

Antiproliferative Activity of *Aloe arborescens* Leaf Skin Extracts Tested on Murine Myeloma Cells: Cytological Studies and Chemical Investigations

Donatella Ceccarelli¹ • Elisa Ovidi¹ • Doriana Triggiani¹ • Carlo F. Morelli² •
Giovanna Speranza² • AnnaRita Taddei³ • Antonio Tiezzi^{1*}

¹ Department for Innovation in Biological, Agro-food and Forest Systems (DIBAF), Tuscia University, 01100, Viterbo, Italy

² Department of Organic and Industrial Chemistry, University of Milan, 20133, Italy

³ Centre of Electron Microscopy (CIME), Tuscia University, 01100, Viterbo, Italy

Corresponding author: * antonio@unitus.it

ABSTRACT

MTT assays showed an intense antiproliferative activity (80%) of *Aloe arborescens* leaf skin extracts tested on murine myeloma cells. Bioassay-guided fractionation carried out by TLC allowed the identification of a spot showing antiproliferative activity; HPLC and NMR investigations showed that the TLC spot consisted of aloenin A and aloins A and B. The effects of the leaf extract and of the TLC spot were evaluated both by immunofluorescence techniques in order to test the microtubular array and at the morphological level by SEM and TEM observations.

Keywords: aloenin A, aloin A, aloin B, microtubules, HPLC, TLC

Abbreviations: DMEM, Dulbecco's minimum essential medium; HPLC, high performance liquid chromatography; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; R_f , retention factor; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TLC, thin layer chromatography

INTRODUCTION

Since ancient times aloe plants, mainly *Aloe barbadensis* Miller and *Aloe arborescens*, were used in traditional medicine for their pharmaceutical properties and for cosmetic uses (Steenkamp *et al.* 2007). Depending also on potential interests for pharmaceutical companies, aloe plants have been investigated in order to characterize their chemical constituents and to evaluate their possible therapeutic role(s) (Cravotto *et al.* 2010). Such investigations showed properties to be ascribed mainly to the inner, colourless, leaf gel in which complex carbohydrates have been found, while 1,8-di-hydroxyanthraquinone derivatives are abundant in the epidermis of leaves (Hamman 2008).

Different authors believe that many of the medical effects of aloe extracts are assigned to a synergistic action of the compounds contained in the inner leaf parenchymatous tissue rather than to a single chemical substance (Dagne *et al.* 2000; Hamman 2008).

Presently, it is well established that compounds from aloe leaves have many different therapeutic properties and, as a consequence, they seem to be good candidates for possible pharmaceutical applications. Immunostimulant and cicatrizant (Heggors *et al.* 1996; Yagi *et al.* 1997; Reynolds and Dweck 1999), anti-fungal (Das *et al.* 2011), antiviral and anti-inflammatory properties have been shown (Davis *et al.* 1989; Yamamoto *et al.* 1991; Pecere *et al.* 2000; Park *et al.* 2009; Das *et al.* 2011), as have antidiabetic (Beppu *et al.* 2003, 2006), chemoprotective (Furukawa *et al.* 2002) and antitumor properties (Shimpo *et al.* 2002; El-Shemy *et al.* 2010; Tomasin *et al.* 2011; Chang *et al.* 2012).

In the past, in our laboratories, some investigations on the biological activities of *A. arborescens* leaf extracts on murine myeloma cells were carried out (Rondini *et al.* 2000). More recently, we (Bedini *et al.* 2009) developed a procedure for *in vitro* micropropagation of *A. arborescens*

plants and provided preliminary data concerning the inhibition activity on murine myeloma cell proliferation of epidermis leaf extracts of three-year old plants. In the present paper we describe significant cytotoxic activities on murine myeloma cells of both *A. arborescens* epidermis leaf extract and of a fraction from the leaf extract; the main components of the active fraction were identified. Treated cells were also investigated at the cytological level by immunofluorescence techniques and both scanning (SEM) and transmission (TEM) electron microscopy.

MATERIALS AND METHODS

Chemicals and reagents

Solvents and other chemicals were purchased from Sigma-Aldrich (St. Louis, MI, USA); Dulbecco's minimum essential medium (DMEM), fetal bovine serum and glutamine were from Lonza Verviers SPRL (Verviers, Belgium); anti α -tubulin antibody was purchased from GE Healthcare (Buckinghamshire, UK), and fluorescein isothiocyanate conjugated anti-mouse IgG secondary antibody from Molecular Probes (Eugene, OR, USA).

