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Original Article

Calycosin Inhibits Cell Proliferation and Induces Apoptosis in Bladder Cancer T24 Cells

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ABSTRACT

Aim/Background: Calycosin is a primary glycoside derived from the Chinese medicinal plant *Radix astragali* (RA). The compound has been demonstrated to suppress cell growth and trigger programmed apoptosis in various cancer cell lines. The current investigation was aimed to examine the impact of Calycosin on the proliferation and death of the bladder cancer cell line and its underlying mechanism. **Materials and Methods:** The present work aimed to investigate the effects of Calycosin on the rate of cell growth, oxidative stress and apoptosis in the T24 human bladder cancer cell line. The viability of Calycosin-treated T24 cells were studied by MTT assay. The apoptosis and ROS production in T24 cells were assessed using fluorescent staining assays. The levels of biochemical markers in the Calycosin-treated T24 cells were examined using kits. **Results:** Results indicated that exposure to BPs could enhance the generation of Reactive Oxygen Species (ROS) and reduce the levels of Superoxide Dismutase (SOD) and Glutathione (GSH) and elevate the TBA Reactive Species (TBARS). **Conclusion:** These results indicate that Calycosin may have chemopreventive potential.

Keywords: Apoptosis, Bladder Cancer, Calycosin, Cell Proliferation, Oxidative Stress.

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INTRODUCTION

Bladder Cancer (BC) causes the mortality of 18,000 individuals annually in the United States.[1] If left untreated; the survival rate for patients is just 15% within two years. The European Association of Urology (EAU) ranks bladder cancer as the ninth most frequently diagnosed cancer globally.[2] In 2020, the American Cancer Society (ACS) documented 80,520 new instances of bladder cancer, with around 61,700 occurring in men and 18,770 in women.[3] There are three phases of superficial: pT1 (high-grade urothelial cancer infiltrating the lamina propria), pTa (papillary urothelial carcinoma) stage, pTis (flat carcinoma *in situ*) stage, Non-Muscle Invasive Bladder Cancer (NMIBC): and stage, where almost 70% of BC cases are categorized.[4] However, despite the positive outlook linked to NMIBC tumors, half of the patients will experience a repetition of the disease four years after their first diagnosis and an estimated 10% will advance to Muscle-Invasive Bladder Cancer (MIBC).[5] The primary therapy for high-grade Non-Muscle Inoperable Bladder Cancer (NMIBC) is surgery using Transurethral Resection of Bladder Tumor (TURBT) along with Bacillus-Calmette-Guerin (BCG).[6] The scarcity of therapeutic and pharmacological alternatives for the management of bladder cancer, along with the significant death rate, necessitates the creation of novel instruments for bladder cancer treatment. Thus, technology offers a unique approach to developing tools to achieve more effective and less cytotoxic therapies.[7,8]

The finding of natural substances with anticancer properties has gained significant interest in recent years. *Radix astragali* (RA) is a naturally occurring substance with over 100 chemical constituents.[9] Calycosin, the primary compound from Rheumatoid Arthritis (RA), has several biological properties, including antibacterial, anti-inflammatory and antiviral actions. Calycosin triggers apoptosis in osteosarcoma cells by increasing the expression of Apaf-1 and cleaved caspase-3 proteins in tumor models.[10,11] Preliminary investigations have shown that induced apoptosis is a significant toxicity mechanism of EDCs.[12] The role of Reactive Oxygen Species (ROS) produced by the environmental milieu in cell death can be validated using ROS scavengers.[13] Prolonged exposure to ROS can decrease the Mitochondrial Membrane Potential (MMP), alter the permeability of the MMP

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and ultimately trigger cell death by releasing apoptotic factors.[14] Prior research demonstrated that BPA, BPS and BPB stimulated the generation of ROS and cellular death in a hippocampus cell line.[15]

