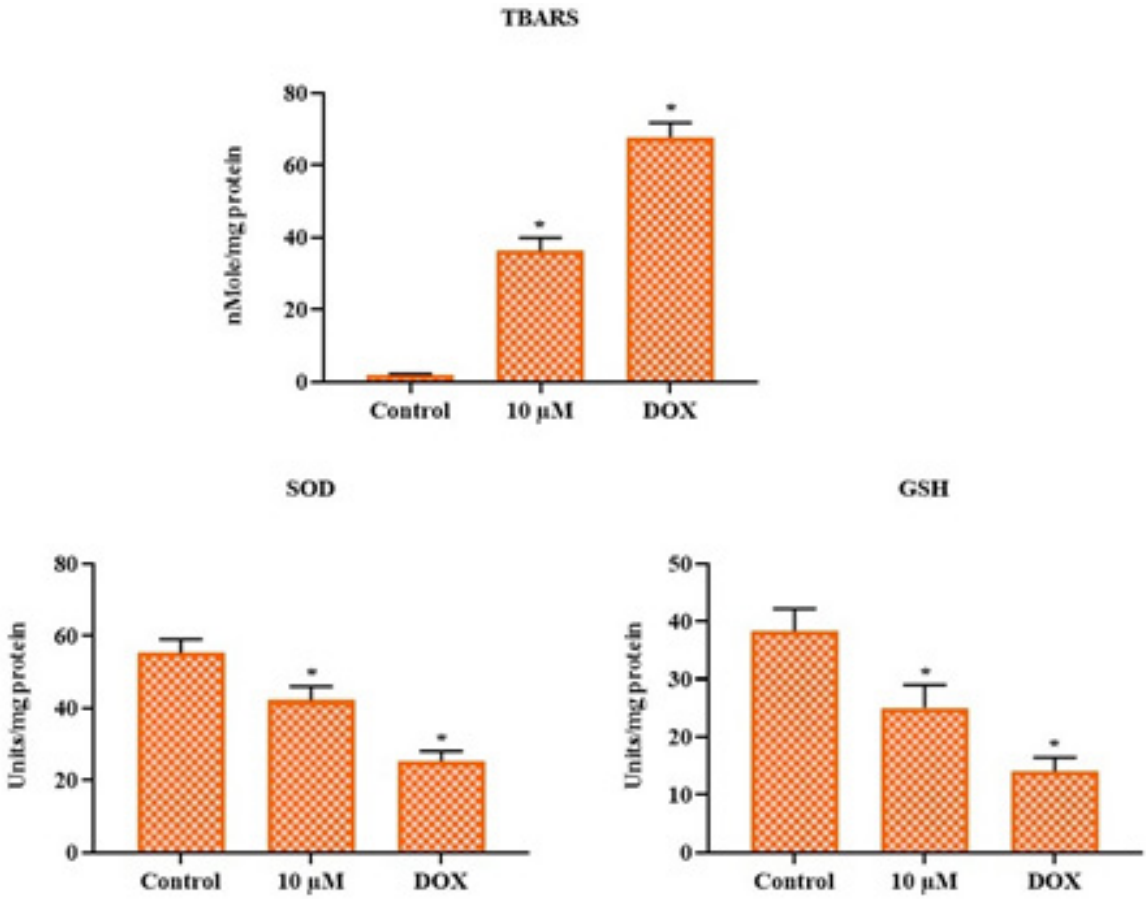

Figure 6: Effect of punicalagin on the oxidative stress marker levels in the bladder cancer T24 cells. The results are presented as a mean±SD of triplicate values. The values are analyzed by one-way ANOVA and Tukey's *post hoc* test using SPSS software. '*' indicates the significance at $p<0.05$ from the control group.

Punicalagin increases apoptotic protein expressions in the bladder cancer T24 cells

The control and punicalagin-treated T24 bladder cancer cells were examined for their apoptotic protein expressions and the findings were revealed in Figure 7. The Bax, caspase-3 and -9 expressions were reduced and Bcl-2 expression was increased in the control cells. However, the treatment of bladder cancer T24 cells with 10 µM punicalagin considerably increased Bax, caspase-3 and -9 activities, while Bcl-2 was reduced when compared to control. These outcomes were supported by the findings of the DOX treatment, which also increased apoptotic protein expression in the T24 cells. Therefore, it was evident that punicalagin promotes apoptosis in the T24 bladder cancer cells.

