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Evaluation of the Anti-Tumoural and Immune Modulatory Activity of Natural Products by Flow Cytometry

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1. Introduction

Flow cytometry has shown to be a very useful tool in the assessment of plant-derived compounds, having potential anti-tumour activity. Our group has focused in the study of fractions and isolated compounds with biological activity over tumour cells and regulatory immune cells, such as dendritic cells. Also we have implemented a flow cytometry screening tests for estimating the biological activity of plant-derived fractions.

The present chapter will discuss important and useful facts to be taken into account for effectively carrying out biological assays in this area. We will start with recommendations about the production of plant fractions and continue with the biological assessment of cell lines or monocyte-derived dendritic cells. Since these protocols are constantly evolving, herein we describe our current experience.

2. Natural product specifications

Natural product specifications will differ depending upon the type of plant material in which the study is to be undertaken: complex fractions or isolated compounds. Similarly, settings will differ depending upon the biological target assigned for the assay e.g. primary tumour cells, tumour cell lines, dendritic cells or antigen presenting cells. Our group has developed plant complex fractions enriched in secondary metabolites, using conventional organic solvent fractionation methods, and primary metabolites-enriched fractions using fractionation of aqueous solutions. Both types of fractions contain a vast variety of compounds, as well as structure diversity.

Our group has been working with fractions enriched in secondary metabolites from *Petiveria alliacea* and *Caesalpinia spinosa* plants. The chemical complexity of the fractions is analysed by dereplication, by comparing the molecular weights of the compounds within the fraction with the molecular weights of compounds known to be present in the plant under study. Among the difficulties we have run into is the autofluorescence display by compounds

present in our fractions, as well as in many other natural products, a condition that interferes with flow cytometry lectures. The autofluorescence displayed by many secondary metabolites, such as flavonoids, covers most of the visible spectrum overlapping the emission range of many flow cytometry fluorochromes. We have overcome such interference by making fraction dilutions and estimating fluorescence values at the overlapping wavelengths. Those values are used as background in the flow cytometry estimations.

Another critical condition to be aware of is the fraction sterility condition with regard to cell culture contamination hazards. The presence of microorganisms in the plant material, such as viruses, bacteria or fungi, must be controlled. Chemical agents such as benzyl chloride, lauryl dimethyl ammonium (benzalkonium chloride) in concentrations between 50 and 100 ppm could be used to prevent the microorganisms' occurrence. Similarly, treatment with short wave ultraviolet radiation (UVC) within the range of 200-280 nm can reduce microbial load. In the case of stock solutions, membrane filtration (0.22 microns) is suggested, taking the precaution to previously check membrane solvent compatibility. The use of high-pressure pasteurisation (600mPa, 20°C) has also been proposed to attain microorganism safe plant material. Chen et al, established that high-pressure pasteurisation for 2 to 5 minutes in *Echinacea purpurea* plant reduced microbial load without affecting biological activity. In addition, the presence of lipopolysaccharide (LPS) in plant-derived fractions must be avoided, since LPS is a major membrane component of gram-negative bacteria. LPS is recognised by macrophages and endothelial cells through CD14-TLR4 (toll like receptor) - MD2 complex, triggering cytokine secretion and expression of co-stimulatory molecules. LPS may appear in the plant material from pathogenic bacteria (*Pseudomonas*, *Xanthomonas*, Burkholderia and Agrobacterium), or beneficial symbiotic Rhizobiaceae (*Pseudomonas fluorescens*).

To estimate LPS presence in the fractions, a limulus amoebocyte lysate (LAL) test is commonly used, although detection of 3 β -hydroxymyristate (a LPS component) can also be used. For LPS removal, an anti-endotoxin synthetic peptide -SAEP- cross-linked with polymyxin B (POLB), can be used to neutralise it. In relation to the fractions eluents, especially in non-polar fractions, it is advisable to use as first choice ethanol then secondly dimethyl sulfoxide (DMSO) for the stock solution preparation. Then proceed to dilute the fraction or isolated compound until the ethanol concentration in the cell culture does not exceed 0,02%.

3. Instrument setup and colour compensation

To ensure data analysis quality in multicolour flow cytometry, daily adjustments are necessary to acquire standard intensity peaks and build up adequate follow-up curves. Currently, there are several quality controls commercially available that can be used to monitor reagents' performance, reproducibility, cell preparation and cell staining. Among the most commonly used are the beads of known concentration and a stable number of fluorochromes per bead (> 6-colour). Also they control parameters such as laser alignment, optics, fluidics, mean fluorescence intensity (MFI) (each bead in solution produces equal amounts of fluorescence), fluorescence signal coefficient variation, autofluorescence threshold and colour compensation. Standardisation of cytometer measurements is essential to accurately make comparisons between experiments.

On the other hand, biological samples (including normal peripheral blood) should be stained with the fluorochrome of interest to compensate the cytometer. The positive cell population must be set as the brightest signal for each fluorochrome. As a negative control, cells without antibody and marked with an irrelevant antibody or isotype control can be used. For tandem fluorochromes, it is important to compensate separately each reagent containing a tandem fluorochrome, because spectral emission properties can vary from lot to lot, between manufacturers and over time. Once the flow cytometer is adjusted and the immunophenotypic panels have been selected, it is essential to optimise and evaluate each reagent in terms of fluorescence intensity to exclude unwanted interactions between them. It is highly recommended to use normal peripheral blood as a sample control since positive and negative cell populations are needed to test the antibody under study.

