

Article

Natural Product Epipyrone A from *Epicoccum nigrum* Exhibits Antiproliferative Activity on Canine Mammary Tumor Cells Through PI3K/Akt/mTOR Pathway Modulation

Consiglia Longobardi ^{1,†}, Daria Lotito ^{1,†}, Alessia Staropoli ¹, Valeria Iervolino ¹,
Nunzio Antonio Cacciola ¹, Serena Montagnaro ¹, Francesco Vinale ^{1,2}, Sara Damiano ^{1,*}
and Roberto Ciarcia ¹

¹ Department of Veterinary Medicine and Animal Production, University of Naples Federico II, 80137 Naples, Italy; consiglia.longobardi@unina.it (C.L.); daria.lotito@unina.it (D.L.); alessia.staropoli@unina.it (A.S.); valeria.iervolino@unina.it (V.I.); nunzioantonio.cacciola@unina.it (N.A.C.); serena.montagnaro@unina.it (S.M.); frvinale@unina.it (F.V.); rciarcia@unina.it (R.C.)

² Institute for Sustainable Plant Protection, National Research Council, Piazzale E. Fermi, 1, 80055 Portici, Naples, Italy

* Correspondence: sara.damiano@unina.it

† These authors contributed equally to this work.

Abstract

Canine mammary tumors (CMTs) are among the most frequent neoplasms in female dogs, with current therapeutic options being limited and non-standardized, prompting the search for alternative treatments such as fungal secondary metabolites. In this study, the fungal pigment Epipyrone A (Epy A) was first isolated from *Epicoccum nigrum* and then tested in vitro on two CMT cell lines, P114 and CF33. The compound significantly reduced cell viability in both lines in a concentration- and time-dependent manner ($p < 0.05$), with the strongest effect observed at 175 $\mu\text{g/mL}$ after 48 h ($p < 0.0001$), while showing no cytotoxicity in MDCK non-tumor cells. Epy A also inhibited cell migration and increased total antioxidant capacity in P114 cells, accompanied by a significant reduction in ROS levels. Western blot analysis revealed modulation of the PI3K/Akt/mTOR pathway, crucial in CMT biology. Specifically, P114 cells showed downregulation of mTOR and p-Akt, indicating inhibition of proliferative signaling, whereas CF33 cells exhibited increased Akt and p-Akt alongside reduced mTOR, consistent with a compensatory feedback mechanism, probably linked to the changing in oxidative balance after treatment. Overall, these results identified Epy A as a promising natural molecule with potential applications in innovative therapeutic approaches for veterinary and comparative oncology.

Keywords: canine mammary tumors; Epipyrone A; *Epicoccum nigrum*; PI3K/Akt/mTOR pathway



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1. Introduction

The percentage of cancer-related deaths in companion dogs ranges from 15% to 30%, depending on the country and breed. In developed nations, cancer often emerges as the primary cause of mortality as life expectancy rises [1]. Because canines and humans live in the same environment and share some genetic features, spontaneous tumours in dogs represent a clinically relevant model of human cancer, as they typically mirror the genetic and biological complexity of human disease, also exhibiting pronounced intra- and inter-patient heterogeneity [2]. Moreover, canine cancers frequently acquire therapeutic

resistance and display a strong propensity to metastasize, further reinforcing their translational significance to humans [3,4]. Among these, canine mammary tumours (CMTs) are the most frequently diagnosed in female dogs, second only to skin tumours, and thus represent a significant challenge in veterinary oncology [5]. Age and reproductive history are major determinants in CMTs development, as the risk rises with advancing age and is substantially higher in females that have not undergone ovariectomy before the first or second oestrus [6]. This occurs because of the tumour's hormone dependency, indicating its influence by ovarian hormones that affect normal breast development, such as estrogen and progesterone [7].

Approximately half of the diagnosed CMTs are malignant and frequently display an aggressive clinical behaviour and variable susceptibility to conventional therapies, constituting one of the principal contributors to cancer-related mortality in female dogs, especially when identified at an advanced stage, with survival often limited to months despite surgical interventions and therapies [8,9].

In veterinary medicine, surgical removal remains the treatment of choice, with the depth of the procedure depending on the size of the tumour, location, extent, and regional lymph node involvement [8,10]. Adjuvant therapies are not yet standardized and are generally reserved for advanced or metastatic cases, where they show variable and often limited efficacy and unfavourable prognosis [11]. This lack of standardization is mostly linked to the biological heterogeneity of CMTs and the lack of large and controlled clinical studies [12]. Thus, discovering new bioactive compounds that work through different and complementary mechanisms of action could be a promising strategy for enhancing treatments and improving the prognosis in specific canine populations.

In recent years, scientific interest in fungal metabolites has grown considerably, thanks to their potential as new sources of molecules with pharmacological activity. Among the diverse taxa of endophytic fungi, *Epicoccum nigrum* (*E. nigrum*) is frequently isolated from a wide range of plants and environments and is known to produce a broad spectrum of secondary metabolites, including pigments, polyketides, and alkaloids with antifungal, antibacterial, and antioxidant activities [13]. Several secondary metabolites from *E. nigrum* have been characterized as bioactive molecules with antitumor, antimicrobial, and antioxidant activities. Recent investigations on *E. nigrum* have led to the identification of novel naphtho- and benzofuran derivatives, in addition to previously described metabolites including epicocconigrone A, epicoccolide B, and epicoccone, all of which exhibit cytotoxic activity against several cancers, with breast cancer showing the greatest susceptibility [14].

To the best of our knowledge, the effects of a polyene with an unfused ring system named Epipyron A (Epy A), the main compound released by an *E. nigrum* strain isolated from oat (*Avena sativa*) [15], have not yet been investigated on CMT cells. Because of the central role of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway for controlling growth, survival, and resistance in CMTs, the aim of this study was to investigate how Epy A modulates this pathway and explicates its antiproliferative and antioxidant activity against P114 and CF33 CMT cell lines.