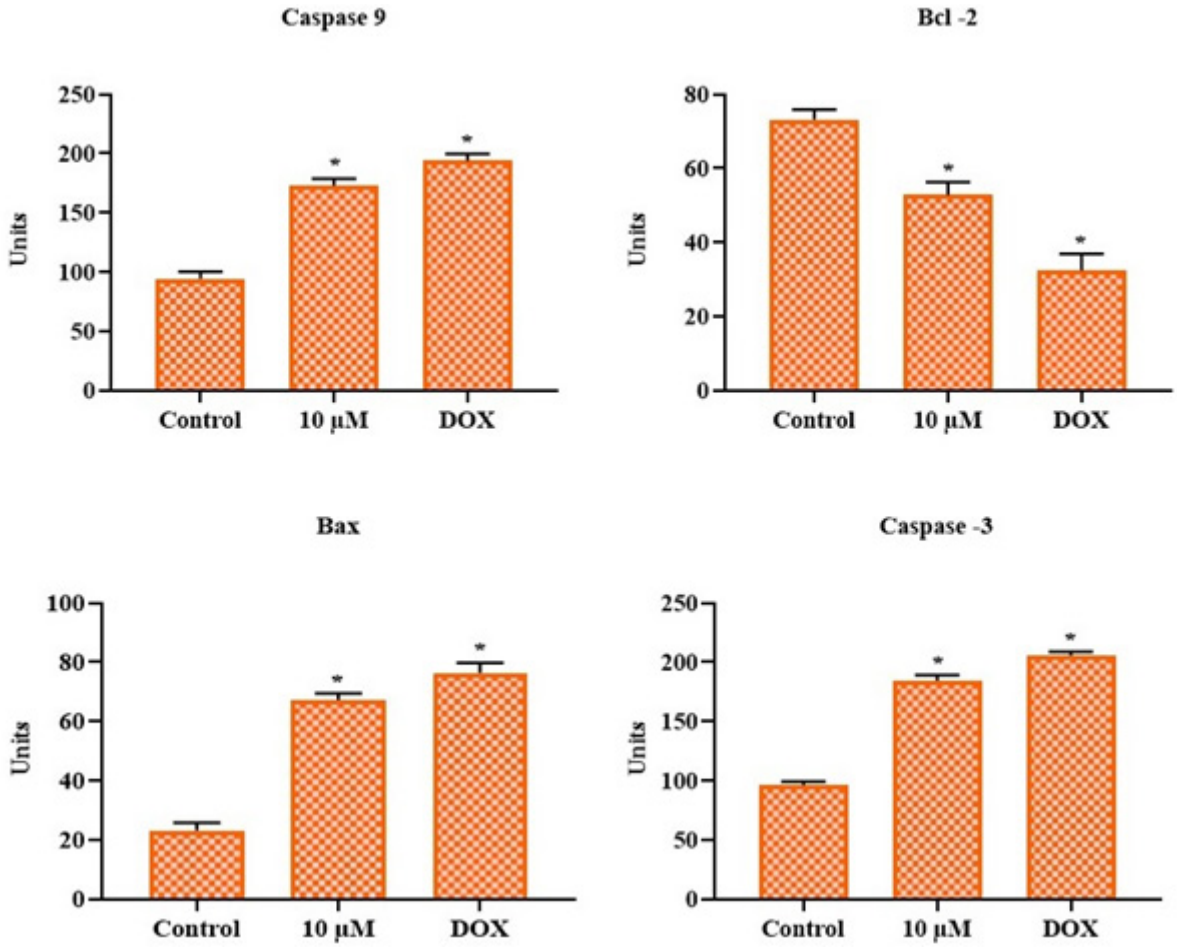

Figure 7: Effect of punicalagin on the apoptotic protein expressions in the bladder cancer T24 cells. The results are presented as a mean±SD of triplicate values. The values are analyzed by one-way ANOVA and Tukey's *post hoc* test using SPSS software. '*' indicates the significance at $p<0.05$ from the control group.