Preparation of leaf extracts

Leaves were collected from three-year old *A. arborescens* plants grown in greenhouses at Tuscia University, Viterbo (Italy). After washing in distilled water, spikes were excised from leaves by a razor blade; epidermis tissue was separated from the leaf gel, weighed, homogenised with an equal volume (w/v) of absolute ethanol and extracted for 20 min in the dark at room temperature. The homogenate was then centrifuged at 2000 rpm for 5 min at room temperature, the pellet was discarded and the supernatant was evaporated to dryness by a nitrogen flow. The resulting residue was diluted with deionized water.

Murine myeloma cell culture

Biological activity of *A. arborescens* leaf extracts was tested on P3X murine myeloma cells (LGC Standards, Milano, Italy). This cell line is a long period viable and stable and grows in DMEM culture medium supplemented with 10% foetal bovine serum and 2 mM glutamine. Cell cultures were maintained in incubator at 37°C with an atmosphere containing 5% CO₂ for two weeks before use. Tests were carried out on cells grown in both 24- and 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany).

Cell treatments and vitality tests (MTT)

For each treatment, 100.000 cells were seeded in 1 ml of DMEM. Cells were treated for 24 h with both 1.2 mg/ml of epidermis extract and with 0.2 mg/ml of a solution containing the content of a spot (named Spot E, see below) obtained from thin layer chromatography (TLC) investigations carried out on *A. arborescens* epidermis leaf extracts. The number of living cells was tested by MTT assays (Slater *et al.* 1963; Berridge and Tan 1993) with slight modifications as follows, in order to carry out the tests on a growing cell suspension. Murine myeloma cells were seeded in 96-well plates and then centrifuged by a plate centrifuge for 5 min at 1500 rpm; the culture medium was discarded and tetrazolium salts added. Cells were incubated for approximately 3 h and subsequently dimethylsulfoxide was added. Samples were then tested by a cell plate spectrophotometer (Sunrise, Tecan Group Ltd., Männedorf, Switzerland).

Experiments were carried out in triplicate (16 each); data are presented as average values with standard deviation. The statistical significance of differences between treatment and control groups were determined by ANOVA test. The level of significance was set at $P < 0.05$.

Separation of *Aloe arborescens* epidermal leaf compounds and their effect on murine myeloma cells using thin layer chromatography

TLC was used both at the preparative level, with the aim of isolating possible active fractions to be tested for their antiproliferative activity, and as an analytical tool to identify and check the purity of compounds used as references.

Preparative TLC was carried out on silica gel (Macherey-Nagel GmbH, Düren, Germany) glass plates (20 × 20 cm), 0.25 mm thick. The plates were spotted with 30 µl (40 mg/ml) of *A. arborescens* epidermal leaf extract using a micrometric syringe. Chromatographic runs occurred by a mobile phase consisting of a mixture of ethanol: chloroform (1: 1). Spots were detected by examination of plates under UV light. The inhibition of proliferation activity of spots separated by TLC on P3X murine myeloma cells was tested.

Starting from the bottom of the preparative TLC plate carried out as stated above, three different regions were identified. The three areas were scraped from the plates by a razor blade and collected separately in plastic tubes. Compounds were extracted from silica gel powder by adding 5 ml of absolute ethanol for each gram of silica gel and mixed by a vortex apparatus; tubes were then centrifuged for 5 min at 14,000 rpm and the supernatants concentrated three times by a nitrogen flow. For biological tests the supernatants were evaporated to dryness by a nitrogen flow. The solutions obtained were diluted with deionized water, filtered through a membrane for sterilization (Merck KGaA, Darmstadt, Germany), and then administered to murine myeloma cells. After incubation, cells were investigated by the MTT assay as previously reported.

After MTT results, new silica gel plates were loaded with *Aloe* leaf extract, TLC runs were carried out by the same operative conditions already described and separated spots were viewed under UV lamps. The single spots were scraped from silica gel plates and eluted by ethanol; the solvent was then evaporated to dryness, the residue dissolved with deionized water, filtered and administered to murine myeloma cells. P3X-treated cells were further investigated by the MTT assay.

Immunofluorescence and electron microscopy investigations for cytological evaluations

Murine myeloma cells treated for 20 h with *A. arborescens* leaf extracts were investigated by immunofluorescence, SEM and TEM in order to evaluate the possible effects at cytological and ultrastructural levels.