2. Materials and Methods

2.1. Fungus Identification and Cultivation

The fungal isolate was grown on PDA at 25 °C in the dark. Colony morphology, including size, color, and hyphal structure, was recorded and photographed. Conidial features were also examined and measured under an Axioskop2 Plus microscope (Zeiss, Milan, Italy). The endophyte isolated from *Avena sativa* leaves displayed morphological traits consistent with *E. nigrum*.

Genomic DNA was extracted from fungal mycelium following a CTAB-based protocol according to Lodhi et al. 1994 [16], with slight modifications. PCR amplifications were conducted using a MiniAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) to amplify the internal transcribe sequences (ITS), in particular, the primers ITS4 (reverse, 5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (forward, 5'-GGA AGT AAA AGT CGT AAC AAG G-3') regions were amplified with polymerase chain reaction (PCR) with the following cycle: 95 °C × 5 min; 35 cicli (95 °C × 30 s; 58 °C × 30 s; 72 °C × 1 min) 72 °C 5 min [17]. DNA quality was checked using 2% agarose gel electrophoresis and quantified using the NanoDrop™ spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA), and the concentration was adjusted accordingly before PCR amplification. The PCR products were purified by Purelink PCR Purification kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). The sequences obtained were compared to database sequences using BLAST version 2.12.0 (NCBI, Bethesda, MD, USA; Gapped BLAST and PSI-BLAST search programs) [17] and have been deposited in GenBank under accession number PX853879.

The *E. nigrum* was initially isolated and cultured on PDA (potato dextrose agar; Hi Media, Thane, India) and subsequently inoculated in eight liters of PDB (potato dextrose broth; Hi Media, Thane, India). The cultures were then incubated at 25° C for 20 days under static condition to obtain bioactive filtrates. After the incubation period, fungal biomass was separated from the culture filtrate by vacuum filtration using Whatman filter paper (Whatman, Brentford, UK).

2.2. Epipyron A (Epy A) Extraction and Identification

The culture filtrate was extracted, and Epy A was purified as reported by Lotito et al. [18]. Briefly, the liquid/liquid extraction was carried out with ethyl acetate, and the combined extracts were dried with anhydrous Na₂SO₄ and concentrated under reduced pressure using a rotary evaporator (IKA RV 10 digital FLEX, Burladingen, Germany), yielding a red crude extract. The organic extract was fractionated with vacuum liquid chromatography (VLC) [19]. A stepwise gradient elution was carried out using mixtures of methanol (MeOH) and water (H₂O) in increasing polarity as reported by Lotito et al. 2026 [18].

The fractions were analyzed with reverse-phase thin-layer chromatography (RP-TLC) using a mobile phase of acetonitrile (ACN) and water 70:30 v/v (plates visualized under UV light at 254 and 365 nm). Subsequently, they were further purified by high-performance liquid chromatography (HPLC). The purification was carried out using a Prodigy ODS (3) C18 column (250 × 10 mm, 10 µm, Phenomenex, Torrance, CA, USA) on an HPLC system equipped with a UV detector (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of solvent A (H₂O + 0.1% formic acid) and solvent B (ACN + 0.1% formic acid), and the following gradient was applied at a flow rate of 2.0 mL/min: from 5% to 70% B in 4 min, isocratic at 70% B for 2 min, from 70 to 80% B in 10 min, isocratic for 4 min, from 80 to 100% B in 2 min, isocratic for 2 min, and finally going back to initial condition (5% B) in 2 min. The injection volume was 200 µL of a concentration of 50 mg/mL, and the elution profile was monitored at 280 nm [18].

Analyses were conducted using an Agilent HP 1260 Infinity LC system coupled to a Q-TOF mass spectrometer with a diode array detector (DAD, Agilent Technologies). Separation was performed on a Kinetex® C-18 column (100 × 2.1 mm, 2.6 µm) at 25 °C, using a flow rate of 0.2 mL/min. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B), with a gradient from 5% to 80% B over 3 min, and from 80% to 98% B over 8 min, followed by 1 min of isocratic elution at 98% B, and going back to 5% B in 2 min. Equilibration time was set to 2 min. Injection volume was 5 µL.

The mass spectrometer operated in positive electrospray ionization modes. Gas flow was 11 L/min at 350 °C, nebulizer pressure 45 psig, and spectra were acquired from m/z 100–1700 at three scans per second.

Mass accuracy was maintained by continuous infusion of purine and phosphazene standards. Instrument control and data acquisition used Agilent MassHunter software (v. B.10.01). Compounds were identified using an in-house fungal database comprising data from the literature, with a mass accuracy of 5 ppm.

^1H and ^{13}C NMR spectra were acquired at 400 MHz and 100 MHz, respectively, on a Bruker Avance spectrometer (Karlsruhe, Germany) using deuterated chloroform (CDCl_3) as both solvent and internal reference.

2.3. Cell Culture and Epy A Treatment

P114 cells (kindly provided by Prof. Dr. Gerard Rutteman, Department of Clinic Science and Companion Animals, University of Utrecht, The Netherlands), derived from a canine anaplastic mammary carcinoma, were cultured in Dulbecco's Modified Eagle's Medium/Nutrient mixture F12 (DMEM/F12, Gibco, Grand Island, NY, USA); CF33MT (purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna), canine mammary tumour epithelial-like cell line, and MDCK (Madin-Darby canine kidney, ATCC) were maintained in DMEM (Corning, NY, USA). The MDCK cell line was used as a non-cancerous control. All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2 , with all media supplemented with 10% fetal bovine serum (FBS, Sial) and 1% antibiotics (penicillin–streptomycin solution; Corning, NY, USA). Cells were passaged when reaching approximately 80% confluence using 0.05% trypsin-EDTA (Corning, NY, USA) and seeded according to experimental requirements.