Using different fluorochromes at the same phenotypic panel can produce fluorescence spectral overlapping. In consequence the emitted light of one fluorochrome may appear in the other detector. To correct overlapping, a compensation protocol could be used. Herein, is the basic protocol that can be used for compensation adjustment using BD™ CompBeads set anti-mouse Igκ (binds mouse Kappa light chain-bearing immunoglobulin) and the BD™ CompBeads negative control (no binding capacity). Before starting the experiment it is necessary to prepare tubes with each conjugate antibody to compensate fluorescence. Briefly, pretitrated monoclonal antibody (MAb) amounts are added to stain buffer aliquots (e.g. phosphate-buffered saline, pH 7.6), the CompBeads anti-mouse Igκ and the CompBeads negative control. In this example: fluorochrome conjugated-fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein-cyanin 5.5 (PerCPCy5.5)/allophycocyanin (APC)/APCCy7- combinations of MAb are used. In addition, a tube containing the CompBeads negative control is used to adjust the photomultiplier voltage settings. After gentle mixing, the different bead aliquots are incubated for 30 min at room temperature (RT) under dark conditions. Then, the beads are washed with 2 mL of staining buffer/aliquot (10 min at 200 g). Immediately after the sample preparation is complete, data acquisition is performed on a FACS Canto II flow cytometer using the FACS DIVA software programme (BD Biosciences). For each MAb combination with CompBeads, 5×10^3 events are acquired and stored. The flow cytometer compensation strategy employed is shown in Figures 1, 2 and 3.

4. Cell lines selection

Biological activity of plant complex fractions or isolated compounds is selective; their effect depends upon the nature of the cell line used at the assay. Our research interest has mostly been on compounds with activity on breast cancer cells, e.g. human breast adenocarcinoma (MCF7) from the American Type Culture Collection (ATCC, Rockville, MD) and mammary murine adenoma carcinoma (4T1), kindly donated by Dr. Alexander Asea, Texas A & M University. Similarly, we have used human melanoma cells (A375) kindly donated by La Universidad del Rosario (Bogotá, Colombia) and murine melanoma (Mel-Rel) donated by Armelle Prevost (Institute Curie, France). We have also used chronic myeloid leukaemia (HL60), human promyelocytic K562 cell lines from the ATCC and finally pancreatic cancer cells (Panc28 and L.3.6PL) donated by Dr. Steven Safe from Texas A & M University as biological agents. In addition we have some others cell lines, obtained from ATCC.

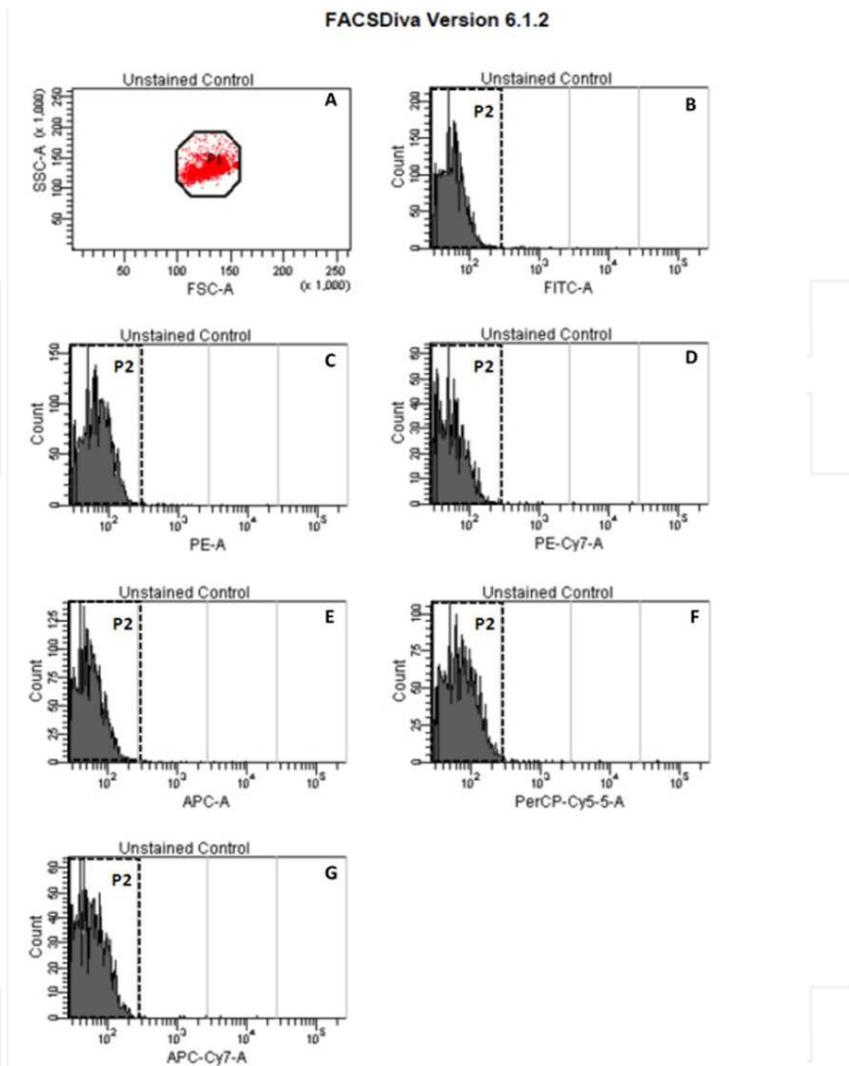


Fig. 1. Dot plot histograms for FACS Canto II cytometer compensation using the commercial BD™ CompBeads negative control (uncoupled with anti-mouse IgG) for voltage adjustment (parameters: Forward Scatter -FSC- and Side-Scatter-SSC- (panel A) and fluorescence detectors (FITC, PE, PERCPY5.5, APC, APCCy7 and PECY7) (panels B-G). Histogram represents the negative value area for each parameter. The beads are gated on FSC and SSC (red dots in P1 region). Acquisition is done only in the P1 region verifying histograms for each fluorochrome and simultaneously adjusting the voltages for each fluorescent channel, so that the peak areas are located in the negative area (region P2 in each histogram). The adjusted minimum voltages in the photomultipliers are used for the BD™ CompBeads set anti-mouse IgG and the BD™ CompBeads negative control acquisition mixture. Y axes units correspond to relative fluorescence intensity