1. Immunofluorescence microscopy

The microtubular component of the murine myeloma cell cytoskeleton was investigated as a possible target for evaluation of *A. arborescens* extract affections. Untreated P3X cells (control; Ctrl), P3X cells treated for 20 h with 1.2 mg/ml of *A. arborescens* epidermis extract (Extr) and P3X cells treated for 20 h with 0.2 mg/ml of the content of a TLC separated spot (Spot E), were seeded in 75 cm³ flasks, approximately 3,000,000 cells/flask in 30 ml of DMEM. Samples were collected by centrifugation at 1000 rpm, supernatants were discarded and the pellets were re-suspended in 100 mM phosphate-buffered saline (PBS) pH 7.4.

Cell suspensions were placed on poly-L-lysine-coated coverslips and incubated at room temperature for 60 min. Excess cell suspension was aspirated and coverslips were rinsed briefly in PBS. Cell-coated coverslips were immersed in ice-cold methanol: acetone (1:1), incubated at -20°C for 10 min and then air dried.

After two washes in PBS, each coverslip was placed on a drop of blocking buffer (1% bovine serum albumin in PBS) for 30 min at 37°C in order to block non-specific binding sites and then washed twice in PBS. Removal of blocking buffer was obtained by holding each coverslip on its edge with forceps and draining it onto a sheet of paper. Then samples were incubated with primary monoclonal antibody to the α -tubulin subunit diluted 1:400 in blocking buffer for 1 h at room temperature using a 60 µl drop of antibody solution for each coverslip. After rinsing three times in PBS, coverslips were dried and incubated with a fluorescein isothiocyanate conjugated anti-mouse IgG secondary antibody diluted 1:100 in blocking buffer. For evaluation of possible unspecific staining, some control samples were prepared omitting the primary antibody. After three washings in PBS, nucleic acid staining was obtained by 5 min incubation with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and then coverslips were mounted on slides with a drop of 5% *n*-propyl gallate in glycerol. The images were captured using a computer-assisted image analysis system, which includes an Axiophot Microscope (Zeiss, Oberkochen, Germany) equipped with a colour video camera (Zeiss) and a software package AxioVision (Zeiss).

2. SEM and TEM

P3X control cells (Ctrl), P3X cells treated for 20 h with 1.2 mg/ml of *A. arborescens* epidermis extract (Extr), and P3X cells treated for 20 h with 0.2 mg/ml of the content of a TLC separated spot (Spot E) were processed for electron microscopy investigations.

Approximately 3,000,000 P3X cells were seeded in 30 ml of DMEM in 75 cm³ flasks. Cells were collected in tubes and centrifuged; the supernatants were discarded and the pellets were fixed with 4% paraformaldehyde and 5% glutaraldehyde, pH 7.2 in 0.1 M cacodylate buffer for 1 h at 4°C (Karnovsky 1963). After rinsing overnight in the same buffer, samples were post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h at 4°C. After two washes in the same buffer, samples were dehydrated in a graded ethanol series.

For SEM, cells were dried by the critical point method using CO₂ in a Balzers Union CPD 020, sputter-coated with gold in a Balzers MED 010 unit, and observed with a JEOL JSM 5200 electron microscope (Jeol Ltd., Tokyo, Japan).

For TEM, samples were fixed and dehydrated as described above and embedded in Epon mixture resin. Thin sections (50-70 nm) were cut with Reichert Ultracut and LKB Nova ultramicrotomes using a diamond knife, collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL 1200 EX II electron microscope (Jeol Ltd.).

Chemical investigations of Spot E

Analytical TLC was performed on silica gel 60 F₂₅₄ pre-coated aluminum sheet (Merck KGaA, Darmstadt, Germany) using AcOEt: EtOH: H₂O (100: 20: 13, v/v) as eluant. Components were detected by UV inspection (254 and 366 nm) and sprayed with 0.5% Fast Blue B salt, followed by 0.1 N NaOH and heated to *ca.* 140°C.

