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Extraction, isolation and *in vitro* evaluation of affinisine from *Tabernaemontana catharinensis* in human melanoma cells

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ABSTRACT

Plant compounds have been identified as new drug prototypes. In this line, this work aimed to isolate the indole alkaloid affinisine from *Tabernaemontana catharinensis* and test its antitumor activity. The alkaloid was isolated by silica gel open column chromatography from the ethanolic extract of the stem of *T. catharinensis*. Afterwards, this molecule was characterized by high-resolution mass spectrometry and nuclear magnetic resonance. In the next step, the cytotoxicity of the compound was tested against human melanoma cell lines (A375, WM1366 and SK-MEL-28) and a normal skin cell line (CCD-1059Sk) using a MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells treated with affinisine were evaluated by flow cytometry to analyze apoptosis and the induction of cell cycle arrest, to evaluate the dead mechanism. The metabolite was isolated in a 0.2% yield relative to the extract. Cytotoxic activity of the molecule was observed at 48 h, resulting in considerable growth inhibition rates in melanoma cells, especially in WM1366, which had the lowest IC_{50} (32.86 ± 2.54 μg/mL). The apoptosis rate was lower in A375 (56.66 and 86.71% with 57 and 65 μg/mL, respectively). Moreover, affinisine was able to significantly induce cell cycle arrest in different phases in the A375 and WM1366 cell lines. However, in SK-MEL-28 cells, cycle arrest was not observed. In summary, this compound significantly decreased the viability of tumor cells in a dose- and time-dependent manner for all evaluated lineages, reduced cell viability by the apoptosis mechanism and presented prominent activities of cell cycle arrest. In this way, the use of antineoplastic agents is among the most widely used therapeutic measures for the control and treatment of cancer. Affinisine is a promising prototype in the search for new drugs to treat cancer.

1. Introduction

Compounds obtained from plants have been recognized as prototypes for the development of new drugs. The adaptation of plants leads to the synthesis of secondary metabolites whose substances also are present pharmacological activities, among which antibacterial, anti-inflammatory, antihypertensive and anticancer activities are the most frequently reported [1,2].

Along this line, indole alkaloids having rich structural diversity have been isolated from natural resources. Many of them possess remarkable bioactivities, as antitumor, antimicrobial, antihypertensive or as a central nervous system stimulant [3]. This compounds been identified as active compounds, with examples being vinblastine and vincristine derived from *Catharanthus roseus*, which are currently used to treat cancer such as Hodgkin's disease [4]. Vinblastine acts on cells in mitosis by blocking β-tubulin, which is unable to polymerise with α-tubulin in microtubules. With the interruption of the formation of the microtubules, the mitotic spindle does not occur, dispersing the chromosomes through the cytoplasm; thus, there are characteristic changes of apoptosis [5].

Tabernaemontana catharinensis A.DC. is an arboreal species belonging to the Apocynaceae family, which is popularly known as "jasmine-vane", "dairy-two brothers", "jasmine", "forquilheira" and "snake bark" [6]. This plant is currently found in the north, northeast, southeast, central-west and southern regions of Brazil, Argentina, Paraguay, Uruguay and Bolivia [7]. The extract of this plant is well characterized

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by antimyionecrotic potential [8,9]. Others medicinal activities, such as anti-cholinesterase[10], antinociceptive [11], anti-inflammatory [12], as well as antitumor activities [9], are mainly due to the presence of indole alkaloids.

In this way, the sarpagine-type indole alkaloid affinisine was isolated from the ethanolic extract of *Tabernaemontana fuchsiaefolia* [13], *Tabernaemontana australis* [14] and *Tabernaemontana hystrix* [15]. This compound exhibits leishmaniacidal activity against the promastigotes of *Leishmania Mexicana* [16], antioxidant activity [17], acethylcolinesterase (AChE) or buthyrylcolinesterase (BuChE) inhibition activity [18].

The search for new cancer chemotherapeutics is constant, with natural products among the most prominent active compounds. Skin cancer is the most common type, and the incidence of both non-melanoma and melanoma skin cancers has been increasing over the past decades. Currently, between 2 and 3 million non-melanoma skin cancers and 132000 melanoma skin cancers occur globally each year [19].

A375 and SKMEL-28 are among the most used human cell lines not only in melanoma drug screening *in vitro,* but also in *in vivo* mouse xenograft transplant models, mainly because they represent well stablished and characterized cell lines, while the WM1366 is not as commonly used [20]. However, they all contain distinct genetic mutations [20] and, therefore, it is of interest to combine their use in the *in vitro* evaluation of compounds aiming to obtain a more reliable anti-melanoma screening.

Based on a bio-guided study conducted by our group [21], this work aimed to isolate the indole alkaloid affinisine from *Tabernaemontana catharinensis* and to screen its *in vitro* antitumor activity against human melanoma cell lines. This compound was chemically characterized using HRMS (high-resolution mass spectrometry), NMR (nuclear magnetic resonance) ^{1}H, ^{13}C and 2D techniques. Moreover, the induction of apoptosis and cell cycle arrest were evaluated.

2. Materials and methods

2.1. Chemicals

Silica gel GF254 (10–40 μm) prepared for TLC, silica gel (0.063–0.200) and silica gel (0.04–0.063) for column chromatography were obtained from Merck® (Germany). All the reagents were HPLC or analytical grade and purchased from Merck®. Ultrapure water was prepared by a Milli-Q water purification system (Millipore®, France). Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and di-methyl sulphoxide (DMSO) were acquired from Sigma-Aldrich (St. Louis, USA). Foetal bovine serum (FBS) was purchased from Cultilab Lab Inc. (Campinas, SP, Brazil).