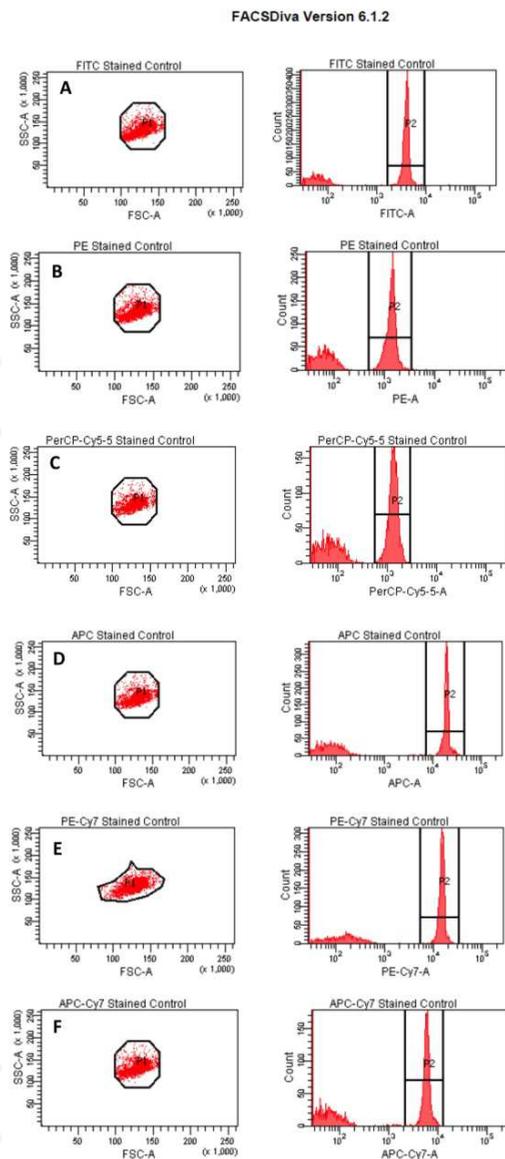


Fig. 2. Dot plot histograms for FACS Canto II cytometer compensation using BD™ CompBeads set anti-mouse Igκ and the BD™ CompBeads negative control commercial mixture. After P1 region acquisition (containing the spheres mixture), histograms have two fluorescence emission peaks, a negative (no fluorescence emission) and a positive (fluorescence emission; P2 region) (panels A –F). The cytometer automatically discriminates positive and negative peaks and calculates the compensation for each and between fluorochromes. The compensation values are stored in the software for future experiments containing the same fluorochrome

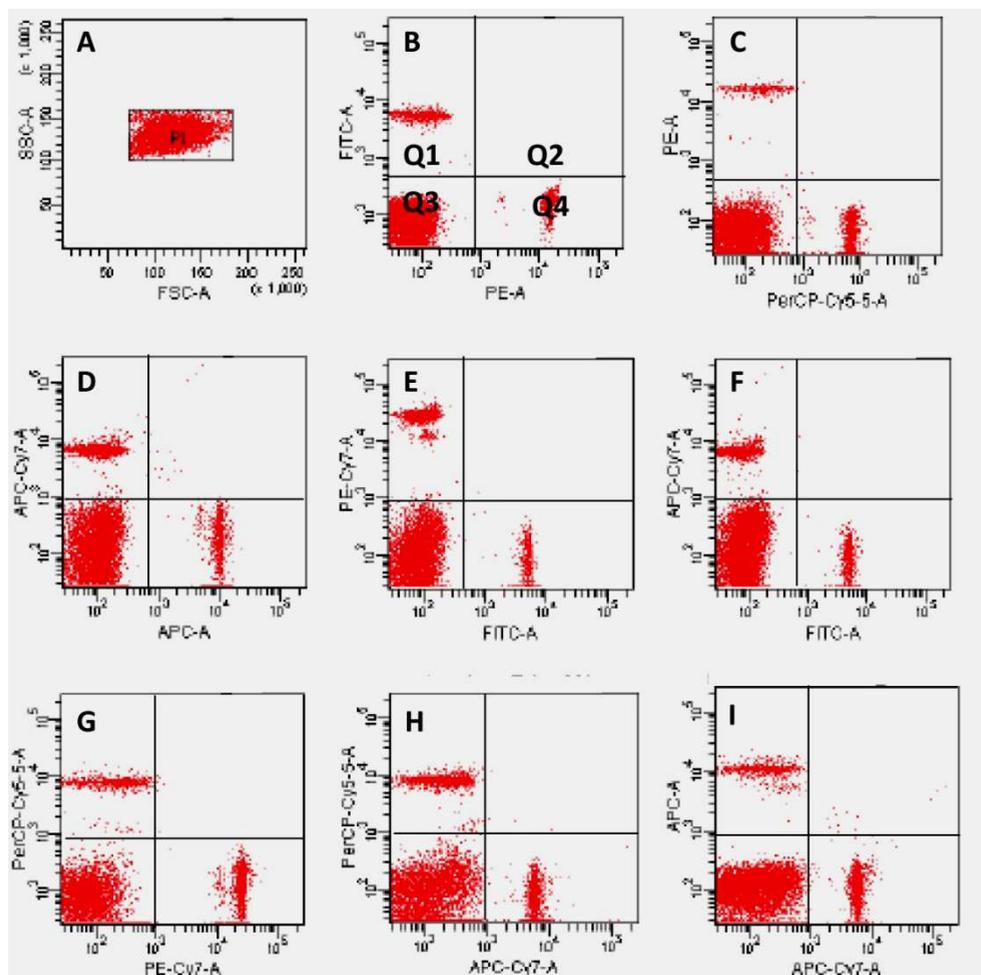


Fig. 3. Optionally in a third step, instrument compensation could be checked by acquiring the bead mixture attached to each antibody conjugate and using the same compensation values calculated by the software as described in figure 2. Creating different two-dimensional dot plots with each fluorochrome on the x-axis and y-axis respectively and verifying that there is no fluorescence overlapping (panels B-I) (e. g. FITC vs PE, PE vs PercPC y 5.5, etc). If the fluorochrome signal is uncompensated, voltage manual adjustment is required

Cells are cultured under humidified conditions with 5% CO_2 in RPMI 1640 media, supplemented with 10% foetal bovine serum (FBS), 0.01 M Hepses, 100 $\mu\text{g}/\text{ml}$ penicillin, 100 U/ml streptomycin, 2 mM glutamine and 0.01 M of sodium pyruvate (Eurobio, France). It is important to determine if the fraction activity displays selectivity on highly proliferating cells, or to the contrary, if its activity can also affect normal cells. Lymphocytes derived from human peripheral blood, separated with Ficoll-Hypaque, or human gingival mucosa fibroblasts can be used as normal cell samples.

