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Croton lechleri sap and isolated alkaloid taspine exhibit inhibition against human melanoma SK23 and colon cancer HT29 cell lines

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ABSTRACT

Ethnopharmacological relevance: *Croton lechleri* Mull. Arg. (Euphorbiaceae) is a traditional medicinal plant which produces a red sap, traditionally known as "Sangre de Drago"; it is used in folk medicine externally for wounds, fractures, and haemorrhoids, internally for intestinal and stomach ulcers and also for the empirical cure of cancers.

Materials and methods: We investigated the effects of *Croton lechleri* sap and taspine in comparison with taxol and vinblastine on the growth of human cancer cell lines of SK23 (melanoma), LoVo and HT29 (colorectal cancer) using MTT and Trypan blue assays. Further, we studied cell cycle by flow cytometry and detected acetylated-α-tubulin by confocal microscope.

Results: *Croton lechleri* inhibited cell proliferation starting from 1 μg/mL in SK23 cells, whereas 10 times higher concentrations were required for growth inhibition of HT-29 and LoVo cell lines. Also taspine (0.1 μg/mL) inhibited the SK23 and HT29 cell proliferation. Further, assay was assessed on SK23 and HT29 cell lines with 24–48 h treatment with sap and taspine. Both sap and taspine inhibited cancer cell proliferation; taspine showed higher activity on SK23 cells, which was significantly increased after 48 h of SK23 treatment. Using confocal microscopy we observed that *Croton lechleri* (1 μg/mL) caused a loss of microtubule structure, whereas taspine (0.5 μg/mL) caused an increase in acetylated α-tubulin and a modification of cellular morphology, mainly in SK23 cells. *Croton lechleri* sap 10 and 50 μg/mL influence cell cycle; 50 μg/mL sap caused a dramatic reduction of cells in G_1/G_0 and S phases with a great increase of $subG_0$ cells.

Conclusions: The data showed that *Croton lechleri* and taspine could inhibit cell proliferation with higher potency against melanoma SK23 cells, supporting the empirical use of the sap as anticancer in ethnomedicine and taspine as a possible anticancer agent.

1. Introduction

Croton lechleri Müll.Arg. (fam. Euphorbiaceae) is a traditional medicinal plant which produces a red sap, traditionally known as "Sangre de Drago" that is obtained carving the bark of plants having two–three or more years. Amazonic natives use this sap to treat several diseases as wound healing, gastrointestinal illness and also cancers (Jones, 2003). Furthermore, the dried sap is marketed in health products in Western countries. The phytochemical characterization of the sap has lead to the finding that the oligomeric proanthocyanidins and flavonols constitute almost the 90% of the dry weight (Cai et al., 1991). But various minor compounds also have been found; one is the alkaloid taspine found in the sap of mature trees (Perdue et al., 1979). Others are the lignan 3′,4-*O*-dimethylcedrusin (Cai et al., 1993a; Pieters et al., 1993), various diterpenoids (Cai et al., 1993b) and norisoprenoid blumenol derivatives (De Marino et al., 2008).

Proanthocyanidins and other condensed tannins are known to be protective against various pathologies, including cancer (Bobe et al., 2009; De Bruyne et al., 1999; Hanausek et al., 2011). *Croton lechleri* has been evaluated in cell lines such as KB, *V*-79, K562 and HeLa for anticancer activity showing low activity (Alfonso-Castro et al., 2012; Chen et al., 1994; Itokawa et al., 1991; Rossi et al., 2003; Vaisberg et al., 1989). This activity was mainly ascribed to the presence of the alkaloid taspine (Fig. 1) which has also been reported as the active compound with wound healing properties (Porras-Reyes et al., 1993; Vaisberg et al., 1989). Taspine is the main alkaloid which characterizes *Croton lechleri* sap, but it is also present in *Leontice eversmanii* Bge (Berberidaceae), *Caulophyllum thalictroides* (*L.*) Michx. and *Calophyllum robustum* Maxim. (Berberidaceae), *Magnolia x soulangiana* Soul.-Bod. (Magnoliaceae). Recently, interest is growing on the *Croton lechleri* sap and on its isolated alkaloid taspine (Fayad et al., 2009; Rollinger et al., 2006; Zhang et al., 2010).

