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Research paper

# Background autofluorescence induced by plant extracts in human lymphocytes: A flow cytometric analysis of a critical bias☆

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ABSTRACT

The presence of background autofluorescence sources is considered as an important problem when performing fluorometric methods, due to the possible spectral overlap between it and the fluorescence emission of probes. Regarding that, we evaluated the presence of background autofluorescence in human lymphocytes after the treatment with extracts from three medicinal plants, including ethanolic extract from aerial parts of *Ageratum fastigiatum*, ethanolic extract from aerial parts of *Eriosema campestre* and the ethanolic extract from stem of *Pseudobrickellia brasiliensis*. Human peripheral blood mononuclear cells were treated with each extract in vitro during 24 h, followed by flow cytometric analysis. Additionally, the fluorescence emission of plant extracts was evaluated by fluorometry, using the same concentrations used in cell cultures. We identified that plant extracts treatment on lymphocytes induced background autofluorescence detectable in several wavelength ranges. Isolated extracts showed no expressive fluorescence emission in fluorometric analyses, suggesting that background autofluorescence was induced in lymphocytes by interactions between cellular components and extracts compounds. Here we discuss the importance to perform previous tests to evaluate a possible background autofluorescence induction after cell treatments with plant extracts or any other substance. In spite of being mandatory, background autofluorescence analysis of cells after treatments and stimulations is still underestimated on literature. In summary, following the precautions herein established should help to reduce the incidence of false positive results.

## 1. Introduction

Fluorometric assays are widely used in biological researches because of their high sensitivity, specificity and quickness (Skoog et al., 2009; Harris, 2007). Although they are very useful, fluorometric techniques have some limitations that can reduce specificity. For instance, when performing such techniques, it is necessary to be careful with possible sources of background autofluorescence, which is characterized by detection of fluorescence that does not belong to the fluorochrome used for staining, resulting in an interference that can impair the detection of addressed structures (Hulspas et al., 2009). Therefore, it is essential to evaluate the fluorescence emission of samples and substances used in experiments, in order to adopt preventive measures that allow to avoid background autofluorescence in analyses.

*Abbreviations:* AFEXT, Ethanolic extract from aerial parts of *Ageratum fastigiatum*; ECEXT, Ethanolic extract from aerial parts of *Eriosema campestre*; PBEXT, Ethanolic extract from stem of *Pseudobrickellia brasiliensis*; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein

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Intracellular interactions with different substances also must be considered, because they can cause the formation of fluorescent products from non-fluorescent molecules (Harrisson et al., 1981; Avelar-Freitas et al., 2014), being such interactions a distinct and discrete source of background autofluorescence.

Considering the important analytical problem of background autofluorescence, especially when staining low-expression molecules, some studies had their focuses in avoid, reduce or eliminate such interferences (Glaser et al., 2015; Vorobjev et al., 2012; Rich et al., 2013; Shilova et al., 2017; Srivastava et al., 2011).

In the present study we verified that the treatments of human lymphocytes with three non-fluorescent plant extracts were capable to induce background autofluorescence on those cells. We believe that the use of controls to verify the inducing of background autofluorescence in cell cultures by different treatments is frequently an underestimated aspect. In this context, we show the relevance of evaluate the fluorescence emission characteristics of a given sample after treatment with a substance or compound of interest.

## 2. Material and methods

### 2.1. Plant extracts

Three different plant extracts were used as cell treatments in the present study, including: Ethanolic extract from aerial parts of *Ageratum fastigiatum* (Gardn.) R. M. King et H. Rob. (AFEXT), ethanolic extract from aerial parts of *Eriosema campestre* var. macrophyllum (Grear) Fortunato (ECEXT), and ethanolic extract from stem of *Pseudobrickellia brasiliensis* (Spreng) R. M. King & H. Rob. (PBEXT). All those extracts were solubilized in dimethyl sulfoxide (DMSO). Such medicinal plants are native from the state of Minas Gerais, Brazil, and their therapeutic properties has been studied by our research group (Avelar-Freitas et al., 2013; Santos et al., 2015; Avelar-Freitas et al., 2015).

The use of such plants was authorized by the Brazilian Genetic Patrimony Management Council (CGEN) under the number A056707.

### 2.2. Ethical aspects

As the present study involved human volunteers, it was firstly approved by the local research ethics committee, and it was registered as CEP CAAE 28665514.2.0000.5108.

### 2.3. Human peripheral blood mononuclear cells (PBMC)

Venous blood samples were collected from 3 healthy volunteers (27.3 ± 1.15 years old) in heparinized tubes at the time of the experiment. The exclusion criteria of the volunteers were: continuous use of drugs or having an infectious disease. Blood was diluted and homogenized in phosphate buffered saline (PBS) in a 1:1 ratio, and then carefully pipetted on a Histopaque®-1077 solution (Sigma, St. Louis, MO, USA) (Bicalho and Gontijo, 1981), in order to obtain PBMC.

### 2.4. Cell cultures

PBMC ($5.0 \times 10^5$ cells/mL) were placed in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with fetal calf serum (Gibco, Invitrogen Corporation, Grand Island, NY, USA) at 10%, L-glutamine (Sigma, St. Louis, MO, USA) at 2 mM and antibiotic/antimycotic solution (penicillin G 100 IU/mL, streptomycin 100 µg/mL and amphotericin B 250 ng/mL) (Gibco, Invitrogen Corporation, Grand Island, NY, USA).

Cells were treated with ethanolic extract from aerial parts (leaves and stem) of *A. fastigiatum*, ethanolic extract from aerial parts of *E. campestre*, or ethanolic extract from stem of *P. brasiliensis*, used always at three different concentrations. In addition, it was made an untreated cell culture (CON) and a cell culture treated with dimethyl sulfoxide at 1.1% (DMSO). Cell cultures were maintained at 37 °C and 5% $CO_2$ during 24 h. After that, cells were washed with PBS and cell viability, using trypan blue exclusion assay (Santos et al., 2015), as well as background autofluorescence analysis were evaluated.