Preparative TLC was carried out on 0.5 mm thick silica gel F254 20 × 20 glass plates (Merck KGaA) using the same eluant as for the analytical analyses. High performance liquid chromatography (HPLC) was carried out on an Amersham Pharmacia Biotech P-900 liquid chromatograph connected to an Amersham Pharmacia Biotech UV-900 detector (GE Healthcare, Buckinghamshire, UK); chromatographic conditions: column, Microcart 254 rp-18, 5 mm; flow rate, 1 mL/min; detector λ 280 nm; mobile phase, linear gradient water-acetonitrile (0 min, 90: 10; 9 min, 90: 10; 55 min, 50: 50). Silica gel 60, 40-63 mm (Merck KGaA) was used for flash chromatography. NMR spectra were acquired in methanol d₄ at 400.133 MHz on a Bruker Advance 400 spectrometer using the XWIN-NMR software package. Chemical shifts were referenced to the solvent signal (δ_H 3.35 ppm from tetramethylsilane (TMS)).

1. Isolation of reference compounds aloenin A (1), aloin A (2) and aloin B (3)

Aloenin A, aloin A and aloin B were from an enriched fraction of Kenya aloe (Duri *et al.* 2004). A pure sample of aloin A was obtained by repeated crystallizations of a commercial sample of amorphous aloin (Merck Millipore) following a previously described procedure (Manitto *et al.* 1990).

2. Identification of aloenin A (1), aloin A (2) and aloin B (3) as the main components of Spot E

An aqueous solution of Spot E (2 ml) was freeze-dried, affording 4 mg of a dark-yellow residue. Due to the paucity of the residue, no separation of the components was attempted; samples were analyzed by HPLC techniques. The residue derived from Spot E was subjected to ¹H NMR analysis.

Confirmation of the identity of the compounds came from direct HPLC comparison of the extract with authentic samples.

RESULTS

Administration of *Aloe* epidermis leaf extract and cell vitality tests (MTT)

The results of the MTT assay after 20 h of treatment with 1.2 mg/ml of *A. arborescens* epidermis leaf extract are reported in Fig. 1. The MTT assay clearly shows that 20 h after the treatment, both untreated cells (Ctrl) had a consistent proliferation activity. Epidermis leaf extract (Extr) significantly affected P3X cell proliferation activity and around 80% of cells died as a consequence of the treatment, $P < 0.0001$.

TLC bioassay-guided fractionation

As reported in Fig. 2, TLC fractionation allowed the *Aloe* epidermis leaf extract to be separated into many fluorescent spots viewed under UV light. We named the spots, respectively, R (the residual that did not migrate during the mobile phase run), A, B, C, D, E, F and G. Each spot was characterized by a specific R_F value.

As reported in the Materials and Methods, to test for the presence of possible biological activities such as the inhibition of murine myeloma cell proliferation, three different areas were considered in the silica gel plate. From the bottom, respectively, the first region consisted of the spots named R, A, B, the second one of the spots named C, D, and the third one consisted of spots E, F, G.

After scraping the areas from the plate, elution with ethanol and substitution of ethanol with water, areas contents were administered to murine myeloma cells and MTT

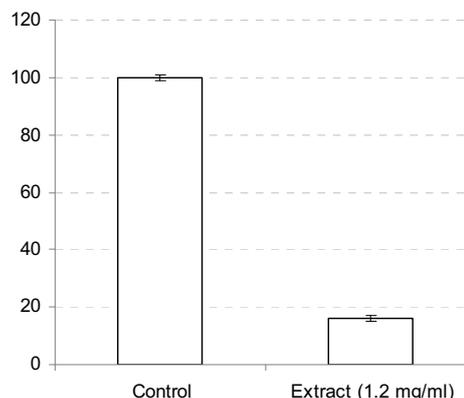


Fig. 1 MTT test for evaluation of inhibition activity of *A. arborescens* leaf extract on P3X cell proliferation. Control, untreated cells; Cells treated with 1.2 mg/ml of *A. arborescens* extract. Y-axis = percentage of living cells. (Test ANOVA; $P < 0.0001$)

| | Name | R_F |
|-----|------|-------|
| III | G | 0.95 |
| | F | 0.92 |
| | E | 0.89 |
| II | D | 0.69 |
| | C | 0.46 |
| I | B | 0.36 |
| | A | 0.28 |
| | R | 0 |

Fig. 2 Thin Layer Chromatography investigation carried out on silica gel plates. *A. arborescens* leaf extract consisted of many spots showing different R_F values. Three different areas were evaluated in the plate.