The recent surge in research interest in traditional Chinese medicines can be attributed to their remarkable efficacy, diverse pharmacological actions and minimal adverse effects on cancer cells.[16] Phytoestrogens, including isoflavones and lignans, are intrinsic non-steroid substances that share structural and functional similarities with estrogen in mammals. They are identified as either estrogenic agonists or antagonists by their ability to bind to estrogen receptors.[17] Calycosin, a functional phytoestrogen isoflavone with the chemical formula 7,3′-dihydroxy-4′-methoxyisoflavone, is the primary bioactive component extracted from *Radix astragali*, a widely used herbal remedy in traditional Chinese medicine.[18] Experimental evidence has demonstrated that Calycosin exhibits several pharmacological characteristics, including hypolipemic, anti-inflammatory, anti-oxidative, neuroprotection, cytoprotection and hypoglycemic actions.[19] Notably, there is extensive documentation indicating that Calycosin has a suppressive impact on several forms of cancer, including hepatocellular carcinoma, osteosarcoma and breast cancer.[20-22] Yet, Calycosin's anticancer properties and underlying mechanism of bladder cancer have not been investigated. Our work aimed to examine the inhibitory impact of Calycosin on the proliferation of the T24 bladder cancer cells and to gain a comprehensive understanding of the processes underlying this effect.

MATERIALS AND METHODS

Chemicals

Unless noted in the procedure, all chemicals were purchased from commercial suppliers and used without further purification. All aqueous solutions were used to ultrapure water from a Milli-Q system. Calycosin and ROS assay kits were obtained from AK Scientific. Dimethyl Sulfoxide (DMSO) and MTT assay kit were purchased from Bio Basic Inc. DMEM cell culture medium was prepared with Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich), 10% (v/v) of fetal bovine serum (FBS, Hyclone) and 1% (v/v) of penicillin/streptomycin (PANBiotech).

Cell line culture and treatment

The human bladder cancer (T24) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Calf Serum (FBS), streptomycin (100 µg/mL), penicillin (100 U/mL) and at 37°C in a humidified atmosphere containing 5% CO_2. All the cells were cultured for 24 hr with proper DMEM medium to treat with the drug formulations for different incubation periods. A stock solution was prepared in Calycosin dissolved in DMSO to maintain the final concentration.

Cell proliferation assay

An MTT colorimetric cell proliferation kit was used to determine cell viability following the manufacturer's guidelines and our previously reported protocols.[23] Briefly, T24 cell lines were planted in a 96-well plate for 24 hr. After further incubation with Calycosin (2.5 to 50 µM/mL) for starting 2 hr in FBS-free DMEM, the resulting cells were washed with PBS twice and incubated for an additional 24 hr in DMEM (resulting in a total treatment lasting 24 hr). Untreated cells were run concurrently as negative controls. After that, 50 µL of MTT reagent solution was added and incubated for 2 hr at 37°C. Finally, the absorbance was measured by using a microplate reader (Bio-Rad 675). All experiments were conducted in triplicate and the OD shown in the following formula was subtracted from the blank value. The cell viability of each sample was calculated as follows, with data representing the mean of three assays with standard deviation.

The measured absorbance values for each treatment were transformed into percentage proliferation. The proliferation data from the MTT experiment was subjected to the resulting equation and was used to calculate the inhibitory concentrations to achieve a 50% decrease in cell growth (IC_{50}). DOX was used as the positive control.

Morphological changes in liver cancer cells

The AO-EtBr technique employs a live and dead stain in which AO diffuses into all live cells. EtBr cannot diffuse across a cell membrane unless it is conceded (leading to necrosis) or the cell is apoptotic. T24 cells were planted in 24-well plates (2×10^3 cells each well) for 24 hr. Later, the cells were cultured with a new medium containing 10 µM of Calycosin. Untreated cells were considered as a positive control group. After 24 hr incubation, all adherent and floating cells were gathered and rinsed thrice with PBS. The apoptotic cells were perceived by fluorescence using an AO-EB Detection Kit. DOX was used as the positive control.[24]

Intercellular ROS generation was measured using a DCFH-DA

One of the significant plant responses to abiotic stress is generating ROS. T24 cells were planted in 24-well plates (2×10^3 cells each well) for 24 hr. Later, the cells were cultured with a new medium containing 10 µM of Calycosin. Untreated cells were considered as a positive control group. After 24 hr incubation, all adherent and floating cells were gathered and rinsed thrice with PBS. The apoptotic cells were perceived by fluorescence using a DCFH-DA Detection Kit. DOX was used as the positive control.[25]

Determination of TBA Reactive Species (TBARS) levels

The amount of lipid-peroxidation membrane products, or TBARS, was examined using the previously reported technique.[26] T24 cells were planted in 6-well plates (2×10^3 cells each well) for

24 hr. Cells were incubated in 3 mL of with or without medium test 10 µM of Calycosin. DOX was used as positive control.