The incidence rate of cutaneous malignant melanoma and colorectal cancer is increasing over the years and classical chemotherapy agents are still partly unsuccessful, therefore there exists a huge need of new therapeutic approaches against these

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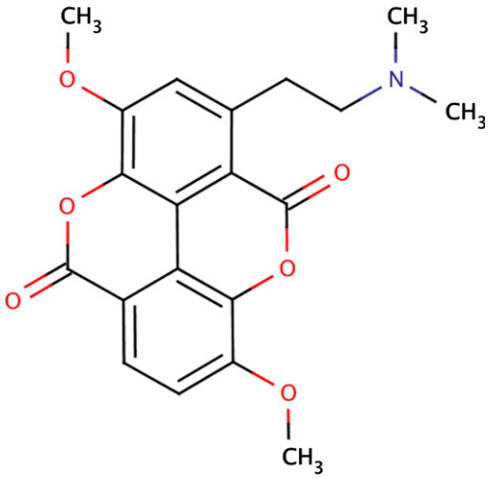

Fig. 1. Chemical structure of taspine.

cancers (Goffin et al., 2005). Further, malignant melanoma is the most serious of all skin cancers and strongly resistant to chemo- and radiotherapy. Epidemiological studies have shown that its incidence is rapidly increasing (Kruijff and Hoekstra, 2012).

In this framework of renewed researches, we have considered the activities of *Croton lechleri* and taspine on epithelial cancer in vitro also in relation with its traditional use, which is mainly for topical application. In this context, *Croton lechleri* sap and isolated taspine were studied in comparison with taxol and vinblastine, two natural anticancer agents, on the cell viability and the cell cycle, and on the α-tubulin structure of melanoma and colon cancer cell lines in culture.

2. Materials and methods

2.1. Plant material

The red sap of *Croton lechleri* was collected by traditional way, carving the bark from trees growing in the province of Napo, Ecuador. The voucher code number (SdD 007) for the crude drug was deposited in the Department of Pharmaceutical and Pharmacological Sciences of Padua University.

The sap was freeze-dried in a lyophilizer and stored at −20 °C. For cell culture experiments, the freeze-dried SdD was solubilised in water.

2.2. Croton lechleri sap and taspine

Before lyophilisation, pH and density of the sap were determined. Dry residue by lyophilisation was also measured. The presence of polyphenols and taspine alkaloid was investigated by TLC. Taspine was isolated and identified by HPLC-DAD, ^{1}H–NMR and mass spectral data as previously reported (Froldi et al., 2009).

2.3. Cell lines and culture conditions

Melanoma (SK-23) and colorectal carcinoma (LoVo and HT29) human cell lines were obtained from Lonza group (Switzerland) and American Type Culture Collections (Rockville, MD, USA), respectively. Cells were grown in DMEM (high glucose), 10% foetal bovine serum (FCS) supplemented with 2% L-glutamine and 1% pen/strep, and maintained in a humidified 5% CO_2 incubator at 37 °C. At confluence, cells were detached with trypsin–EDTA. Harvested cells were grown in 6-well tissue culture plates (1×10^6 cells/well) for viability. Cells were allowed to grow to confluence over 24 h before use. For proliferation studies, cells were plated in 96-well plates.

2.4. Cell viability

Cell viability was determined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 200 μL medium in 96 well microtiter plates at a density of 2500/well. Following overnight incubation, cells were exposed to a range of different concentrations of *Croton lechleri* sap and testing compounds, according to the experimental protocol. After 24 h, cells were replaced with fresh medium and cells were added with 20 μL of MTT (5 mg/mL) and incubated for 4 h at 37 °C. The cells were then lysed by adding 200 μL of acidified isopropanol (0.04 N HCl in isopropanol). The absorbance at 570 nm was determined using a Victor x3 multilabel counter, PerkinElmer (USA). The absorbance was directly related to viable cells.

The Trypan blue assay was performed using a standard 0.4% Trypan blue solution in phosphate buffered saline without calcium or magnesium. Cell suspension (1 mL per well) was seeded in flat-bottom 24-well culture plates at 500,000 cells per mL and incubated overnight at 37 °C in a humidified incubator containing 5% CO_2. Cells were allowed to attach and recover for 24 h before they were exposed to drug treatment. The cells were then trypsinised and counted with a haemocytometer in a 1:1 dilution of cells in a 0.4% Trypan blue solution and the percentage of non-viable cells was calculated.

2.5. Cell cycle analysis by flow cytometry

The cells were seeded at a density of 1.5×10^5 cells/well. After 24 h cells were treated according to the experimental protocol as previously described (Montopoli et al., 2009). At the end of the treatment the cells were collected with trypsin, counted and fixed for 30 min on ice with cold ethanol at 70% at a density of 1×10^6 cell/mL, washed, resuspended with a solution of 0.05 mg/mL propidium iodide (Molecular Probes, Invitrogen, UK) and 0.2 μg/mL RNAase-DNAase free in PBS and incubated for about 30 min at room temperature in the dark. About 3×10^4 cells/sample were analysed on Epics XL-flow cytometer (Beckman Coulter, USA), with an Argon Laser λexc= 488 nm and a photomultiplier PMT2, at λem=575 ± 20 nm. The percentage of cells in the different phases of cell cycle was calculated with the Multicycle software provided by the manufacturer, considering diploid cycle and correcting for cell clusters.