2.2. Plant material

T. catharinensis stems (17 kg) were collected in Ijuí, Rio Grande do Sul, Brazil, in November 2017 (28°26'06.4"S and 53°56'15.7"W). A voucher specimen has been identified by Felipe Gonzatti and deposited in the Caxias do Sul University Herbarium (HUCS 34038-34057/g1669) at Caxias do Sul, Brazil.

2.3. Extraction and isolation

The stem samples of *T. catharinensis* (1880g) were extracted according to the methodology of our group [21], resulting in 9.3g of extract rich in alkaloids. This extract was partitioned successively with hexane (3000 mL), hexane:chloroform (1:1 v/v; 3500 mL) and chloroform (2500 mL). The extract was fractionated in an open chromatographic column (25 cm) using silica gel 60 (0.063–0.200mm), chloroform:methanol (1:1 v/v; 500 mL) and methanol (1000 mL).

The chloroform:methanol extract (3.628g) was subjected to silica gel 60 (0.063–0.200mm), with $CHCl_3$/MeOH (75:25 to 0:100 v/v) to obtain five fractions (A1–A5), which were combined according to TLC analysis. Fraction A3 (2.0495 g) was chromatographed on silica gel 60 (0.063–0.200mm) with a gradient of $CHCl_3$/MeOH (100:0 to 0:100 v/v) to obtain six fractions (B1–B6). Fraction B4 (1.7435 g) was chromatographed on silica gel 60 (0.063–0.200mm) eluted with $CHCl_3$/MeOH (100:0 to 0:100 v/v) to yield seven fractions (C1–C7). C2 (1.300g) was subjected to silica gel 60 (0.04–0.063) with $CHCl_3$/MeOH (99:1 to 0:100 v/v) to obtain five fractions (D1–D5). Fraction D2 (0.246 g) was chromatographed on silica gel 60 (0.04–0.063mm) with a gradient of $CHCl_3$/MeOH (99:1 to 0:100 v/v) to obtain six fractions (E1–E6). E3 was purified by preparative TLC (CH_2Cl_2/MeOH 95:5 v/v) to affinisine (0.02697g).

2.4. Nmr

The NMR experiments were performed on an Ultrashield300 Bruker® 9.4 Tesla (300 MHz for hydrogen frequency, 75.48 MHz for carbon frequency and 75.48 MHz for 2D frequency) spectrometer with a 5-mm internal diameter BBI probe, with reverse detection and field gradient coils in the coordinate. The chemical shifts are stated relative to TMS and expressed in δ values (ppm), with coupling constants reported in Hz. The spectra were acquired at a temperature of 303 K, which were recorded in $CDCl_3$ solutions using 5 mm quartz tubes.

2.5. Hrms

The mass analysis was conducted in a quadrupole-time of flight system (Bruker, Q-TOFII® Billerica, USA). ESI(+)MS and tandem ESI (+)MS/MS were acquired using a hybrid high-resolution and high accuracy (5 μL/L) microTof (Q-TOF) mass spectrometer (Bruker Scientific®) under the following conditions: capillary and cone voltages were set to +3500 V and +40 V, respectively, with a de-solvation temperature of 200 °C. The collision-induced dissociation energy (CID) for the ESI(+)MS/MS was optimized for each component. For data acquisition and processing, TOF control and data analysis software (Bruker Scientific®) were used. The data were collected in the *m/z* range of 70–1000 at a speed of two scans per second, providing a resolution of 50000 (FWHM) at *m/z* 200. ESI(+)-MS data is shown in the *m/z* 180 − 750 range.

2.6. Determination of cytotoxic activity

The WM1366, SK-MEL-28, A375 and CCD-1059Sk cell lines were cultured in DMEM-supplemented media with antibiotics (1% penicillin and streptomycin) and 10% FBS (fetal bovine serum) (Gibco BRL/Life Technologies, Carlsbad, CA, USA) at 5% CO_2 and 37 °C. The cytotoxic activity of affinisine was assessed as follows: the cells were seeded in 96-well flat-bottom microplates, at a density of 2×10^4 cells/mL for the WM1366 and A375 cell lines, and 1×10^4 cells/mL for the SK-MEL-28 and CCD-1059Sk cell lines, with 10% FBS in DMEM. After cell attachment, serial dilutions of the compound, with increasing concentrations (5, 10, 25, 50 and 100 μg/mL) at 37 °C in the culture medium, were added to the cells for 24 and 48 h. Aliquots were removed and assayed by the MTT method. The MTT solution was removed after 3 h of incubation and the formazan crystals were dissolved by adding 100 μL DMSO to each well, followed by agitation on a rotary shaker for 30 min, protected from light. The absorbance was read on a microplate reader (Victor X5, PerkinElmer, USA) at a test wavelength of 492 nm, and the results were expressed as percentage viability of the negative control. At least three independent experiments in triplicates for each experiment were performed for each cell line. IC_{50} (%) values (dose causing 50% cell survival) were determined as the means ± SEM [22].