Estimation of Glutathione (GSH)

The Glutathione (GSH) level quantification was performed using Ellman's reagent.[27] The assay mixture comprised phosphate buffer, Dextran Nitrate Buffer (DTNB) and cell extract. The reaction was observed at a wavelength of 413 nm and the quantity of GSH was presented in nmol GSH/mg protein units. DOX was used as positive control.

Assessment of Superoxide Dismutase (SOD)

The Superoxide Dismutase (SOD) activity measurement was conducted using a technique outlined in the earlier publication.[28] Sodium pyrophosphate buffer, reduced Nicotinamide Adenine Dinucleotide (NADH), Phenazine Methosulphate (PMS) and Nitroblue Tetrazolium (NBT), the necessary volume of cell extract was included in the assay combination. A single unit of SOD activity is the quantity of enzyme needed to reduce chromogen synthesis (measured by optical density at 565 nm) by 50% within 1 min under test circumstances. It is represented as a specific activity in units/minutes/milligrams of protein. DOX was used as the positive control.

RESULTS

Calycosin suppresses cell growth

The initial assessment of the impact of Calycosin on T24 cell growth was conducted using the MTT assay. This study cultivated cells with or without Calycosin (2.5 to 50 µM/mL) for 24 hr, 48 hr and 72 hr. The MTT test was conducted daily following the procedures outlined in the Materials and Methods section. Significant reduction in proliferation was seen under our experimental settings, particularly after treatment with 24 hr, 48 hr and 72 hr, compared to the control growth rate of 100% (Figure 1). A regression analysis was conducted to determine the doses of Calycosin needed to achieve a 50% decrease in cell growth (IC_{50}) using data from MTT assays (Figure 1). The IC_{50} value increases with the duration of treatment (IC_{50} for 24 hr: 27.35±2.35 µM/mL, IC_{50} for 48 hr: 7.99±3.05 µM/mL and IC_{50} for 72 hr: 5.21±1.95 µM/mL).

Nuclear morphologic changes

A double staining procedure using ethidium bromide and acridine orange was conducted to investigate the potential of Calycosin to trigger apoptosis and necrosis mechanisms. This staining technique employs a mixture of AO-EB staining to visualize cells exhibiting abnormal chromatin arrangement. The DNA-binding fluorescent dye acridine orange intercalates into the DNA, causing it to appear greenish fluorescent and binds to the RNA, dyeing it reddish orange. EB is exclusively absorbed by dead cells and its fluorescent intensity surpasses AO's. The staining characteristics allowed for the identification of three distinct cell populations: green nuclei show viable cells, nuclei contracted; colored orange reveals apoptotic cells and reddish nuclei display necrotic cells. Our study revealed that using Calycosin at all concentrations evaluated (10 µM) for 24 hr reduced the viable cell percentage