2.6. Immunofluorescent detection of microtubules

Cells were collected by trypsinization and centrifugation, washed with PBS, seeded at approximately 30% confluence in 12-well plates on glass cover slips and incubated overnight. After 12 h, the adherent cells were cultured for 12 h in fresh media with or without the compounds studied. For detection of acetylated-α-tubulin and *F*-actin, cells fixed with 4% formaldehyde were permeabilized with 0.1% Triton *X*-100 in PBS and stained with polyclonal antibody against acetylated-α-tubulin (Santa Cruz Biotechnology, Inc. Heidelberg, Germany). Alexa Fluor 488-conjugated antibodies (Molecular Probes, Invitrogen, Carlsbad, CA) were used to detect acetylated-α-tubulin proteins. For *F*-actin, cells were stained with phalloidine-Alexa Fluor 456-conjugated. For nuclear counterstaining, cells were treated with 20 μg/mL DNase-free RNase for 10 min at room temperature and then stained with red-fluorescent propidium iodide. Cover slips were mounted on glass slides by using Mowiol 40–88 at the final concentration of 0.5 μg/mL. Images were acquired with a Nikon C1 confocal microscope and analyzed using Nikon EZ-C1 (version 2.10) (Nikon Corporation, Tokyo, Japan).

2.7. Chemicals and reagents

Unless otherwise stated, all reagent grade chemicals and the rabbit polyclonal anti-α-tubulin antibody were purchased from Sigma-RBI (USA). The purity of all compounds was >98%. All cellular reagents and culture medium were from Gibco BRL (Gaithersburg, MD, USA).

2.8. Statistical analysis

Each experiment was performed at least three times and results are presented as the mean ± S.E.M. Sigmoid curve fitting was performed using the GraphPad Prism program (GraphPad Software, San Diego, CA, USA). The half maximal inhibitory concentration (IC_{50}) represents the concentration (μg/mL) of a test substance that lowers MTT reduction or the cell number by 50% compared to the untreated controls. Statistical comparisons between treatment and control data were performed by ANOVA followed by Bonferroni *t* test. In each case, $p < 0.05$ was considered significant.

3. Results

3.1. MTT assay

The pH of the sap from *Croton lechleri* was 3.9 and the density was 1.08 g/mL according with the literature data (Risco et al., 2003). The chemical characterization has been performed through the determination of the alkaloid taspine as previously reported (Froldi et al., 2009). To allow the conservation, we freeze-dried the fresh sap obtaining a crystal brown powder with a dry residue of 27.5 ± 0.6%.

We studied the activity of *Croton lechleri* sap and taspine on cellular proliferation using MTT test. Thus SK23, HT29 and LoVo cells were treated for 24 h with the sap in the range concentration from 0.01 μg/mL to 100 μg/mL; at 0.5 μg/mL the sap significantly decreased cell proliferation to 67.5 ± 3.4% in SK23 cells, but without significant action on HT29 and LoVo cells (Fig. 2). Whereas the inhibition of HT29 and LoVo cell proliferation started with 10 μg/mL *Croton lechleri* (Fig. 2). The sap was not used at higher concentration because we observed foam formation when cells were treated with concentrations >100 μg/mL. The 50% viability inhibition (IC_{50}) for SK23 cells was >5 μg/mL, while for HT29 and LoVo cells was about 10 times higher. The alkaloid taspine isolated from the sap was evaluated from 1 ng/mL to 1 μg/mL, showing a significant inhibition activity at 0.1 μg/mL in SK23 and HT29 cell lines (Fig. 2). The IC_{50} was 0.6 μg/mL, 0.8 μg/mL and >1 μg/mL in SK23, HT29 and LoVo cell lines, respectively.

In comparison to *Croton lechleri* sap and taspine, taxol and vinblastine were evaluated in the range of 0.1 ng/mL to 1 μg/mL. As expected, taxol greatly decreased viability in all the cell lines tested, with an $IC_{50} < 0.03$ μg/mL (Fig. 2). Also, vinblastine inhibited the viability of the human cancer cell lines; for HT29 and LoVo with an $IC_{50} < 0.01$ μg/mL, while SK23 cell line was less sensitive, with an IC_{50} of about 1 μg/mL (Fig. 2).