### 2.5. Evaluation of the presence of background autofluorescence

After washing, cells were evaluated on an unmodified 3-laser, 4-2-2 configuration BD FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA). Cell doublets were excluded and ten thousand lymphocytes were obtained from each cell culture, using BD FACSDIVA™ software (BD Biosciences, San Jose, CA, USA). The fluorescence emission profile of such cells was recorded in all of the eight detection channels of the flow cytometer.

The analysis strategy adopted consisted in comparing the emitted fluorescence intensity between lymphocytes untreated and treated with plant extracts or DMSO. Thus, for all cell cultures, fluorescence intensity histograms were plotted according to the channel in which they were evaluated. Histograms median of each cell culture were obtained. Data analysis was performed using FlowJo software, version 10.0.7 (Tree Star, Ashland, OR, USA).

### 2.6. Fluorometry

The spectral absorption and the fluorescence emission profile of each plant extract and DMSO were evaluated at the same concentrations used in cell cultures, in a flat bottom 96-well polystyrene plate, using a Multiskan™ GO spectrophotometer (Thermo Fisher Scientific, MA, USA) and a Spectramax Paradigm® multi-mode microplate reader (Molecular Devices, CA, EUA), respectively.

Blank control was established as DMSO diluted in ultrapure water to exclude its contribution in the absorption and fluorescence emission results. Thus, a concentration of 1.25% of DMSO was used in all wells. Blank controls were automatically discounted from the extracts analysis. Additionally, fluorochrome-associated antibodies were used as positive controls for fluorescence emission. Fluorochromes selection was made based in the optimal absorption and the wavelength range of the fluorescence emitted by each extract, so different fluorochromes could be used in each case.

### 2.7. Flow cytometric analysis of $CD3^+$ and $HLA\text{-}DR^+$ lymphocytes after treatment with the ethanolic extract from aerial parts of Eriosema campestre

PBMC treated or not with 220 µg/mL of the ethanolic extract from aerial parts of *E. campestre* during 24 h were stained with a PE-conjugated anti-human CD3 monoclonal antibody (clone HIT3a, BD Pharmingen, San Diego, CA, USA) or a PE-conjugated anti-HLA-DR monoclonal antibody (clone LN3, eBioscience, San Diego, CA, USA). BD FACSCanto™ II and BD FACSDIVA™ were used in cell acquisition, while the analysis was performed using FlowJo software.

### 2.8. Statistical analysis

Values were expressed as mean ± standard deviation. Data was evaluated by Shapiro-Wilk test, followed by ANOVA one-way with Tukey's post-hoc for normally distributed data or Kruskal-Wallis with Dunns post-hoc for asymmetrically distributed data. It was adopted a significance level of $p < 0,05$. Softwares used for such analysis were IBM SPSS® (IBM Corp., Version 22.0, Armonk, NY, EUA) and GraphPad Prism 5® (GraphPad Software Inc., San Diego, CA, EUA).

## 3. Results

### 3.1. Effect of the plant extracts treatment on cell viability

Before evaluate the presence of background autofluorescence in cell

**Table 1**
Effect of the plant extracts treatment on cell viability.

| Cultures | | Cell viability (%) |
|---|---|---|
| CON | | 96.8 ± 2.48 |
| DMSO | | 97.5 ± 0.55 |
| AFEXT | 12.5 μg/mL | 96.3 ± 2.80 |
| | 25 μg/mL | 94.8 ± 2.14 |
| | 50 μg/mL | 92.7 ± 1.75 |
| | 75 μg/mL | 75.0 ± 3.57* |
| ECEXT | 140 μg/mL | 91.7 ± 0.52 |
| | 180 μg/mL | 89.17 ± 4.07 |
| | 220 μg/mL | 84.0 ± 5.65 |
| | 260 μg/mL | 52.4 ± 22.60* |
| PBEXT | 25 μg/mL | 98.5 ± 0.50 |
| | 50 μg/mL | 98.2 ± 0.51 |
| | 100 μg/mL | 97.1 ± 1.26 |
| | 200 μg/mL | 97.0 ± 0.97 |

CON: untreated cells. DMSO: cells treated with dimethyl sulfoxide; AFEXT: cells treated with ethanolic extract from aerial parts of *Ageratum fastigiatum*. ECEXT: cells treated with ethanolic extract from aerial parts of *Eriosema campestre*. PBEXT: cells treated with ethanolic extract from stem of *Pseudobrickellia brasiliensis*.

* $p < .05$ when compared to CON.

cultures after treatment with plant extracts, it was firstly necessary to evaluate its effect on cell viability. Such care is very important, because dead cells can be a significant source of background autofluorescence (Zamai et al., 1993; Dittmar et al., 2012).

As observed in Table 1, the concentrations that maintain PBMC viability are: 12.5, 25 and 50 μg/mL for cells treated with ethanolic extract from aerial parts of *Ageratum fastigiatum*; 140, 180 and 220 μg/mL cells treated with ethanolic extract from aerial parts of *Eriosema campestre* and 25, 50, 100 and 200 μg/mL for cells treated with ethanolic extract from stem of *Pseudobrickellia brasiliensis*.

### 3.2. Background autofluorescence induced by plant extracts in lymphocytes

In order to investigate the presence of background autofluorescence on cultures, we firstly evaluated qualitatively the fluorescence emission capacity of the cells using all the detection channels of BD FACSCanto™ II flow cytometer. It was observed that lymphocytes treated with ethanolic extract from aerial parts of *A. fastigiatum* increased fluorescence intensity in 5 of the 8 wavelength ranges evaluated (Fig. 1). The most evident increase was observed between 425 and 535 nm after violet laser excitation.

Similarly, the treatment of cells with the ethanolic extract from aerial parts of *E. campestre* induced them to emit fluorescence in 5 of the 8 wavelength ranges tested (Fig. 2). The most evident histograms shift for lymphocytes treated with that extract, when compared to fluorescence intensity histogram of untreated cells, is seen in the 485–535 nm channel, after violet laser excitation.

Finally, the treatment of cells with ethanolic extract from stem of *P. brasiliensis* also caused background autofluorescence in the flow cytometric analysis (Fig. 3), but in a smaller number of channels (3 of 8).

In order to convert the qualitative results expressed in Figs. 1–3 in quantitative data, it was analyzed the median average of the histograms of the cell cultures treated with the ethanolic extracts from aerial parts of *A. fastigiatum*, from aerial parts of *E. campestre* and from stem of *P. brasiliensis*.