Epy A was first dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution, which was then diluted in medium supplemented with 5% FBS. The compound was maintained in the culture medium of each cell line for 24 and 48 h at increasing concentrations (17.5, 30.0, 55.5, 100, and 175 $\mu\text{g/mL}$), except for the vehicle-treated group (Ctr/DMSO).

2.4. Cell Viability Evaluation

Cell viability was assessed by measuring mitochondrial metabolic activity using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay in 96-well plates, as described in previous works [20,21]. Briefly, MTT powder was dissolved in Roswell Park Memorial Institute (RPMI) medium at a final concentration of 0.5 mg/mL. An aliquot of 100 μL was added to each well, and the plate was incubated at 37 °C for 4 h. Subsequently, 100 μL of DMSO was added to each well to solubilize the formazan crystals, and the plate was further incubated at 37 °C for 10 min. The absorbance was then measured at 570 nm using a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. PI (Propidium Iodide)/DAPI Staining

After 48 h of treatment, the supernatant was removed, and cell monolayers were washed twice with phosphate-buffered saline (PBS), fixed, and sequentially incubated with propidium iodide (PI) solution (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature in the dark, followed by DAPI staining for 3 min. After 3 washes with PBS, fluorescence images were acquired by ZOE™ Fluorescent Cell Imaging System (Bio-Rad, Milan, Italy).

2.6. TAC (Total Antioxidant Capacity) Assay

TAC assay (Abcam, Cambridge, UK) was performed according to the manufacturer's instructions. Briefly, treated cell monolayers were harvested, pelleted, and washed once

with cold PBS. Pellets were then resuspended in 0.05% Triton X-100 and homogenized by pipetting. After incubation on ice for about 10 min, the suspensions were centrifuged at maximum speed at 4 °C. The resulting supernatants were collected and incubated with the Cu²⁺ working solution. After 15 min of incubation at room temperature in the dark, absorbance was measured spectrophotometrically at 570 nm. Data were analyzed using a Trolox standard curve.

2.7. DCHF-DA (2',7'-Dichlorofluorescein diacetate) Staining

Cell monolayers, appropriately treated, were incubated with DCFH-DA (10 µM in DMEM, Sigma, St. Louis, MO, USA) at 37 °C. After 30 min incubation, cells were washed twice with DMEM and once with PBS to remove excess dye. Images were acquired with the ZOE™ Fluorescent Cell Imaging System (Bio-Rad, Milan, Italy).

2.8. Western Blot Analysis

Cell pellets obtained from the experimental procedures described above were lysed using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with an appropriate concentration of protease and phosphatase inhibitors (Roche, Basel, Switzerland). Twenty-five micrograms of proteins were loaded, separated by SDS-page, and blotted on nitrocellulose membranes (Bio-Rad, Milan, Italy). Membranes were incubated overnight at 4 °C with primary antibodies (1:1000) targeting mTOR (Cell Signaling Technology, Danvers, MA, USA), p-mTOR (Cell Signaling Technology, Danvers, MA, USA), PI3K (Cell Signaling Technology, Danvers, MA, USA), Akt (Santa Cruz Biotechnology, Dallas, TX, USA), p-Akt (Cell Signaling Technology, Danvers, MA, USA), β-Actin (Santa Cruz Biotechnology, Dallas, TX, USA). After washing, membranes were incubated for 1 h with species-specific peroxidase-conjugated secondary antibodies. Protein bands were visualized with a ChemiDoc Imaging System (Bio-Rad, Milan, Italy), and densitometric analysis was performed by Image Lab software version 6.1 (Bio-Rad, Milan, Italy).

2.9. Wound Healing Assay

Cells were seeded in 6-well plates, and after 24 h, a linear scratch was created across the cell monolayer using a sterile pipette tip. Detached cells and debris were gently removed by washing twice with fresh medium. Subsequently, cells were adequately treated with serum-free medium containing the treatment (Epipyrone A) to inhibit proliferation-related effects. Images of the wounded area were captured immediately after scratching (0 h) and after 12 h (12 h) with ZOE™ Fluorescent Cell Imaging System (Bio-Rad, Milan, Italy).

2.10. Statistical Analysis

All experimental procedures were conducted in duplicate. Statistical analyses were performed using GraphPad software (version 8.0.2). Differences between groups were assessed through ordinary one-way ANOVA, and results with *p*-values less than 0.05 were deemed statistically significant.

3. Results

3.1. Isolation of Epy A from *E. nigrum*

The culture filtrate was analysed by mass spectrometry coupled to liquid chromatography (LC-MS). Interestingly, the most abundant compound was putatively identified as Epy A, a brownish pigment with known antioxidant activity [18].

Following further separation by HPLC, the fraction containing the main compound was identified (Figure 1) and confirmed by one-dimensional NMR spectroscopy to be Epy

A (Table S1). The structure was validated by comparison with previously reported in the literature [22,23].