2.7. Apoptosis assay using flow cytometry

An Annexin V-7AAD apoptosis detection kit was used (Guava Technologies, Millipore Corporation). WM1366 cells were treated with 32 and 45 μg/mL of affinisine for 48 h, A375 cells were treated with 57 and 65 μg/mL of affinisine for 48h and SK-MEL-28 cells were treated with 46 and 55 μg/mL of affinisine for 48 h. Briefly, 1×10^5 cells were added to 100 μL of Guava Nexin reagent. Cells were incubated in the dark at room temperature for 20 min, and were then acquired by flow cytometry (Muse™ Cell Analyzer, Merck Millipore Corporation) and analyzed using Guava Software.

In the apoptosis assay, annexin V-negative and 7-AAD-positive cells indicate the presence of nuclear debris; annexin V positive and 7-AAD-positive indicate late apoptotic cells; annexin V negative and 7-AAD-negative indicate live healthy cells; and annexin V positive and 7-AAD-negative indicate early apoptotic cells. Results were reported as the percentage of cells in each apoptotic phase (early and late) using GraphPad.

2.8. Cell cycle changes measured by flow cytometry

WM1366 cells were seeded at a density of 2×10^5 cells/well in 12-well plates for 24 h, then growth was synchronized without serum for an additional 24 h before the affinisine compound was added at the concentrations of 32 and 45 μg/mL for 48 h. A375 cells were seeded at a density of 2×10^5 cells/well in 12-well plates for 24 h, then growth was synchronized without serum for an additional 24 h before the affinisine compound was added at the concentrations of 57 and 65 μg/mL for 48 h. SK-MEL-28 cells were seeded at a density of 2×10^5 cells/well in 12-well plates for 24 h, then growth was synchronized without serum for an additional 24 h before the affinisine compound was added at the concentrations of 46 and 55 μg/mL for 48 h.

The cell cycle evaluation was performed using a propidium iodine staining (Guava Technologies). The samples were read in a flow cytometry system (Muse™ Cell Analyzer, Merck Millipore Corporation).

2.9. Data analysis

Data sets were analyzed using a two-way ANOVA, followed by a Tukey test for multiple comparisons. Significance was considered at $p < 0.05$ in all analyses. Data were expressed as mean ± SEM (standard error of the mean).

3. Results and discussion

Numerous alkaloids classified as indole terpenoids, of common biosynthesis, have different pharmacological properties. Affinisine is a monoterpenoid indole alkaloid that can be isolated from plants of the genus *Tabernaemontana*. Structurally, it can be considered a member of the sarpagine alkaloid family [14,23].

Chromatographic methods are used for the isolation of bioactive substances. Chromatography is a more versatile technique for the separation of complex mixtures, such as plant extract. The most frequently applied chromatographic techniques for the isolation of natural products are: thin layer chromatography (TLC), column chromatography (CC) and high performance liquid chromatography (HPLC) [24].

The indole alkaloid affinisine has been previously isolated from *Ervatamia hirta* leaves and root bark using silica gel open column chromatography [23]. Andrade et al. (2005) used the same method to isolate this compound from the chloroform extract of *Tabernaemontana australis* stalk [14]. In the same way, Mathias et al. (2005) also isolated indole alkaloid affinisine from the methanolic extract of *Tabernaemontana hystrix* root bark, with a 0.11% yield, using CC [15]. Santos et al. (2009) also isolated this indole alkaloid from the ethanolic extract of *Peschiera affinis* roots [17] and from the methanolic extract of *Alstonia angustifolia* [16], with yields of 0.2% and 0.08%, respectively, using silica gel open column chromatography and HPLC. In this work, the extract rich in indole alkaloids from *T. catharinensis* stems resulted in a 0.07% yield (plant dry mass). After the partition and fractionation by silica gel open column chromatography, affinisine (Figure 1) was obtained in a 0.3% yield (relative to the extract).

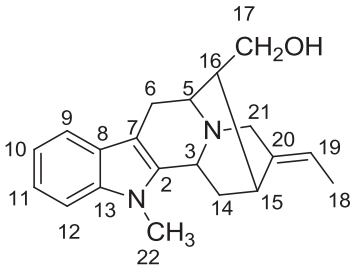

Figure 1. Chemical structures of affinisine isolated from the stem of *T. catharinensis*.

The compound was isolated as a brown solid and was alkaloid-positive to Dragendorff's reagent tested over silica gel TLC plates. The HRMS analysis and the NMR data indicated $C_{20}H_{25}N_2O^+$ as the molecular formula and an ion peak at *m/z* 309.1970 $[M + H]^+$ (error – 2.9 ppm; isotopic ratio fit – 13.8 msig) was observed in the HRMS spectrum (Figure 2). The fragmentation MS/MS(%) was 291.1863 (6.58), 170.0969 (5.01), 158.0977 (16.64), 138.0931 (31.37), 120.0832 (2.23), 108.0824 (0.92), which corresponds to the ions $C_{19}H_{22}N_2^+$, $C_{11}H_{12}N_2^+$, $C_{11}H_{12}N^+$, $C_8H_{12}NO^+$, $C_8H_{12}N^+$ and $C_7H_{10}N^+$, respectively. The affinisine fragmentation by HRMS showed the same results as previously reported [10,18].