DISCUSSION

Tumor recurrence was frequently observed in the bladder cancer patients after therapy. Distant metastases and cancers that have migrated to neighboring organs present the greatest challenges to treatment.[27] Cell division and DNA replication are blocked by radio chemotherapy. Despite the fact that this treatment does inhibit bladder cancer cell proliferation and cause the cells to undergo senescence, bladder cancer are resistant to it since they can recover their viability after being damaged. Potentially useful chemotherapy for the treatment of bladder cancer exists, with some medications being able to decrease cancer cell growth by inducing cell death or preventing the self-renewal of bladder cancer stem cells.[28]

High rates of cell growth, invasion and extreme resistance to apoptosis have all been linked to the widespread resistance of bladder cancer to several therapies.[29] This migration, which often occurs within 1-2 cm of the original tumor mass, makes it difficult, if not impossible, to remove the entire tumor during surgery and leads to a high probability of tumor recurrence afterwards.[30] Therefore, it is essential to investigate the causes of bladder cancer and find effective treatments for the disease. The current exploration was aimed to investigate the anticancer potentials of punicalagin against bladder cancer T24 cells. In this study, the impacts of punicalagin against the bladder cancer T24 and normal Vero cells was assessed. The results clearly showed that punicalagin effectively reduced the growth of T24 cells while not disturbing Vero cell viability. These findings demonstrated that punicalagin was effective in inhibiting T24 bladder cancer cell growth.

Apoptosis is a cell death mechanism that serves a central role in tumor progression. It was highlighted that the bladder cancer cells can overcome apoptosis and survives treatment with conventional and targeted therapies.[31] This suggests that novel strategies aimed at compensatory or resistance mechanisms may be necessary to accomplish a potent anticancer effect. The morphological alterations that occur during apoptosis, including chromatin condensation and DNA destruction,[32] make it a potentially useful target for anticancer therapy. Inducing tumor cell death, or apoptosis, is a major goal of cancer treatment and a lack of apoptosis is often given as an explanation for drug resistance. Therefore, apoptosis resistance is an important part

of oncogenesis and treatment resistance. In this work, followed by treatment with punicalagin, T24 cells showed signs of early apoptosis, including nuclear shrinkage, membrane blebbing, cytoplasmic vacuolization and pyknotic nuclei. Indicators of late apoptosis include the presence of an increased number of apoptotic bodies and nuclear fragments were also seen in punicalagin-treated T24 cells.

Cancer cells have high metabolic activity, making them more vulnerable to treatments that raise oxidative stress. Increases in ROS and decreases in antioxidant levels have been shown to be particularly harmful to cancer cells.[33] In cancer cell lines, oxidative damage products have been found at significantly elevated levels. Malignant tumor cells also display notable changes in the amounts of ROS-scavenging systems, including GSH and SOD. Increased levels of ROS can lead to imbalances that manifest as oxidative stress. An important function of ROS is to trigger apoptosis in cancer cells. A key mechanism in the study of anticancer medicines is the triggering of apoptosis in tumor cells by upsetting the antioxidant mechanism and encouraging the formation of ROS.[34] The accumulation of ROS, directly involved in controlling mitochondrial processes, has been said to cause cell death through several mechanisms, including cellular injury and cell cycle arrest brought on by oxidative DNA damage. Anticancer medications frequently upregulate ROS levels in bladder cancer cells, which in turn triggers cell signaling and even cell death. Overactivation of oncogenes and aberrant metabolism lead to an increase in endogenous ROS in cancer cells.[35] Therefore, cancer cells are more susceptible to ROS because they must endure an environment with more oxidative stress.