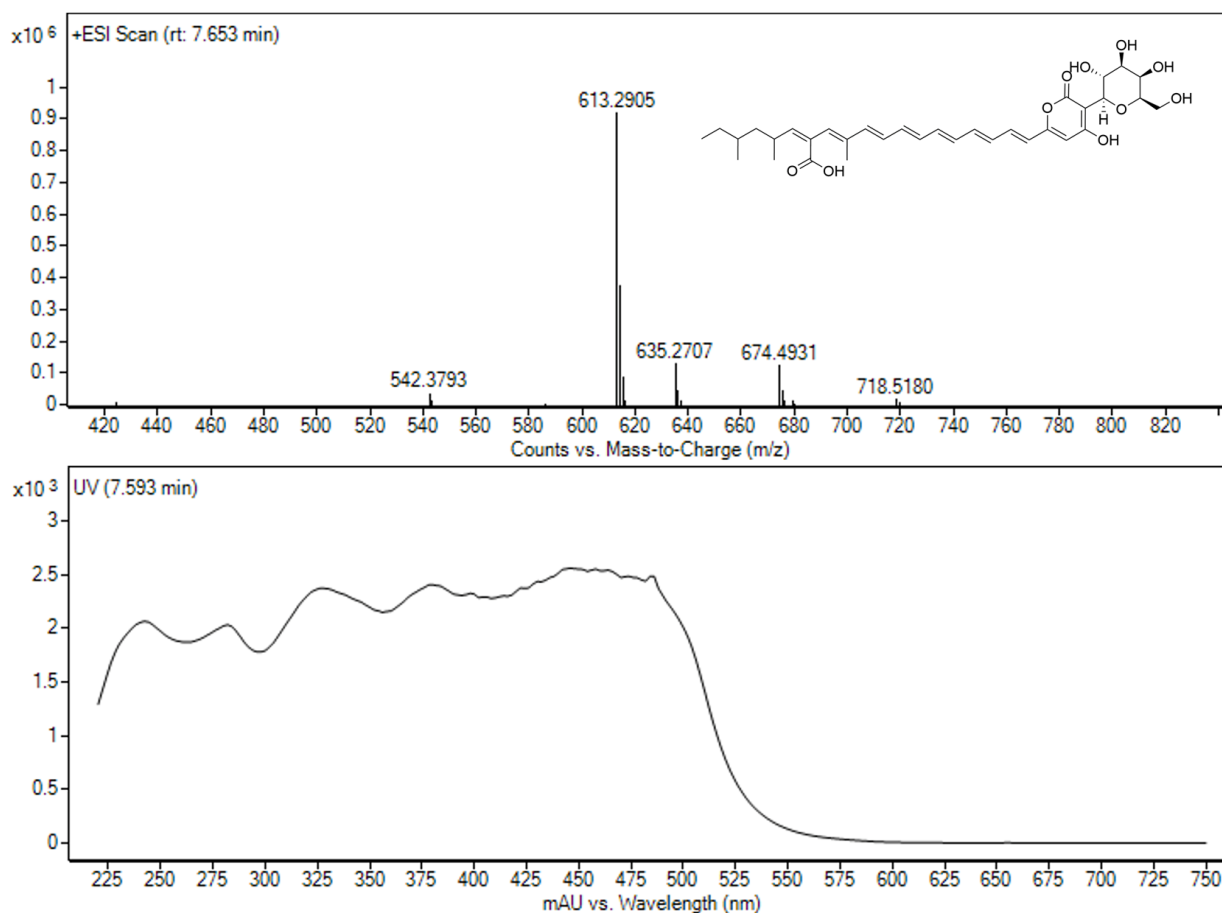


Figure 1. Mass (top) and UV 360 nm (bottom) spectra of Epipyron A isolated from *Epicoccum nigrum* extract obtained from liquid culture. Mass spectrum was recorded in positive ionization mode.

3.2. Epy A Reduces CMTs Cells Viability

The P114, CF33, and MDCK cell lines were treated with five different concentrations of Epy A and assessed at two time points: 24 and 48 h post-treatment (Figure 2). The MDCK line, used as a control, did not exhibit any significant reduction in cell viability compared to the vehicle-treated group at either time point (Figure 2A).

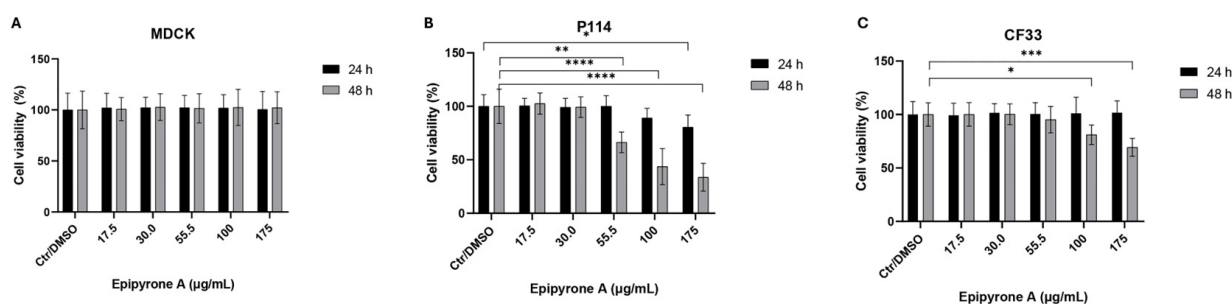


Figure 2. Antiproliferative effects of Epipyron A (17.5, 30.0, 55.5, 100 and 175 µg/mL) on (A) MDCK, (B) P114, and (C) CF33 cell lines at 24 and 48 h post-treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ versus Ctr/DMSO.

In contrast, CF33 cells showed a statistically significant decrease in viability only after 48 h of treatment, with reductions observed at 100 µg/mL (* $p < 0.05$) and 175 µg/mL (** $p < 0.001$) (Figure 2C). In this case, the calculated IC₅₀ value is 80.77 µg/mL.

P114 cells appeared more sensitive to Epy A. A significant reduction in viability was already evident at 24 h at the highest concentration tested (175 µg/mL, * $p < 0.05$). After 48 h, the effect became more pronounced, with viability decreasing significantly from 55.5 µg/mL (** $p < 0.01$) to 100 µg/mL (**** $p < 0.0001$) and 175 µg/mL (**** $p < 0.0001$), indicating a clear concentration- and time-dependent cytotoxic effect (Figure 2B), and exhibiting an IC₅₀ of 64.64 µg/mL.

Due to the marked decrease in cell viability observed at the highest concentration of Epy A after 48 h of exposure, and based on the calculated IC₅₀, subsequent experiments were conducted using the 100 and 175 µg/mL doses for both P114 and CF33 cell lines.

