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Article

# AC3™ Reduces Cell Proliferation and Migration by Modulating the TNF/TNFR1 Axis and Improves the Immune Response by Downregulating CD39/CD73/Ado in Cutaneous Melanoma Cell Lines

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**Simple Summary:** Cutaneous melanoma (CM) is an aggressive neoplasm with high recurrence and therapeutic resistance. This study evaluated the antineoplastic effect of Advanced Curcumin C3 Complex (AC3™), a *Curcuma longa* extract enriched with Bisdemethoxycurcumin, on A375 and SK-MEL-28 cell lines. Cell viability tests (MTT), migration (wound healing assay), reactive oxygen species (ROS) production and gene expression by RT-PCR demonstrated that AC3™ inhibits cell proliferation and migration. In A375 cells, the compound reduced ROS levels and increased caspase-3 expression. In SK-MEL-28 cells, there was an elevation of caspases-3 and -8, activating intrinsic and extrinsic apoptotic pathways. AC3™ also modulated inflammation, increasing Tumor Necrosis Factor Alpha (TNF- $\alpha$ ), Interleukin 6 (IL-6) and NLR Family Pyrin Domain Containing 3 (NLRP3) in A375 and reducing NLRP3 in SK-MEL-28. Furthermore, it regulated ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5'-nucleotidase (CD73), indicating an influence on purinergic signaling. These data suggest the therapeutic potential of AC3™ against melanoma, acting on multiple cellular pathways.

**Abstract:Background/Objectives:** Cutaneous melanoma (CM) poses a critical public health concern due to its high recurrence rates and resistance to standard treatments. This study investigated the antineoplastic potential of *Curcuma Longa L.* extract enriched with Bisdemethoxycurcumin (AC3™) on human melanoma cell lines A375 and SK-MEL-28. **Methods:** The A375 and SK-MEL-28 cell lines were cultured according to the manufacturer's recommendations and were subsequently treated with different concentrations of AC3™. The MTT test and fluorescence microscopy were used to assess cell viability. The wound healing assay was used to verify cell migration after treatment with the compound. To determine reactive oxygen species (ROS) levels, we used the fluorescence assay with 2,7-dichlorodihydrofluorescein diacetate. Subsequently, the molecular pathways associated with apoptosis were evaluated using RT-PCR, including the modulation of the inflammatory profile and the components of purinergic signaling. **Results:** The results revealed that AC3™ effectively inhibited cell proliferation and migration in both melanoma cell lines. In A375 cells, the compound reduced ROS levels and significantly modulated apoptotic pathways, including upregulation of caspase-3 expression. Similarly, in SK-MEL-28 cells, increased expression of caspase-8 and caspase-3 indicated the activation of both intrinsic and extrinsic apoptotic mechanisms. Additionally, AC3™ influenced the inflammatory response by increasing the expression of Tumor Necrosis Factor Alpha (TNF- $\alpha$ ), Interleukin 6 (IL-6) and NLR Family Pyrin Domain

Containing 3 (NLRP3) in A375 cells while downregulating NLRP3 expression in SK-MEL-28 cells, highlighting its differential immune modulatory effects. Furthermore, the compound modulates the expression and enzymatic activity of ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5'-nucleotidase (CD73), key regulators of purinergic signaling, suggesting its ability to impact immune evasion mechanisms in melanoma. **Conclusions:** These findings position AC3™ as a promising therapeutic candidate for melanoma, targeting critical pathways related to tumor progression, immune regulation, and cellular survival.

**Keywords:** Curcuminoids. Cutaneous melanoma. Apoptosis. TNF/TNFR1 axis. Purinergic signaling. Antitumor Therapy

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## 1. Introduction

Since ancient times, humans have used natural compounds to prevent and treat various diseases. Over time, several studies have been carried out, and the functional properties of these products have been sought, as well as their route of action [1]. There is currently a wealth of evidence highlighting the potential of natural compounds in the prevention or treatment of aging [2], obesity and non-alcoholic fatty liver disease (NAFLD) [3,4], neurodegenerative diseases [5], inflammatory bowel diseases [6], kidney disease [7], osteoporosis [8], chronic pain [9], and numerous types of cancer [10–12]

Polyphenols and flavonoids, such as curcumin, resveratrol, and phenolic acids, stand out among the compounds with the most significant scientific evidence. These compounds have numerous pharmacological properties, including antioxidant, anti-inflammatory, antibacterial, immunomodulatory, anticancer, antiproliferative, antimutagenic, and antithrombotic activities [13–27].

Curcumin, derived from *Curcuma longa L.*, a plant belonging to the Zingiberaceae family, stands out as one of the most extensively investigated polyphenols. In addition to curcumin (CUR) (about 75–80%), the plant contains approximately 50 other curcuminoids, including demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC), which together comprise the primary bioactive constituents [28].

Despite its potent pharmacological activities, the low bioavailability of curcumin limits its effectiveness which has prompted the researchers to work with other curcuminoids, such as DMC and BDMC [29]. These compounds differ significantly from curcumin in both their chemical composition and biological activity, offering superior pharmacological effects [30]. BDMC, in particular, has been shown to possess more favorable properties than curcumin, in terms of bioavailability, and bioaccessibility, leading to more potent therapeutic effects [31]. Moreover, these curcuminoids support curcumin's biological activities and physical stability improving its therapeutic potential. The combination of these three curcuminoids was found to be more effective since they act synergistically [28].

Among the therapeutic properties of curcuminoids, their antineoplastic activity has garnered significant attention [32]. Cancer remains a major global health challenge, with certain types, such as cutaneous melanoma, demonstrating resistance to conventional therapies and high recurrence rates, contributing to elevated mortality [33]. To solve this problem, several natural compounds, such as curcuminoids, and the modulation of alternative signaling pathways, such as purinergic signaling, have been studied as adjuvant strategies for treating the disease [34–36]. Cutaneous melanoma is characterized by its aggressive nature and ability to evade immune responses, often mediated by purinergic signaling pathways involving ectonucleotidases such as CD39 and CD73 [37]. These enzymes regulate the extracellular levels of ATP and adenosine, which play opposing roles in tumor immunity [38]. Targeting these pathways has emerged as a promising strategy for overcoming therapeutic resistance in melanoma.