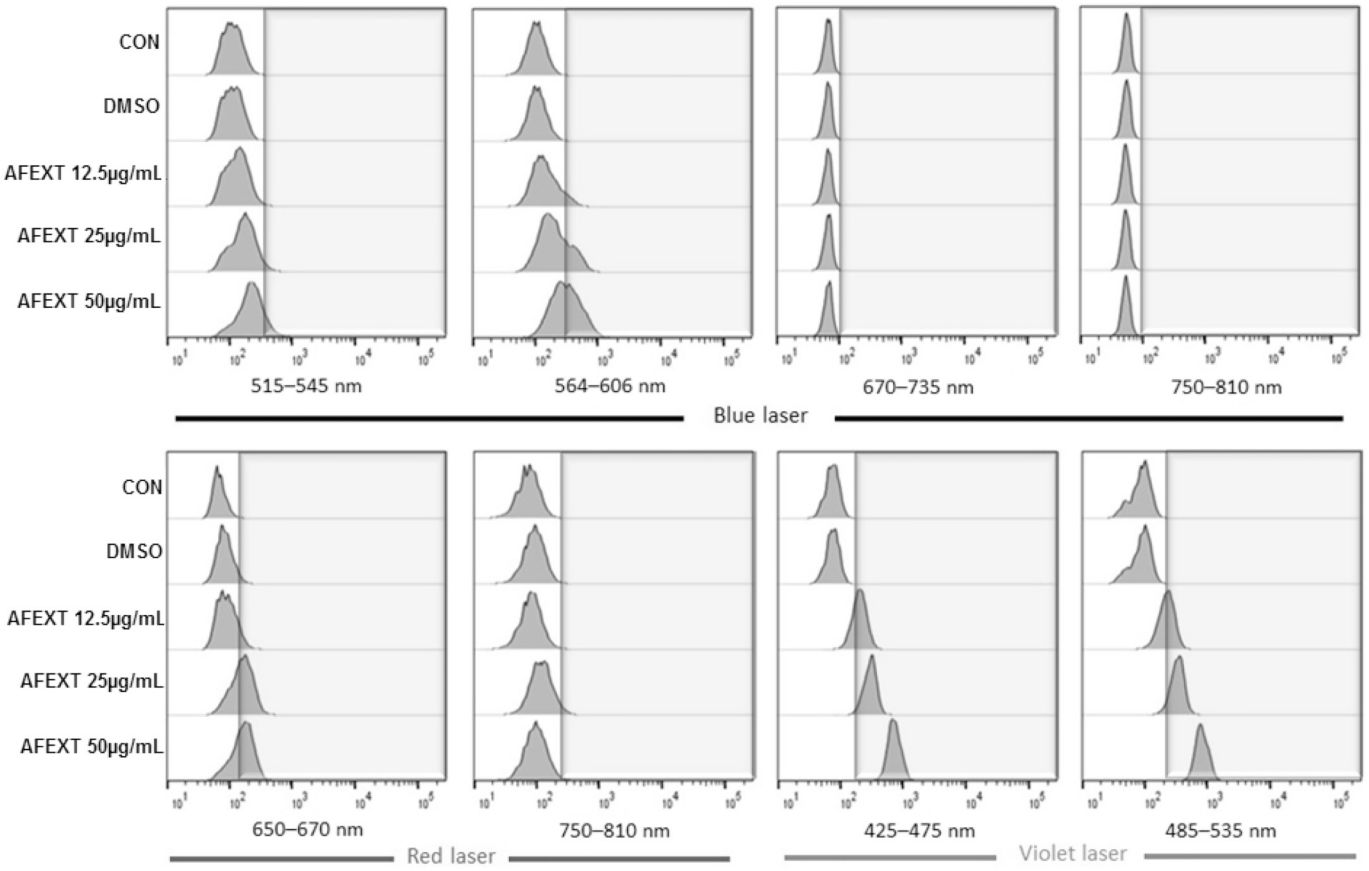


**Fig. 1.** Background autofluorescence in cells treated with the ethanolic extract from aerial parts of *Ageratum fastigiatum* (AFEXT). Fluorescence emission profiles of untreated human lymphocytes (CON), and lymphocytes treated with AFEXT or dimethyl sulfoxide (DMSO). Histograms displaced toward gray box indicate increasing in fluorescence intensity when compared to fluorescence intensity of untreated cells.