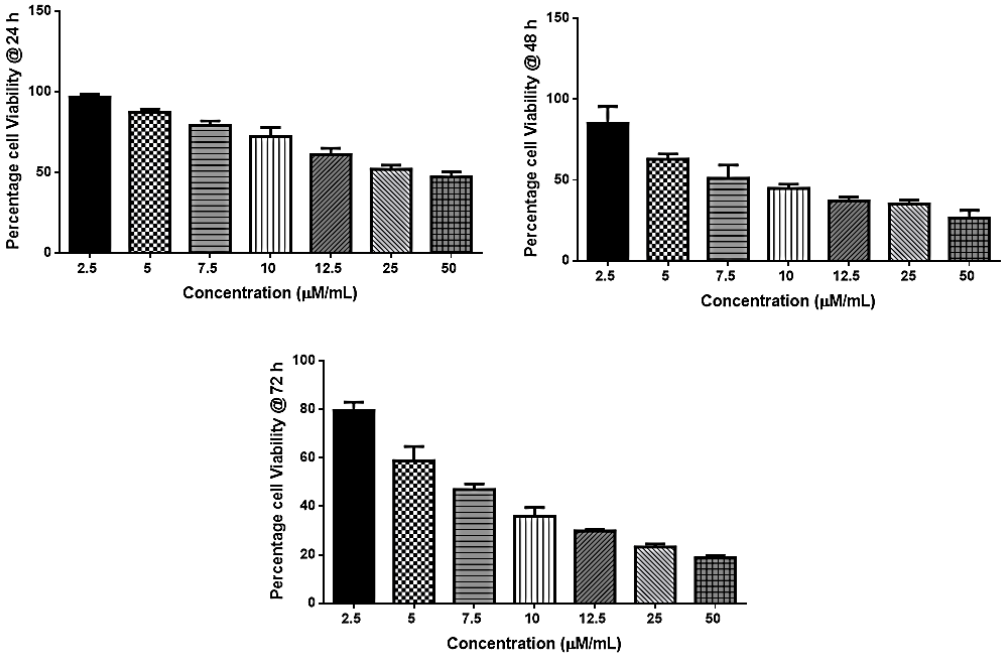

Figure 1: Effect of Calycosin on the viability of bladder cancer T24 Cells. The periplocin treatment at various concentrations (0, 2.5, 5, 7.5, 10, 12.5, 25 and 50 µM) effectively inhibited the Bladder cancer T24 Cells viability in a dose and time-dependent manner. The results are presented as a mean±SD of triplicate values. The values are analyzed by one-way ANOVA and Tukey's *post hoc* test using SPSS software.

in the T24 cell line (Figure 2). T24 cells were cultured for 24 hr with the specified concentrations or left untreated (control) and then dyed with a combination of AO-EB compared to the DOX (positive control). A fluorescence microscope was assessed.

Generation of ROS

The significant contribution of mitochondria in generating free radicals within live cells and their substantial significance as a primary source of ROS is widely recognized. Numerous studies have indicated that mitochondria play a crucial part in the apoptotic signal pathway, essential for cell survival by generating ATP through oxidative phosphorylation. Apart from providing energy, mitochondria control cell death processes, including incorporating and distributing death signals that trigger apoptosis and oxidative stress. Our investigation shows that the Calycosin at concentrations (10 µM) for 24 hr reduced the viable cell percentage in the T24 cell line. T24 cells with 10 µM concentration effectively generate the ROS compared to the DOX and positive control. A fluorescence microscope was assessed (Figure 3).

Induced oxidative stress

The oxidative stress-inducing potential of Calycosin was assessed by quantifying the concentrations of TBARS, GSH and SOD in T24 cells. Calycosin activates oxidative stress by reducing TBARS and GSH levels (Figure 4) and the increase of SOD levels, depending on the 10 µM concentration of Calycosin (Figure 4).

DISCUSSION

The primary objective of this work was to assess the inhibitory impact of Calycosin on the proliferation of the T24 bladder cancer cell line and to gain a strong understanding of the processes underlying this effect.[21] Initially, we observed that the administration of Calycosin significantly reduced cell proliferation, particularly at 24 hr, 48 hr and 72 hr post-drug therapy. An IC_{50} at 24 hr, 48 hr and 72 hr might be assessed by MTT experiments. This conclusion concurred with a prior study demonstrating that aldehyde natural compounds, including Calycosin, exhibit an inhibitory effect on the proliferation of the different cancer cells.[29-31] Furthermore, the analysis of the morphological characteristics of T24 cells exposed to the three chosen dosages revealed that the increased dosage had a more pronounced lytic effect than the other two doses.[32] This result implies that the inhibitory effect on cell proliferation is not associated with necrosis's induction of cell death. Cell death induction by Calycosin was confirmed by cytochemical labeling of nuclei with AO-EB. This evidence was consistent with the reported changes in cell survival as determined by the MTT test and morphological aspect of cells. Thus, AO-EB staining showed that Calycosin employs its antiproliferative impact on T24 cell lines by inducing apoptosis at 10 µM concentration.