Propidium iodide (PI) staining was used to detect loss of cell viability and membrane integrity. No noticeable changes in fluorescence intensity were observed in MDCK cells at any of the tested Epy A concentrations, indicating the absence of cell death in the non-tumor cell line. In contrast, CF33 and, more markedly, P114 showed increased PI uptake following Epy A treatment, evidenced by a stronger red fluorescence signal, consistent with an increase in nonviable cells and those with damaged membranes (Figure 3). These results suggest preliminary evidence of cytotoxic activity, necessitating additional investigation through specific assays to determine the cell death mechanism.

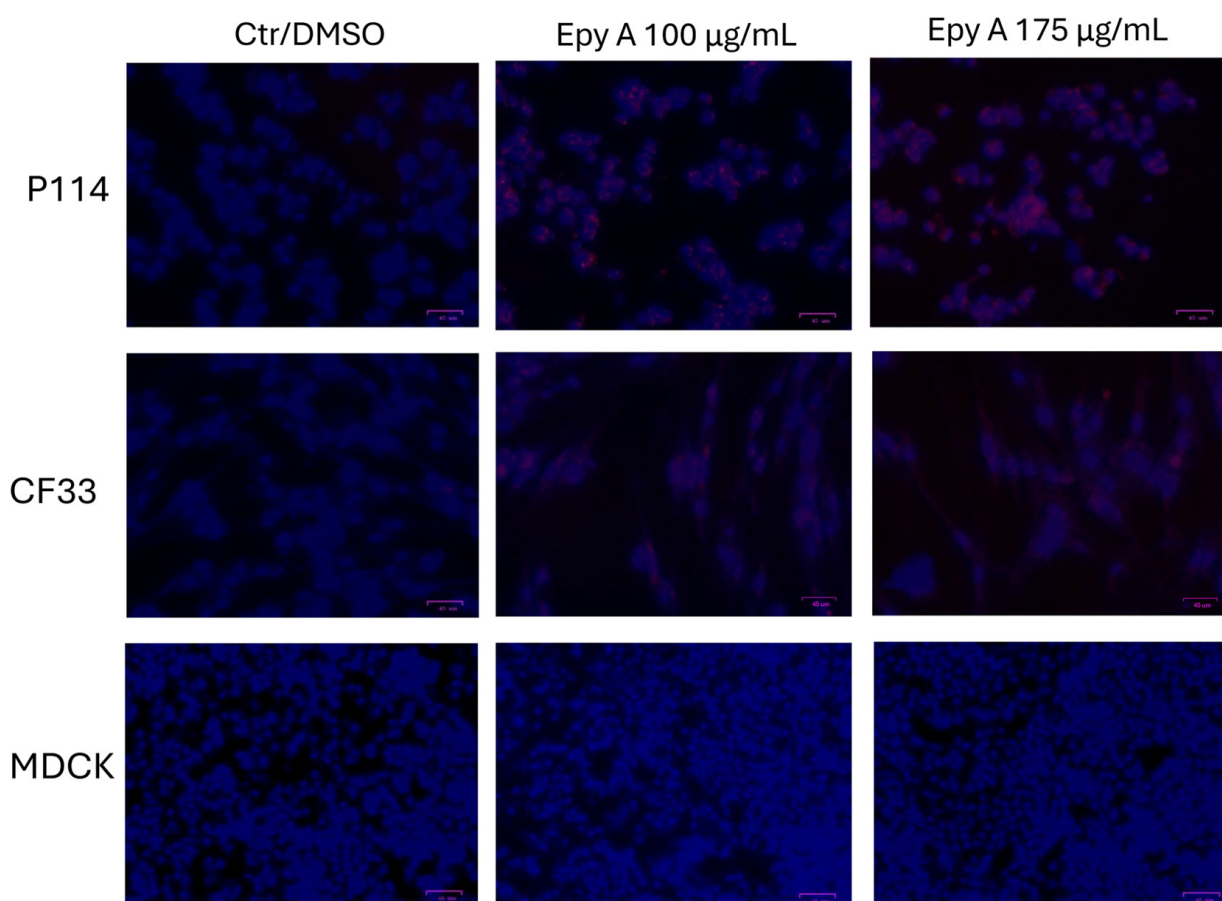


Figure 3. Propidium iodide (PI) and DAPI dual staining on MDCK, P114, and CF33 cell lines after 48 h of Epipyrrone A (Epy A) treatment (100 and 175 µg/mL). Scale bar = 45 µm.

3.3. Epy A Exerts Modulatory Effects on the PI3K/Akt/mTOR Pathway in P114 and CF33 Cell Lines

Upon treatment with Epy A, both cell lines exhibited a concentration-dependent response.

In P114 cells, Epy A significantly reduced total mTOR and its phosphorylated form (p-mTOR), with the greatest inhibition observed at 175 µg/mL (* $p < 0.05$; *** $p < 0.001$). Total Akt levels were also markedly decreased at 175 µg/mL (*** $p < 0.001$), while p-Akt was significantly reduced at both concentrations (** $p < 0.01$).

In contrast, CF33 cells displayed an opposite pattern. Epy A significantly increased total Akt and p-Akt levels at 175 µg/mL (* $p < 0.05$), while p-mTOR was significantly upregulated at 100 and 175 µg/mL (* $p < 0.05$). Conversely, total mTOR expression was significantly decreased at both concentrations (** $p < 0.01$; **** $p < 0.0001$).

For PI3K, expression was significantly decreased in CF33 cells at both concentrations (*** $p < 0.001$; * $p < 0.05$), whereas in P114 cells, a significant reduction was observed only at 175 µg/mL (* $p < 0.05$).

Together, these findings indicate that Epy A exerts reciprocal, cell context-dependent modulation of the PI3K/Akt/mTOR signaling pathway (Figure 4). Whole blots can be found in Figure S1.