The ^{1}H NMR spectrum showed hydrogen atoms related to an indole ring **H10** δ 7.09 ppm (1H, td, J^1 = 7.02 and J^2 = 1.2 Hz,), **H11** 7.19 ppm (1H, td, J^1 = 7.0 and J^2 = 1.2 Hz,), **H12** 7.28 ppm (1H, d, J = 8.1 Hz,), **H9** 7.47 ppm (1H, d, J = 7.8 Hz,); a quartet related to **H19** δ 5.42 ppm (1H, q, J = 6.9 Hz); a doublet of doublets related to **H3** δ 4.24 ppm (1H, dd, J = 10 and 1.8 Hz,); δ 1.65 ppm related **H18** (3H, dt, J^1 = 6.6 and J^2 = 1.8 Hz,) and δ 3.64ppm (3H, s, **H22**); δ 3.53ppm (1H, m, **H17b**) and δ 3.59ppm (1H, m, **H17a**) related to methylene; δ 1.71ppm (1H, m, **H14a**) and δ 2.12pmm (1H, ddd, J = 1.8 and 10.2 Hz, **H14b**); and signals of the respective hydrogen δ 1.87ppm (1H, m, **H16**); δ 2.67ppm (1H, d, J = 15.6 Hz, **H6a**); δ 2.86ppm (1H, m, **H5**); δ 2.82 (1H, m, **H15**); δ 3.07ppm (1H, dd, J = 10.5 and 4.8 Hz, **H6b**) and δ 3.60ppm (2H, m, **H21**).

The ^{13}C NMR spectrum exhibited 20 carbon signals, consisting of two unsaturated carbon atoms δ_C 118.28ppm (**C19**) and δ 135.26 ppm (**C20**), eight unsaturated carbon atoms related to an indole ring δ127.43 ppm (**C8**), δ 117.09 ppm (**C9**), δ 118.91 ppm (**C10**), δ 121.15 ppm (**C11**), δ 108.28 ppm (**C12**), δ 137.34 ppm (**C13**), δ 139.11 ppm (**C2**), δ 103.50 ppm (**C7**), and 10 aliphatic carbon atoms δ 49.19 ppm (**C3**), δ 53.84 ppm (**C5**), δ 26.79 ppm (**C6**), δ 32.74 ppm (**C14**), δ 27.44 ppm (**C15**), δ 44.17 ppm (**C16**), δ 64.96 ppm (**C17**), δ 12.83 ppm (**C18**), δ 29.71 ppm (**C22**) and δ 55.82 ppm (**C21**).

The HSQC spectrum exhibited δ 1.65ppm and δ 12.83ppm, related to the correlation between **C18** and **H18**. In δ 1.71 ppm and δ 2.12 ppm, coupling of **C14** (δ 32.74 pm) to **H14b** and **H14a**, respectively, were shown. There was a correlation between signals **C16** (δ 44.17 ppm) and **H16** at δ 1.87ppm. In region δ 2.67 ppm, **C6** (δ 26.79 ppm) coupled to **H6a** and in δ 3.07 ppm, **C6** coupled to **H6b**. There was a correlation between signals **C15** (δ 27.44 ppm) and **H15** at δ 2.82 ppm and, there was a correlation between signals **C5** (δ 53.84 ppm) and **H5** at δ 2.826 ppm. In δ 3.53 ppm and δ 3.59 ppm, **C17** (δ 64.96 ppm) coupled to **H17b** and **H17a,** respectively. The spectrum exhibited a correlation between **C21** (δ 55.82 ppm) and **H21** (δ 3.60 ppm); **C22** (δ 26.71 ppm) and **H22** (δ 3.64 ppm); **C3** (δ 49.19 ppm) and **H3** (δ 4.24 ppm); **C19** (118.28 ppm) and **H19** (δ 5.42 ppm); **C10** (δ 118.91 ppm) and **H10** (δ 7.09 ppm); **C11** (δ 121.15 ppm) and **H11** (δ 7.19 ppm); **C12**