The treated cells exhibited a notably increased level of membrane leakage of LDH, another indicator of cytotoxicity, compared to the control group. Several bioactive compounds may induce oxidative stress as one of their cytotoxic mechanisms. The phenomenon of oxidative stress triggers a diverse range of

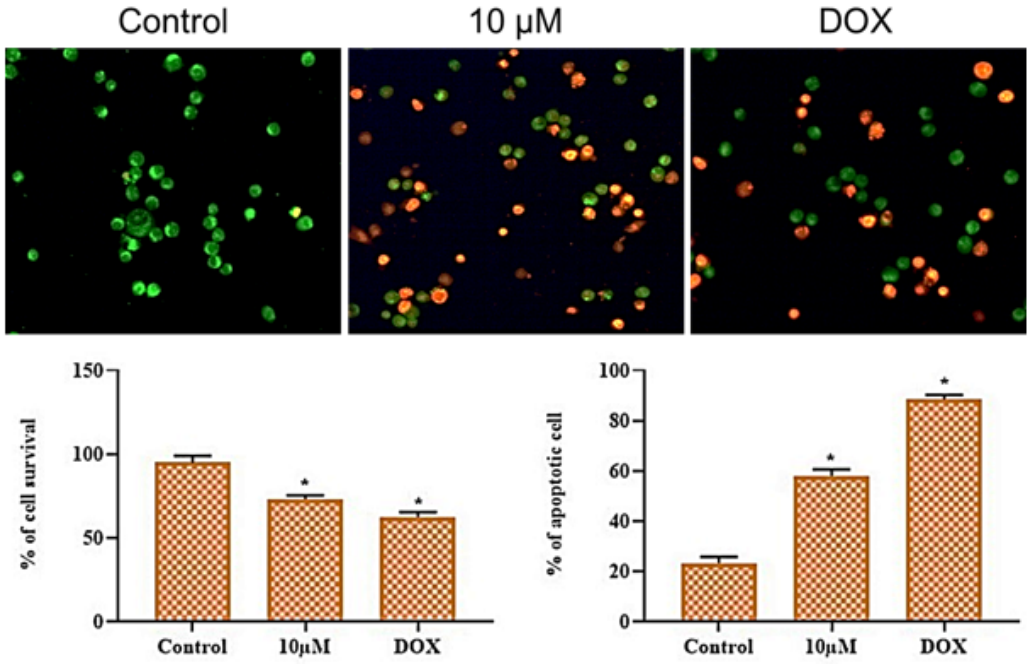

Figure 2: Effect of Calycosin on the apoptotic cell death in the bladder cancer T24 cells. Cells produced more yellow and orange fluoresced cells after the treatment with 10 µM of Calycosin, confirming early and late apoptosis in the T24 cells. The results are presented as a mean±SD of triplicate values. 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.