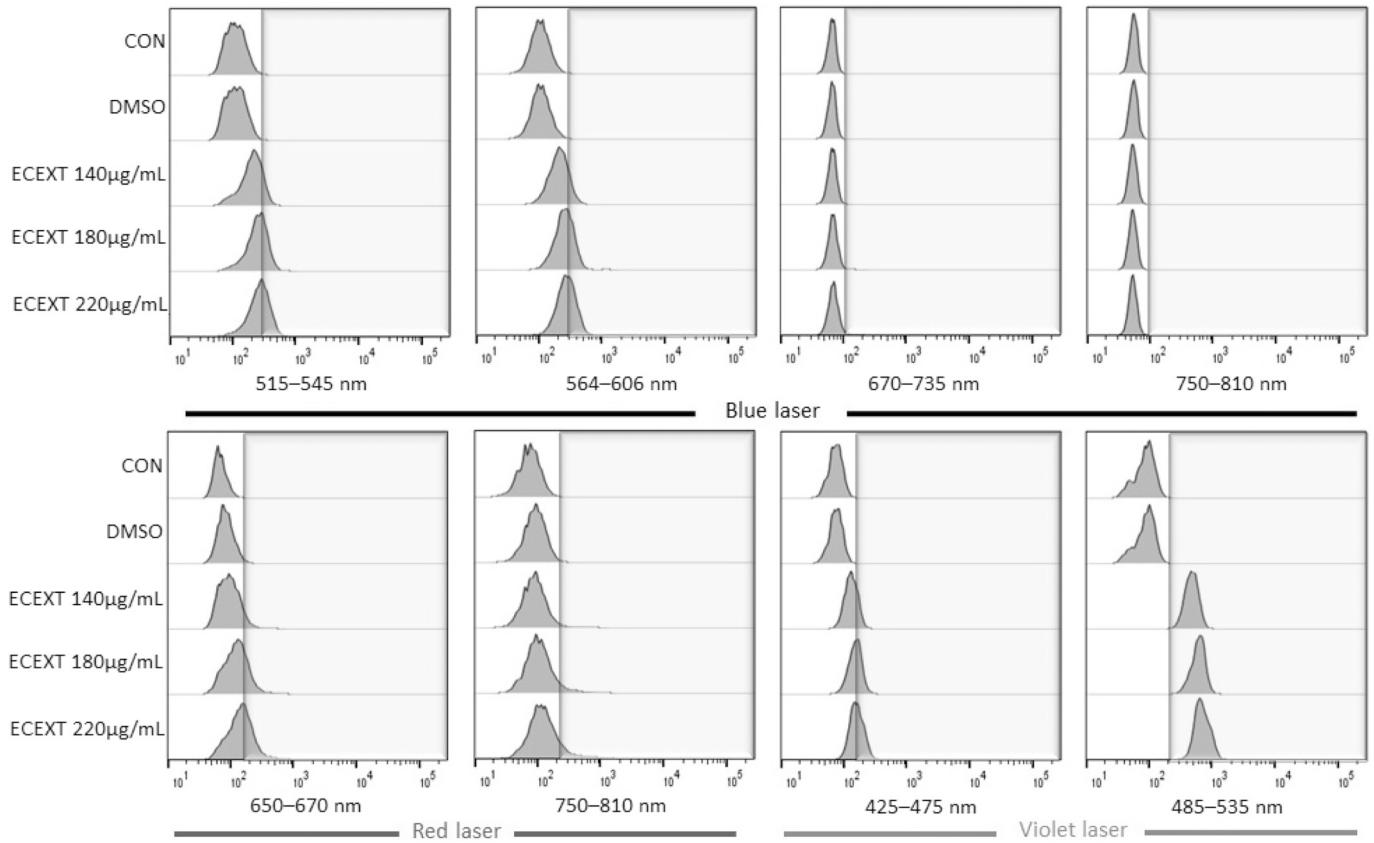


**Fig. 2.** Background autofluorescence in cells treated with the ethanolic extract from aerial parts of *Eriosema campestre* (ECEXT). Fluorescence emission profiles of untreated human lymphocytes (CON), and lymphocytes treated with ECEXT or dimethyl sulfoxide (DMSO). Histograms displaced toward gray box indicate increasing in fluorescence intensity when compared to fluorescence intensity of untreated cells.

No statistical difference was identified when comparing medians averages of cells treated with DMSO and untreated cells. Thus, fluorescence emission of cells treated with plant extracts was compared to the emission of cells treated with DMSO.

Analyzing the data on Tables 2–4, it was seen that the treatment of cells with each one of the plant extracts induced a background autofluorescence in the cells detectable in, at least, 4 wavelength ranges [515–545 nm (excitation by blue laser), 650–670 nm (excitation by red laser), 425–475 nm and 485–535 nm (both with excitation by violet laser)]. Additionally, the treatment of cells with ethanolic extract from aerial parts of *A. fastigiatum* can lead to a fluorescence emission detectable in the 564–606 nm channel, when excited by the blue laser (Table 2). Nevertheless, the ethanolic extract of aerial parts of *E. campestre* induced background autofluorescence in lymphocytes on a larger number of wavelength ranges, leading those cells to emit not only in the channels already mentioned, but also in the 670–735 nm channel, after excitation with the blue laser (Table 3).

Furthermore, it was observed in some cases, a tendency of increasing fluorescence intensity when higher concentrations of the extracts are used to treat lymphocytes. For example, in the 485–535 nm channel (Table 3), cells treated with the ethanolic extract from aerial parts of *E. campestre* at 220 µg/mL emitted fluorescence in a higher intensity (688.0 ± 56.2) than cells treated with 180 µg/mL (615.3 ± 14.2) ($p < .05$). These cells, in turn, emitted fluorescence in higher intensity than cells treated with the lowest concentration, 140 µg/mL (477.3 ± 27.5) ($p < .05$).

### 3.3. Fluorometry of the plant extracts

The absorption scanning of each plant extract and the evaluation of their fluorescence emission were performed to investigate spectral characteristics of the extracts.

The ethanolic extract from aerial parts of *A. fastigiatum* and the ethanolic extract from stem of *P. brasiliensis* showed their optimal absorption in the range of 300 to 450 nm (Supplementary fig. 1A and 1B, respectivelly), while the ethanolic extract from aerial parts of *E. campestre* presented its optimal absorption profile between 300 and 530 nm (Supplementary fig. 1C).

The excitation wavelength of 405 nm was used to obtain the fluorescence emission profile of the ethanolic extract from aerial parts of *A. fastigiatum* and the ethanolic extract from stem of *P. brasiliensis*. Both extracts were not able to emit fluorescence in the same range where background autofluorescence was detected in flow cytometry (425–535 nm) (Fig. 4A and B).

For the ethanolic extract from aerial parts of *E. campestre*, two wavelengths were chosen for excitation (405 and 488 nm), considering its longer absorption range. As well as occurred for the other extracts previously mentioned, no significant fluorescence emission was detected when the ethanolic extract from aerial parts of *E. campestre* was excited at 405 nm (Fig. 4C). In contrast, when excited at 488 nm, however, fluorescence emission peaks (between ~660–760 nm) were observed for each tested concentration (Fig. 4D). Nevertheless, the fluorescence intensity of this extract is much lower than the fluorescence intensity of the positive control, suggesting that the fluorescence emission of the extract in the evaluated parameters is not significant.