3.2. Trypan blue assay

Further, to deeply study the activity of the sap and taspine on cell viability, we performed the Trypan blue assay in SK23 and HT29 cell lines (Fig. 3), excluding LoVo because less sensitive to taspine between the two colon cancer lines studied. The sap and taspine were used in the same range, from 1.0 μg/mL to 25.0 μg/mL, for 24 h

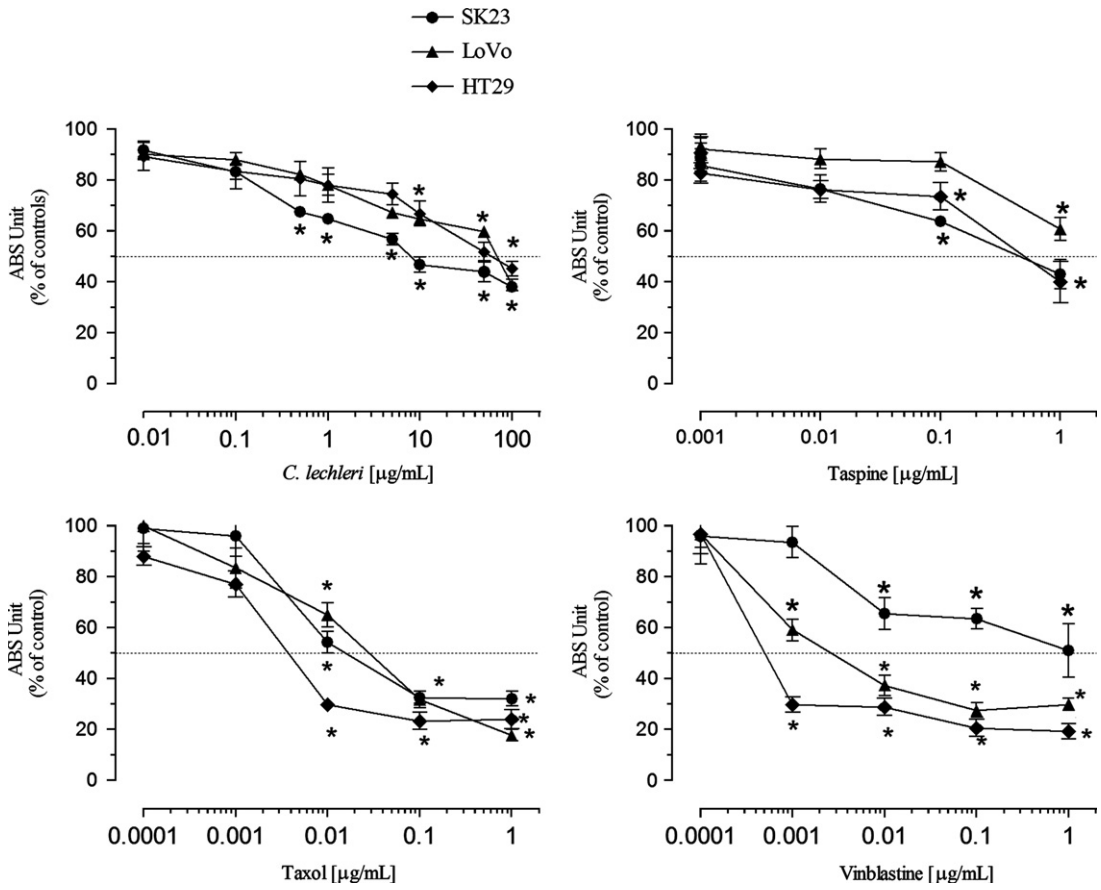

Fig. 2. Effects of 24 h treatment with *Croton lechleri* sap, taspine, taxol and vinblastine on SK23, LoVo and HT29 growth determined by MTT assay. Values are expressed as means ± S.E.M. of 3–5 experiments. * $p < 0.05$ vs. controls.