Considering the evidence presented above, the objective of the present study was to evaluate the antineoplastic activity of the Advanced Curcumin C3 Complex (AC3™) (Sami-Sabinsa Group Limited), in two cutaneous melanoma cell lines, A375 and SK-MEL-28. Unlike regular turmeric extracts, it is a specialized formulation derived from *Curcuma longa*, enriched with DMC and BDMC for enhanced physiological benefits. Additionally, we sought to elucidate the key signaling pathways

modulated by AC3<sup>TM</sup>, focusing on its effects on purinergic signaling, oxidative stress, and apoptotic mechanisms.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Chemicals and reagents used in this study were high analytical grade purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), Merck (Darmstadt, Germany), Thermo Fisher Scientific (Grand Island, NY, USA), Invitrogen Life Technologies (Carlsbad, CA, USA) and Sami-Sabinsa Group Limited (India). The cell lines A375 and SK-MEL-28 were purchased from the Cell Bank of Rio de Janeiro (BCRJ) (Brazil). Cell culture medium was purchased from Vitrocell<sup>TM</sup> (Brazil), and plates and flasks used for culture procedures were obtained from Kasvi<sup>TM</sup> (Brazil).

### 2.2. Cell Culture and Exposure to AC3<sup>TM</sup>

The A375 and SK-MEL-28 cell lines were cultured following the Bank of Cell of Rio de Janeiro (BCRJ) recommendations. For A375, Dulbecco's Modified Eagle's Medium (DMEM) containing 2mM of L-glutamine, 4500 mg/L glucose, and 10% of fetal bovine serum was used. For SK-MEL-28, we used Dulbecco's Modified Eagle's Medium (DMEM) with 2mM L-glutamine, 1.0 g/L glucose, and 10% of fetal bovine serum. The cells were maintained three times a week and kept in a humidified and controlled atmosphere of 5% carbon dioxide (CO<sub>2</sub>) at 37°C until reaching the desired confluence. AC3<sup>TM</sup> was dissolved in 0.2% dimethyl sulfoxide (DMSO); the non-toxic dosages used (0.78 µg/mL, 1.56 µg/mL, 3.12 µg/mL, 6.25 µg/mL) were defined through a previous study [32]. Cells belonging to the negative control (CT) group were treated with a culture medium only. After treatment, all treatment groups were incubated for 24h.

### 2.3. Cell Viability by MTT Assay

To assess cell viability, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used as previously described by Mosmann [39]. After the treatment exposure time, the treatment was removed, the cells were washed with phosphate-buffered saline (PBS) (0.1 M, pH 7.4), and 100 µL of MTT dissolved in PBS was added. The cells were incubated at 37°C for 2 hours, during which time the viable cells reduced the MTT to formazan crystals, which were subsequently dissolved in DMSO and read at 570 nm in a microplate reader (Thermo Scientific<sup>TM</sup> Varioskan<sup>TM</sup> LUX).

### 2.4. Cell Viability by Fluorescence Microscopy Assay

Cell viability was also measured using the fluorophore acridine orange (AO), which is taken up only by viable cells and stains both double-stranded (ds) and single-stranded (ss) nucleic acids. Consequently, viable cells emit green fluorescence, which is read under a fluorescence microscope (Nikon® Eclipse TS2-FL) (480–490 nm) in triplicate at 200× magnification and adjusted for brightness and contrast linearly by Imagej® software [40].

### 2.5. Measurement of Mitochondrial Transmembrane Potential ( $\Delta\Psi_m$ )

The mitochondrial transmembrane potential was measured using tetramethylrhodamine ethyl ester (TMRE), accumulating in active mitochondria and emitting red fluorescence proportional to the mitochondrial transmembrane potential when excited at 550 nm. All samples were read in triplicate under a fluorescence microscope (Nikon® Eclipse TS2-FL) at 200× magnification and adjusted for brightness and contrast linearly using Imagej® software [41].

### 2.6. Wound-Healing Migration Assay

Cell migration assay was performed according to Justus et al. [42] cells were seeded in monolayer in 6-well plates, at a density of  $1 \times 10^6$  cells/well. When they reached 100% confluence, a scratch was made using a sterile 200 µL tip, and an initial image was recorded by optical microscopy at 4× magnification. After treatment, the supernatant was discarded, the cells were washed with saline solution, and a new image was obtained. The final images were linearly adjusted for brightness and contrast using ImageJ® software. The results were expressed as percentage (%) of wound closure compared to the control.

### 2.7. Detection of Reactive Oxygen Species (ROS)

ROS levels were checked by a fluorescent assay using 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA), as previously described by Manica et al. [43]. The reading was performed on a fluorescence plate reader (Thermo Scientific™ Varioskan™ LUX) at Ex./Em. = 488/525 nm. All tests were performed in quadruplicate, and results were expressed as a percentage (%) of relative fluorescence compared to the control.

### 2.8. Gene Expression

To extract total RNA from cells, TRIzol® reagent (Invitrogen) was used according to the manufacturer's instructions. Subsequently, RNA quantification and purity verification were performed by the absorption ratio of 260/280 nm using a  $\mu$ Drop™ spectrophotometer (Varioskan LUX™, Thermo Fisher Scientific, USA). RNA was treated with DNase (Thermo Scientific, USA) following the manufacturer's recommendations. cDNA was prepared according to the instructions of the cDNA High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, USA). To evaluate gene expression, each reaction consisted of 6  $\mu$ L of cDNA sample, 10  $\mu$ L of PowerUp™ SYBR™ Green Master Mix for qPCR (Thermo Scientific, USA), 2  $\mu$ L of primer F (500 nM), and 2  $\mu$ L of primer R (500 nM) for a final volume of 20  $\mu$ L. Amplification occurred in the QuantStudio™ 7 Pro Real-Time PCR System (Thermo Scientific, USA). Melting curve analysis was performed to verify product identity. Tests were conducted in quadruplicate, and GAPDH was used for housekeeping. A calibrator was used, and data were calculated by the  $\Delta\Delta C_q$  method. The forward and reverse (5'-3') oligo sequences used for each gene are described in Table 1.