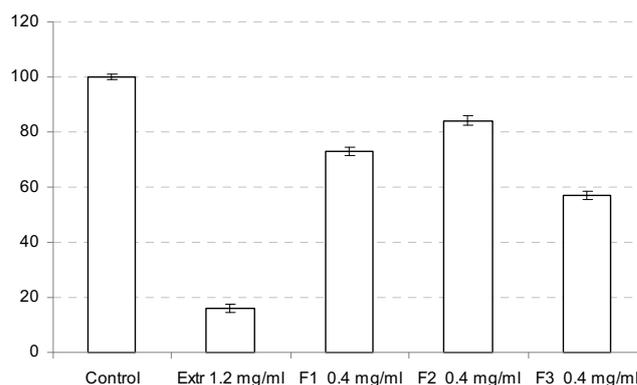


Fig. 3 MTT test for evaluation of inhibition activity of the spot collected from the three different plate areas. F3 area shows higher activity than F1 and F2 areas. Activity of the leaf extract is higher than single areas. Y-axis = percentage of living cells. (Test ANOVA; $P < 0.0001$)

assays were carried out after 20 h of treatment. Results are reported in Fig. 3 and a marked difference between the activity of epidermis leaf extract and the TLC-separated fractions occurred. Among the three TLC-separated fractions, area III had a more consistent activity of inhibition of P3X cell proliferation than other areas, $P < 0.0001$. As reported in Fig. 4, further MTT assays carried out by administering single spot contents (E, F, G) collected from area III showed that Spot E was the more active spot, inhibiting murine myeloma cell proliferation, $P < 0.0001$. In Fig. 5, a com-

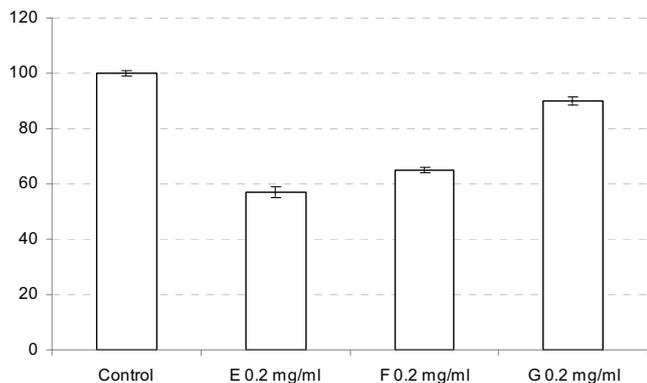


Fig. 4 MTT test for evaluation of inhibition activity of the spots collected from F3 plate area. Spot E shows higher activity respect to the other spots. Activity of the leaf extract is higher than spot E. Y-axis = percentage of living cells. (Test ANOVA; $P < 0.0001$)

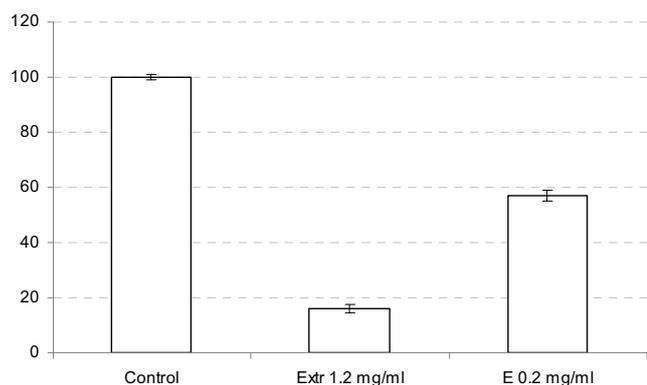


Fig. 5 MTT test for comparison of inhibition activity of the same volume of leaf extract and spot E. Activity of leaf extracts is higher than spot E. Y-axis = percentage of living cells. (Test ANOVA; $P < 0.0001$)

parison of the activity on P3X cells of epidermis leaf extract with respect to that exerted by Spot E is reported: the activity of Spot E was clearly lower, $P < 0.0001$.

Immunofluorescence investigations

An immunofluorescence study carried out by fluorescence microscopy using an antibody against the α -tubulin subunit allowed the microtubular array in untreated P3X murine myeloma cells (Ctrl) and after treatments with epidermis leaf extract to be viewed. In addition, in all samples nuclear material was investigated by DAPI. As reported in **Fig. 6A**, untreated cells clearly revealed the presence of a microtubule organizing centre from which microtubular elements originated and were distributed in the cytoplasm; as shown by DAPI staining (**Fig. 6B**), DNA was precisely localized in a defined zone of the cytoplasm. Merging MTs and DAPI staining (**Fig. 6C**) allowed a clearer view of staining specificity.