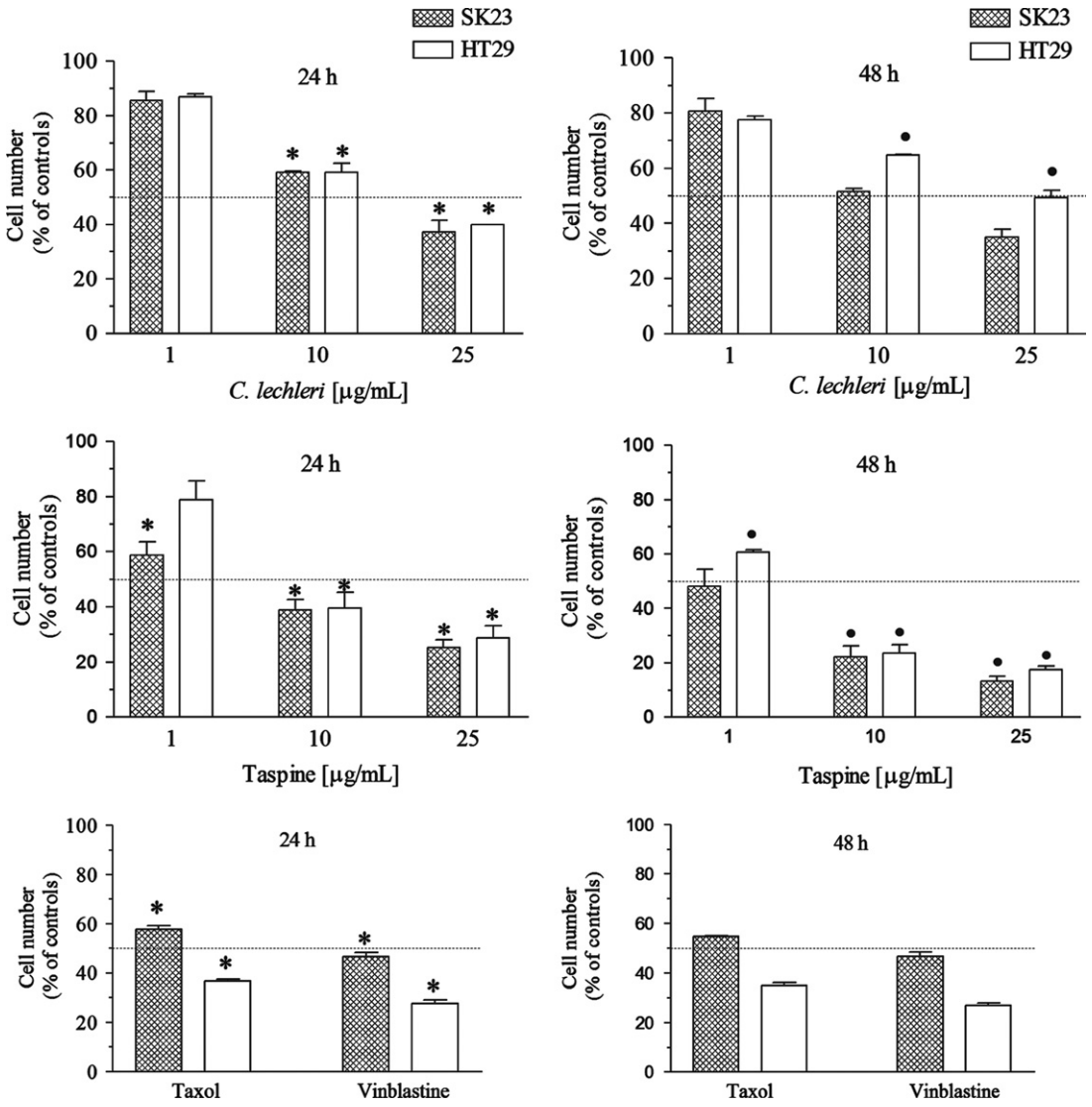

Fig. 3. Effects of 24–48 h treatments with *Croton lechleri* sap, taspine, taxol (0.01 μg/mL) and vinblastine (0.01 μg/mL), on SK23 and HT29 growth determined by Trypan blue assay. Values are expressed as means ± S.E.M. of 3–5 experiments. * $p < 0.05$ vs. controls. ● $p < 0.05$ vs. 24 h treatment.

and 48 h treatment. The sap showed an $IC_{50} > 10$ μg/mL in both SK23 and HT29 cell lines; partially, the HT29 cells recovered after 48 h of treatment. Otherwise, taspine showed an inhibition which increased with the incubation time in both cell lines; the IC_{50} was ≥1 μg/mL in SK23 cells (Fig. 3). The differences observed between the inhibitory concentration values of MTT and Trypan blue exclusion assay could be related to the specificity of the methods, since Trypan blue test evaluates the structural integrity of cell membrane, while the reduction of MTT assesses the mitochondrial function through the activity of succinate dehydrogenase (Mosmann, 1983). In comparison, taxol (0.01 μg/mL) and vinblastine (0.01 μg/mL) showed a higher inhibition of SK23 and HT29 cell lines, already for 24 h treatment (Fig. 3).