**Table 1.** Primer sequences for RT-qPCR.

Gene	Forward	Reverse
GAPDH	CTCCTCACAGTTGCCATGTA	GTTGAGCACAGGGTACTTTATTG
CASP-8	AGGAGCTGCTCTTCCGAATT	CCCTGCCTGGTGTCTGAAGT
CASP-3	TTTGAGCCTGAGCAGAGACATG	TACCAGTGCATATGGAGAAATG G
TNF- $\alpha$	CAGGCAGTCAGATCATCTTC	GCTTGAGGGTTTGCTACAAC
IL-6	TCATCCCATAGCCCAGAGCA	CTGGCATTGTGGTTGGGTC
NLRP3	AACATGCCCAAGGAGGAAGA	GGCTGTTACCAATCCATGA
CD39	GCCCTGGTCTTCAGTGTATTAG	CTGGCATAACCTACCTACTCTTT C
CD73	GTGCCTTTGATGAGTCAGGTAG	TTCCTTCTCTCGTGTCTTTG

Abbreviations: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Caspase-8 (CASP-8). Caspase-3 (CASP-3). Tumor Necrosis Factor Alpha (TNF- $\alpha$ ). Interleukin 6 (IL-6). NLR Family Pyrin Domain Containing 3 (NLRP3). Ectonucleoside triphosphate diphosphohydrolase 1 (CD39). Ecto-5'-nucleotidase (CD73). Adenosine deaminase (ADA).

### 2.9. Assessment of CD39 and CD73 Enzymatic Activities

The enzymatic activities of CD39 and CD73 were performed according to Pilla et al. [44] and Lunkes et al. [45] with adaptations. The reactions were performed in quadruplicate, prepared as described [46], and subsequently read at 630 nm using the Varioskan LUX™ spectrophotometer (Thermo Fisher Scientific, USA). A standard curve with KH<sub>2</sub>PO<sub>4</sub> was used to calculate the enzymatic activity, and the results were expressed as nmol/Pi /min/mg of protein.

### 2.10. Protein Determination

The Bradford method [47] was employed for protein determination, using bovine serum albumin as the standard. The protein samples were adjusted according to each assay in mg/mL.

### 2.11. Statistical Analysis

All the parameters were statistically evaluated by analysis of variance followed by the appropriate post hoc test using GraphPad Prism 9 software. All data were expressed as mean  $\pm$  standard deviation. The differences between the groups in terms of the studied variables were evaluated through one-way ANOVA analysis. The differences in the probability of rejecting the null hypothesis as  $< 5\%$  ( $P < 0.05$ ) were considered statistically significant. Statistical significance was defined for p values of \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ), \*\*\* ( $P < 0.001$ ) and \*\*\*\* ( $P < 0.0001$ ).

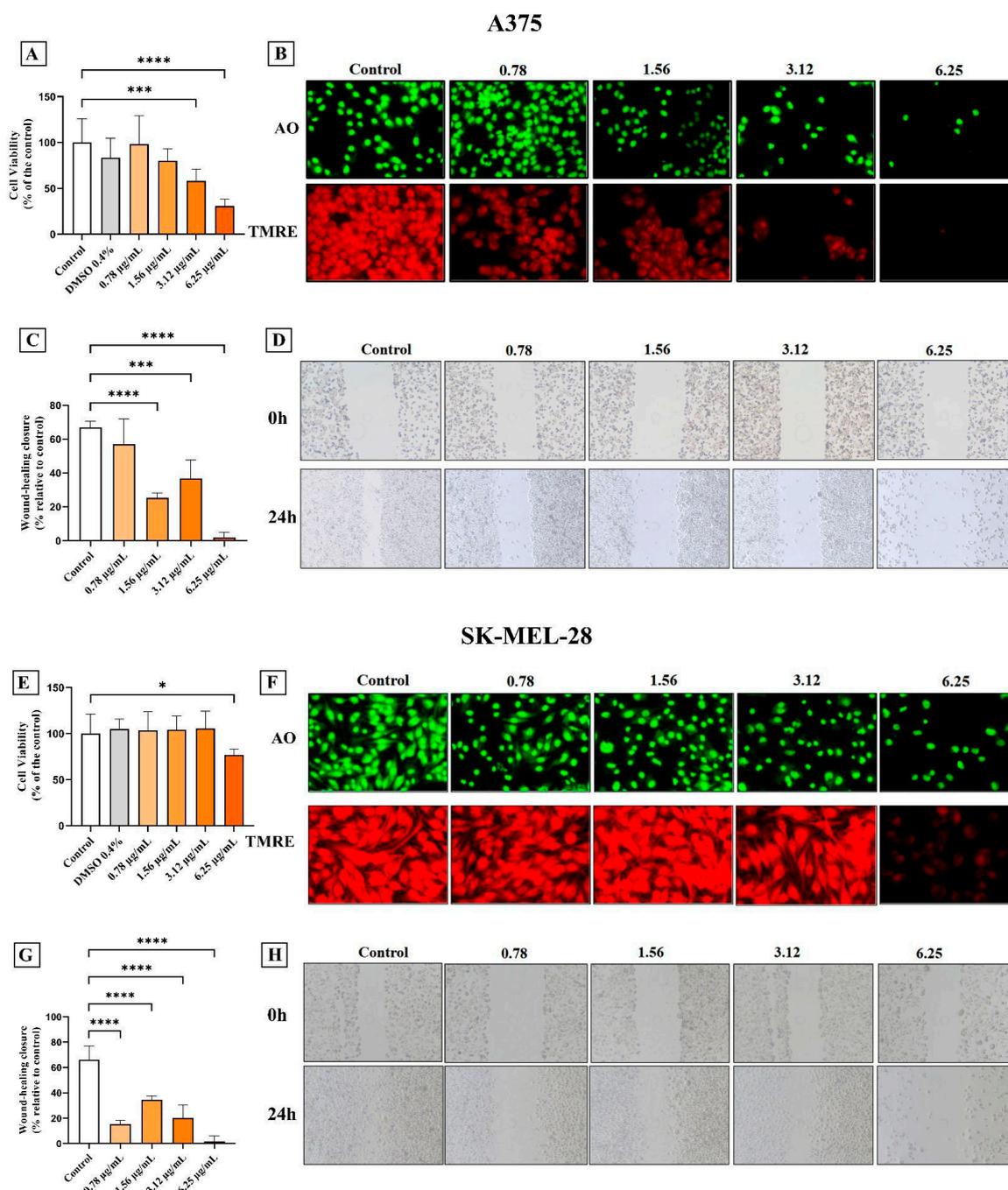
## 3. Results

### 3.1. AC3<sup>TM</sup> Decreased Viability and Migration of Melanoma Cells

Figure 1 presents the antineoplastic effect of AC3<sup>TM</sup> on the SK-MEL-28 and A375 cells. AC3<sup>TM</sup> significantly inhibited the proliferation and migration of A375 and SK-MEL-28 cells. In the A375 cell line, a significant reduction in cell viability was observed at concentrations of 3.12  $\mu\text{g/mL}$  ( $P = 0.0009$ ) and 6.12  $\mu\text{g/mL}$  ( $P < 0.0001$ ) (Figure 1A).

