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# *Antiproliferative and cytotoxic effect of abietane diterpenes in urothelial carcinoma cell line*

ARTIGO CIENTÍFICO ORIGINAL

ÁREA CIENTÍFICA DE BIOMEDICINA/FISIOPATOLOGIA

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***Antiproliferative and cytotoxic effect of abietane diterpenes in urothelial carcinoma cell line***

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**ABBREVIATION LIST**

AB® – Alamar blue®

ATCC – American type culture collection

AV – Annexin V

BC – Bladder cancer

BCG - Bacillus Calmette–Guerin

DMEM-HG – Dulbecco's modified Eagle's medium-high glucose

DMSO – Dimethyl sulfoxide

EAU – European Association of Urology

EMA - European Medicines Agency

FDA – Food and Drug Administration

MIBC – Muscle-invasive bladder cancer

NMIBC – Non-muscle-invasive bladder cancer

PI – Propidium iodide

TCC – Transitional cell carcinoma

TURBt - Transurethral resection of the bladder tumor

UCC – Urothelial cell carcinoma

UTUC – Upper urinary tract urothelial carcinoma

## ABSTRACT

**Introduction**: In the realm of urological oncology, bladder cancer (BC) stands out as the most common form of urothelial cancer and ranks among the most aggressive urological malignancies. Characteristically, 75-80% of BC cases are non-muscle-invasive (NMIBC), marked by high recurrence rates and significant risk of developing into muscle invasive bladder cancer (MIBC). The prevailing adjuvant treatment, intravesical Bacillus Calmette–Guerin (BCG) administration, aims to prevent this progression. However, the efficacy of BCG therapy is limited, with 30 to 50% of patients exhibiting resistance and an elevated risk of progression to MIBC. This therapeutic shortfall underscores the urgent need for novel pharmacological interventions. Diterpenes, particularly those derived from the genus *Plectranthus* L´Hér., have emerged as a promising class of biologically active secondary metabolites. This study is dedicated to exploring the cytotoxic capabilities of the naturally occurring abietane diterpenoids: 7α-Acetoxy-6β-hydroxyroyleanone (Roy), 7β,6β-dihydroxyroyleanone (DiRoy), and Parvifloron D (ParvD) against urothelial cancer cells.

**Material and methods**: The compounds (Roy, DiRoy and ParvD) were isolated from South African plants belonging to the Lamiaceae family: Roy and DiRoy were isolated from the acetonic extract of leaves and stems of *Plectranthus hadiensis* Schweinf, and ParvD was isolated from the acetonic extract of *Plectranthus ecklonii* Benth. The cytotoxic/antiproliferative profile of these diterpenes in 639-V cell line was investigated. Cell viability was evaluated by Alamar blue® assay, while cell death and cell cycle's impact were assessed by flow cytometry. The half-inhibitory concentration ($IC_{50}$) was determined using GraphPad Prism.

**Results and discussion:** The diterpenes Roy and ParvD induced a significant cytotoxic/antiproliferative effect, contrarily to DiRoy. Roy also showed an increase in early cell apoptosis and also interfered with cell proliferation, inducing cell cycle arrest in the S/$G_2$/M phase.

**Conclusion**: Roy and ParvD exert a robust cytotoxic and antiproliferative action against the cancer cells evaluated. As Roy induced cell cycle arrest in the S/$G_2$/M phase, it is worth of future evaluation in order to understand its mechanist effect in the cells.

## KEYWORDS

Urothelial carcinoma, bladder cancer, phytochemicals, abietane diterpenes, cytotoxic/antiproliferative profile

## INTRODUCTION

In the landscape of oncology, malignancies of the urinary system are rising sharply in prevalence, particularly in developed nations. Among these, urothelial cell carcinoma (UCC), also known as "transitional cell carcinoma (TCC)," emerges as a significant cancer type affecting the urinary tract system, encompassing the renal pelvis, ureter, bladder, and urethra. Ranking as the sixth most common cancer in Western countries, UCC represents a critical focus of medical research and intervention.

