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Antifungical and immunomodulatory activities from *Caesalpinia pulcherrima* extracts

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Abstract.
Caesalpinia pulcherrima is a species widely used in folk medicine for various diseases such as fever, infections and mouth ulcers. In addition, scientific studies have reported medicinal properties such as antitumor, antiviral, antibacterial and antifungal. Therefore, the objective of this work was to evaluate if organic extracts obtained from leaves of *C.*

pulcherrima have medicinal properties. Three organic extracts were obtained (hexane, ethyl acetate and ethanol) from the leaves collected from the Soxhlet equipment. The characterization was made by GC-MS and UPLC-MS and biological properties as antioxidant (AAT, DPPH, ABTS and lipid peroxidation radicals), antimicrobial, cytotoxicity and immunostimulant (using splenocytes of mice Balb/c) were investigated. Results showed several classes of secondary metabolites, but the ethyl acetate showed more phenols and flavonoids than the other extracts. Extracts showed good results in antioxidant tests, especially the ethyl acetate, but did not show antibacterial activity. The fungal evaluation showed high antifungal properties, especially the hexanic and the ethyl acetate against *Candida tropicalis*, *Aspergillus terreu*, *Candida krusei* and *Cryptococcus neoformans* strains. Cytotoxicity evaluation showed that under 25 µg/mL are the safety doses that can be used for *in vitro* tests, besides that, extracts were able to induce cytokine stimulation. These results show that the ethyl acetate extract was the best formulation found in this study to be used against fungal infections, for antioxidant proposes and for promote immunostimulation.
Keywords: Anti-inflammatory profile; Antioxidant; Antimicrobial; Immunostimulation

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Introduction

The study of medicinal plants has been growing in different parts of the world. Associated with the progression of research in this area, also increases interest in the advanced knowledge about natural chemical compounds obtained from these medicinal plants used by communities for the maintenance of health and quality of life (Asadi-Samani et al., 2013; Porta et al., 2018)

The Fabaceae family has several species with medicinal properties, among them *Caesalpinia pulcherrima* (L.) Sw. (Figueiredo and Sáber, 2016). In Brazil, it is used as an ornamental plant and as a therapeutic agent in popular medicine for various diseases, such as fevers, infections and mouth ulcers (Moteriya and Chanda, 2017; Santos et al., 2013; Silva et al., 2015).

Studies have been reported the great potential of *C. pulcherrima* for some biological activities as antitumor, antiviral (Chiang et al., 2003), antibacterial (Ragasa et al., 2003), antifungical (Figueiredo and Sáber, 2016) and anti-inflammatory (Matiz et al., 2011), recently, Aguiar et al. (2019) reported the immunomodulatory effect from *C. pulcherrima* saline extract. On the present work, we aimed to investigate the phytochemical composition, antioxidant potential, cytotoxicity actions and antimicrobial properties of three organic extracts obtained from leaves of *Caesalpinia pulcherrima*. Thus, our objective was to evaluate the biotechnological potential of different *C. pucherrima* extracts, used in folk medicine for the treatment of diseases, to discover new sources of bioactive natural molecules.

Materials and Methods

Plant material and extracts preparation

Caesalpinia pulcherrima leaves were collected at Federal University of Pernambuco – Brazil (8º 3' S, 34º 50' W), an esxicate was deposited in the Geraldo Mariz Herbarium from Federal University of Pernambuco, under the voucher 82.702. For the extract preparations, the leaves were kept at room temperature for 2 days to remove humidity, later 10g of dried leaves was post in contact with different solvents: n-hexane, ethyl acetate, and ethanol in a Soxhlet apparatus. After 73 hours, the solvents were removed using a rotary evaporator under low pressure.

Phytochemical analysis

Total phenolic compounds measurement

The Folin-Ciocalteu method was used to measure total phenols content in accordance with Li et al. (2008) with modifications. Folin solution (2 mL; 1:10 v/v) was added to 0.2 mL of the diluted in water (1 mg/mL). After 4 min, the solution was added 1.6 mL of sodium carbonate (7.5%) and incubated for 120 min in the dark and room temperature. Absorbances of the samples were measured at 765 nm against a blank (reagent added to the sample solvent). A calibration curve was prepared by plotting the absorbance as a function of the gallic acid concentration (0-500 µg/mL) and then finding the linear equation $y = 0,0287x + 0,1657$; $R^2 = 0,9773$). The assay was performed in five replicates and the phenols contents are expressed in gallic acid equivalent (mg/g GAE).

Investigation of flavonoid content

Flavonoid content was determined using the methodology already described by Woisky and Salatino (1998) with modifications. In this method, 1 m L of aluminum chloride solution at 3% is added to the tubes containing the samples. After 30 minutes of incubation, at room temperature and protected from light, absorbance was measured at 425 nm. The curve with the quercetin compound (0-500 µg / mL) was performed to obtain the equation ($y = 0.003x + 0.0617$; $R^2 = 0.985$). The trials were done in quintuplicate. Flavonoids are expressed in equivalent to quercetin (mg QE/g extract).