The decrease in cell viability was further supported by fluorescence microscopy analysis, where nuclei were stained with the fluorescent agent acridine orange (AO, green), and mitochondria were stained with Tetramethylrhodamine Methyl Ester (TMRE, red). A decrease in fluorescence intensity indicated impaired cellular proliferation. Moreover, the wound-healing assay demonstrated an inhibition of A375 cell migration following treatment with AC3<sup>TM</sup>, with significant effects observed at concentrations of 1.56  $\mu\text{g/mL}$  ( $P < 0.0001$ ), 3.12  $\mu\text{g/mL}$  ( $P = 0.0003$ ), and 6.25  $\mu\text{g/mL}$  ( $P < 0.0001$ ) (Figure 1C-D).

Similarly, in the SK-MEL-28 cutaneous melanoma cell line, AC3<sup>TM</sup> demonstrated a significant reduction in cell proliferation at the highest concentration tested ( $P = 0.0304$ ) (Figure 1E-F). Furthermore, the wound-healing assay indicated an inhibition of cell migration, as evidenced by a significant reduction in wound closure across all tested concentrations ( $P < 0.0001$ ) (Figure 1G-H).

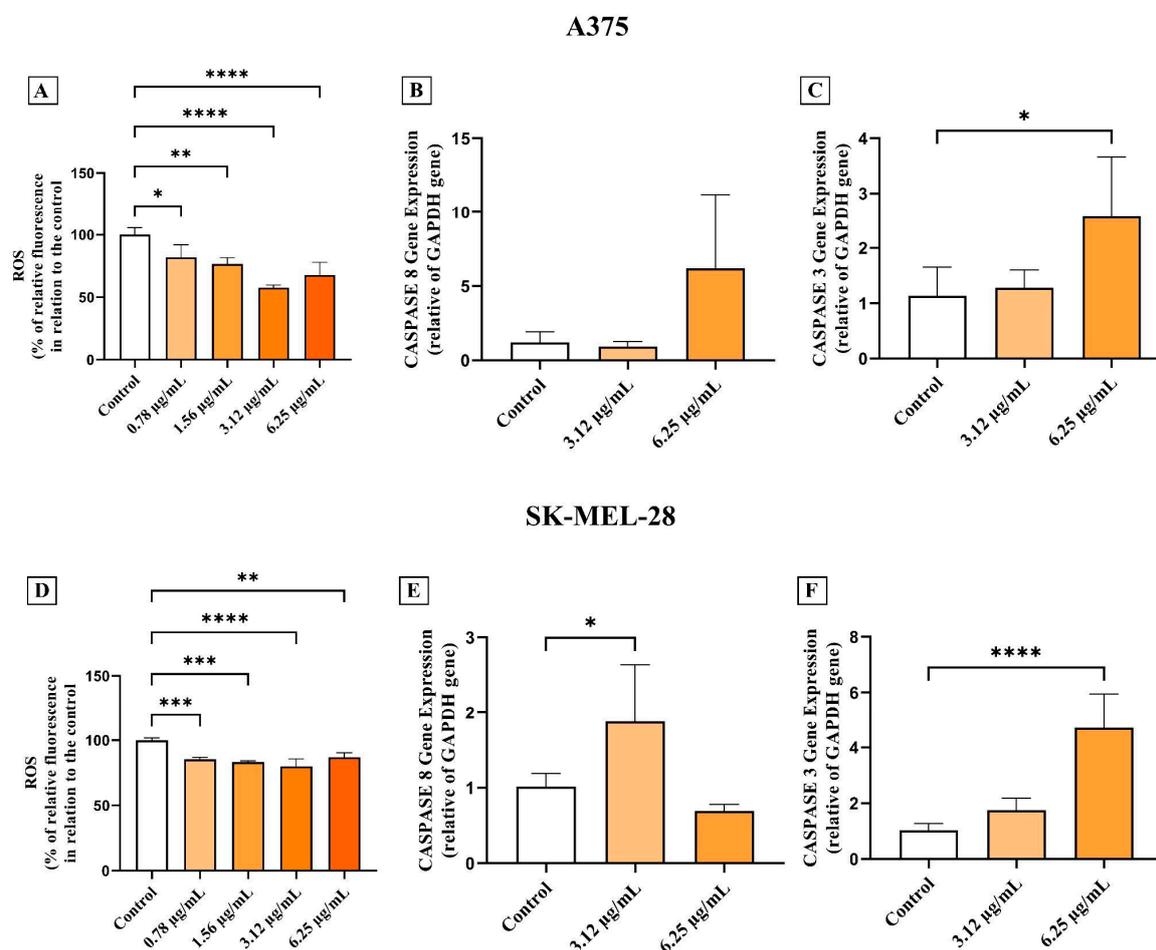


**Figure 01.** Antiproliferative and migratory activity of AC3™ in CM A375 and SK-MEL-28 cells. In the A375 cell line, the MTT assay indicated that treatment concentrations with 3.12 µg/mL and 6.12 µg/mL of AC3™ reduced cell viability after 24 hours of treatment (A); similar results were found in fluorescence microscopy (B). In the cell migration assay, we observed a reduction in cell migration at concentrations of 1.56 µg/mL, 3.12 µg/mL, and 6.25 µg/mL (C, D). In the SK-MEL-28 cell line, we found that treatment with 6.25 µg/mL of AC3 decreased cell viability (E, F). Furthermore, all treatment concentrations effectively reduced CM cell migration (G, H). Data are presented as mean ± SD. Statistical analysis: ANOVA. Values with  $P < 0.05$  were considered statistically significant. \* ( $P < 0.05$ ) \*\* ( $P < 0.01$ ) \*\*\* ( $P < 0.001$ ) \*\*\*\* ( $P < 0.0001$ ).