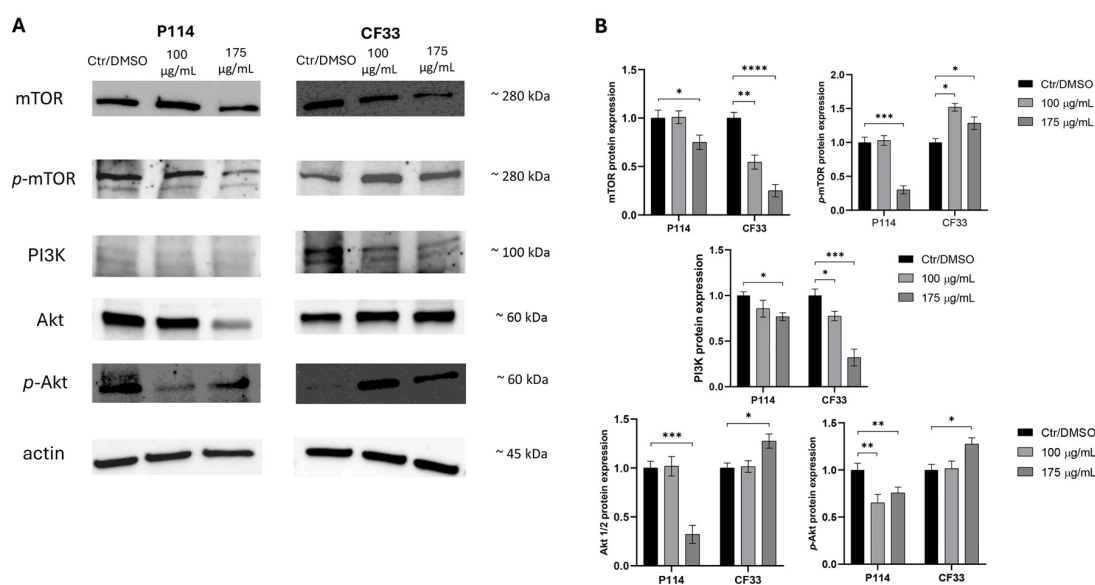


Figure 4. Effects of Epipyron A (Epy A) on the PI3K/Akt/mTOR pathway in P114 and CF33 cells. (A) Representative Western blots showing total and phosphorylated levels of proteins after treatment with Epy A (100 and 175 µg/mL) compared with control (Ctr/DMSO). Actin was used as a loading control; (B) densitometric analysis of the blots expressed as protein/actin ratio. Data are presented as mean $\pm$ standard deviation; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Wound Healing Impairment by Epy A

Treatment with Epy A compromised the wound healing capacity of both CF33 and P114 cell lines (Figure 5A,B). Cells in the control group (Ctr/DMSO) progressively migrated to close the scratch as early as 12 h post-treatment. In contrast, Epy A-treated cells exhibited a marked delay in wound closure, with the remaining gap area visibly wider compared to controls. This inhibitory effect was concentration-dependent, with higher concentrations of Epy A resulting in a more pronounced reduction in cell migration and wound scratch closure.

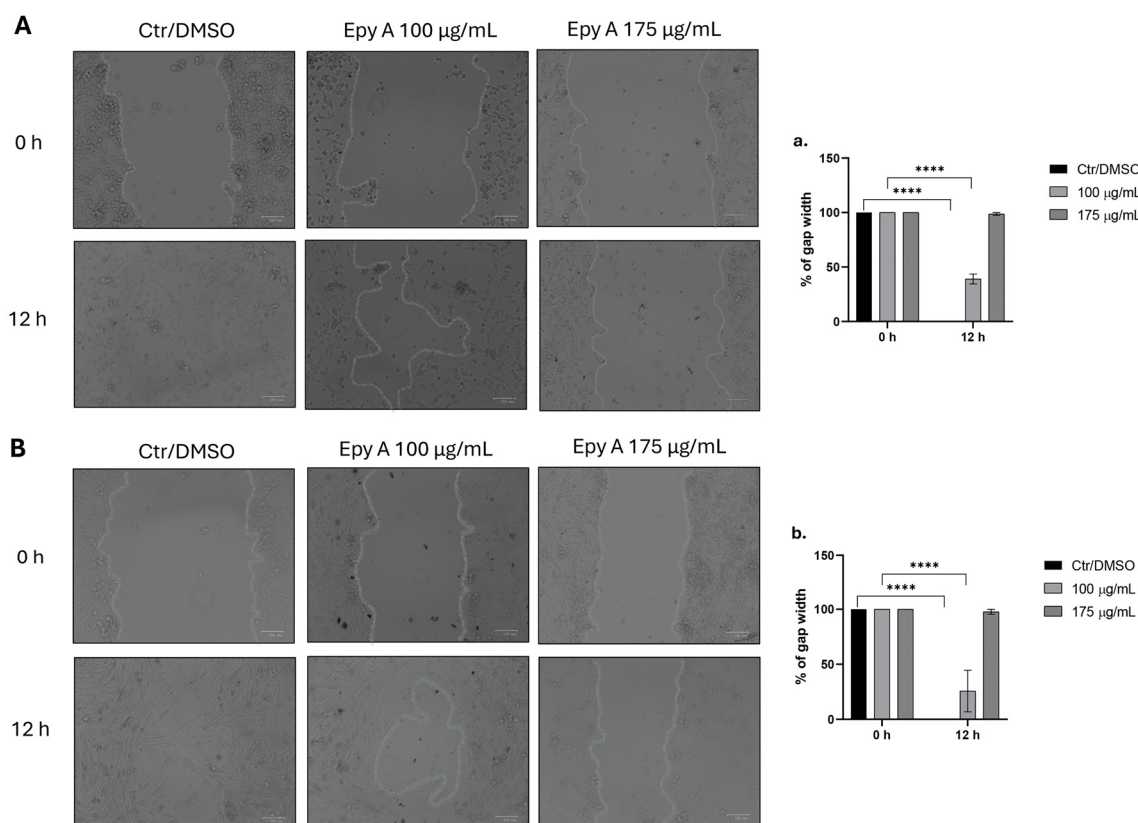


Figure 5. (A,B) Representative images of the wound healing assay performed on P114 (A) and CF33 (B) at 0 h and 12 h after scratch in Ctr/DMSO and Epipyrone A (Epy A)-treated cells (100 and 175 µg/mL). Scale bar = 100 µm. (a,b) Graphical representation of the relative migration of treated and untreated P114 (a) and CF33 (b) cells after 12 h, expressed as a percentage of gap width reduction compared to time zero (0 h), designated as 100%. Data are presented as mean values $\pm$ SD derived from a minimum of five measurements across at least six fields for each experimental condition, representing two separate studies that exhibit comparable trends; **** $p < 0.0001$.