Upper tract urothelial carcinoma (UTUC) and bladder carcinoma (BC) represent the majority of UCC. Despite their historical grouping based on similar histological features, UTUC and BC are fundamentally different, not only in their embryological origins but also in their responses to therapeutic interventions. Diving deeper, BC stands out as the most prevalent urothelial cancer, manifesting in both non-muscle-invasive (NMIBC) and muscle-invasive (MIBC) forms, each with distinct clinical outcomes. (1-5) This dynamic and complex nature of urothelial carcinomas poses both challenges and opportunities for advancing our understanding and treatment of these increasingly common malignancies.

The main risk factor for BC is cigarette smoking, but occupational exposure to a variety of carcinogens (from petroleum products, paints, dyes, rubber), long-term exposure to cyclophosphamide or analgesics, infection with *Schistosoma haematobium* in endemic areas, and pelvis radiation have also been described in literature as risk factors for BC incidence. BC prognosis depends on BC histopathology and the depth of tumor infiltration into bladder wall (NMIBC or MIBC) provide an acceptable risk stratification. (6, 7)

In the intricate group of bladder cancer (BC) management, the initial transurethral resection of the bladder tumor (TURBT) reveals that a striking 80% of newly diagnosed BC cases fall under the category of non-muscle-invasive bladder cancer (NMIBC). This form of BC, confined to the bladder's mucosa or muscularis mucosae, navigates a complex treatment pathway steered by risk stratification and the extent of muscle invasion. Particularly for patients classified as intermediate and high-risk, the journey involves adjuvant therapies designed to thwart disease progression and recurrence.

In the realm of high-risk NMIBC, the battle against progression to muscle-invasive bladder cancer (MIBC) is waged with intravesical adjuvant treatments. For decades, the standard bearer in this fight has been the intravesical administration of Bacillus Calmette–Guerin (BCG), an agent originally crafted as a vaccine against tuberculosis, now serving as a gold-standard in BC immunotherapy. Yet, this treatment is not infallible: about 40% of patients witness a recurrence post-treatment, and 15% see their condition escalate to MIBC. Tumors that resist BCG, either by recurring within six months of treatment or by developing carcinoma in situ within a year, are now branded as BCG-unresponsive by the European Association of Urology

(EAU), rendering further BCG treatments futile. Alternatives like mitomycin C, docetaxel, gemcitabine, valrubicin, and pembrolizumab have entered the fray, but their impact remains muted (6-9). This landscape underscores a pressing, unfulfilled demand for newer, more potent drugs capable of confronting high-risk NMIBC with the efficacy it demands.

Nowadays, 25% of herbal medicines in the modern pharmacopeia are plant-based and other synthetic medicines are produced from chemicals isolated from plants (10, 11). The search for improved antitumor agents validates the phytochemical studies by the scientific community. (12) In fact, about 60% of the chemotherapeutic drugs approved by Food and Drug Administration (FDA) or European Medicines Agency (EMA) are derived from natural compounds, such as the diterpene paclitaxel or the alkaloids vinblastine and vincristine, used in the ovarian and breast cancer and leukemia treatments, respectively. (13)

A large number of species of the genus *Plectranthus* L'Hér., which belongs to the Lamiaceae family and consists of 300 species distributed in Australia, Asia and Africa, are widely used in traditional medicine and the bioactivities of these plants are becoming progressively important in drug research and development. The first described *Plectranthus* species was *Plectranthus hadiensis* Schweinf. (*P. hadiensis*), in 1775. This plant has antibacterial, antifungal, antiviral and antitumor properties. Regarding the latter, it has been documented for its use against abdominal, intestinal, uterine, prostate, brain, breast, skin, and throat cancers, as well as leukemia. (14, 15)