### 3.4. Flow cytometric analysis of $CD3^+$ and $HLA\text{-}DR^+$ lymphocytes presenting background autofluorescence

In order to show the impact of the background autofluorescence in a

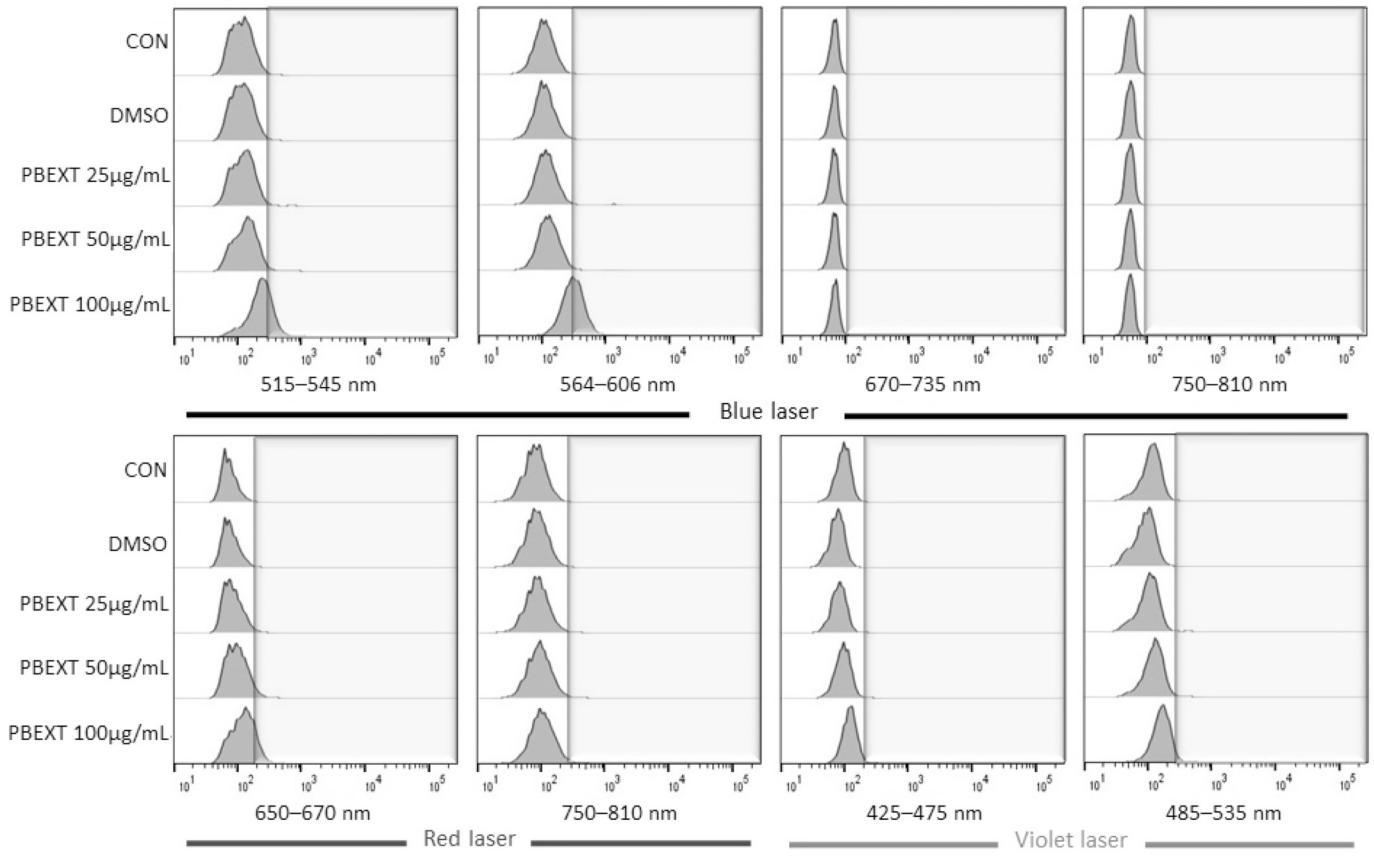


**Fig. 3.** Background autofluorescence in cells treated with the ethanolic extract from stem of *Pseudobrickellia brasiliensis* (PBEXT). (A) Fluorescence emission profiles of untreated human lymphocytes (CON), and lymphocytes treated with PBEXT or dimethyl sulfoxide (DMSO). Histograms displaced toward gray box indicate increasing in fluorescence intensity when compared to fluorescence intensity of untreated cells.

**Table 2**
Medians average of the fluorescence emission histograms of cells treated with the ethanolic extract from aerial parts of *Ageratum fastigiatum* or dimethyl sulfoxide.

| Wavelength range evaluated | Fluorescence intensity medians | | | |
|---|---|---|---|---|
| | DMSO | AFEXT | AFEXT | AFEXT |
| | | 12.5 µg/mL | 25 µg/mL | 50 µg/mL |
| 515–545 nm$^{B}$ | 117.0 ± 10.8 | 139.3 ± 12.1 | 175.0 ± 14.8$^{a}$ | 236.7 ± 34.1$^{a,b,c}$ |
| 564–606 nm$^{B}$ | 107.0 ± 3.6 | 144.0 ± 12.1 | 194.7 ± 21.2$^{a}$ | 305.0 ± 46.2$^{a,b,c}$ |
| 670–735 nm$^{B}$ | 65.0 ± 1.1 | 64.7 ± 0.7 | 65.7 ± 0.5 | 66.3 ± 0.6 |
| 750–810 nm$^{B}$ | 54.0 ± 1.1 | 53.0 ± 1.0 | 53.0 ± 1.0 | 53.0 ± 1.0 |
| 650–670 nm$^{R}$ | 73.4 ± 8.0 | 92.0 ± 7.0 | 145.0 ± 16.1$^{a,b}$ | 195.0 ± 29.5$^{a,b,c}$ |
| 750–810 nm$^{R}$ | 82.7 ± 7.6 | 84.7 ± 5.5 | 106.4 ± 14.2 | 112.6 ± 19.2 |
| 425–475 nm$^{V}$ | 79.3 ± 6.8 | 205.3 ± 17.9 | 352.3 ± 93.4$^{a}$ | 646.0 ± 150.6$^{a,b,c}$ |
| 485–535 nm$^{V}$ | 96.0 ± 9.5 | 233.3 ± 23.1 | 393.0 ± 105.1$^{a}$ | 728.0 ± 166.4$^{a,b,c}$ |

DMSO: cells treated with dimethyl sulfoxide; AFEXT: cells treated with ethanolic extract from aerial parts of *Ageratum fastigiatum*. $^{B}$Excitation by blue laser, $^{R}$Excitation by red laser, $^{v}$Excitation by violet laser. $p < .05$ when $^{a}$compared to DMSO, $^{b}$compared to AFEXT 12.5 µg/mL, $^{c}$compared to AFEXT 25 µg/mL.

flow cytometric analysis, CD3, a lymphocyte marker of high frequency, and HLA-DR, present in lesser frequency, were evaluated on lymphocytes treated or not with ethanolic extract from aerial parts of *E. campestre*.