3.5. Epy A Modifies TAC and ROS Production in CF33 and P114 Cell Lines

To evaluate the potential antioxidant activity of Epy A on cells after treatment, the TAC assay was used together with DCFH-DA staining, with green fluorescence reflecting ROS production. Figure 6A shows a marked decrease in fluorescence in the P114 cell line following Epy A treatment compared to the control (Ctr/DMSO). In contrast, the CF33 cell line exhibited a less pronounced reduction.

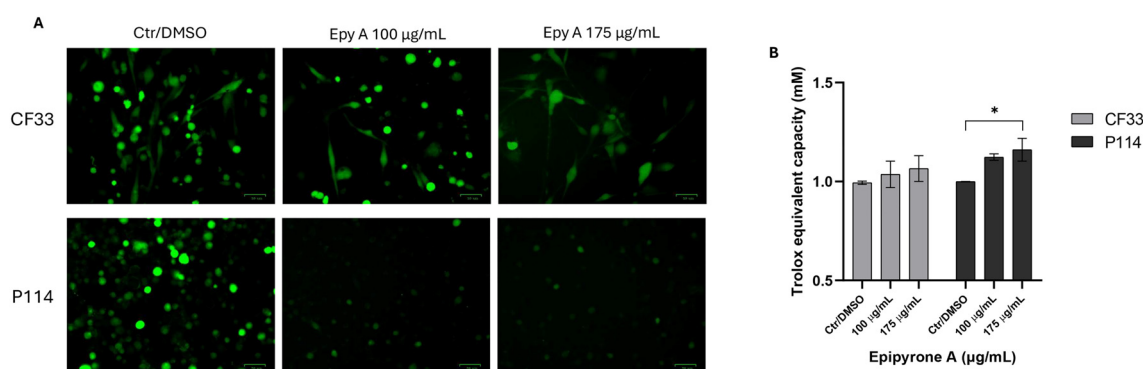


Figure 6. (A) Representative DCFH-DA (2',7'-Dichlorofluorescein diacetate) stain, scale bar = 50 µm; (B) total antioxidant capacity (TAC) relative to nmol of Trolox. * $p < 0.05$.

These findings align with the observed increase in the antioxidant capacity of P114 cells treated with Epy A. Specifically, a statistically significant increase in TAC was observed at a concentration of 175 $\mu\text{g/mL}$ (* $p < 0.05$, Figure 6B). No statistically significant changes were observed in CF33 cells, although a trend toward increased TAC was noted (Figure 6B).

4. Discussion

CMTs are among the most frequent tumours affecting female dogs, for which no standardized and effective therapies are currently available [24]. Naturally derived compounds are attracting interest as potential anticancer agents, given their ability to modulate pathways different from those targeted by conventional chemotherapeutics [25,26]. Such features make them promising molecules to develop alternative or complementary strategies in veterinary medicine.

Metabolomic profiling of both the culture filtrate led to the identification of different compounds, including epicoccolide B, epicolactone, epicoccone A/B, epicoccalone, and epicoccin D, but the major compound was Epy A [18]. Epy A was identified as the dominant secondary metabolite in the culture filtrate through LC-MS, with the highest mass (m/z 613.2950) and longest retention time, with a prominent signal corresponding to molecular formula $\text{C}_{34}\text{H}_{45}\text{O}_{10}$, confirmed by NMR analysis. The compound is a complex polyketide associated with antimicrobial and antioxidant activities. Epy A, in addition to its well-known antifungal activity, has also been shown to possess antiviral effects, including inhibition of HIV-1 Rev protein, telomerase, and influenza A (H1N1) virus [27]. Its biosynthesis has been clarified through genomic studies, which revealed the essential role of the *epnABCD* gene cluster [28]. Moreover, the compound's yellow-orange pigmentation and sensitivity to heat have sparked interest in its potential application as a natural colorant, although comprehensive evaluations of its stability under different processing conditions are still required [29].

This study presents the preliminary evidence that Epy A induces a selective, concentration- and time-dependent antiproliferative effect on CMT cells. P114 cells, derived from an anaplastic carcinoma, the most aggressive CMT subtype, exhibited the highest sensitivity. In contrast, CF33 showed a moderate response, while MDCK non-tumor cells were predominantly unaffected. This pattern highlights the biological diversity among CMT subtypes and suggests a degree of tumour selectivity for Epy A, with an encouraging preliminary safety profile against non-transformed epithelial cells. A similar pattern was observed in other studies with Epy A showing the highest cytotoxicity against the KA3IT tumor cell line, whereas its activity against non-transformed lines like MDCK, HSCT6, and HEK293 was significantly lower [30].