Diterpenes are the most prominent biologically active group of secondary metabolites found in this genus, and their properties have already been well studied. Particularly, abietane diterpenes are the most diverse group and have long been studied for their variety of bioactivities. (3, 16, 17) These compounds deserve special attention because of their anti-proliferative, anti-tumoral and/or cytotoxic activity against several cancer cell lines including breast, liver, lung, pancreatic, colon, endometrial and prostate carcinoma cell lines. (15, 18)

There are some abietane diterpenes, such as 7α-Acetoxy-6β-hydroxyroy-leanone (Roy), 7β,6β-dydroxyroyleanone (DiRoy), and Parvifloron D (ParvD), whose genotoxic and cytotoxic characteristics have been already evaluated in a few published studies, showing strong activity in different human cancer cell lines. (19)

The focus of this research is to investigate the anticancer properties of specific natural compounds – Roy, DiRoy, and ParvD – extracted from the leaves and stems of *P. hadiensis* and *Plectranthus ecklonii* Benth. (*P. ecklonii*), respectively. We aim to assess their efficacy in the 639-V cell line, derived from urothelial cell carcinoma and frequently employed as a model in bladder cancer studies. Our goal is to evaluate the potential of these compounds as promising candidates for drug development in the treatment of bladder cancer, thereby contributing to the advancement of oncological therapeutics.

## MATERIALS AND METHODS

### Plant material

The species *P. hadiensis* was cultivated in the Botanical Park of Tapada da Ajuda (Lisbon, Portugal), and the species *P. ecklonii* was provided by the Faculty of Pharmacy of the University of Lisbon. The Kirstenbosch National Botanical Garden (Kistenbosch, South Africa) supplied the seeds of both species.

The voucher specimens for *P. hadiensis* (833/2007 and 438/2010) were deposited in the Herbarium João de Carvalho e Vasconcellos, ISA/UL, Lisbon, Portugal (LISI), while the voucher specimens for *P. ecklonii* (S/No.LISC) were deposited in the Herbarium of the Tropical Research Institute, Lisbon, Portugal. (13, 15)

In order to maintain stability, the plant was air dried at room temperature and then stored in cardboard boxes protected from humidity and light. A plant taxonomist confirmed the plants' names and follow the repository http://www.worldfloraonline.org/. (20)

### Isolation of abietane diterpenes

The abietane diterpenes used in this work: 7α-Acetoxy-6β-hydroxyroy-leanone (Roy), 7β,6β-dydroxyroyleanone (DiRoy), and Parvifloron D (ParvD) were isolated and chemically characterized in the laboratory of Professor Patrícia Rijo. (21)

### Cell culture

The cell line used in this work was acquired from the American Type Culture Collection (ATCC). Human 639-V cell line (transitional cell carcinoma of the ureter) was kindly provided by João Vinagre PhD (i3S, Institute for Research and Innovation in Health, University of Porto). Cells were cultivated in Dulbecco's Modified Eagle's Medium-high glucose (DMEM-HG) (Biowest, Nuaillé, France) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (Biowest, Nuaillé, France) and 1% (v/v) of penicillin-streptomycin (Sigma, St. Louis, MO, United States), being maintained at 37°C and 5% $CO_2$.

### Cell viability

The 639-V cell line was seeded, with a density of $1x10^4$ cells/well, in 96-well plates, 24 h before treatment. Cells were treated either with Roy, DiRoy and ParvD, using a range of concentrations of 0.390625, 1.5625, 6.25, and 25 µg/mL, or with the vehicle control (Dimetyl sulfoxide (DMSO)), and further incubated for 24, 48 and 72 h. Cell viability was assessed through a modified Alamar blue® (AB®) assay (22). Briefly, a solution was prepared of DMEM-