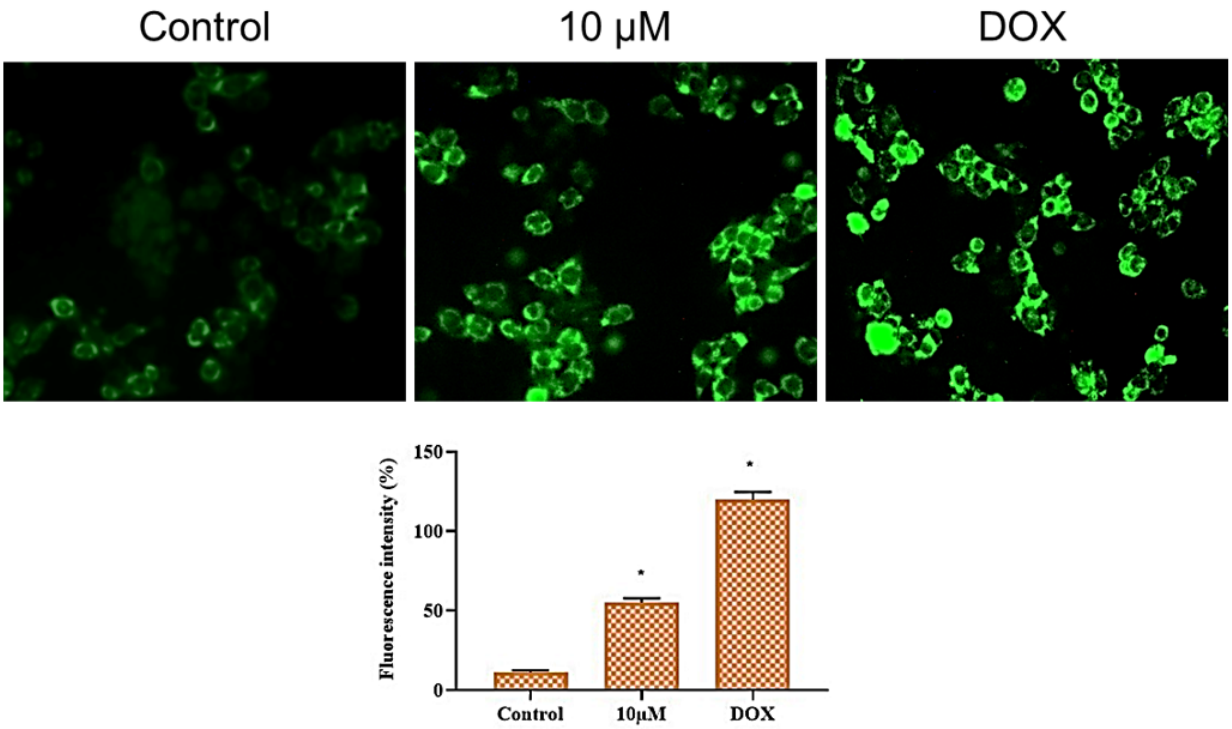

Figure 3: Effect of Calycosin on the intracellular ROS production in the T24 cells. T24 cells treated with 10 µM of Calycosin showed increased green fluorescence compared to the control, which evidences higher endogenous ROS production. The results are presented as a mean±SD of triplicate values. The 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.

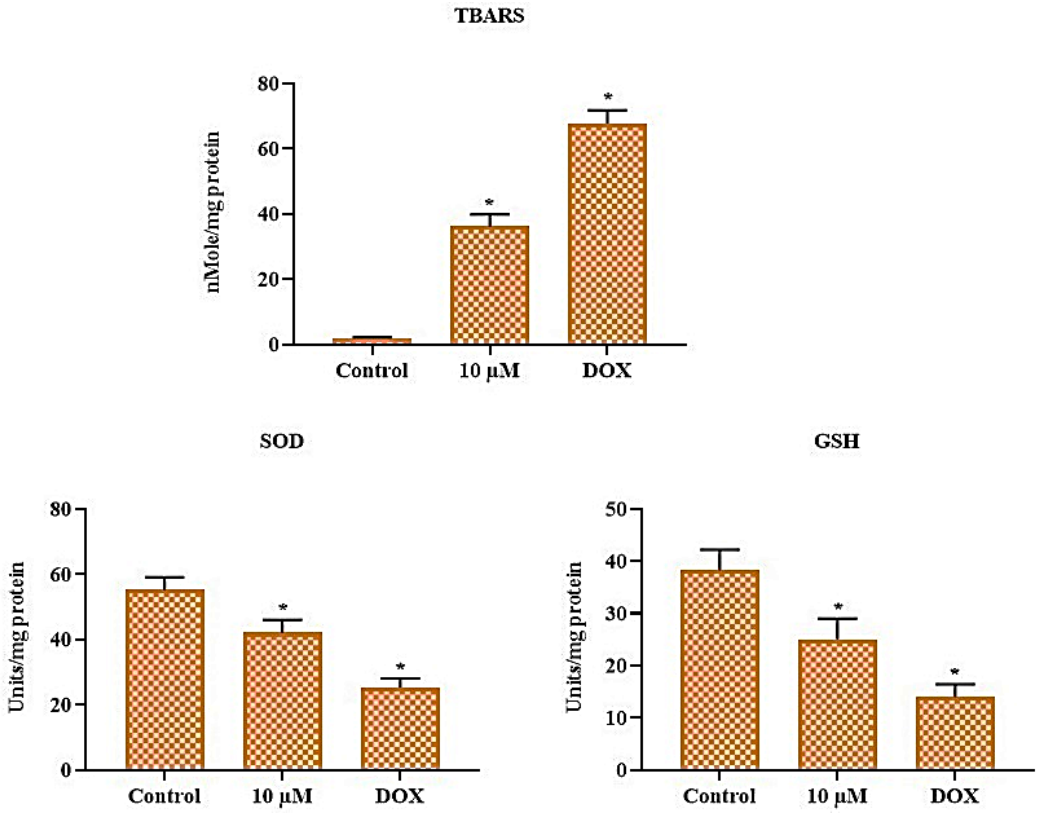

Figure 4: Effect of Calycosin on the oxidative stress marker levels in the Bladder Cancer T24 cells. The results are presented as a mean±SD of triplicate values. The values are analyzed by one-way ANOVA and Tukey's *post hoc* test using SPSS software. '*' indicates the significance at $p<0.05$ from the control group.