3.3. Microtubule morphology

In order to highlight a possible action of *Croton lechleri* sap on the cytoskeletal proteins, a set of experiments have been conducted using confocal microscopy and antibody markers of acetylated α-tubulin and *F*-actin in comparison with taxol and vinblastine, considered as reference drugs which inhibit the dynamics of microtubules with different mechanisms. In SK23 cells, 24 h treatment with *Croton lechleri* (1 μg/mL) caused a loss of microtubule structure (Fig. 4) while no modification of *F*-actin was observed (data not shown). In HT29 cells, the sap has left unchanged microtubule cytoskeleton (Fig. 4). Taspine (0.5 μg/mL) caused an increase in acetylated α-tubulin and a modification of cellular morphology, mainly in SK23 cells (Fig. 4). As expected from literature data, taxol (0.01 μg/mL) induced an evident increase of acetylated α-tubulin, whereas vinblastine (0.1 μg/mL) decreased this one, both in SK23 and HT29 cell lines (Fig. 4). As expected, the two anticancer drugs disrupted the cytoskeletal elements although in different ways.

3.4. Cell cycle

On the basis of the experimental results reported above in which SK23 cells were most sensitive to treatments, we chose to analyse the cell-cycle and apoptosis by flow cytometry only in SK23 cell line exposed for 24 h to *Croton lechleri* sap, taspine, and also to taxol and vinblastine. The sap used at 1.0 μg/mL had not influence on cell cycle, but higher concentration of 10.0 μg/mL increased cells in G_1/G_0 phase of 17.7 ± 3.5%, while decreased S and G2/M phases of 7.9 ± 3.1% and 11.7 ± 2.8%, respectively (Fig. 5). Whereas when the sap was used at 50.0 μg/mL we observed a strong decrease of G_1/G_0 and S phases with a shocking

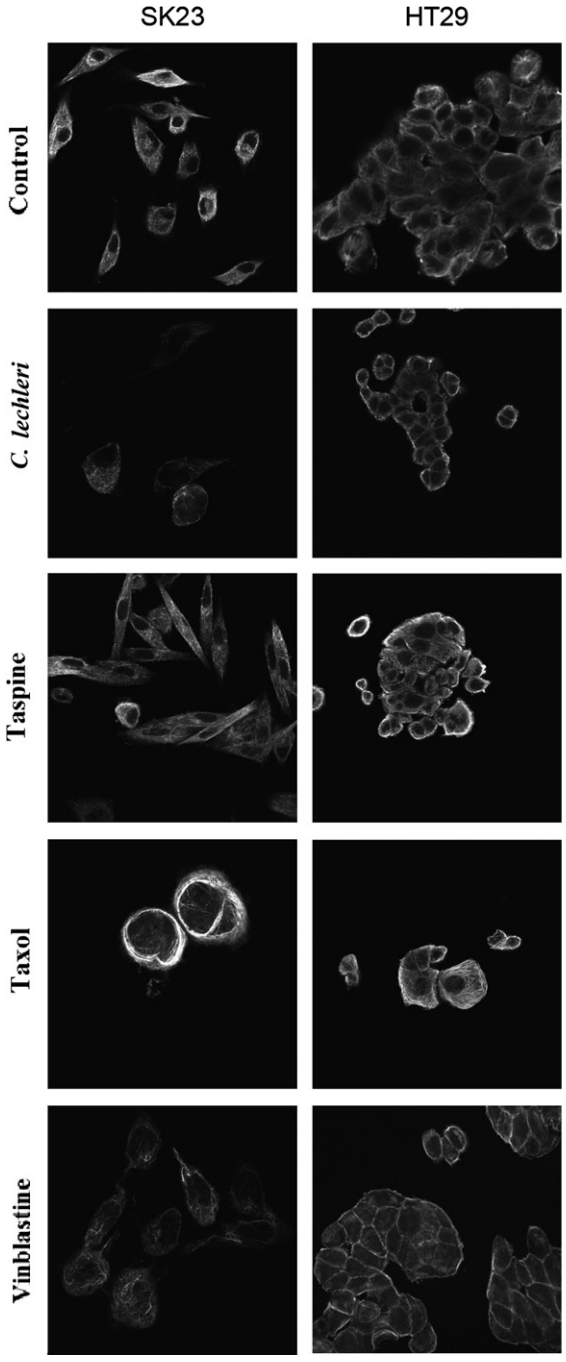

Fig. 4. Effects of *Croton lechleri* sap, taspine, taxol and vinblastine on SK23 and HT29 cell morphology and cytoskeletal organization. Data are from one experiment representative of three.