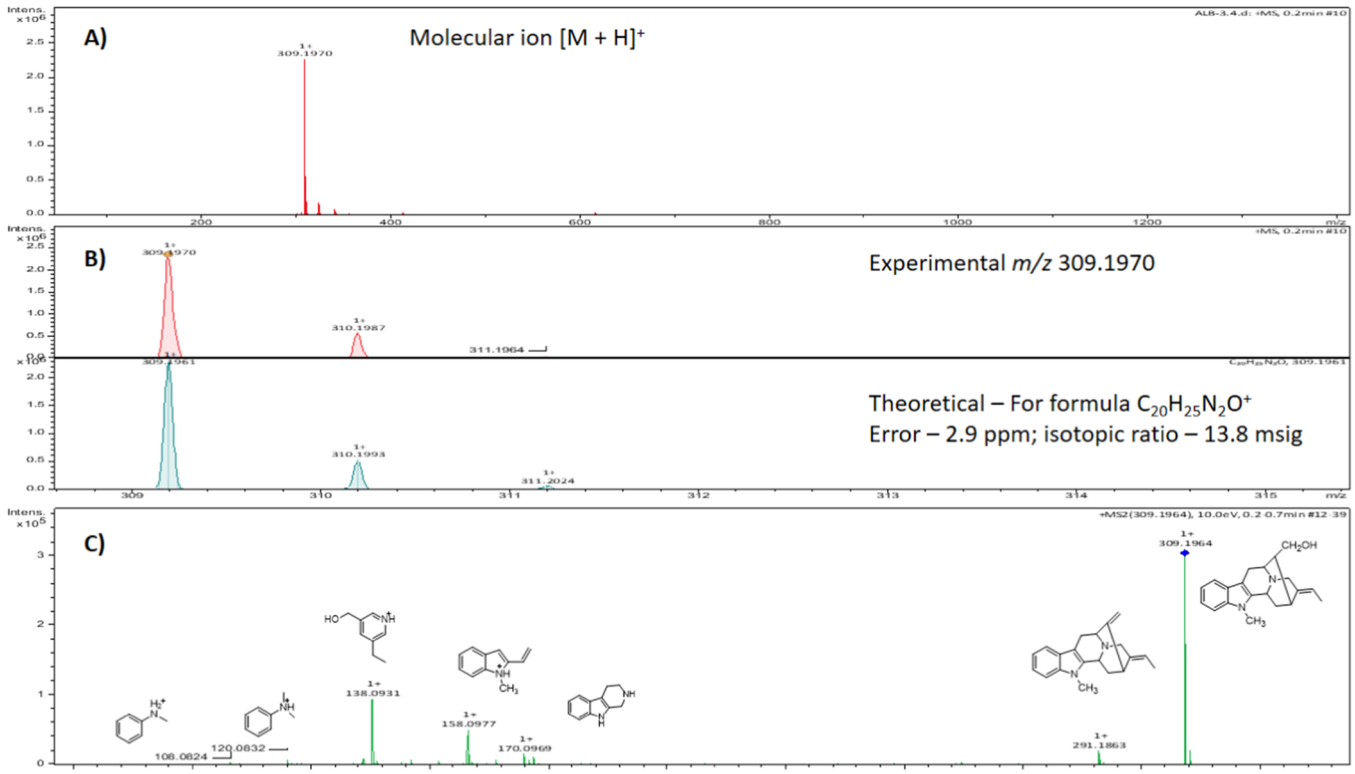

Figure 2. Full mass spectrum (HRMS) in A) affinisine in the positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 325.1975 in MS-MS mode HRESIMS spectrum of affinisine.

(δ 108.28 ppm) and **H12** (δ 7.28 ppm); and **C9** (δ 117.09 ppm) and **H9** (δ 7.47 ppm). This NMR information is similar to that reported for affinisine [13,15–17].

In this line, the bioactive compounds from plants have showed pharmacological properties in animals. Several drugs have already been discovered from the natural products, especially to treatments of cancer. Some of the new active natural compounds have been reported in recent years, such as the indole alkaloids, which had showed a wide spectrum of pharmacologic activities [25].

Melanoma, the most aggressive type of skin cancer, is a malignant tumor originating from melanocytes, the pigment-producing cells [26]. The most commonly used compounds for the treatment of melanoma are dacarbazine, temozolomide and cisplatin [27]. However, new chemotherapeutic agents, which could minimize the treatments' side effects, are a continuous approach in cancer research. Thus, we investigated the effects of affinisine from *T. catharinensis* on cell proliferation, the cell cycle and the potential to trigger cell death by apoptosis against three different human melanoma cell lines (A375, WM1366 and SK-MEL-28) and in a non-tumor skin cell line (CCD-1059Sk).

The compound decreased the viability of tumor cells in a dose- and time-dependent manner for the evaluated lineages (Figure 3). This compound was able to inhibit 50% of cell viability in the three investigated melanoma cell lines (Table 1). After 24 h of exposure, the IC_{50} values ranged from 48.22 ± 1.74 to 75.04 ± 4.03 μg/mL, whereas after 48 h, these values changed to 32.86 ± 2.54 and 57.69 ± 3.94 μg/mL. On the other hand, in the skin non-tumor cell line (CCD-1059Sk), the IC_{50} value (77.81 ± 13.26 μg/mL) was higher than in all tested melanoma cells, which could indicate some selectivity of affinisine towards tumor cells. Due to the best results being observed at 48 h in the tumor cell lines, this exposure time was chosen to be used in the following experiments, including the cytotoxicity assay in the non-tumor cell line. Along the same line, affinisine isolated from the stem bark of *Alstonia angustifolia* had previously demonstrated antiproliferative activity against HT-29 human colon carcinoma and HeLa cell lines, with IC_{50} values > 20 μM [16].

In order to investigate the cause/mechanism of the cellular growth inhibition observed in the melanoma cell lines, as demonstrated in the cell viability assay, we performed an annexin assay to test whether the compound triggered cell death by apoptosis. The treatment of melanoma cell lines with affinisine at concentrations near to the IC_{50} value after 48 h of treatment increased the percentage of total apoptotic cells, compared to the control group (Figure 4). The cell line A375 was treated at concentrations of 57 and 65 μg/mL, with 58.16 and 87.16% of total apoptotic cells, respectively. Treatment of WM1366 cells at 32 and 45 μg/mL significantly increased the percentage of total apoptotic cells, compared to the control group (11.37 and 20.06%, respectively). The melanoma cell line SK-MEL-28 was treated at 46 and 55 μg/mL, which led to 38.83% and 60.69% apoptotic cells, respectively. Affinisine induced significant apoptosis in all the human melanoma cell lines used in this study.

The apoptosis rate was evaluated by the flow cytometry assay Annexin, which is based on the phosphatidylserine externalization, one of the most prominent characteristics of this mechanism of death. Apoptosis is an important mechanism involved in the inhibition of tumor growth, with distinct morphological characteristics, such as chromatin condensation, fragmentation and cellular membrane blebbing [28]. Moreover, tumor cells can acquire resistance to apoptosis by the expression of anti-apoptotic proteins (e.g. Bcl-2), down-regulation/mutation of pro-apoptotic proteins (e.g. Bax) or impaired p53 function, which is a worsening factor to malignant tumor [29,30].