### 3.2. AC3™ Decreases ROS Levels and Modulates Caspase Expression in CM A375 and SK-MEL-28 Cells

Following a 24-h treatment with AC3™, a significant reduction in reactive oxygen species (ROS) levels was observed across all tested concentrations in both A375 (Figure 2A) and SK-MEL-28 (Figure 2D) melanoma cell lines. To further elucidate the AC3™ mechanism of action, the gene expression of caspases 8 and 3 was evaluated. In SK-MEL-28 cells, treatment with AC3™ at 3.12 µg/mL resulted in

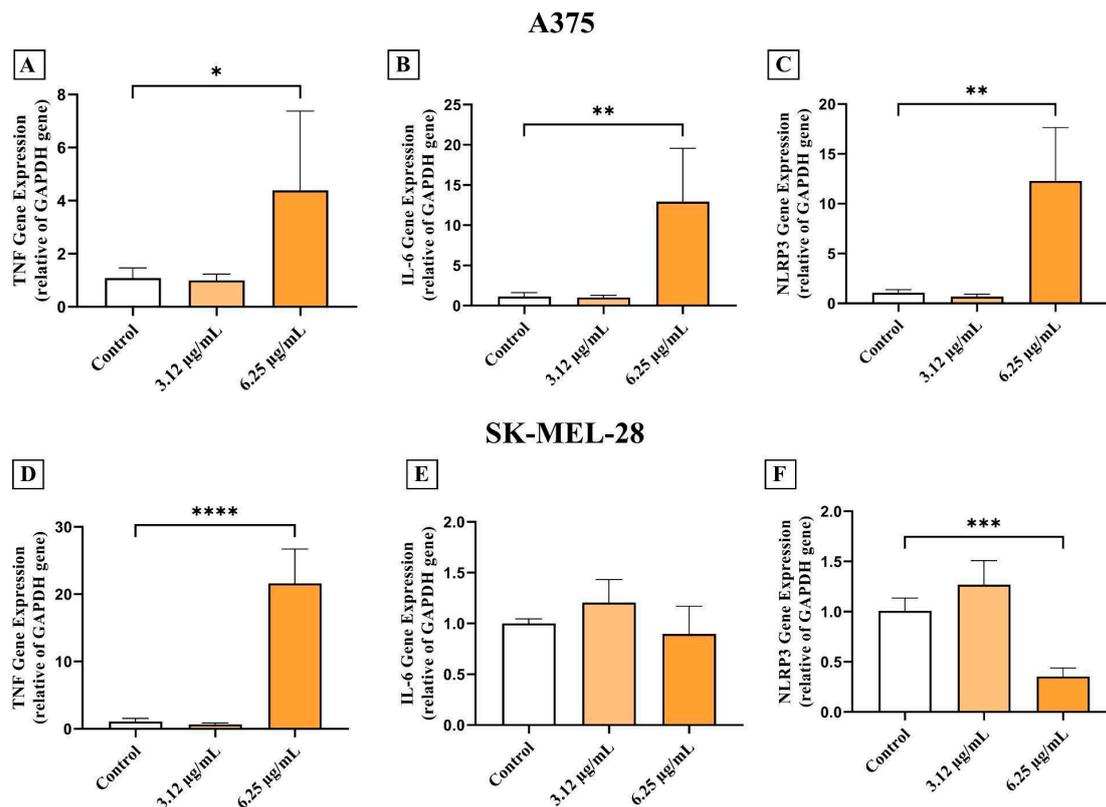
a significant upregulation of caspase-8 gene expression ( $P = 0.0435$ ) (Figure 2E). In contrast, no significant alterations in caspase-8 expression were detected in A375 cells when compared to the control (Figure 2B). Regarding caspase 3, we found a substantial increase in caspase-3 gene expression after treatment with 6.25  $\mu\text{g}/\text{mL}$  of the compound in the A375 ( $P = 0.0150$ ) (Figure 2C) and SK-MEL-28 ( $P < 0.0001$ ) (Figure 2F) cell lines.



**Figure 02.** AC3<sup>TM</sup> modulates ROS levels and caspase expression in CM A375 and SK-MEL-28 cells. Treatment with all AC3<sup>TM</sup> concentrations reduced ROS levels in A375 (A) and SK-MEL-28 (D) cell lines. We did not observe a significant change in Caspase-8 gene expression levels in A375 (B) cells, whereas, in SK-MEL-28 cells, treatment with 3.12  $\mu\text{g}/\text{mL}$  of AC3<sup>TM</sup> increased gene expression (E). Caspase-3 gene expression significantly increased after treatment with 6.25  $\mu\text{g}/\text{mL}$  of AC3 in A375 (C) and SK-MEL-28 (F) cells. Data are presented as mean  $\pm$  SD. Statistical analysis: ANOVA. Values with  $P < 0.05$  were considered statistically significant. \* ( $P < 0.05$ ) \*\* ( $P < 0.01$ ) \*\*\* ( $P < 0.001$ ) \*\*\*\* ( $P < 0.0001$ ).

### 3.3. AC3<sup>TM</sup> Modulates the Inflammatory Cascade

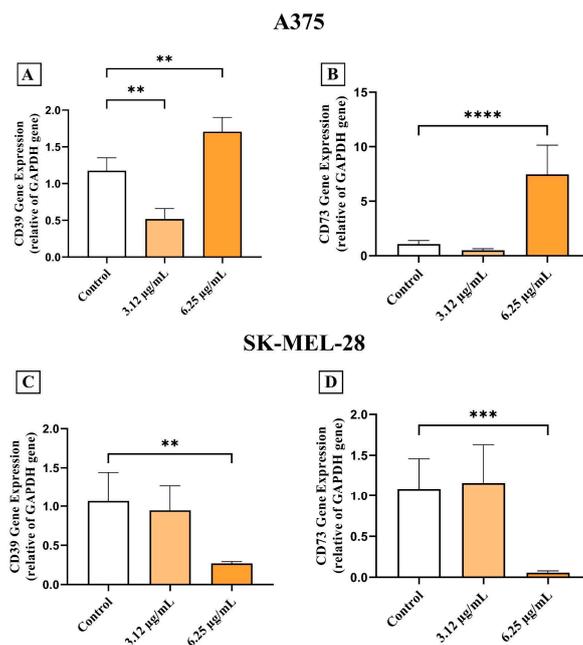
We used RT-qPCR to evaluate TNF- $\alpha$ , IL-6, and NLRP3 gene expression, as shown in Figure 3. After 24h of treatment with 6.25  $\mu\text{g}/\text{mL}$  of AC3<sup>TM</sup>, there was an increase in the gene expression of TNF- $\alpha$  ( $P = 0.0446$ ), IL-6 ( $P = 0.0035$ ) and NLRP3 ( $P = 0.0012$ ) in the A375 cell line (Figure 3A-C). In the SK-MEL-28 cell line, an increase in the gene expression of TNF- $\alpha$  ( $P < 0.0001$ ) was observed at the highest treatment concentration (Figure 3D). In contrast, in this same cell line and treatment concentration, a reduction in the levels of NLRP3 was observed ( $P = 0.0006$ ) (Figure 3F).