HG medium with 10% (v/v) of a resazurin salt dye stock solution (Sigma. St. Louis, MO, United States) at a 0.1 mg/ml concentration, which was further added to each well after 24, 48 and 72 h treatment. After 2 h of incubation at 37°C and 5% $CO_2$, the absorbance of the plate was read at 570 and 600 nm in a BioTeck (BioTek Instruments, Inc., Winooski, VT, United States). The absorbance results were obtained by the Gen5 program. Cell viability was then calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{(A570-A600)\ of\ treated\ cells}{(A570-A600)\ of\ control\ cells} \times 100\%$$

Half-maximal inhibitory concentration ($IC_{50}$) values were further calculated using GraphPad Prism Software v.10.1.0 (GraphPad Software, Inc.). (23)

**Annexin V assay**

Assessment of cell death was achieved using the Annexin V (AV)/propidium iodide (PI) assay by flow cytometry. 24 h before treatment, 639-V cells ($20\times10^4$ cells/well) were seeded in 12-well plates. Cell medium was replaced, and 6.25 µg/mL of Roy was added to the cells. After 48 h of treatment, cells were co-stained with AV-APC and PI according to the manufacturer's protocol (Biolegend, San Diego, CA, United States). Cells were resuspended in binding buffer (100 µl) and incubated with AV-APC solution (5 µl) and PI solution (2 µl). Then, cells were diluted in a binding buffer (400 µl). Data were acquired and treated as previously described. (24)

**Cell cycle analysis**

Cell cycle was analyzed by flow cytometry using PI/RNase solution, according to the manufacturer's protocol (Immunostep, Salamanca, Spain). 639-V cells ($30\times10^4$ cells/well) were seeded 24 h before treatment. Afterward, cells were treated with 6.25 µg/mL of Roy. Cells were detached, fixed in 70% ethanol for 60 min at 4°C, washed twice with PBS, and then stained with 500 µl PI/RNase solution. Results were acquired using CellQuest software and <0.05 was considered significant analyzed to calculate the percentage of the cell population in each cell cycle phase.

**Statistical analysis**

Data were analyzed using GraphPad Prism v.10.1.0. All experiments were performed in triplicate and acquired results were expressed as mean ± SD. Statistical analysis was performed by one-way and two-way ANOVA, using the multiple comparison test Dunnett. A value of $p<0.05$ was considered significant.

## RESULTS

### Metabolic activity and $IC_{50}$ values after treatment with Roy, DiRoy and ParvD

The 639-V cell line was treated for 24, 48, and 72 h either with a serial dilution scope of concentrations (25, 6.25, 1.5625, and 0.390625 µg/mL) of each abietane diterpene or with vehicle (DMSO).

Although Roy and DiRoy came from the same species, their effect in 639-V cell line's metabolic activity showed different patterns (Fig. 1). In all concentrations tested, DiRoy did not significatively affect the metabolic activity in the tested cells, showing always a cell viability above 80%, and in most cases near to 100%. On the other hand, cells treated with Roy revealed a decrease in the metabolic activity at the highest concentrations in all incubation time points. This decrease in cell viability was directly correlated to the duration of the treatment.

After 24 h of treatment, the metabolic activity was reduced by 50% with the highest concentration. At 48 h, the reduction was in the metabolic activity dropped to less than 20%. At 72 h, the tendency was maintained and there was again a further decrease in the metabolic activity for the same concentrations (with cell viability of 10-15% for 6.25 µg/mL, and near 0% for 25 µg/mL).

The compound ParvD from *P. ecklonii* exhibited an effect similar to that evidenced with Roy (Fig. 1). It also induced a decline in the metabolic activity at the highest concentrations in all time points. At 24 h, the metabolic activity was reduced and the cells presented a viability of about 30% with the highest concentration tested. At 48 h, cell viability was around 50% for the concentration of 6.25 µg/mL, and between 10-15% for the concentration of 25 µg/mL. At 72 h, the cell viability almost reached 0%.

The $IC_{50}$ (inhibitory concentration necessary to decrease cell viability by 50%) values determined for these compounds are shown in Table 1.