4.1 Mononuclear cell isolation by Ficoll-Hypaque gradient

Mononuclear cells can be separated from heparin, EDTA, sodium citrate or defibrinated anti-coagulated blood. Equal parts of phosphate buffered saline (PBS) are used to dilute the blood sample at RT. In a 15 ml conical tube, 3 ml of ficoll are carefully placed and 4 ml of diluted blood are placed on top, avoiding damage to the gradient (the sample must be poured slowly along the tube walls). The tube is centrifuged ($300 \times g$) for 30-40 minutes at RT. After centrifugation, four layers should appear in the tube. Granulocytes and erythrocytes are the cells found at the bottom of the tube, next is the ficoll hypaque layer, monocytes, lymphocytes and platelets are at the upper intermediate phase, and finally, at the top layer is the plasma. The layer containing the lymphocytes has to be carefully removed, transferred into a new tube, diluted with three parts of PBS (6 ml) and centrifuged ($100 \times g$) for 10 min at RT. The latter washing procedure should be done twice and at the end cells must be suspended in supplemented culture medium.

4.2 Gingival fibroblast generation

Gingival fibroblasts are obtained from the gingival tissue of healthy volunteers. Briefly, a gingival sample is cut into small pieces and cultured in RPMI 1640 supplemented media with 10% FBS, 0.01 M Hepes, 100 $\mu\text{g/ml}$ penicillin, 100 U/ml streptomycin, 2 mM glutamine and 0.01 M sodium pyruvate (Eurobio, France) under humidified conditions and 5% CO_2 . The culture medium is replaced every three days until the fibroblasts are derived from the tissue. After two weeks of culture, a confluent fibroblast culture is obtained.

5. Determination of fraction concentration and anti-tumour bioassays

5.1 Fraction's cytotoxic activity on tumour cells

The fraction's cytotoxic effect is evaluated by trypan blue and MTT assays (Sigma, St. Louis MO, USA) using tumour and normal cell cultures. Suspension or adherent tumour cells (5×10^3 cells/well or 3×10^3 cells/well, respectively) and fibroblasts (3×10^3 cells/well) are cultured in 96-well plates at different plant fraction concentrations (from 125 to 0.975 $\mu\text{g/ml}$) in ethanol (0.02% final concentration). Aqueous ethanol solution is used as a negative control and doxorubicin, etoposide, vincristine, taxol and camptothecin (10 μM) as positive controls, during 48 h at 37°C . Peripheral blood mononuclear cells (2×10^5 cells/well) are incubated for 12 h with phytohemagglutinin (Invitrogen Corp, Grand Island, NY, USA) before treatment. At the end of the incubation time, the culture media of the tumour and normal cells is removed and replaced with new RPMI 1640 media lacking phenol red dye (Eurobio, Toulouse, FR). 50 μl of MTT (1 mg/ml) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, St. Louis MO, USA), is placed in each well and incubated for 4 h at 37°C . The formazan crystals are dissolved with DMSO and their optical density at 540 nm is measured in a MultiskanMCC/340 (Labsystems, Thermo Fisher Scientific, Waltham, USA). Probit analysis is used to calculate the inhibitory concentration 50 (IC50) (MINITAB® Release14.1 Statistical Software Minitab Inc. 2003). In addition, cell viability is determined using Newbauer haemocytometer and trypan blue dye.

For these types of procedures the preparation of negative controls is extremely important. Since some plant fractions can reduce MTT reagent, a negative control containing plant fraction, culture media and MTT without cells, should be prepared.

5.2 Tumour cells' mitochondrial membrane potential assessment

For many years mitochondria was considered only as the cell energy source, generating ATP through oxidative phosphorylation and lipid oxidation. Now, is clear that this organelle has a pivotal role in apoptosis, a form of cell death characterised by morphological and biochemical specific changes. The mitochondrial membrane potential ($\Delta\Psi$), addresses the oxidative phosphorylation process and mitochondrial calcium uptake. If the electron transport ceases, as happens in ischemia, the inner mitochondrial membrane potential is developed at the expense of ATP hydrolysis. A decrease in $\Delta\Psi$ is often considered as an early apoptotic signal, an event that can be detected by flow cytometry. Nonetheless, the relationship between the mitochondrial membrane depolarisation and apoptosis is still controversial. Some researchers even consider a decrease in $\Delta\Psi$ an irreversible sign of apoptosis, while others say it is an apoptotic late event.

Changes in mitochondrial membrane potential can be assessed using a lipophilic cationic probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). The probe potential depends on its mitochondrial accumulation, which is indicated by a change in green fluorescence (525nm) to red (590 nm) emission. The mitochondrial membrane depolarisation is indicated by a decrease in fluorescence intensity through the red/green ratio. The change in colour is due to the "red fluorescent J aggregates" accumulation which in turn depends on the concentration. One advantage of JC-1 dye is the possibility to be used in a wide variety of cell types, including myocytes, neurons and tumour cells, among others.