physiological and cellular processes, including apoptosis, DNA damage, inflammation and oxidative stress.[33] Oxidative stress is a well-documented method by which Calycosin exerts its cytotoxic effects. Administration of Calycosin increases intracellular ROS levels.[34] It eliminates antioxidants like antioxidant enzymes TBA Reactive Species (TBARS) or Glutathione (GSH), such as Superoxide Dismutase (SOD) and catalase, resulting in the creation of DNA adducts. Intracellular ROS is considered a vital marker for detecting different toxic effects caused by various natural products. Exposure to natural products creates a pro-oxidant milieu in cancer cells, disrupts the balance of redox potentials and induces negative biological outcomes ranging from inflammation to apoptosis.[35] The present work established a strong correlation between the cytotoxic properties of Calycosin on T24 cells and ROS generation. The phenomenon of oxidative stress arises when the production of ROS surpasses the ability of antioxidant defense mechanisms. ROS refers to oxygen-containing compounds, including Hydrogen peroxide (H_2O_2), Hydroxyl radical (OH•) and Superoxide anion (O^2), which possess higher chemical activity compared to molecular oxygen, for example. Reduction of the GSH and generation of ROS have been linked to oxidative damage of cellular components.[36] In our results, the oxidants' ROS levels were markedly elevated compared to the DOX drug. In contrast, the level of antioxidant GSH was reduced significantly in human bladder cancer cell lines exposed to Calycosin. Glutathione (GSH) plays a crucial role in the front line of the oxidative damage. This work establishes a strong association between the development of ROS and TBA Reactive Species (TBARS) Glutathione (GSH) depletion. This suggests that an increase in ROS production results in an increase in antioxidants such as GSH, which are responsible for scavenging these free oxygen radicals. The underlying mechanism of Calycosin-induced cytotoxicity may be attributed to oxidative stress in human bladder cancer T24 cells. The Calycosin-induced suppression of T24 cell growth is probably facilitated by a route not dependent on ROS. These findings suggest a potential scenario for the cytotoxicity of Calycosin in T24 cells, whereby Calycosin may induce immunotoxicity in MCF-7 cells through at least two primary mechanisms. Increased production of ROS is recognized to augment the synthesis of proinflammatory cytokines and the activation of the inflammasome, ultimately resulting in the onset of pulmonary fibrosis.[37] The definitive correlation between the surface area, capacity to generate ROS and proinflammatory effects of natural products has been consistently demonstrated.[38] Ultimately, the increased toxicity of Calycosin seems to be associated with oxidative stress, proinflammatory effects and genotoxic potential.[39] Based on these results, it is proposed that the effects caused by Calycosin could have a chemopreventive impact. These promising *in vitro* findings provide a rationale for additional investigation to clarify its *in vivo* activities.

CONCLUSION

In conclusion, our findings indicate that Calycosin induces cytotoxic responses in human bladder T24 cells, maybe by producing Reactive Oxygen Species (ROS) and oxidative stress. These findings suggest that the industrial use of Calycosin should be thoroughly evaluated for its possible adverse impacts on the environment and human health.

FUNDING SOURCE

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

RA: *Radix astragali*; **ROS:** Reactive oxygen species; **SOD:** Superoxide dismutase; **GSH:** Glutathione; **TBARS:** TBA reactive species; **BC:** Bladder cancer; **EAU:** European association of urology; **NMIBC:** Non-muscle invasive bladder cancer; **NAC:** N-acetylcysteine; **MMP:** Mitochondrial membrane potential; **DMSO:** Dimethyl sulfoxide; **DMEM:** Dulbecco's modified eagle medium; **FBS:** Fetal bovine serum.

SUMMARY

The present work aimed to investigate the effects of Calycosin on the rate of cell growth, oxidative stress and apoptosis in the T24 human bladder cancer cell line. Results indicated that exposure to BPs could enhance the generation of Reactive Oxygen Species (ROS) and reduce the levels of Superoxide Dismutase (SOD) and Glutathione (GSH) and elevate the TBA Reactive Species (TBARS). Based on these results, it is proposed that the effects caused by Calycosin could have a chemopreventive impact.

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