As reported in **Fig. 6D**, many of the cells treated with 1.2 mg/ml of *A. arborescens* extract for 20 h showed the disappearance of the microtubule organization centre and the presence of a few short microtubules together with a diffuse fluorescence staining, probably due to the presence of unpolymerized tubulin in the cytoplasm. DAPI staining (**Fig. 6E**) was more diffused with respect to control cells; merging images of MTs and DAPI staining (**Fig. 6F**) allowed for a clearer view of staining specificity.

Similar results were observed by treating P3X cells with Spot E for 20 h, although in this case the microtubular apparatus still consisted of microtubules and in some cases microtubules organizing centers (MTOCs) were clearly visible (**Fig. 6G**). DAPI staining was less diffused (**Fig. 6H**) and merging images of MTs and DAPI staining (**Fig. 6I**) allowed for a more lucid view of staining specificity.

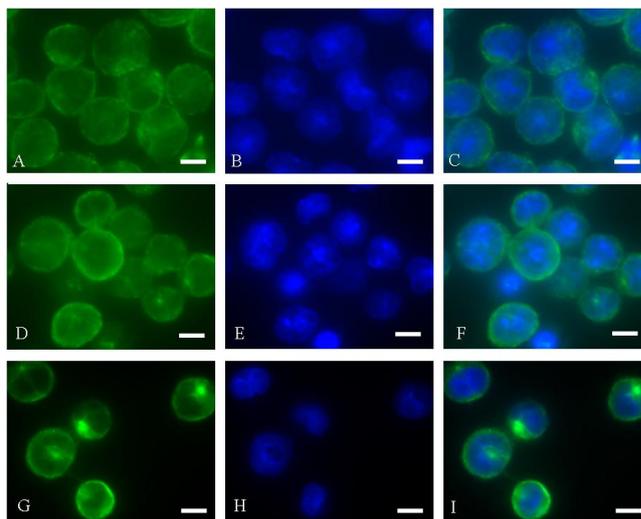


Fig. 6 Immunofluorescence investigations using an antibody to the α tubulin subunit and DAPI staining. (A-C) Ctrl; (D-F) cells treated with *A. arborescens* extract; (G-I) cells treated with Spot E. Bars = 10 μ m.

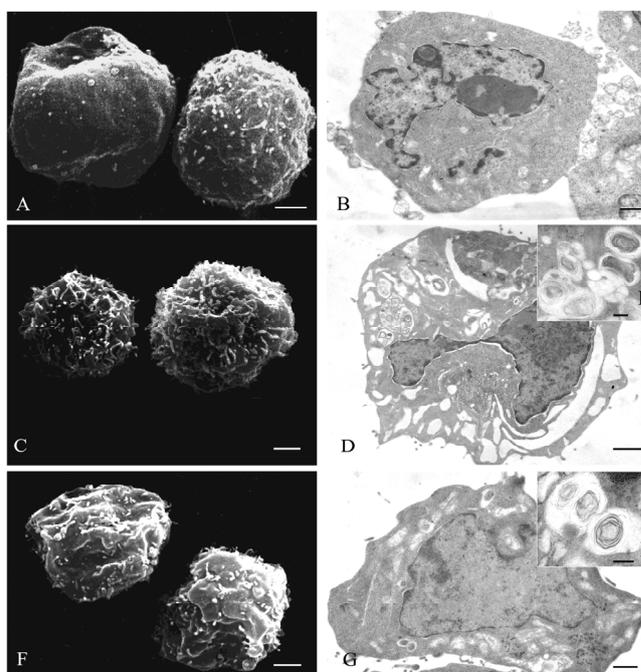


Fig. 7 Electron microscopy investigations. (A, B) SEM and TEM images of untreated cells; (C, D) SEM and TEM images of cells treated with *A. arborescens* extract; (E-G) SEM and TEM images of cells treated with Spot E. Bars = 5 μ m (A, C, F); 1 μ m (B, D, G); 200 nm (E, H).

Electron microscopy investigations

1. Control

Murine myeloma cells, when observed by SEM, showed a spherical shape 8-10 μ m in size; short filamentous structures were observed on the cell surface (**Fig. 7A**). Investigations carried out by TEM (**Fig. 7B**) showed a regular cell surface of P3X myeloma cells, organelles and vacuoles in the cytoplasm, and a characteristically large nucleus containing disaggregated chromatin.