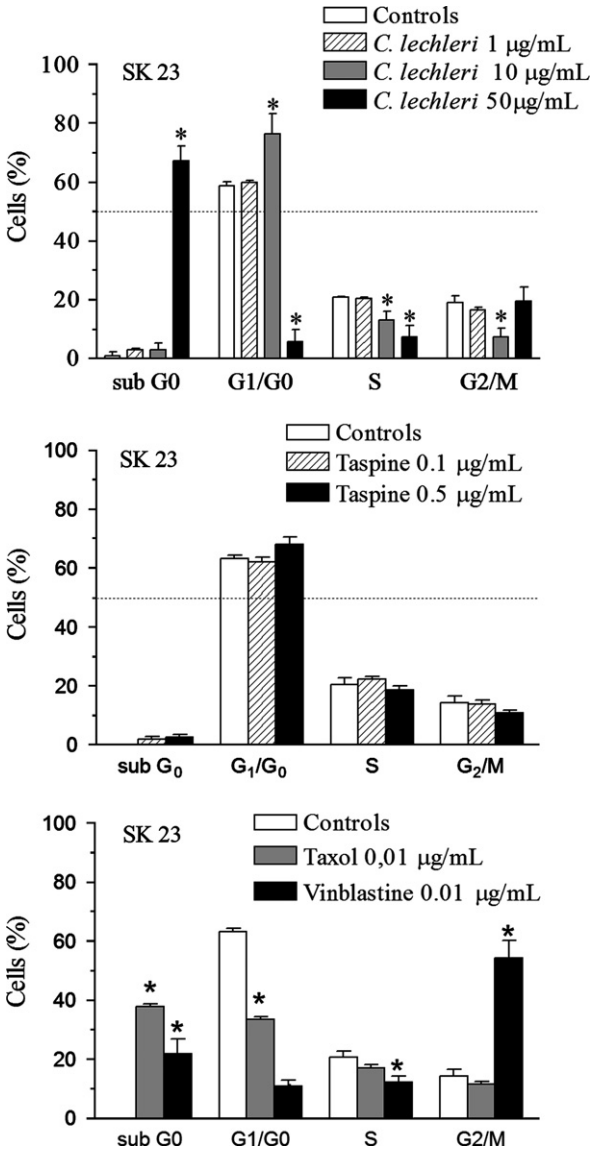

Fig. 5. Cell-cycle analysis performed by flow cytometry of SK23 cells exposed for 24 h with different concentrations of *Croton lechleri*, taspine, taxol, and vinblastine. Values are expressed as means ± S.E.M. of 3 experiments. * $p < 0.05$ vs. controls.

increase of subG$_0$ cells (Fig. 5), suggesting a cytotoxic action of *Croton lechleri* sap. We also studied the alkaloid taspine (0.1 and 0.5 µg/mL) which did not modify cell cycle (Fig. 5). Taxol and vinblastine (0.01 µg/mL) significantly reduced the percentage of cells in G$_1$/G$_0$ and dramatically increased subG$_0$ phase in SK23 cells. Vinblastine greater increased the G$_2$/M phase in comparison with taxol (Fig. 5).

4. Discussion

In this research, we studied the potential anticancer activity of *Croton lechleri* sap and its isolated alkaloid taspine in comparison with taxol and vinblastine on the growth of melanoma and colon cancer cell lines, studying cell proliferation, α-tubulin structure and cell-cycle. The sap (1–100 µg/mL) showed antiproliferative activity against all cell lines with a higher inhibition of SK23 cells growth, determined by MTT assay. Also taspine showed antiproliferative activity from 0.1 µg/mL against both SK23 and HT29 cell lines. In the same experimental conditions, taxol started to be effective at 0.01 µg/mL, which is a ten times lower concentration in comparison to taspine. These antiproliferative effects were confirmed by Trypan blue analysis, showing also that the prolongation of incubation time, from 24 h to 48 h, of taspine increases its effect on SK23 and HT29 proliferation, differently from taxol and vinblastine. *Croton lechleri* caused also a loss of microtubule structure more evident in SK23 cells. Whereas taspine appears to increase the α-tubulin acetylation and change the morphology of SK23, suggesting its action on specific targets of cytoskeleton.