Overproduction of ROS causes oxidative stress, which in turn damages macromolecules, triggers various signaling cascades and ultimately results in cell death via apoptosis.[36] Cancer cells express large amounts of antioxidants to maintain pro-tumorigenic genes and apoptotic resistance restore redox balance and suppress ROS production.[37] The redox equilibrium of cancer cells is often maintained by a more robust antioxidant defense system and a greater basal level of ROS than in non-malignant cells. Consequently, the formation of excessive ROS is a direct cause of cell death brought on by some ROS-inducing anticancer drugs.[38] In line with the above statement, the results of the present investigation evidenced that the punicalagin treatment effectively promoted ROS accumulation in T24 bladder cancer cells. In addition, punicalagin also improved the TBARS level while decreasing the SOD and GSH in the bladder cancer T24 cells. These findings supported the hypothesis that punicalagin promotes oxidative stress and thereby facilitates apoptosis in T24 bladder cancer cells.

Tumor development and treatment resistance are both influenced by apoptosis. Previous research has linked apoptosis to the onset and progression of bladder cancer.[39] Both Bcl-2 and Bax, both members of the Bcl-2 family, play key functions in controlling apoptosis. The pro-apoptotic Bax promotes apoptosis, while the anti-apoptotic Bcl-2 suppresses apoptosis. Overexpression of Bcl-2 is common in cancer cells, allowing them to survive and multiply by avoiding apoptosis. Reduced expression of Bax in cancer cells is one factor that can contribute to apoptosis resistance.[40] Caspase-9 is the primary regulator of mitochondrial-mediated apoptosis, while caspase-3 is widely regarded as the most critical regulator of apoptosis overall. The activation of caspase-9 occurs in early apoptosis.[41] The decreased activation of caspase-3 contributes to apoptotic resistance in bladder cancer cells and promotes cancer growth.[42] It is well known that the ratio of Bax/Bcl-2 proteins controls apoptosis and caspase-3 in bladder cancer cells. Bcl-2 enhances cell survival by blocking caspase-3 activation, while pro-apoptotic Bax encourages caspase-3 activation and apoptosis. An imbalance caused by the disruption of Bcl-2 and Bax can reduce caspase-3 activity and aid in apoptosis resistance. Therapeutic strategies for bladder cancer have been proposed that target Bcl-2 and/or activate Bax expression.[43] Modulating Bax/Bcl-2 expression and reviving caspase-3 activity in bladder cancer cells has shown promise in preclinical investigations.[44] Chemotherapeutic drugs can activate Bax expression and other pro-apoptotic genes.[45] The activation of caspases and Bax genes was said to decrease bladder cancer tumor invasion and the number of malignant glial cells.[46] Similarly, the results of the present study also highlighted that the punicalagin treatment effectively enhanced the Bax, caspase-3 and -9 expressions while reducing the Bcl-2 expression in the T24 bladder cancer cells. These outcomes supported the apoptosis-inducing potential of the punicalagin on the bladder cancer T24 cells. The anticancer activities of punicalagin on the bladder cancer T24 cells were also supported by the results of the standard drug DOX treatment.

CONCLUSION

Altogether, the current exploration discovered that punicalagin has anticancer properties against the bladder cancer, inhibiting viability and promoting apoptosis in the T24 cells via upregulating oxidative stress and apoptotic protein expressions. The punicalagin treatment considerably inhibited cell viability, increased endogenous ROS production, promoted apoptosis, increased oxidative stress markers and upregulated the apoptotic markers in the T24 bladder cancer cells. Therefore, the current results suggest the punicalagin as a promising drug candidate for bladder cancer treatment in the future. Moreover, further studies are highly recommended in the future to fully comprehend the precise therapeutic roles of the anticancer effects of punicalagin against bladder cancer.

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ABBREVIATIONS

FRAP: Ferric-reducing antioxidant power; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **CL:** Chemiluminescence**; ORAC:** Oxygen radical absorbance capacity.

SUMMARY

High rates of cell growth, invasion and extreme resistance to apoptosis have all been linked to the widespread resistance of bladder cancer to several therapies. Cancer cells have high metabolic activity, making them more vulnerable to treatments that raise oxidative stress. The punicalagin treatment considerably inhibited cell viability, increased endogenous ROS production, promoted apoptosis, increased oxidative stress markers and upregulated the apoptotic protein expressions in the T24 bladder cancer cells.

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