**Table 1.** $IC_{50}$ values for Roy, DiRoy and ParvD in 639-V cell line.

| **$IC_{50}$ (µg/mL)** | **Roy** | **DiRoy** | **ParvD** |
|---|---|---|---|
| **24 h** | 7.52 | - | 7.15 |
| **48 h** | 5.99 | - | 6.27 |
| **72 h** | 3.44 | - | 6.74 |

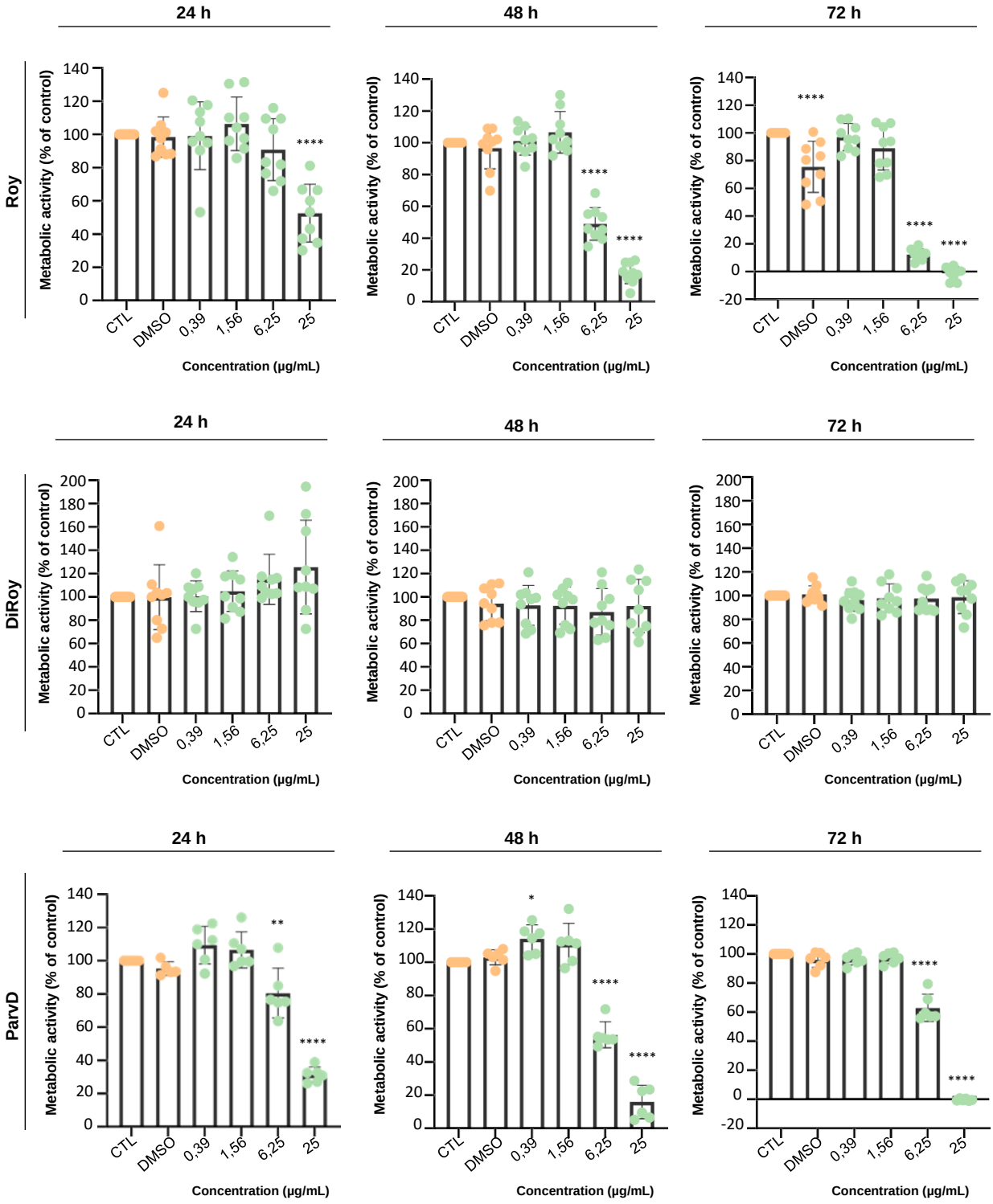


**Figure 1.** Effect of Roy, DiRoy, and ParvD in the metabolic activity of 639-V cell line. Cells were treated with the compounds for 24, 48, and 72 h and after Alamar blue® assay was performed. Metabolic activity was expressed as percentage of control (CTL) (untreated cells). Asterisks (*p<0.05, **p<0.01, and ****p<0.0001) represent the values that significantly differ from the CTL.

**Cell death assessment after Roy's treatment and its impact on cell cycle regulation**

To further understand the cellular mechanisms underlying the cytotoxicity of Roy, the 639-V cells were incubated for 48 h with a concentration of 6.25 µg/mL and afterwards undergo analysis by flow cytometry. The results obtained concerning apoptosis induction and