Corroborating previous results (Fig. 2A and Table 3), it was possible to observe that unstained cells treated with the plant extract increased its fluorescence intensity at 564–606 nm (65.7%), when compared to untreated cells (0.55%) (Fig. 5). When evaluating CD3 labeling, it was observed that there is positivity for most of the untreated cells (85.3%), and such positivity slightly increases in cells treated with the plant extract (91.3%). However, in the analysis of HLA-DR labeling, it was observed that 22.1% of the untreated cells were positive, while the treated cells presented 82.3% of positivity for such marker.

## 4. Discussion

In the present study, it was demonstrated that the treatment of human lymphocytes with different plant extracts generated non-specific fluorescence in different wavelength ranges, when evaluated by flow cytometry. However, such background autofluorescence cannot be attributed to something intrinsic to the plant extracts, because they were not able to emit fluorescence in the same parameters, when evaluated

**Table 3**
Medians average of the fluorescence emission histograms of cells treated with ethanolic extract from aerial parts of *Eriosema campestre* or dimethyl sulfoxide.

| Wavelength range evaluated | Fluorescence intensity medians | | | |
|---|---|---|---|---|
| | DMSO | ECEXT | ECEXT | ECEXT |
| | | 140 μg/mL | 180 μg/mL | 220 μg/mL |
| 515–545 nm[B] | 117.0 ± 10.8 | 217.0 ± 12.1[a] | 253.7 ± 7.09[a] | 299.0 ± 36.6[a,b] |
| 564–606 nm[B] | 107.0 ± 3.6 | 220.3 ± 13.9[a] | 260.7 ± 9.2[a,b] | 306.0 ± 41.7[a,b] |
| 670–735 nm[B] | 65.0 ± 1.1 | 66.7 ± 0.6 | 68.0 ± 0.0 | 69.37 ± 0.5[a] |
| 750–810 nm[B] | 54.0 ± 1.1 | 52.6 ± 0.5 | 52.9 ± 0.0 | 52.9 ± 0.0 |
| 650–670 nm[R] | 73.4 ± 8.0 | 105.6 ± 12.4[a] | 132.3 ± 10.1[a,b] | 153.3 ± 9.3[a,b] |
| 750–810 nm[R] | 82.7 ± 7.6 | 96.1 ± 8.0 | 110.0 ± 9.5 | 125.0 ± 7.6 |
| 425–475 nm[V] | 79.3 ± 6.8 | 139.0 ± 9.9 | 155.0 ± 4.4 | 196.3 ± 59.8[a] |
| 485–535 nm[V] | 96.0 ± 9.5 | 477.3 ± 27.5[a] | 615.3 ± 14.2[a,b] | 688.0 ± 56.2[a,b] |

DMSO: cells treated with dimethyl sulfoxide; ECEXT: cells treated with ethanolic extract from aerial parts of *Eriosema campestre*. [B]Excitation by blue laser, [R]Excitation by red laser, [v]Excitation by violet laser. $p < .05$ when [a]compared to DMSO, [b]compared to ECEXT 140 μg/mL, [c]compared to ECEXT 180 μg/mL.

**Table 4**
Medians average of the fluorescence emission histograms of cells treated with ethanolic extract from stem of *Pseudobrickellia brasiliensis* or dimethylsulfoxide.

| Wavelength range evaluated | Fluorescence intensity | | | |
|---|---|---|---|---|
| | DMSO | PBEXT | PBEXT | PBEXT |
| | | 25 μg/mL | 50 μg/mL | 100 μg/mL |
| 515–545 nm[B] | 117.0 ± 10.8 | 121.7 ± 13.5 | 136.7 ± 12.2 | 189.0 ± 40.4[a,b] |
| 564–606 nm[B] | 107.0 ± 3.6 | 112.3 ± 6.5 | 125.0 ± 7.6 | 206.3 ± 85.2 |
| 670–735 nm[B] | 65.0 ± 1.1 | 65.1 ± 0.0 | 65.4 ± 0.5 | 66.3 ± 0.6 |
| 750–810 nm[B] | 54.0 ± 1.1 | 53.6 ± 0.6 | 53.6 ± 0.6 | 53.6 ± 0.6 |
| 650–670 nm[R] | 73.4 ± 8.0 | 75.7 ± 4.2 | 82.7 ± 9.0 | 114.0 ± 23.0[a,b] |
| 750–810 nm[R] | 82.7 ± 7.6 | 80.7 ± 4.9 | 83.7 ± 8.1 | 97.7 ± 15.0 |
| 425–475 nm[V] | 79.3 ± 6.8 | 82.7 ± 6.7 | 93.7 ± 2.6 | 120.3 ± 8.5[a,b,c] |
| 485–535 nm[V] | 96.0 ± 9.5 | 104.7 ± 8.0 | 121.7 ± 7.8 | 161.0 ± 12.5[a,b,c] |

DMSO: cells treated with dimethyl sulfoxide; PBEXT: cells treated with ethanolic extract from stem of *Pseudobrickellia brasiliensis*. [B]Excitation by blue laser, [R]Excitation by red laser, [v]Excitation by violet laser. $p < .05$ when [a]compared to DMSO, [b]compared to PBEXT 25 μg/mL, [c]compared to PBEXT 50 μg/mL.

by fluorometry.

A possible explanation for that finding is an interaction between cellular components and compounds present in the plant extracts. It was already seen in previous works some cases of non-fluorescent compounds that became capable of emitting fluorescence after being complexed to other molecules, such as terpenes, which can emit fluorescence when complexed to cyclodextrins (Ochocka et al., 1992; Kfoury et al., 2014). Thus, considering the presence, not only of terpenes, but also of other compounds in the plant extracts used, we suggest the occurrence of some interaction (by complexation or chemiluminescence) between lymphocytes components and plant extracts, forming products capable of emitting background autofluorescence. Nevertheless, such hypothesis still must be clarified in future works.