For optimal results tumour cell concentration in the culture should be one in which confluence is reached only after incubation for 48 hours. Usually, 2.5×10^5 cells/well are placed in 12-well plates. Adherent cells should be allowed to adhere before the treatment begins. Cells are tested with different plant fraction concentrations, using ethanol aqueous solution as a negative control and valinomycin as a positive inductor of membrane

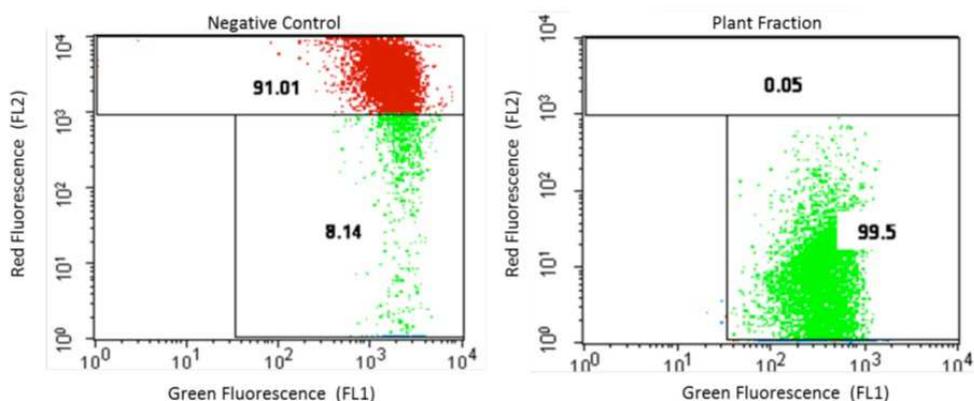


Fig. 4. *C. spinosa*-derived fraction induces mitochondrial membrane depolarisation. K562 cells treated with the fraction or the negative control. The left panel shows the eluent effect (ethanol), the right panel shows the fraction effect on the cell mitochondrial membrane depolarisation

depolarisation. The assay is carried out in kinetics of 4, 8, 12, 24 and/or 48 h. At the end of the treatment, cells are collected in cytometry tubes and JC-1 dye (2.5 mg/ml in PBS) is added for 10 minutes at 37°C. JC-1 fluorescence intensity is quantified in a flow cytometer FACSDiVa (Becton Dickinson, New Jersey, USA) and data analysis is performed using FlowJo software (Tree Star Inc., Ashland, USA). All treatments are done in triplicate and results are expressed as mean +/- SEM. Figure 4.

5.3 Analysis of apoptosis vs necrosis - annexin V/PI staining

There are two main cell death mechanisms: apoptosis or programmed cell death and necrosis or trauma cell death. Both types of cell death have different morphological and biochemical characteristics. Changes as phosphatidylserine (PS) externalisation, chromatin and nuclear condensation, appearance of apoptotic bodies, among others, can be seen in apoptotic cells. Necrotic cells show nuclear swelling, chromatin flocculation, loss of nuclear basophilia, breakdown of the cytoplasmic structure, organelle function and swelling cytolysis.

Plasma membrane phospholipids display asymmetrical distribution. Most of the phosphatidylcholine and sphingomyelin are mainly located at the outer side, while PS is mostly located at the inner side of the plasma membrane. During apoptotic cell death, phospholipid asymmetry is disturbed and PS begins to expose at the membrane outer side. Annexin V is an anticoagulant protein with the property of binding PS with high affinity; in conjugation with fluorochromes it is used to detect apoptotic cells by flow cytometry. Since apoptotic cells begin to react with annexin V before the plasma membrane loses the ability to exclude cationic dyes as propidium iodide (PI), marking the cells with a combination of annexin V-FITC/PI, allows us to distinguish four populations: non-apoptotic cells (annexin V-FITC negative/PI negative), early apoptotic cells (annexin V-FITC positive/PI negative), late apoptotic cells (annexin V-FITC positive/PI positive) and necrotic cells (annexin V-FITC negative/PI positive).

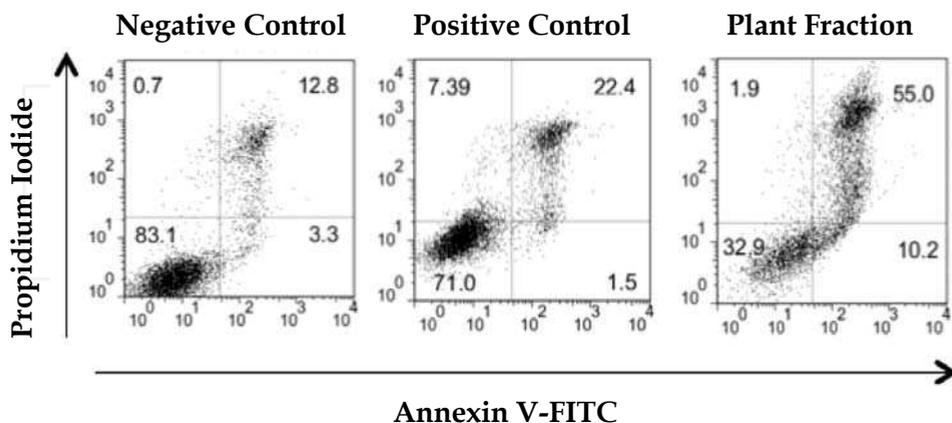


Fig. 5. 4T1 cells marked with annexin V and PI after treatment with *C. spinosa* fraction. Ethanol solution is used as a negative control and doxorubicin as a positive control

Tumour cells (3×10^5) are treated with plant fractions at different concentrations, or doxorubicin (positive control) or ethanol solutions (negative control), for 24 or 48 h. After the treatment, cells are suspended in an annexin V buffer (Hepes 100 mM, NaCl 140 mM, CaCl_2 2.5mM), incubated with annexin V-Alexa fluor 488 for 8 min at 37°C and then incubated with IP for 2 min at 37°C . Assays are made in triplicate. Cells are acquired in a FACSAria I (Becton Dickinson, New Jersey, USA) and FlowJo (Tree Star Inc, Ashland, USA) software for analysis (Figure 5).