cell cycle regulation, displayed in Fig. 2, revealed that 48-hour Roy treatment increased the percentage of cells in early apoptosis and promoted an increase of tumor cells in S/$G_2$/M phases.

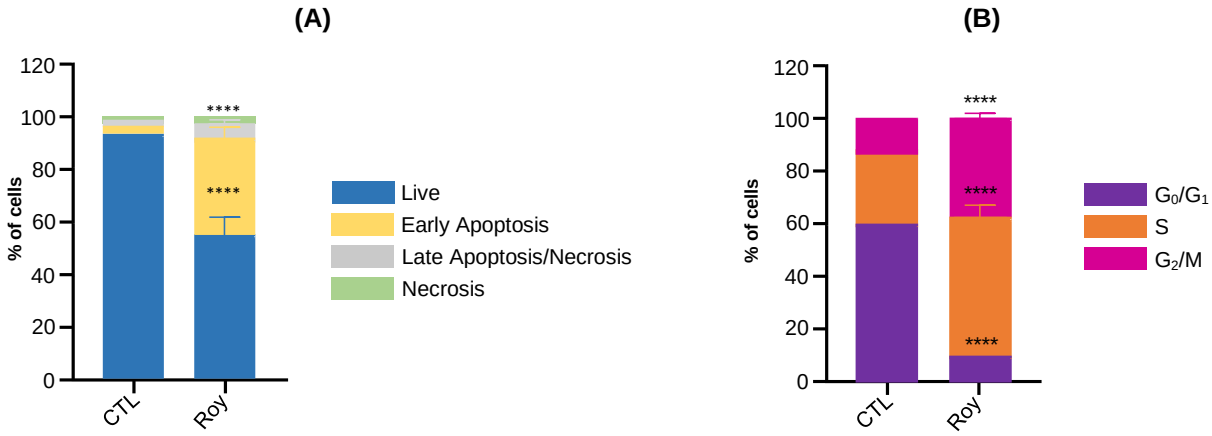


**Figure 2.** Effect of treatment with 6.25 µg/mL of Roy on apoptosis induction and cell cycle regulation, in 639-V cell line, after 48h, presented as percentage of total cell population. (A) Proportion of live, early and late apoptotic, and necrotic cells. (B) Proportion of individual cell cycle phases ($G_0/G_1$, S and $G_2$/M). Asterisks (****p<0.0001) traduce the significant difference between the values obtained for treated cells comparatively to untreated cells (CTL).

The proportion of cells in early apoptosis after Roy treatment increased to roughly 40% (****p<0.0001) compared to the control. Although there is a slight increase in cells in late apoptosis/necrosis and in necrosis, the rise percentages of treated cells are below 5-10%, not much higher than the control cells.