Moreover, the background autofluorescence verified in the present study also cannot be attributed to dead cells, because the extracts concentrations used in cultures were able to maintain cell viability and, through a labeling with propidium iodide, we verified by flow cytometry that dead lymphocytes, after the treatment with extracts, had average fluorescence intensity similar to that of viable cells (Data not shown).

The results indicate the relevance of performing previous tests aiming to investigate, under work conditions, the appearance of background autofluorescence sources. Once such interference is not a rare event, it may be an important source of false-positive results. An example is the study of Manrique et al. (2011), where the existence of FOXP3$^+$ macrophages was reported (Manrique et al., 2011). However, other authors demonstrated that what was observed in that study was actually a false-positive result, caused by macrophages autofluorescence, whose wavelength range overlaps with green fluorescent protein's (GFP), fluorophore used in that study for FOXP3 labeling (Li et al., 2012; Put et al., 2012; Mayer et al., 2012).

Here, it was shown the presence of background autofluorescences detectable in many channels of a flow cytometer, which imposes important limitations in the choice of fluorochromes that can be used in antigen staining. We believe that the present findings are not particular to the treatment with plant extracts, but it can possibly happen also with other compounds. We highlight the importance of using controls for compounds to be tested in cell cultures in different concentrations, in order to check a possible induced background autofluorescence. The using of such controls should mandatory, but is often observed in literature an underestimating of such aspect in experiments. The implementation of those preventive procedures should avoid significantly the occurrence of false-positive results on fluorescent assays.

In the study of Glaser et al. (2015), the presence of background autofluorescence was detected in different wavelength ranges for monocytes and lymphocytes treated with different surfactant preparations (Glaser et al., 2015). In this case, the background autofluorescence was associated to residual particles from their own object of study. However, the knowledge of the background autofluorescence source allowed the authors to use a substance to eliminate it, thus, avoiding problems in the flow cytometric analysis.

In a previous works performed by Quan et al. (2010), in which the interaction mechanism between doxorubicin and somatostatin for tumor cell killing was evaluated, an assay was performed to evaluate the type of cell death using Annexin V and propidium iodide by flow cytometry (Quan et al., 2010). In that case, the authors firstly

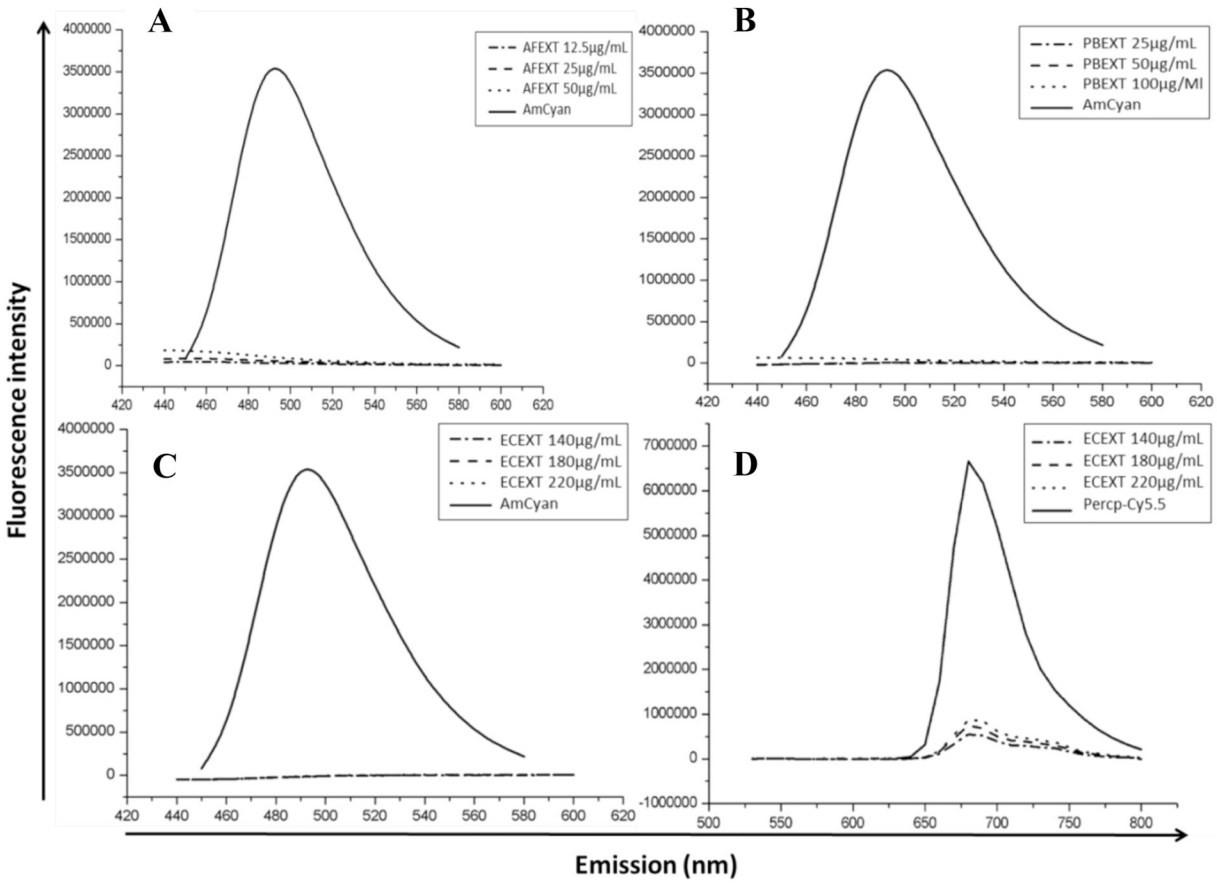


**Fig. 4.** Plant extracts fluorescence emission spectra. (A) Ethanolic extract from aerial parts of *Ageratum fastigiatum* (AFEXT) excited by 405 nm laser. (B) Ethanolic extract from stem of *Pseudobrickellia brasiliensis* (PBEXT) excited by 405 nm laser. (C) Ethanolic extract from aerial parts of *Eriosema campestre* (ECEXT) excited by 405 nm laser. (D) ECEXT excited by 488 nm laser. The fluorescence emission control used in A, B and C was AmCyan, while in D, it was used Percp-Cy5.5.

investigated the fluorescent properties of doxorubicin, analyzing cells treated only with that drug. A strong background autofluorescence was seen, but it was still possible to set a gate to exclude false-positive results.