Croton lechleri represents an important ethnomedicine effectively used in the treatment of skin and gastrointestinal disorders, for this we carried out the experiments on SK23 (melanoma)

and HT29 and LoVo (colorectal cancer) cell lines. Although colorectal cancer is more prevalent in developed countries, an increasing number of colon cancer cases are also being reported where Western dietary habits have taken hold. These epidemiological observations push to develop new cytotoxic drugs potentially useful in therapy. In this view, *Croton lechleri* could be considered of interest as such or as a precious source of compounds against gastrointestinal and skin diseases, also in light of its traditional uses. To study whether colorectal cancer cells could be influenced by *Croton lechleri* sap and taspine, one cell line with high metastatic potential (LoVo) and one with low metastatic potential (HT29) were chosen for experimental studies. On the basis of MTT assay, the sap had similar activity against HT29 and LoVo cell lines, whereas taspine showed relatively lower activity against LoVo. In the present data, taspine has an IC_{50} of 0.6 μg/mL and 0.8 μg/mL in SK23 and HT29 cell lines, while the IC_{50} was not detectable in LoVo cells. In SK23 cell line, the activity of taspine is comparable to that of taxol used at concentrations 10-fold lower. Other authors have studied taspine on various cancer cell lines. In human squamous carcinoma cell line (A431) Hou et al. have found for taspine an IC_{50} of 15.4 μg/mL, with significant inhibition starting from 0.5 μg/mL (Hou et al., 2011; Zhang, et al., 2011). In estrogen-receptor-positive breast cancer ZR-75–30, taspine inhibited cell growth with an IC_{50} of 1.69 μM (Zhan et al., 2011). Further, the alkaloid (5 μg/mL) showed high embryotoxicity, but no teratogenic activity, in the cultured rat embryos (Kennelly et al., 1999). The present IC_{50} values are also in agreement with literature data obtained in HUVEC cells (Zhang et al., 2008).

Through confocal microscopy we studied acetylated α-tubulin, which is a marker of stable, long-lived, nondynamic microtubules (Belletti et al., 2008; Cambray-Deakin and Burgoyne, 1987; Perdiz, et al., 2011). The *Croton lechleri* sap caused reduction of acetylated α-tubulin mainly in SK23 cells, whereas taspine showed a trend to increase the acetylated α-tubulin with change in cellular morphology, mainly of SK23 cells. By evaluating these observations, the action of the sap may resemble that of vinblastine, whereas taspine could act on a different site on the microtubules in comparison with taxol and vinblastine. Also taspine had not influence on SK23 cell cycle, differently to reference anticancer drugs. These observations suggest the idea of a different pathway from taxol and vinblastine; anyway, further research needed to be undertaken. Thus, taspine may be a lead compound for a new class of anticancer drugs, and the present data suggest, for the first time, selectivity against melanoma cancer cells. It is of interest to note that 10 μg/mL *Croton lechleri* has also antiproliferative action against SK23 and HT29, suggesting that other compounds in the sap, over taspine, could have anticancer activity given that more than 90% of sap consists of proanthocyanidins (Cai et al., 1991a, 1991b; Jones, 2003). Very few authors studied antiproliferative activity of *Croton lechleri*; in KB cancer cells the sap showed an IC_{50} of 187 μg/mL (Chen et al., 1994), while in K562 cells the IC_{50} was of 2.5 μg/mL (Rossi et al., 2003). In the present experimental data, *Croton lechleri* inhibited cell proliferation with an $IC_{50} \leq 10$ μg/mL in SK23, and > 50 μg/mL in HT29 and LoVo cell lines. Thus, *Croton lechleri* sap seems undoubtedly to contain biologically active components endowed with antitumor properties. Other authors studied the activity of a sap from *Croton palanostigma* with $IC_{50} > 100$ μg/mL against stomach and colon cancer cells (Sandoval et al., 2002). Recently, Alfonso-Castro et al. (2012) showed that methanolic extract of leaves of *Croton lechleri* exerts antitumor effects in mice bearing cancer. While for taspine has been described antitumor activity in the mouse S180 sarcoma and ZR-75–30 breast cancer animal-transplanted tumour models (Zhan et al., 2011; Zhang et al., 2007). Therefore, other in vivo studies are required to fully assess the antitumoral potential of the sap and of its isolated constituents.

The induction of apoptosis and microtubule damage in SK23 cells suggest that *Croton lechleri* sap should be evaluated further as a potential source of anticancer agents, while taspine could be a selective potential anticancer with its activity below the micromolar concentrations both in SK23 and HT29 cell lines. In this context, all data suggest that the *Croton lechleri* sap has the potential for providing biologically active compounds to reduce malignant cell proliferation, and it deserves additional evaluation even in clinical trials as a chemotherapeutic agent mainly against skin or gastrointestinal cancers.

The present research shows for the first time an extensive comparison between *Croton lechleri* sap and taspine antiproliferative activities on SK23 and HT29 cell lines, using various in vitro methods. Both substances show high potency of inhibition on human melanoma SK23 cell line. This original observation could be the cue to develop a new strategy against melanoma cancer since an effective therapy against this tumour has not been fully established. However, further studies in vivo are necessary to support this suggestion.

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