5.4 Cell cycle analysis

Cell division is an evolutionarily conserved process involving series of molecular events. The mammalian cell cycle has five phases: three gaps, a G₀ phase where the quiescent or resting cells are; G₁ and G₂ phases where RNA and protein synthesis takes place, the S phase is where DNA replicates and the M phase is where mitosis and cytokinesis happens. G₀, G₁, S and G₂ are collectively known as interphase. Cell cycle control is a fundamental cell process. The progression from G₁ to S to G₂ and mitosis is coordinated at checkpoints. Before mitosis begins, the cell must check if the DNA is fully replicated and free of damage. The spindle mitotic control stops the cell cycle if the spindle is not properly formed or if the chromosomes are not properly attached. DNA aneuploidy or mutations are the result if the cell avoids these controls. A gap in any of the control sites allows the cell to repair DNA and enter mitosis, otherwise it goes into apoptosis. In cancer, the control repair of the cell cycle is damaged and this is precisely where new drugs based on natural products or their derivatives can achieve a stop in tumour cell division and perhaps induced apoptosis.

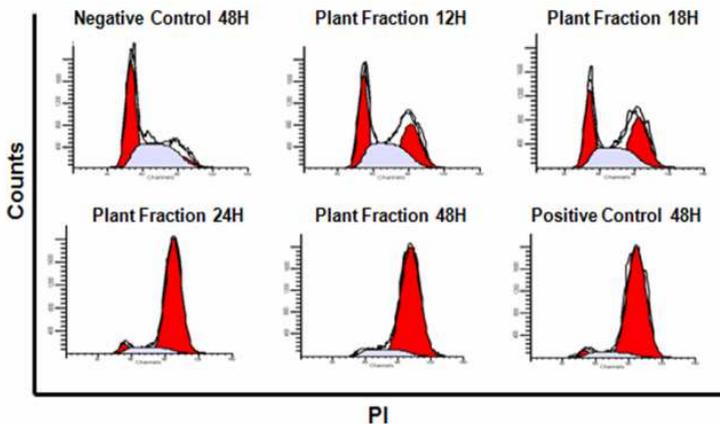


Fig. 6. Effect of a *P alliacea*-derived fraction on cell cycle distribution. A375 cells treated with plant fractions during 12, 18, 24 and 48 h. Ethanol is used as a negative control and vincristine as a positive control

A common and simple way to evaluate cell cycle phases is to measure the DNA cell content. DNA cell content allows the discrimination between cell populations in G₀/G₁, S and G₂/M cell cycle phases. The DNA can be labelled with fluorescent dyes and cell fluorescence can be measured by flow cytometry. Dyes such as PI, Hoechst, 7-AAD and DAPI can be used for this purpose. To start, cells must be synchronised in the G₁ phase, a

process that can be done by depriving the cells of serum. Mel Rel, A375 and K562 cells are deprived of FBS for 72 h are seeded in 12-well plate (4×10^5 cells/well) and treated with different plant fraction concentrations or vincristine (positive control) or ethanol (negative control) for 12, 18, 24 and 48 h under humidified conditions 37°C and 5% CO_2 . Next cells are washed and fixed with ethanol (70% ice-cold) for 18 h, then suspended in PBS 1X with 100 U/ml RNase A, 50 $\mu\text{g}/\text{ml}$ PI (Sigma, St Louis, MO, USA) at RT for 30 min. DNA cell content is measured by flow cytometry using a FACSAria I (Becton Dickinson, New Jersey, USA). The cell cycle distribution percentages are calculated by FlowJo (Tree Star Inc., Ashland, USA) and ModFit LT software. Treatments are done in triplicate (Figure 6).

6. Immunomodulatory evaluation on human-derived dendritic cells

Natural products are good immunostimulating agents and can be evaluated using human monocyte-derived dendritic cells. Although the model cannot be used as a screening test, once standardised, it allows a large number of compounds to be tested in a more physiological way. The test measures the plant fractions real activity on normal human cells. Following we will describe the procedures carried out in our laboratory which includes dendritic cell separation and flow cytometry measurements of the biological activity of plant-derived fractions.

6.1 Monocyte-derived dendritic cell differentiation

Peripheral blood monocytes are attained from concentrated packs (15 ml) of leukocytes from healthy volunteers attending the blood bank and who previously gave informed consent. The mononuclear cells are separated from the peripheral blood using the Ficoll Hypaque density gradient (Amercham, GE Healthcare Europe GmbH). $\text{CD}14^+$ population cells are separated with a MiniMACS positive selection kit, using the protocol suggested by the manufacturer, without any modifications (Miltenyi Biotech GmbH, Bergisch, Gladbach, United Kingdom). Cell purity is determined by flow cytometry. Eluted cells (10 μl) are labelled with anti- $\text{CD}14\text{-APC}$ (Pharmingen, San Diego, CA, USA). Monocytes with purity over 98% are grown for five days in 24-well plates in RPMI 1640 (1 ml) supplemented with 5% FBS, 2 mM glutamine and 100 IU/ml penicillin/streptomycin (Eurobio, Paris, France). The differentiation stimulus used is 800 UI/ml of Granulocyte-Macrophage colony-stimulating factor and 1000 IU/ml of interleukin 4 (R & D Systems, Minneapolis, MN, USA). After three days of differentiation, one half of the media culture is replaced with fresh medium and supplemented with half of the stated cytokines concentration.

At day five, monocyte-derived dendritic cells (MDDC) are stimulated with different concentrations of the plant fractions dissolved in ethanol, DMSO or culture medium, depending upon the fraction solubility, for 48 h. Control DCs (immature) are incubated without stimulus or with the plant fractions eluents. As maturation control, DCs are stimulated with LPS $1\mu\text{g}/\text{ml}$ in PBS (Sigma, St Louis, MO, USA). Additionally, plant fractions are pre-treated with agarose beads coated with POLB (Sigma, St Louis, MO, USA) to eliminate possible LPS contamination. To discard LPS presence in the plant fractions, all reagents are tested with LAL (Bio Whittaker Inc., Walkersville, MD, USA) and used according to manufacturer's instructions. Cell viability is estimated to monitor the fractions' or compounds' potential toxicity using the trypan blue exclusion test.