To determine whether affinisine regulates the cell cycle phases, flow cytometry was used. Melanoma cells were exposed to the compound at concentrations close to the IC_{50} value for 48 h. The separation of cell populations into phases G0/G1, S and G2/M was based on their linear fluorescence intensity after propidium iodide staining. Affinisine was able to significantly induce cell cycle arrest in different phases in the A375 and WM1366 cell lines. However, in SK-MEL-28, cell cycle arrest

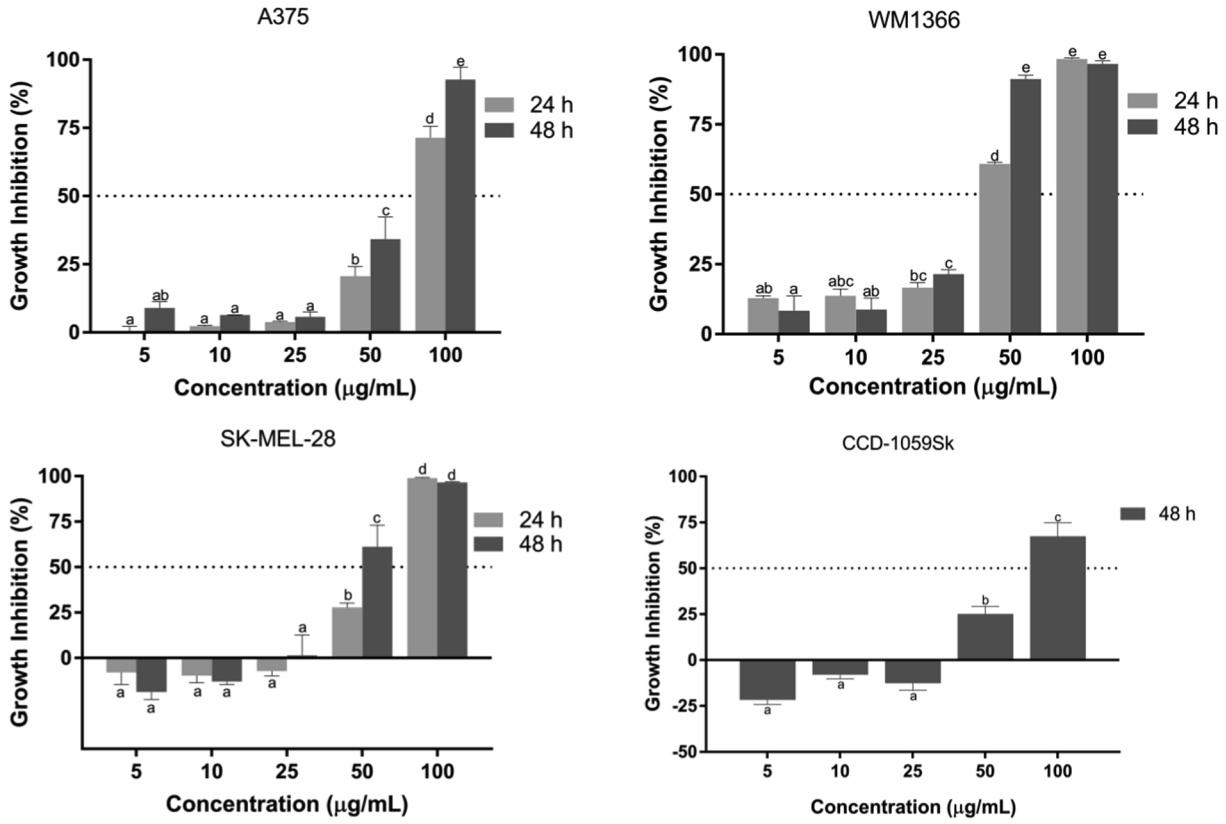

Figure 3. Affinisine inhibits cell growth of melanoma cell lines A375, WM1366 and SK-MEL-28. Growth inhibition in melanoma cells and non-tumoral skin CCD-1059Sk were measured by MTT assay. Data are presented as mean ± SEM from three independent experiments. Different letters indicate significant differences among means. Significance was considered at $p < 0.05$.

Table 1
IC_{50} values (μg/mL ± SEM) of affinisine in A375, WM1366, SK-MEL-28 and CCD-1059Sk cell lines after 24 and 48h of exposure. Data are expressed as mean ± SEM from three independent experiments.

	IC_{50} concentration (μg/mL)	
	24h	48h
A375	75.04 ± 4.03	57.69 ± 3.94
WM1366	48.22 ± 1.74	32.86 ± 2.54
SK-MEL-28	57.84 ± 23.03	41.51 ± 5.82
CCD-1059Sk	N/A	77.81 ± 13.26

N/A: not applicable. CCD-1059Sk cell was evaluated only after 48h of treatment exposure.

was not observed (Figure 5). The compound induced cell cycle arrest in the G2/M phase in A375 cells. After treatment, the number of cells in this phase increased from 8.66% in the control to 14.56 and 14.73% with 57 and 65 μg/mL of affinisine, respectively. With respect to the control, this indole alkaloid significantly increased the percentage of WM1366 cells in the G0/G1 phase, from 69.56% to 78 and 77.53% at the concentrations of 32 μg/mL and 45 μg/mL, respectively.