Gas chromatography coupled to mass spectrometry (GC-MS)

GC-MS is routinely used to identify non-polar compounds in hexane extraction. The oven temperature was adjusted to 70º C, with an increase of 4º C / min to 280 º C and maintained for 15 min. Helium gas was used to drag, with a constant flow of 1.4 ML / min. The ionization temperature was maintained at 280 º C, the temperature of ionization energy was maintained a70 Ev and the ionization current at 0.7 Kv. The mass spectra were recorded from 300 m / z to 450 m /z. Individual components were identified by combining the mass spectraof 70 eV with those of the spectrometer database, using Wiley L-Built library.

Ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS)

UPLC-MS is made to identify polar compounds in ethyl acetate extracts and ethanolic

extract. To this end, a high-performance liquid chromatograph Acquity H-Class (Waters) (UPLC) was used, and a BEH column and particles were employed. The moving phases used were based on aqueous solution containing MeOH at 2%, ammonia format 5 Mm, 0.1% formic acid (eluent A) and metabolic solution containing formic acid, the eluent B.The temperature of the column was maintained at 40º C and the auto injector at 10ºº C. The UPLC system was coupled to the SQ Detector 2 quadruple mass spectrometer (Waters). Data acquisition was performed in complete scanning mode, seeking masses between 100 and 1000 Da. in ionization of the negative type. Subsequently, the software Mass Lynx TM (Waters) was used to obtain the chromatograms and spectra. The database of the Center for Strategic Technologies in the Northeast (CETENE), the Wiley L-Built library and the MS library of the NIST computer were used to interpret the spectra.

Antioxidant investigation

AAT, DPPH and ABTS methods

Extracts ability to free radical sequestration were measured using the AAT, DPPH and ABTS methods, as described by Cruz-Filho et al. (2018)

Lipid peroxidation assay

The antioxidant activity was determined using the ferric thiocyanate method described by Jayaprakasha et al. (2001) Aliquots of 0.2 mL (1 mg/mL) of the extracts were individually mixed with 2.5 mL linoleic acid emulsion (1 mg of linoleic acid and 50 mL of ethanol), 0.4 mL phosphate buffer 2.5 M (pH 7.0) and 0.196 mL of distilled water packed in screw-capped glass vials and incubated at 50 °C in an air circulation. Every 24h, 50 µL of this solution was removed and added to 50 µL of 75 % (v/v) ethanol, 50 µL of 30 % (w/v) ammonium thiocyanate and 50 µL of ammonium chloride solution of 0.02 M iron in 3.5 % HCL. After exactly 3 minutes at room temperature the absorbance at 500 nm was measured in spectrophotometer. The degree of inhibition of linoleic acid peroxidation was calculated using algebraic expression, Inhibition (%) = [(Ac – As) / Ac] × 100. Assay was performed in triplicate and Ascorbic acid and the BHT were used as standard molecules in the same pectin concentrations.

Antimicrobial assay

Obtainment of fungal and bacterial strains and sample preparation for microbial assay

Fungal strains were obtained from the culture collections of the Mycology Department and Microorganism Collection of Antibiotics Department of UFPE (UFPEDA), the isolated bacteria were provided from the culture collection of the Antibiotics Department, both belonging to the Universidade Federal de Pernambuco, Brazil. The pathogenic microorganisms used in this investigation and its respective register numbers were *Candida guilliermondii* (URM6558), *Candida tropicalis* (URM1150), *Aspergillus terreu* (URM4347), *Aspergillus tamarii* (URM4348), *Candida trusei* (URM4263), *Candida krusei* (ATCC6258), *Candida albicans* (URM4990), *Candida parapsilosis* (URM22019), *Staphylococcus aureus* (UFPE02); *Enterococcus fecalis* (UFPEDA09); *Pseudomonas aeruginosa* (UFPEDA416); Esch*erichia coli* (UFPEDA224); and *Klebsiella pneumoniae* (UFPEDA396).

Determination of minimum inhibitory concentration (MIC)

For evaluation the MIC of the extracts against the strains of bacteria, the method described by CLSI (2017) was used, where the strains were grown on Mueller Hinton agar. For the analysis of MIC in relation to fungal isolates, we followed the methodology described by CLSI (2008), using RPMI 1640 sterilized in 0.22µm membranes (Millipore, Darmstadt, Germany), with L-glutamine, without sodium bicarbonate and pH 7.0 ± 0.1 buffered with Morpholine Propane Sulfonic Acid (MOPS), 0.165 mol.L-1. In the 96-well microtiter plates, extracts were added in different concentrations (1.4 - 750 µg / mL), and after testing they were incubated at 35ºC. For bacterial isolates, the optical density was measured at zero time and after 24 hours of incubation, using a spectrophotometer (600 nm). For fungal isolates, MIC was defined visually with 50% inhibition in relation to the negative control, after incubation.