**Figure 03.** AC3<sup>TM</sup> modulates the levels of pro-inflammatory markers in CM A375 and SK-MEL-28 cell lines. In A375 cells, treatment with 6.25 µg/mL of AC3<sup>TM</sup> increased the gene expression of TNF (A), IL-6 (B), and NLRP3 (C). In SK-MEL-28 cells, we observed similar results in TNF levels (D). In contrast, IL-6 levels (E) did not show significant differences, while there was a reduction in NLRP3 gene expression levels after 24 hours of treatment with 6.25 µg/mL of AC3<sup>TM</sup> (F). Data are presented as mean ± SD. Statistical analysis: ANOVA. Values with  $P < 0.05$  were considered statistically significant. \*( $P < 0.05$ ) \*\*( $P < 0.01$ ) \*\*\*( $P < 0.001$ ) \*\*\*\*( $P < 0.0001$ ).

#### 3.4. AC3<sup>TM</sup> Modulates Gene Expression of CD39 and CD73

We evaluated the gene expression of ectonucleotidases after 24 h of treatment with AC3<sup>TM</sup> in A375 and SK-MEL-28 cells, as shown in Figure 04 (A-D). In A375 cells, the treatment concentration of 3.12 µg/mL ( $P = 0.0011$ ) decreased CD39 expression. In comparison, at the concentration of 6.25 µg/mL ( $P = 0.0078$ ), there was an increase in CD39 gene expression (Figure 4A). Regarding CD73, the concentration of 6.25 µg/mL increased gene expression in A375 cells ( $P < 0.0001$ ) (Figure 4B). Differently, for SK-MEL-28 cells, the treatment was able to decrease CD39 gene expression at the concentration of 6.25 µg/mL ( $P = 0.0016$ ) (Figure 4C). Similarly, treatment with 6.25 µg/mL of AC3<sup>TM</sup> decreased CD73 gene expression, compared to the control ( $P = 0.0009$ ) (Figure 4D).

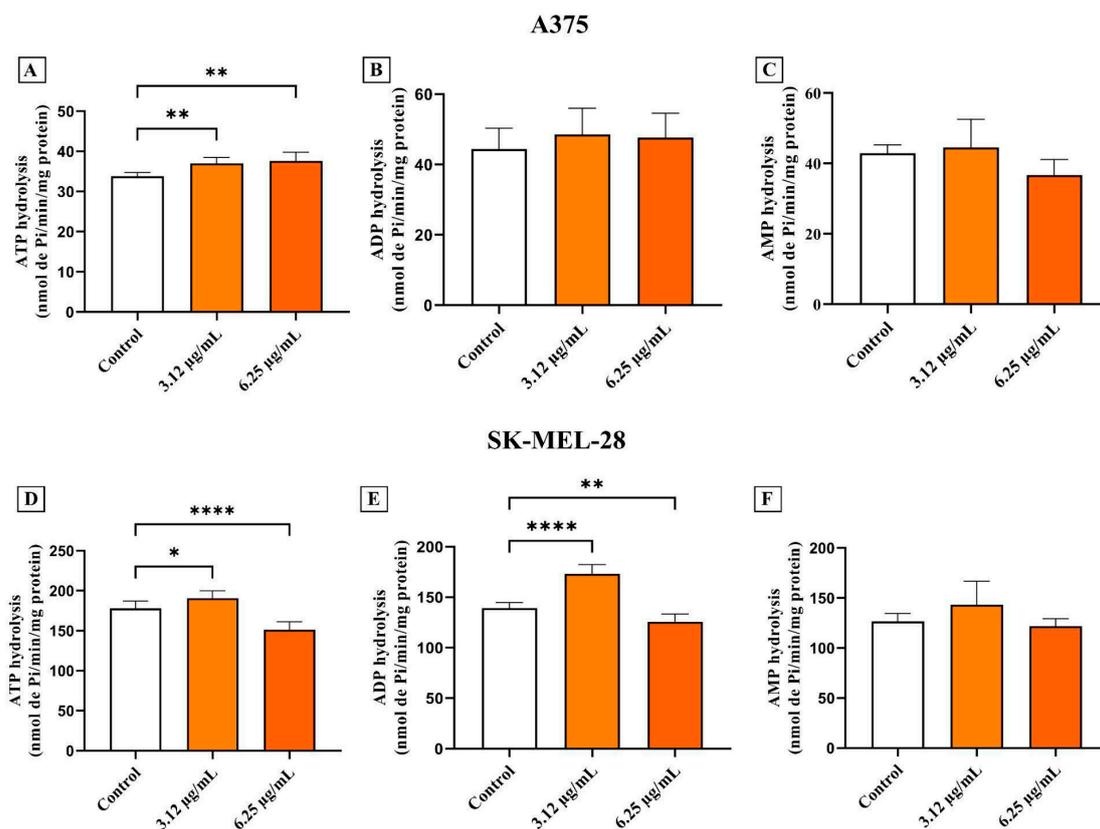


**Figure 04.** AC3<sup>TM</sup> modulates the levels of pro-inflammatory markers in CM A375 and SK-MEL-28 cell lines. CD39 gene expression levels increased after AC3<sup>TM</sup> (A) treatment in A375 cells. The same occurred in CD73 levels after treatment with 6.25 µg/mL of the compound (B). The highest concentration tested in the SK-MEL-28 cell line decreased CD39 (C) and CD73 (D) gene expression. Data are presented as mean ± SD. Statistical analysis: ANOVA. Values with  $P < 0.05$  were considered statistically significant. \*( $P < 0.05$ ) \*\*( $P < 0.01$ ) \*\*\*( $P < 0.001$ ) \*\*\*\*( $P < 0.0001$ ).