6.2 Dendritic cell phenotype analysis

Expression of membrane surface markers in immature DCs or those treated with LPS, or with compounds or fractions, are assessed after seven days. Phenotypic analysis is performed using anti-CD1a Pacific Blue, anti-CD86 PE, anti-CD83 FITC, anti-HLA-DR APC-H7, anti-CD209 PerCP-Cy5 and anti-CD206 APC (BD Biosciences). The cells are suspended in a PBS buffer (0.1% sodium azide and 2% FBS) at 4°C. To discriminate living from dead, cells are marked with LIVE/DEAD® Fixable Aqua Dead Cell Stain (Invitrogen, Carlsbad, CA, USA). Also 5 to 20 µl of each antibody is added according to the manufacturer's instructions and incubated for 30 min at RT. After incubation, cells are washed twice with buffer and suspended in 400 µl, to be read on the FACSaria. The analysis is carried out with FACSDiva™ 6.1 (BD Immunocytometry Systems, BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo 8.7 (Tree Star Inc., Ashland, USA). As an example, Figure 7 shows how a galactomannan derived from the *C.spinosa* plant, induces maturation of human dendritic cells shown by the increase in expression of surface membrane markers such as CD86 and HLA-DR.

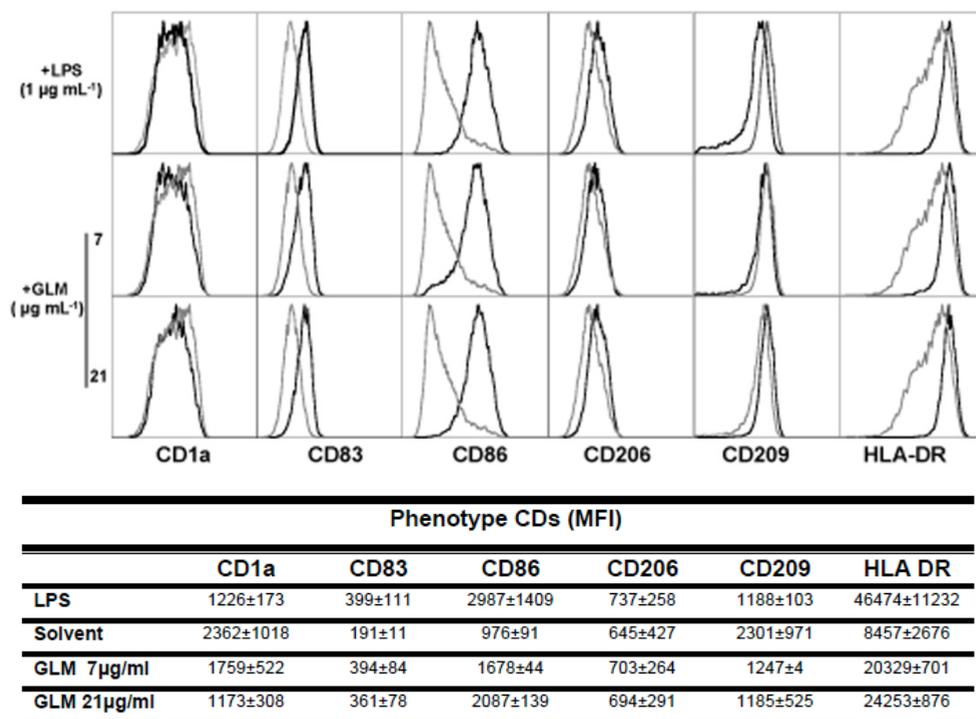


Fig. 7. Galactomannan induces phenotypic and functional maturation of DCs. DC cultured for 48 h with LPS (1 µg/mL) and galactomannan (7 and 21 µg/mL). An increase in expression of membrane surface molecules such as HLA-DR, CD86 and CD83 is shown after treatment with galactomannan (black) as compared to the controls (grey). Histograms represent one of four independent experiments. Differences on the mean fluorescence are shown on the table

6.3 Phagocytosis assays

To examine DC's phagocytic capacity, 100,000 DCs/well are grown in RPMI medium supplemented with 5% FBS and stimulated for 48 h with the different treatments. Immature and stimulated DCs are washed with Hanks buffer and suspended in 100 μ l (0.5 μ g/ml) of *E. coli* pHrodo™ (Invitrogen) bioparticles, for 3 h at 37°C. Bio-particles fluorescence is read in a fluorometer using an excitation filter of 535 nm and an emission filter of 595 nm (Dynex Technologies, Chantilly, VA, USA). The phagocytic cell percentage is estimated by flow cytometry. DCs are labelled with anti-CD11c-APC and the double labelled cell population is analysed. Assays are performed in triplicate and the analysed as previously described. Figure 8 illustrates the phagocytic activity induced by a galactomannan derived from *C. spinosa*.