The proportion of cells in $G_0/G_1$ phase after Roy treatment suffered a significant decrease (approximately 50%, ****p<0.0001) compared with control cells. There was spotted an increased proportion of treated cells in S phase (about 20-25%, ****p<0.0001) and in G2/M phase (almost 30%, ****p<0.0001), relatively to the control group.

## DISCUSSION AND CONCLUSION

### Discussion

In the ongoing quest to enhance anti-cancer therapies, our study presents a novel exploration of natural compounds, particularly focusing on the structural uniqueness and mechanisms of action of abietane diterpenes. This research offers a perspective on the potential applications of these compounds in oncological treatments, providing the first insights into the effects of abietane diterpenes. Our findings demonstrate a cytotoxic impact of these phytochemicals on urothelial cancer cells, thus highlighting their potential as effective agents in cancer therapy, especially in BC.

This work combines two fundamental chapters: cellular viability, and cell death assessment and impact on cell cycle regulation.

The results offered a clear contrast between the compounds DiRoy and Roy. Even at the highest concentrations, regardless the incubation time, DiRoy barely induced cytotoxicity against the 639-V cell line, whereas Roy induced the most pronounced cytotoxic effect at the highest concentrations, across all time points (**** $p<0.0001$). The lowest metabolic activity of tumor cells treated with Roy was achieved with the concentration of 25 µg/mL, after 72 h of treatment. To reach a metabolic activity as low as possible in the tumor cells was the principal aim of testing these compounds and Roy exceeded that expectation. The values expressed in Table 1 also supports this encouraging finding: Roy's $IC_{50}$ values were significantly lower as the incubation time increased.

DiRoy in all tested concentrations in this cell line did not reach less than 50% viability, so it was not possible to determine $IC_{50}$ values.

As evidenced in the results, the cytotoxic behavior of ParvD was parallel to Roy, which also strengthens ParvD as an interesting compound. At 24 and 48h time points, ParvD even demonstrated better metabolic activity scores than Roy in the highest concentrations. Nonetheless, the $IC_{50}$ values pattern is not linear: $IC_{50}$ decreased from the lower incubation to the intermediate incubation time, which means that a lower concentration was necessary to reach the same effect, but at the longer incubation time the $IC_{50}$ has risen again. Comparing the $IC_{50}$ values of ParvD and Roy, the latter requires lower concentrations (almost half) to achieve a cytotoxicity of 50% of the cells and can be considered more effective.

Our abietane diterpenes have been shown to have a potential therapeutic effect for multiple cancers in different past studies. For example, the contrast between the diterpenes Roy and DiRoy, found in our results, was not so surprising, considering that Domínguez-Martín *et al*. also performed a similar study with comparable results. Five glioblastoma cell lines (U87, A172, H4, U118, and U373) were treated with a serial dilution range of the same

concentrations of *P. hadiensis* stem extracts, and Roy also induced the most pronounced antiproliferative effect across all tested cell lines, although it was possible even at the lowest concentrations. Likewise, DiRoy did not significantly affect cell viability, except for the U118 cell line. The differences in chemical structure or physicochemical properties may be at the origin of that different bioactivity between these two compounds. (15) Sitarek *et al*. also studied the viability of primary H7PX glioma cells with nine different concentrations of our three abietane diterpenes, isolated from *P. madagascariensis* (Roy and DiRoy) and *P. ecklonii* (ParvD). In this study the cells were most sensitive to Roy and ParvD compared to DiRoy, and ParvD was the most active compound. (25) ParvD also displays cytotoxic activity against a lung cancer cell line (A549), (19) pancreatic cell lines (BxPC3, PANC-1 and Ins1-E) (26) and leukemia cell lines (HL-60, U-937, K-562 and MOLT-3). (27) The pronounced antiproliferative effect of ParvD from *P. ecklonii* in glioblastoma cells, compared with its current first-line treatment, in five glioma cell lines (U87, U118, H4, A172 and U373), showed much lower $IC_{50}$ values than those of the corresponding drug used as first-line treatment. (13) Other studies have been consistent with the strong cytotoxic activity of Roy and ParvD, for example, in a leukemia cell line (CCRF-CEM), a lung adenocarcinoma cell line (A549) and melanoma cell lines (B16V5 and A375), showing their strong cytotoxicity, against the denoted weak results of DiRoy in both cell lines (19, 29). Furthermore, the anticancer effect of ParvD in MDA-MB-231 cells, a model of human triple negative breast cancer, proved a remarkable decrease of cell viability. (28)