2. Epidermis leaf extract

After treatment with epidermis leaf extract, murine myeloma cells viewed by SEM microscopy (**Fig. 7C**) maintained their spherical shape; however, the treatment affected cell size and morphology since a reduction in cell size with

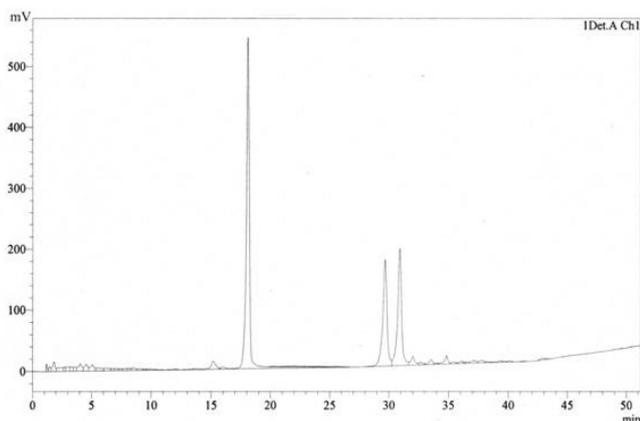


Fig. 8 HPLC separation of the Spot E content. Three major peaks are well separated.

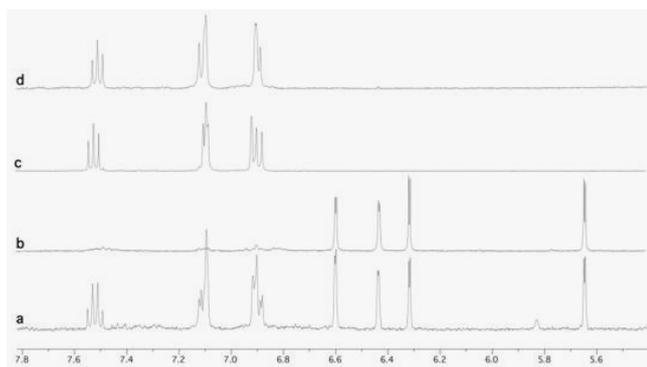


Fig. 9 Superimposition of the 5.5-7.8 ppm region of ^1H NMR spectra showing the signals of aromatic protons of Spot E (A); aloenin A (B); aloin A (C) and aloin B (D). Spectra recorded at 400 MHz in CD_3OD .

respect to control cells occurred and many lobes were present on the cell surface. In addition, many filamentous structures were observed on the cell surface. Investigations carried out by TEM (Fig. 7D) showed an irregular cell surface and evidenced numerous membranous inclusions, thus suggesting degenerative processes within the cytoplasm; furthermore, large vacuoles containing membranous structures were present (Fig. 7E). A large nucleus containing disaggregated chromatin was observed.

3. Spot E

When investigated by SEM, murine myeloma cells treated with Spot E showed a reduction in cell size relative to control cells and the presence of lobes and filamentous structures on the cell surface (Fig. 7F). TEM showed the cell surface to appear more regular than the extract leaf-treated cells (Fig. 7G). In the cytoplasm large vacuoles containing membranous structures were present (Fig. 7H) and a large nucleus containing disaggregated chromatin was observed.

Identification of aloenin A, aloin A and aloin B as main components of Spot E

Removal of the solvent from a solution of Spot E afforded 4 mg of a dark-yellow, solid residue. HPLC analysis of the residue revealed that Spot E consisted of three compounds with retention times corresponding to that of aloenin A (1), aloin A (2) and aloin B (3) (Fig. 8), also isolated from Kenya *Aloe* (Duri *et al.* 2004). Analytical TLC comparison of the *A. arborescens* total leaf extract with fractions from Kenya *Aloe* enriched in these compounds (data not shown) led to the hypothesis that aloenin A (1) was one of the major components of the extract and that it was present also in Spot E. In addition, due to the small difference in RF between aloenin A (1) and the aloins A (2) and B (3) on the

TLC plate, it was supposed that Spot E contained a mixture of these three compounds. Therefore, authentic samples of each were obtained by known procedures (Manitto *et al.* 1990; Duri *et al.* 2004) and were used as reference compounds.

An ^1H NMR spectrum of the Spot E mixture showed two sets of signals, suggesting that two out of the three components should be very similar, thus confirming our hypothesis. Signals were indeed super-imposable with those of the reference compounds (Fig. 9). Moreover, by integration of the signals in the ^1H NMR spectrum, it was possible to establish that the three species were present in approximately a 4: 1: 1 molar ratio. Direct HPLC comparison of Spot E with the reference compounds confirmed finally that aloenin A and aloins A and B constituted the so-called Spot E.