The PI3K/Akt/mTOR pathway is an important driver of spread, survival, migration, and resistance to therapies in CMTs, where this pathway is often activated and promotes epithelial-mesenchymal transition (EMT) [31]. Dysregulation of this pathway is associated with numerous human malignancies and is therefore considered a critical therapeutic target for the development of novel antitumor agents [32]. Its pharmacological inhibition in vitro with BYL719 alpelisib reduces CMTs cell viability, highlighting this axis as a druggable target in veterinary oncology [33]. The downregulation of the PI3K/Akt/mTOR axis in P114 Epy A-treated cells, together with the PI stain highlighting cell membrane damage, supports loss of tumor cell viability. These data align with results on other fungal secondary metabolites that suppress this pathway and trigger cell death [34]. In contrast, CF33 cells exhibited increased total Akt and p-Akt levels, along with diminished total mTOR levels. This opposite response is consistent with conventional feedback circuitry: the attenuation of mTOR could generate a rebound in Akt activation, an effect widely observed after mTOR inhibition in some cancer models and in patients treated with rapalogs [35]. Moreover,

it is well known that physiologically, mTOR phosphorylates Akt; however, alterations in the homeostasis may preserve Akt phosphorylation even when mTOR expression is reduced [36], resulting in the apparent decoupling observed in CF33. Although further research is needed to clarify the Epy A mechanism of action, this framework could account for the reduced sensitivity of CF33 to Epy A, evidenced by loss of cell viability occurring only after 48 h at the highest concentration tested, corroborated by the PI staining. In this case, co-inhibiting PI3K/Akt together with mTOR could limit the rebound [37].

This pathway is also linked to cell migration, as it regulates the cytoskeletal dynamics and motility, making it an important factor in cancer progression. In fact, when the Akt/mTOR pathway is activated, the activity of matrix metalloproteinases, which degrade the extracellular matrix and cell basal membrane, is increased and facilitates migration [38]. Thus, downregulation of the axis could contribute to the impaired migration observed in wound-healing assays, more pronounced in P114 than in CF33 cells. This behaviour is consistent with the anti-migratory effects reported for other fungal metabolites, such as FTY720, which has been shown to inhibit cell migration in prostatic cancer cells [39].

Oxidative stress has been increasingly recognised as a major cause of mammary tumours in dogs. In CMTs, levels of ROS are higher than in normal mammary tissue [40], and such alterations are often associated with increased cancer risk and worse clinical outcomes [41]. In this study, the capacity of Epy A to modulate ROS levels and promote TAC in P114 cells suggests that the redox modulation could be associated with its anti-proliferative activity. While a direct causal relationship cannot be established based on the current data, it is plausible that changes in cellular redox equilibrium may affect survival signalling pathways, such as PI3K/Akt/mTOR, which are regulated by redox-sensitive processes [42]. In this context, mitochondria are pivotal in connecting redox homeostasis, PI3K/Akt/mTOR signaling, and determining whether cells maintain survival signaling or undergo cell death [43]. This well-established interconnection provides a biological framework to elucidate the simultaneous modulation of redox balance and survival signaling observed in the present study. In fact, when oxidative stress levels are high, mitochondrial ROS production can keep Akt activated, promoting cancer cells' survival; conversely, restoration of redox impairment could reduce mitochondrial support to cell survival, thereby altering bioenergetic homeostasis [44]. Therefore, the reduction of intracellular ROS following Epy A treatment may signify a shift in the mitochondrial redox signaling that accompanies increased susceptibility to loss of cell viability. In this context, Epy A could therefore indirectly affect survival pathways such as PI3K/Akt/mTOR by contributing to an intracellular environment less permissive to tumour cell survival and migration, likely through mechanisms involving redox-sensitive regulators [42]. Although mitochondrial function was not directly assessed in this study, the different post-treatment susceptibilities indicate a possible correlation between redox reactivity and the differential sensitivity of the cells to Epy A. In fact, the simultaneous reduction in ROS levels, inhibition of PI3K/Akt/mTOR signaling, and loss of cell viability observed in P114 cells align with a cellular environment less permissive to sustained survival signaling. Conversely, the lower susceptibility of CF33 correlates with persistent Akt phosphorylation and diminished cytotoxicity. These results support the idea that Epy A doesn't function through a single dominant route, but rather, it affects multiple interconnected processes to shape tumor cell response.

Chemotherapeutic agents commonly administered in veterinary oncology induce systemic toxicity and are known to act primarily through direct cytotoxic mechanisms [45]. On the other hand, Epy A has been characterized as a biologically active natural product associated with the modulation of intracellular signaling pathways and redox homeostasis *in vitro*. Such properties distinguish Epy A from conventional chemotherapeutics and

suggest a mode of action that may influence tumor cell behavior without relying solely on pronounced cytotoxicity.

Unfortunately, the development of natural product-based therapeutics in veterinary medicine is associated with a number of challenges [46,47]. Structural complexity could complicate large-scale production, standardization, and reproducibility. Importantly, the bioavailability, stability, and pharmacokinetics of natural products remain poorly characterized, particularly in veterinary species [46]. In addition, natural compounds that display moderate biological activity *in vitro*, such as Epy A, can be used as a useful biological scaffold for further molecular optimization or combination strategies, while preserving a safety profile [48].

Despite the limitations of an *in vitro* explanatory study, this work falls within a translational perspective and underscores Epy A value as a candidate for further mechanistic investigation. Indeed, canine tumor cell lines provide an excellent preclinical model for testing anti-tumor responses, discovering cancer-specific signaling pathways, and identifying genes involved in cancer genesis, such as oncogenes or tumor suppressors.

Further investigations, including mechanistic and *in vivo* studies, will be necessary to confirm the specificity of its action and evaluate whether the described properties could translate into clinical benefit.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox15020173/s1>. Table S1: ¹H NMR spectroscopic data for the isolated compound Epipyron A (400 MHz, CD₃OD). Figure S1: Uncropped and unedited whole western blot from which the cropped images shown in the main manuscript were derived.

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Abbreviations

The following abbreviations are used in this manuscript:

CMT	Canine mammary tumor
DAD	Diode array detector
DCHF-DA	2',7'-Dichlorofluorescein diacetate
EPY A	Epipyron A
HPLC	High-performance liquid chromatography
LC-MS	Mass spectrometry coupled to liquid chromatography
NMR	Nuclear magnetic resonance
PDA	Potato dextrose agar
PDB	Potato dextrose broth

PI	Propidium iodide
PI3K/Akt/mTOR	Phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin
TAC	Total antioxidant capacity

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