As Roy seemed to have the most promising cytotoxic profile of all three abietane diterpenes tested, we then enquired the potential mechanism by which Roy induced cell death on 639-V cell line. Cell death assessment and cell cycle regulation were measured by flow cytometry, using one of the most applied experiments to detect apoptosis, the AV/PI assay+ (30), and PI/Rnase assay, respectively.

The results after Roy treatment showed a cytotoxic effect in 639-V cells sustained by a significant increase of cell death, specifically in early apoptosis, (****$p<0.0001$, Fig. 2 – A). Roy also caused cell cycle arrest in $S/G_2/M$, as evidenced by the increase in the percentage of cells at these stages, accompanied by a decrease in cell population in $G_0/G_1$ phases. (Fig. 2 - B). Our results suggested that Roy induced cell cycle arrest and apoptosis.

From what we know about molecular and cellular dogmas, this effect could be associated with downregulation of key proteins, such as cyclins, or interference with signaling pathways, such as the retinoblastoma pathway, which play critical roles in the $G_1/S$ transition. An increase S phase may possibly indicate an arrest in DNA replication, as a result of enabling replication checkpoints. The increase in the $G_2/M$ phase could suggest a possible blockage in the $G_2/M$

transition, attributed to the activation of DNA damage checkpoints or to a direct interference in the activation of complexes that regulate entry into mitosis. (31)

Tumor cells entering into apoptosis seems to be a good therapeutic goal. (19) Many published works have shown that compounds isolated from plants are able to induce apoptosis in different cancer types. (32, 33, 34) In one of the above-mentioned studies on abietane diterpenes for cell viability, Roy and ParvD induced early and late apoptosis (53% with Roy and 73% with ParvD) in H7PX cells. Besides, an increase in $G_2$/M phase with the compounds Roy and ParvD (58% and 60%, respectively) was verified. (25) In another one, ParvD caused an increase of sub$G_0$/$G_1$ in in three glioma cell lines (U87, A172 and H4), and an arrest in S/$G_2$/M phases in the other two (U373 and U118), corroborating a cytotoxic effect by significant increase of early apoptosis, and early but also late apoptosis, respectively, in glioblastoma cells. (13)

We acknowledge the limitations of our study, namely, that 639-V cells are not full representative of all urothelial cancers. Besides, in vitro studies also do not accurately reflect the effect of abietane diterpenes in BC.

Further investigation would be necessary to unveil the molecular mechanisms underlying these compounds. Gene and protein expression analyses could identify specific targets of these natural compounds. Additionally, assessment of apoptosis markers, such as caspase expression, may clarify whether the increase in S/$G_2$/M phases is associated with apoptotic events.

## Conclusion

The outcomes of our study have elucidated that Roy, a natural diterpene, exhibits a highly promising cytotoxic profile. This compound notably influences cell cycle regulation by inducing arrest at the S/$G_2$/M phase, coupled with a marked increase of cells in early apoptosis. Moreover, Roy and ParvD have demonstrated a robust cytotoxic and antiproliferative effect against tumor cells. These findings pave the way for future research, in order to understand the role of diterpenes and enhancing our comprehension of the intricate interactions between these natural compounds and cellular mechanisms.

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