The apoptosis rate induced by affinisine was significantly different between the control and treated cells for all the melanoma cell lines studied in this work, according to the data shown in Fig. 4. However, this result was more prominent in the A375 cell line, in which both tested concentrations induced significant late apoptosis/dead (56.66 and 86.71% at 57 and 65 μg/mL, respectively). Moreover, the levels of apoptosis in SK-MEL-28 were also expressive, corresponding to approximately 60% of the total cells. On the other hand, the apoptosis rate was lower in WM1366 (9.18 and 19.4% at 32 and 45 μg/mL, respectively), which could be explained by the high level of cell cycle arrest that indole alkaloid caused in this cell line.

Previous studies have demonstrated that indole alkaloids induce apoptosis in a variety of malignant cells, such as glioblastoma, colon, breast and leukemia cells [31–33]. Moschamine, an indole alkaloid isolated from the seeds of *Centaurea* species, exerts cytotoxic and cytostatic effects on glioma cells *in vitro* [31]. Among the indole alkaloids isolated from the stem-bark extract of *Tabernaemontana corymbosa,* two compounds, ervatensines A and B, inhibit the proliferation of HCT-116 and MDA-468 cells, evoking apoptotic and necrotic cell death [32]. Cathachunine, an indole alkaloid isolated from *Catharanthus roseus* (L.), is cytotoxic to human leukemia cells and capable of antiproliferation and pro-apoptosis activities [33].

Affinisine triggered significant G0/G1 phase arrest in WM1366 cells after treatment at 32 and 45 μg/mL, associated with a significantly decreased population in the S phase of the cell cycle, the phase in which DNA replication occurs [34]. Cell cycle arrest at the G1 phase may decrease cell proliferation, which is consistent with our results. In general, cell cycle progression is regulated in different ways, as the activation/inactivation of cyclin-dependent kinases (cdks) [35]. The p21 protein controls the progression rate of the cell cycle in the G1 phase by the inhibition of cdk2 and cdk4, which are necessary for cell cycle progression [36]. Thus, up-regulation of p21 accompanied by down-regulation of cdk2 and cdk4 are tumor suppressors of cell cycle regulation in melanoma cells [37].

The capacity of indole alkaloids to induce cell cycle arrest in the G0/G1 phase has been previously reported, such as the indole alkaloid moschamine, which induces an increase in the cell populations in sub-G0/G1 and S-phase arrest in a dose-dependent manner [30]. Also, isorhynchophylline, a component of *Uncaria rhynchophylla*, exhibits a cytotoxic effect against HepG2 cells, in addition to inducing apoptosis, as characterized by the accumulation of cells in the sub-G1 phase [38].

In this study, affinisine induced a short cell cycle arrest in the G2/M phase, the last phase of the cell cycle, in the A375 cell lines. This data

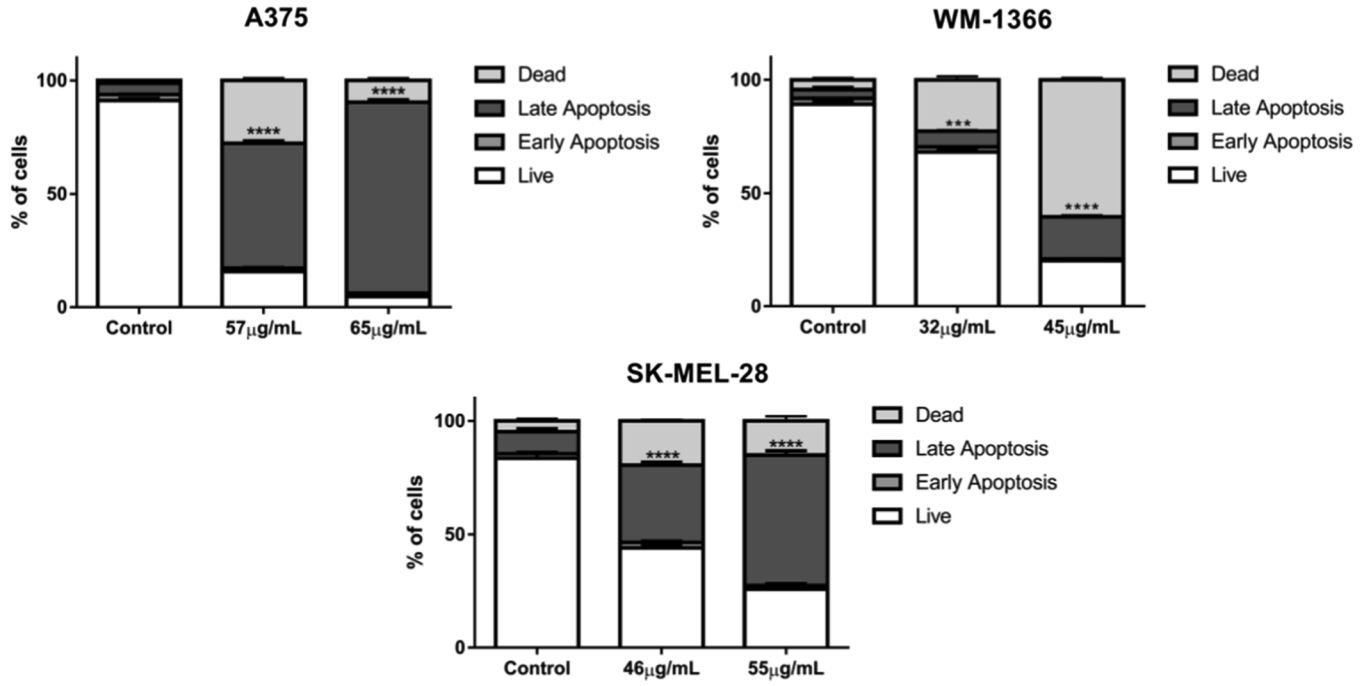

Figure 4. Affinisine increases apoptosis rate in melanoma cell lines A375, WM1366 and SK-MEL-28 treated at IC_{50} concentration for 48h. Percentage of apoptotic cells were determined by Annexin assay. The graphics presents the percentage of dead, late and early apoptosis and live cells. *indicates significant difference among treated groups and control group. Significance was considered at * $p < 0.05$; *** $p < 0.0006$; **** $p < 0.0001$.