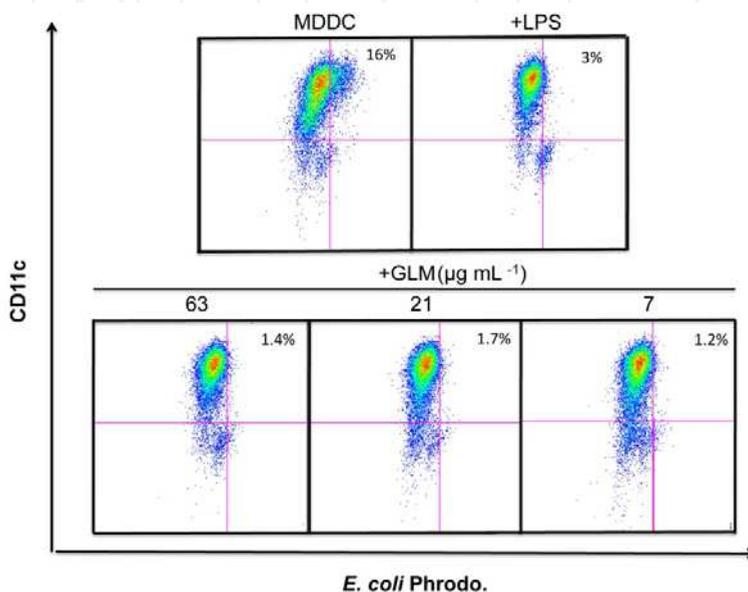


Fig. 8. Phagocytosis is evaluated by staining DCs (MDDC) with CD11c-APC antibody. The scatter plots show the cells after the treatments. DCs (MDDC), DCs (MDDC) plus LPS, DCs with galactomannan (GLM) 63, 21 and 7 (μ g mL⁻¹). Dot plots are representing one of four independent experiments

6.4 Mixed leukocyte reaction

Immunostimulatory activity of natural products can be evaluated by measuring the DCs' ability to induce allogeneic response in an assay named mixed leukocyte reaction. Human monocyte-derived DCs are stimulated for 48 h with LPS (1 μ g/ml) or plant-derived fractions at different concentrations in RPMI supplemented medium. The stimulated DCs are recovered, washed and cultured in fresh RPMI medium supplemented with 5% of human AB serum GemCell™ (Gemini Bio-Products, West Sacramento, CA). 500,000 CD4⁺ T cells/well are cultured at different ratios of allogeneic peripheral blood mononuclear cells (PBMC) (1:2, 1:5 and 1:10) previously obtained through Ficoll Hypaque gradient and labelled with

2.5 μM of carboxyfluorescein succinimidylester (CFSE) according to the manufacturer's recommendations (Invitrogen). Cells are collected after five days of co-culture; the first cells are marked with fluorescent viability marker aqua reactive dye (Invitrogen) and the second with anti-CD3 PerCP, anti-CD4 APC-H7 and anti-CD8 Pacific Blue (BD Biosciences). Samples are acquired on a FACSaria (BD Biosciences) and analysed as described above. As an example, Figure 9 shows T cell proliferation after treating DCs with a galactomannan derived from *C. spinosa*.

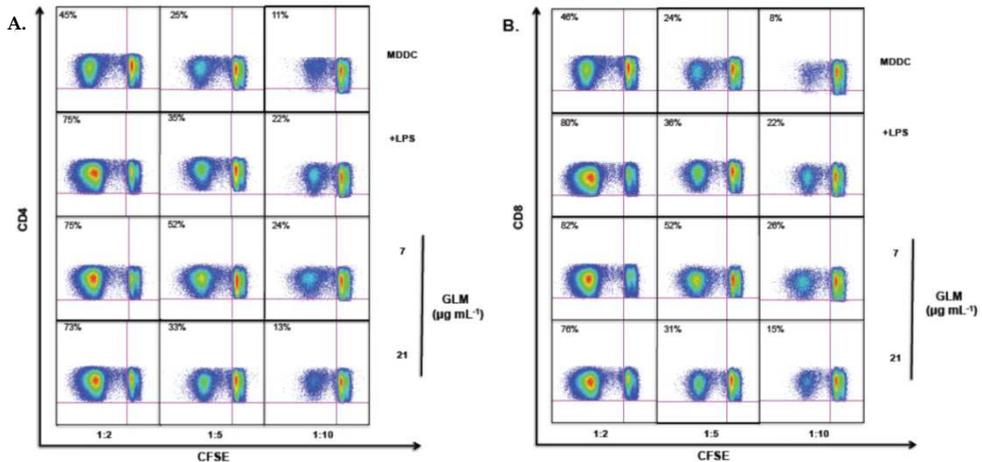


Fig. 9. (A) Dendritic cells (MDDCs) induce allogenic CD4 and CD8 T cell proliferation after treatment with a galactomannan derived from *C. spinosa*. Antigen presenting DCs (MDDC) after stimulation with LPS ($1\mu\text{g mL}^{-1}$) or GLM (7 or 21 $\mu\text{g mL}^{-1}$) determined by T cell proliferation assays. PBMCs stained with CFSE (2,5 μM) and co-cultured with DCs (MDDC) in ratios 1:2, 1:5 and 1:10 (DCs: PBMC) over five days. CD4+ and CD8+ (B) cell proliferation is determined by specific antibodies and analysed through flow cytometry. Dot plots are representing one of four independent experiments

6.5 Cytokine secretion measurement

Cytokine secretion can be assessed by flow cytometry after stimulating with sterile and LPS-free plant fractions or compounds for 48 h as indicated above. The CBA kit (human inflammation cytometric bead array kit) (BD Biosciences) is used to evaluate levels of proinflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12p70, and TNF- α , in the culture supernatant according to the manufacturer's instructions (CBA, BD Biosciences). The CBA application allows flow cytometry users to quantify simultaneously multiple proteins based on the cytometer capability to detect fine levels of fluorescence. The beads are coupled to antibodies that can capture different substances. Each bead has a unique fluorescence intensity allowing them to mix and record all bead signals at the same time and in a single tube. The analysed supernatants are obtained from 1×10^6 cells/per well and can be stored at -80°C until analysis. Marking is done according to the supplier instructions and the tubes are analysed as described above.

7. Conclusion

In general terms, flow cytometry constitutes a powerful tool to evaluate the anti-tumour and immunomodulatory activities of natural products, always keeping in mind the correct use of the controls and the instrument settings.

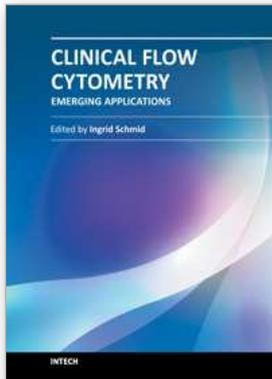
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