### 3.5. AC3<sup>TM</sup> Modulates Enzymatic Activity of CD39 and CD73

Figure 5 shows the results of the enzymatic activity of CD39 and CD73 after 24 h of treatment with AC3<sup>TM</sup> (Figure 5A-F). In the A375 cell line, an increase in ATP hydrolysis was observed after treatment with 3.12 µg/mL ( $P = 0.0059$ ) and 6.25 µg/mL ( $P = 0.0015$ ) (Figure 5A). No significant difference was observed in the levels of ADP (Figure 5B) and AMP (Figure 5C) hydrolysis in this cell line after treatment with the compound.

In SK-MEL-28 cells, after 24 h of treatment with AC3<sup>TM</sup>, we observed an increase in ATP hydrolysis at the concentration of 3.12 µg/mL ( $P = 0.0251$ ), followed by a decrease at the concentration of 6.25 µg/mL ( $P < 0.0001$ ) (Figure 5D). Similar results were obtained in AMP hydrolysis, where an increase in CD39 activity occurred at a concentration of 3.12 µg/mL ( $P < 0.0001$ ), followed by a decrease at a concentration of 6.25 µg/mL ( $P = 0.0061$ ) (Figure 5E). Similarly to the A375 cell line, CD73 activity did not show significant differences after treatment with AC3<sup>TM</sup>.



**Figure 05.** AC3<sup>TM</sup> modulates the enzymatic activity of CD39 and CD73 in CM A375 and SK-MEL-28 cell lines. Regarding enzymatic activity, we observed an increase in ATP hydrolysis after treatment with AC3<sup>TM</sup> in the A375 cell line (A). In contrast, in SK-MEL-28 cells, we observed an increase in ATP hydrolysis at a concentration of 3.12 µg/mL, followed by a decrease in the levels of 6.12 µg/mL (D). The same occurred in the levels of ADP hydrolysis (E). We did not observe significant differences in the levels of ADP (B) and AMP (C) hydrolysis in the A375 cell line or AMP hydrolysis (F) in SK-MEL-28 cells. Data are presented as mean ± SD. Statistical analysis: ANOVA. Values with P<0.05 were considered statistically significant. \*(P<0.05) \*\*\*(P<0.001) \*\*\*\*(P<0.0001).

#### 4. Discussion

Curcuminoids have been used for many years as a spice. Among them, the most studied curcuminoid is CUR, which has shown antitumor effects in several types of cancer, such as breast [48] and ovarian [49] and esophagus [50]. In melanoma cell lines, it was proved that curcumin induces apoptosis by activating cell death pathways involving caspases 3, 7, 8, and 9, and AKT/mTOR. It also can decrease the expression of factors associated with angiogenesis and invasion, such as Vascular Endothelial Growth Factor (VEGF), Matrix Metalloproteinase-2 (MMP- 2), Matrix Metalloproteinase-9 (MMP-9), and Cyclooxygenase (COX), which are predictive of metastasis [43,51–53]. The others curcuminoids, such as DMC and BDMC, also have antitumor activity and have been shown to act synergistically with curcumin [32,54–56]. Considering the previous antineoplastic activity of available commercial curcuminoids, we verified the antineoplastic properties of a mixture of phytonutrients that expounds the benefits of BDMC, as the major ingredient, along with DMC and CUR standardized in ACM<sup>TM</sup> in two cutaneous melanoma cell lines.

Furthermore, in our study, we chose to use two cutaneous melanoma cell lines, A375 and SK-MEL-28. These cell lines are widely studied and present different levels of aggressiveness. A comparative study between cutaneous melanoma cell lines using an aggressiveness scale (Melanoma Aggressiveness Score: MAGS) showed that the A375 cell line was classified as the most aggressive and the SK-MEL-28 cell line the least aggressive [57].

We found that the AC3<sup>TM</sup> was able to decrease cell proliferation and migration in both melanoma cell lines. According to Huang et al. [32], the three curcuminoid constituents, CUR, DMC, and BDMC, act synergistically. Our results corroborate previous findings, higher doses of curcuminoids showed beneficial effects on cell proliferation and migration of both the cutaneous melanoma cell lines, A375 and SK-MEL-28 [54,58,59]. Similarly, curcumin-loaded nanoparticles have

also demonstrated antiproliferative and anti-migratory effects and marked cell viability reduction in breast cancer models [60]. In this study, we observed a significant decrease in cell viability at lower doses (Figure 1).

In addition to reducing cell viability, previous studies have found that curcuminoids increase ROS levels, causing damage to DNA as a way of inducing apoptosis [61,62]. In contrast to the results found here, we found that when in association, these compounds decrease ROS levels in A375 and SK-MEL-28 cells and increase caspases-8 and -3 expression independent of ROS levels (Figure 2). Sandur et al. [28] found similar results, in which, regardless of ROS levels, curcuminoids induced cell death via nuclear factor- $\kappa$ B (NF- $\kappa$ B) and TNF, that is, apoptosis through the extrinsic pathway. Our data suggest that AC3<sup>TM</sup> exerts its antineoplastic effects, at least in part, by reducing oxidative stress and modulating the apoptotic machinery by regulating caspase gene expression in melanoma cells.

An increase in TNF- $\alpha$  gene expression was observed after treatment with AC3<sup>TM</sup> in the A375 and SK-MEL-28 cells (Figure 3). In these cases, apoptosis is mediated via TNF and carried out by the TNF receptor 1 (DR1/TNFR1), in which interaction occurs between TNF and TNFR1 leading to the recruitment of the TNFR-associated death domain (TRADD) protein, through its DD [63]. Subsequently, interaction occurs with the Fas-associated death domain (FADD), which recruits pro-caspase-8 and cleaves it, transforming it into active caspase-8, which finally activates caspase-3, an effector of cell death [64]. In A375 cells, Tyciakova et al. [65] demonstrated that TNF overexpression is correlated with an increase in IL-6 and the pro-apoptotic tumor necrosis factor ligand gene (TRAIL) mediated by TNF/TNFR1 signaling. Similar results were found in our study, where we observed an increase in the inflammatory cascade and the overexpression of TNF, IL-6, and NLRP3 in cell line A375.