In our study, it was also performed a qualitative and quantitative analysis of the results obtained by flow cytometry. However, it is important to mention that in some cases it is possible to observe a visible difference of shifting between histograms and, nevertheless, have no statistical difference between their fluorescence intensity values. An example is the fluorescence emission of lymphocytes treated with 100 µg/mL of the ethanolic extract from stem of *P. brasiliensis* in the 564–606 nm channel (Fig. 3A), where there was a visible increasing in fluorescence intensity when compared to histograms of lymphocytes treated with DMSO or lower concentrations of that extract, but when the same data was statistically analyzed, no difference between the fluorescence intensities was observed (Table 4). Possibly, the reason for this divergence was the elevated standard deviation for the fluorescence emission of cells treated with the extract. Thus, both qualitative and quantitative results must be analyzed together for a correct interpretation about the presence of background autofluorescence and whether it is capable or not to overlap a fluorescence signal of a probe.

Another important issue is that autofluorescence may impair differently in the analysis of antigens of distinct frequency in a given cell population. When probing a highly frequent antigen, background autofluorescence may impact more discretely on a given assay, as exemplified by the labeling of CD3, a pan T-cell marker, on lymphocytes treated with the ethanolic extract from aerial parts of *E. campestre* (Fig. 5). Otherwise, the labeling of lesser frequent antigens on cells with an existing background autofluorescence may impact much more critically, as exemplified here in HLA-DR staining on non-activated lymphocytes (Fig. 5), because of the high number of false-positive events generated. Also, the fluorescent labeling of such antigens generally results in low intensity fluorescent signals, and can, therefore, be easily overlapped by a background autofluorescence.

In this context, Vorobjev et al. (2012) showed how procedures can be adopted to increase the signal of rare events of interest or reduce the detection of background autofluorescence (Vorobjev et al., 2012). For that, it was proposed the optimization of a flow cytometer based on tests of different filter sets, in order to select the most suitable for the detection of transgenic *Plasmodium* lines $GFP^+$ with the minimum interference of erythrocytes autofluorescence.

## 5. Conclusions

In summary, when performing a fluorometric assay, it is fundamental to know about the presence of background autofluorescence in a given sample at experimental conditions. Such practice is important to be performed even when it is known that the sample and other substances are not naturally fluorescents, once it is possible that the interaction between them may result in an autofluorescence induction in the cell. Hence, adopting routine assays to detect background autofluorescence, as proposed here, should lead to an important reduction of erroneous results in studies involving fluorometric analyses.

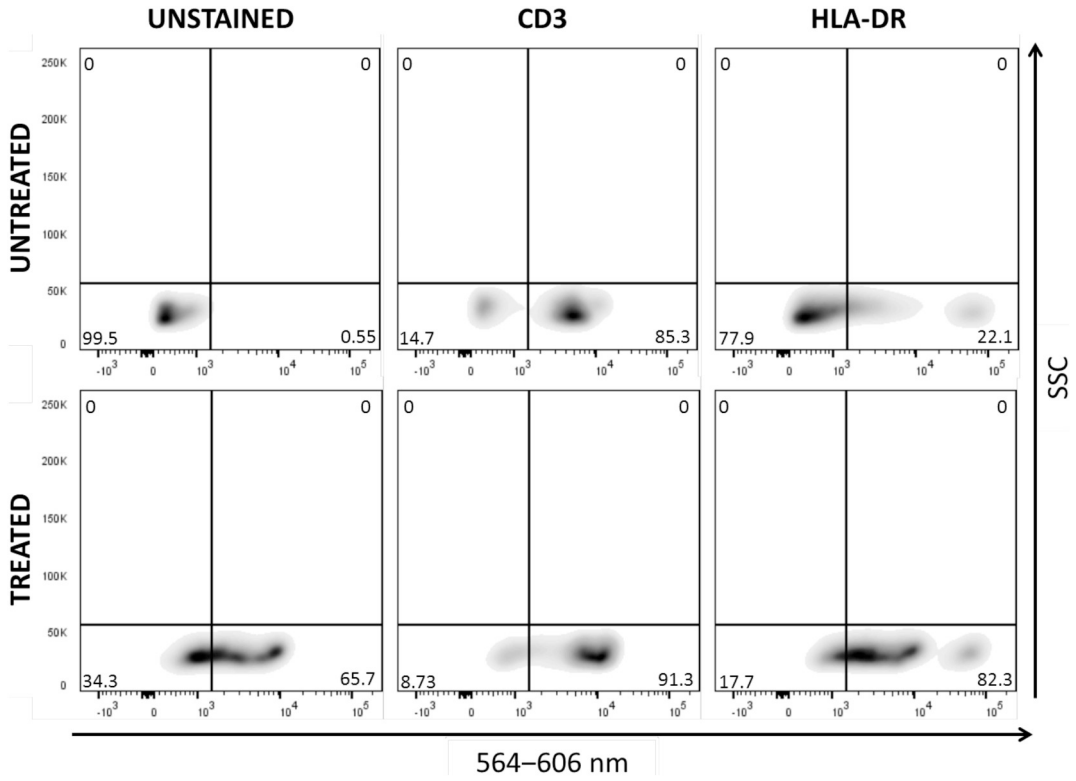


**Fig. 5.** Fluorescent staining of lymphocytes with or without the presence of a background autofluorescence induced by plant extract treatment. Lymphocytes were treated or not with 220 μg/mL of the ethanolic extract from aerial parts of *Eriosema campestre*. In the first column, it is shown unstained lymphocytes. At CD3 column, lymphocytes were stained with PE-conjugated anti-human CD3 monoclonal antibodies, and at HLA-DR column, lymphocytes were stained with PE-conjugated anti-HLA-DR monoclonal antibodies.

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The authors report no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.02.007.

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