DISCUSSION

From ancient times a relation between plant molecules and pharmaceuticals has been reported (Triggiani *et al.* 2006). In more recent times, in order to evaluate new molecules as possible active principles for pharmaceuticals, research activities have been carried out in the field of natural substances.

Aloe plants have been used for humans for their medicinal properties and recently, as a consequence of an intense research activity, many *Aloe* pharmaceutical products are present on the market. We already provided some contributions and in our previous investigations (Rondini *et al.* 2000; Bedini *et al.* 2009), *A. arborescens* epidermis leaf extracts were shown to affect the proliferation of murine myeloma cells thus suggesting the presence of chemical compounds playing such a role. In light of this, we planned further investigations in order to provide new results both at the biological and chemical level.

MTT assays (Fig. 1) confirmed that *A. arborescens* is a potential source of cytotoxic compounds for P3X murine myeloma cell line, since within the extract, more molecules, alone or in synergy, could be responsible of cell death. In order to evaluate if such an activity could be related to specific molecules, we carried out TLC investigations to separate molecule groups (spots) and after MTT tests we were able to confirm Spot E as consisting of P3X cell affecting molecules. As shown by our results (Figs. 4, 5), the cell affection activity of Spot E was lower than the cell affection activity of epidermis leaf extract. Presently we can only speculate about the possibility that such antiproliferative activity could also be dependent on other molecules present in the epidermis leaf extracts that may have been lost during the separation procedures; to confirm this, some experiments are currently under way.

In order to evaluate affection modalities at the cytological level, we carried out investigations administrating both leaf extract and the isolated Spot E to murine myeloma cells. In both cases, after treatment many cells died although, as mentioned before, in a different ratio. This finding could provide a clue about the possible molecular mechanisms of drug resistance occurring in surviving treated cells. In comparison to control cells (untreated cells), as shown by immunofluorescence techniques using a monoclonal antibody to the α -tubulin subunit (Fig. 6), leaf extract treatment affected the microtubular cytoskeleton of P3X cells inducing (i) modification of the organization and distribution of microtubules, (ii) disruption of the centre of origin of microtubules (the MTOC), and (iii) the presence of diffuse fluorescent staining in the cytoplasm probably related to the presence of free unpolymerised tubulin molecules. Spot E treated cells were less affected being MTOC still visible. Additional information emerged from both SEM and TEM. After leaf extract treatment, P3X cells showed a reduced cell diameter and the presence of lobes and filamentous structures on the cell surface; in addition, within the cytoplasm large vacuoles containing membranous structures occurred. Spot E treated cells showed a

more regular surface and a minor number of vacuoles containing membranous structures.

Therefore, taken together immunofluorescence (Fig. 6) and electron microscopy (Fig. 7) investigations showed a different level of P3X affection after leaf extract or Spot E treatments. Our observations do not fully contribute to identify the cellular target of both leaf extract and the molecules making up Spot E.

Chemical investigations carried out by chromatographic and spectroscopic methods revealed that Spot E specifically consisted of three components: a phenylpyrone derivative and two C-glucosylanthrones: aloenin A (1), aloin A (2) and aloin B (3), respectively. Such molecules, identified in the TLC Spot E affecting murine myeloma cells, have been already investigated in other cell models. Treatment of Jurkat cells with aloin (Buenz 2007) resulted in a reduction in cell size, compromised membrane integrity, and loss of mitochondrial membrane potential in a dose-dependent manner. Additionally, treatment with aloin resulted in alteration of the cell cycle, specifically a block at the G2/M phase. Importantly, the loss of cell membrane integrity was preceded by a loss of mitochondrial membrane potential, suggesting a mitochondrial-dependent pathway for aloin-induced apoptosis.

Antioxidant effects of aloin on DNA has been also reported (Tian and Hua 2004), whereas aloin A, aloin B and its derivative aloemodin are reported to have anti-inflammatory (Yamamoto *et al.* 1991; Park *et al.* 2009) and antitumor activities inducing apoptosis (Lee *et al.* 2001; Shimpo *et al.* 2003), and increasing antioxidant enzymes such as SOD and GPX (El-Shemy *et al.* 2010).

Together with all these findings, our observations raise new questions for additional investigations on possible cellular target(s) in mammalian cell models and to the modalities of induction of P3X cell death.

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