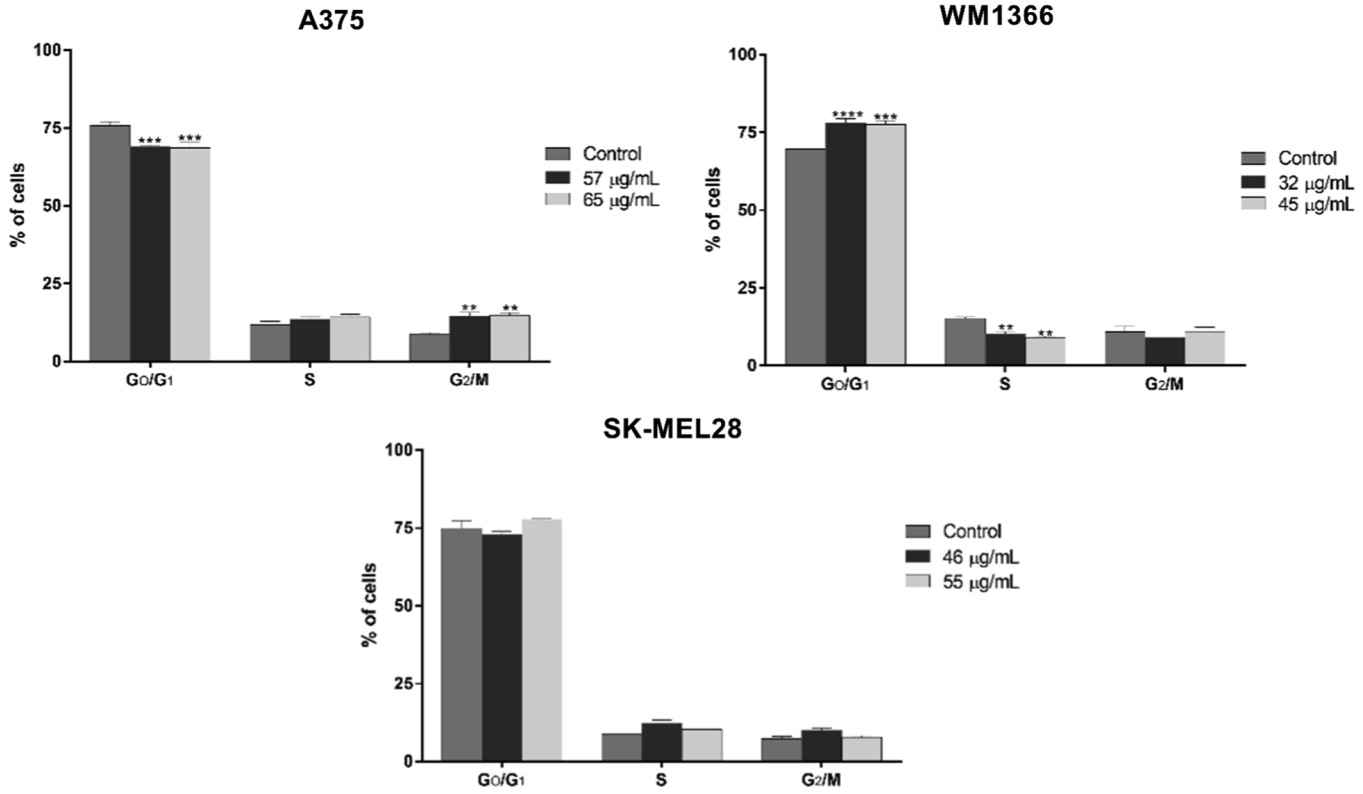

Figure 5. Affinisine induces cell cycle arrest of A375 and WM1366 cells treated at IC_{50} concentration for 48h. No significant difference was observed in SK-MEL-28 cell line after treatment. Percentage of cells in each cell cycle phase was determined by flow cytometry. *indicates significant difference among treated groups and control group. Significance was considered at * $p < 0.05$; *** $p < 0.0006$; **** $p < 0.0001$.

could indicate that affinisine inhibited A375 cell growth by the apoptosis mechanism, not by cell cycle arrest.

4. Conclusion

The challenge of cancer chemotherapy has stimulated several research groups to study the natural products of countless plants. In the present study, we isolated the compound affinisine from *T. catharinensis* using an easy and efficient method. Furthermore, we tested the antitumor activity of this compound against cell lines of melanoma. We found that treatment with this compound could inhibit the proliferation of three different human melanoma cell lines (A375, WM1366 and SK-MEL-28) in a dose- and time-dependent manner, as was evidenced by the cell viability assay. Moreover, the induction of apoptosis and the arrest of cells at the G0/G1 phase indicate that the compound could be a potential molecule or prototype to the development of new chemotherapeutic agents against melanoma.

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Conflict of Interest

No conflict of interest declared

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No conflict of interest declared.

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