Another essential component of the inflammatory cascade is the NLRP3 inflammasome, considered a double-edged sword in several types of cancer, being able to exert a protective antitumorigenic effect and a pro-tumorigenic role [66]. In CM, the NLRP3 inflammasome is highly expressed, acting on tumor progression through activating and cleavage of caspases and IL-1 $\beta$ , decreasing the immune response and consequently suppressing anti-melanoma T cell responses [64,67,68]. In our previous study on the SK-MEL-28 line treated with CUR [36], we observed that the substance could decrease the expression of NLRP3 and consequently increase the efficacy of anti-melanoma therapies, particularly those targeting the anti-programmed cell death 1 (anti-PD-1) pathway. Contrary to what was found in A375 cells, in the SK-MEL-28 cell line, we observed a decrease in the gene expression of the NLRP3 inflammasome, supporting the hypothesis of the previous study that in this type of melanoma, treatment with CUR or a mix of curcuminoids CUR, DMC and BDMC, in this case, AC3<sup>TM</sup> can improve the response to therapies, acting mainly in the inhibition of tumor suppression, that is, positively regulating the immune response [69].

Another important therapeutic target for the treatment of CM, capable of modulating the immune response, are the components of purinergic signaling, in particular, the purinergic mediators of the extracellular environment, adenosine triphosphate (ATP) and adenosine (Ado) [70,71]. ATP present in the extracellular environment can be released in different ways. One of them is cell lysis, a process that is directly related to apoptosis or by non-lytic mechanisms such as exocytosis of ATP-containing vesicles via nucleotide-permeable channels (connexin/pannexin hemichannels, maxi-anionic channels, volume-regulated anion channels, and P2X7 receptor channels), through transport vesicles that deliver proteins to the cell membrane or through lysosomes [72,73]. Ado comes from the dephosphorylation of ATP by the ectonucleotidases CD39 and CD73, the former being responsible for the conversion of ATP into adenosine monophosphate (AMP) and the latter converts the AMP resulting from CD39 activity into Ado [74]. High levels of ATP in the tumor microenvironment act as a Danger-Associated Molecular Pattern (DAMP), inducing the immune response. Otherwise, Ado inhibits the immune response, causing immunosuppression [75,76]. Antitumor immunity induced by Ado is one of the significant challenges encountered in the treatment of CM since the newly available therapies that act by inhibiting immune checkpoints (ICIs) and represent a more attractive prognosis depend on the negative regulation of the CD39/CD73/Ado axis [77,78].

Patients with CM have higher levels of CD39 and CD73 expression, which are associated with resistance to therapies and worse prognosis [37,79,80]. In this study, we found that treatment with 6.25  $\mu$ g/mL of the AC3<sup>TM</sup> complex was able to decrease the gene expression of the ectonucleotidases CD39 and CD73 in the SK-MEL-28 cell line, considered the least aggressive (Figure 4). Furthermore, in this same lineage we observed a decrease in the activity of the CD39 enzyme (Figure 5). In contrast,

in the A375 cell line, there was an increase in the expression of the CD39 and CD73 enzymes and in the enzymatic activity of CD39 (Fig 4-5), demonstrating that the compound can act in different ways in cell lines, possibly due to the different levels of aggressiveness of the tested strains [81].

Lower levels of adenosine in the extracellular environment, due to lower expression of CD73, can decrease immunosuppression, increasing the response to therapies [82]. One of the therapies for treating cutaneous melanoma with the highest levels of efficacy inhibits an immune checkpoint via PD-1/PD-L1 blockade [83]. However, high resistance rates are still observed, so recent studies support associated therapies [84]. This association can be observed in the study by Tu et al., who found that the combination of anti-PD-L1 and anti-CD73 antibodies improved the response to treatment of non-small cell lung cancer [85].

In melanoma, we observed that treatment with AC3<sup>TM</sup> decreased CD73 expression in SK-MEL-28 cells. This agrees with our previous study, in which CUR exerted similar activity in SK-MEL-28 cells. Thus, both CUR and the curcuminoid mixture are strong candidates for adjuvant therapy of MC (17).

## 5. Conclusions

In this study we showed that AC3<sup>TM</sup> reduces cell viability, inhibits cell migration, increases caspase-3 levels, and modulates the levels of pro-inflammatory markers and components of the purinergic system in melanoma cell models A375 and SK-MEL-28. This complex compound positively regulated the TNF pathway, promoting apoptosis of melanoma cells. In addition, regulating the inflammatory cascade results in lower levels of immunosuppression, a key factor contributing to therapeutic resistance in melanoma. Finally, AC3<sup>TM</sup> emerges as a promising candidate for further investigation in CM, given its ability to modulate purinergic signaling through the CD39/CD73 axis, which controls extracellular ATP levels, and through the antagonistic action on P1 receptors. Taken together, our findings position AC3<sup>TM</sup> as a multifaceted and innovative therapeutic candidate capable of enhancing the biological and therapeutic activity of curcuminoids, while overcoming the limitations associated with curcumin's poor bioavailability. By targeting critical mechanisms such as tumor cell survival, immune evasion, and purinergic signaling, AC3<sup>TM</sup> offers a promising strategy to improve treatment outcomes and address therapeutic resistance in melanoma.

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## Abbreviations

The following abbreviations are used in this manuscript:

CM	Cutaneous melanoma
AC3 <sup>TM</sup>	Advanced Curcumin C3 Complex
ROS	Reactive oxygen species
TNF- $\alpha$	Tumor Necrosis Factor Alpha
IL-6	Interleukin 6
NLRP3	NLR Family Pyrin Domain Containing 3
CD39	Ecto-nucleoside triphosphate diphosphohydrolase 1
CD73	Ecto-5'-nucleotidase

CASP-8	Caspase-8
CASP-3	Caspase-3
NAFLD	Non-alcoholic fatty liver disease
CUR	Curcumin
DMC	Demethoxycurcumin
BDMC	Bisdemethoxycurcumin
BCRJ	Bank of Cell of Rio de Janeiro
DMEM	Dulbecco's Modified Eagle's Medium
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
AO	Acridine orange
TMRE	Tetramethylrhodamine ethyl ester
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ADA	Adenosine deaminase
VEGF	Vascular Endothelial Growth Factor
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9
COX	Cyclooxygenase
MAGS	Melanoma Aggressiveness Score
NF- $\kappa$ B	Nuclear factor- $\kappa$ B
DR1/TNFR 1	TNF receptor 1
TRADD	TNFR-associated death domain
FADD	Fas-associated death domain
TRAIL	Tumor necrosis factor ligand gene
anti-PD-1	Anti-programmed cell death 1
ATP	Adenosine triphosphate
Ado	Adenosine
AMP	Adenosine monophosphate
DAMP	Danger-Associated Molecular Pattern
ICIs	Inhibiting immune checkpoints

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