Cytotoxic and immunostimulatory analysis

Animals, culture and in vitro stimulation of mouse splenocytes

Female BALB/c mice (6-8 weeks old) were raised and maintained at the animal facilities of the Keizo Asami Immunopathology Laboratory – LIKA located in Federal University of Pernambuco, Brazil. Mice were kept under standard laboratory conditions (20–22°C and 12 h day and night cycle) with free access to a standard diet (Labina/Purina, Campinas, Brazil) and water. Mice splenocytes obtention procedure was made in accordance with Melo et al. (2011) protocol. Briefly, mice spleens were obtained, macerate in douncer and the homogenate was centrifugate (1600 rpm / 30 min) in Ficoll 10.77 gradient (BD®). After cell ring obtention, cells were centrifuged twice in PBS 1X (1000 rpm / 10 min) and counted using tripan blue in Neubauer chamber. Cells (10^6) were used in each well of culture plate. All experimental procedures were performed with the approval of Ethics Committee of Animal Use (CEUA) of Federal University of Pernambuco (protocol number: 0048/2016).

Analysis of cell viability

This procedure was performed in accordance to Melo et al. (2011)protocol. The cultures of splenocytes treated with the extracts, as well untreated cells were centrifuged at 450 x g and 4°C for 10 min. After discarding the supernatant, the pellet was resuspended in 1 ml of PBS 1X, and the cells were centrifuged again (450 x g, 4°C, 10 min).

The pellet was resuspended in 300 µL of binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$), transferred to a labelled cytometer tube and incubated with propidium iodide (PI, 20 µg/mL). Flow cytometry was performed in a FACSCalibur platform and the results analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA, USA). PI-positive cells were considered necrotic while PI-negative cells were considered viable cells.

Cell proliferation analysis

This procedure was performed in accordance to Aguiar et al. (2019) protocol. Cells obtained (10^6) were stained with CFSE dye, treated with different concentrations of extracts (12.5, 6 and 3 µg/mL), and incubated in CO_2 chamber for 24 hours. After culture time, cells were centrifuged (300 x g/5 min), washed in PBS 1X and were carried to acquisition on FACS Calibur platform (BD®) using 30000 events. Results were analyzed using Flowing 2.5 software.

Measurement of cytokine levels

Supernatants collected from cultures treated with three extracts (in 12.5 µg/mL) and untreated cultures (cells + medium) were collected to quantify cytokines. The evaluation was performed using the Th1/Th2/Th17 Human Cytometric Bead Array (CBA) cytokine kit (BD®) for the detection of interleukins IL-4, -6, -10, -17A TNF-α and IFN-γ. Measurements were performed according to the manufacturer's instructions, all data were acquired on the FACS Calibur platform and analyzed using Flowing 2.5 software.

Statistical analysis

To test the normal hypothesis on the variable involved in this study we applied the the Shapiro–Wilke test. Means of samples were analyzed using non-parametric tests. The statistical difference between two groups was analyzed by Wilcoxon test and that among more than three groups by one-way analysis of variance (ANOVA). All the conclusions were considered with a significance level of 5%. For statistical analysis was used GraphPad Prim 8® software.

Results and discussion

Phytochemical analysis

The phytochemical analysis revealed the presence of 3 compounds on the ethanolic extract, galic acid, syringic acid and isoquercetin, while the ethyl acetate extract has shown the presen of 4 compounds, besides isoquercetin and gallic acid , it also presented the quercetin and acacetin, as seen in table 1). Our results corroborate with the literature, that reports the presence of flavonoids and phenolic compounds in *C. pulcherrima* extracts (Sharma and Rajani, 2011). This class of compounds are widely used as natural antioxidants in several industries, besides being frequently associated with anti-inflammatory and antimicrobial effects (Bouarab-chibane et al., 2019; Maleki et al., 2019; Nohynek et al., 2001; Pietta, 2000; Procházková et al., 2011). As for the hexanic extract, the analysis revealed the presence on 1,4-dimethyl Benzene being the major compound identified (table 1).

As for phenolic and flavonoids content, the ethyl acetate extract presented the highest concentration of both class of molecules, with 58.53 mg GAE/g of phenolic compounds, and 45.05 mg QE/g (table 2), followed by ethanolic extract and hexanic extract. As stated before, the phenolic and flavonoids compounds present several biological activities, and ours results shows that *C. pulcherrima* is rich in phenolic compounds and flavonoids. The biological activities further investigated may vary according to the metabolites founded on our extract, as well the total phenolic and flavonoid content.

Table 1. Phytochemical analysis of hexanic, ethyl acetate, ethanolic and hexanix extracts of *C. pulcherrima*.

Extract	Compound name	Retention time	Formula	Mass	Area
Ethanolic	Gallic acid	3.09	$C_7H_6O_5$	170	4042.9
	Syringic acid	5.42	$C_9H_{10}O_5$	198	1738.1
	Isoquercetin	6.34	$C_{21}H_{20}O_{12}$	464	5088.4
Ethyl acetate	Acacetin	8.85	$C_{16}H_{12}O_5$	284	7479.0
	Gallic acid	3.09	$C_7H_6O_5$	170	6666.5
	Isoquercetin	6.34	$C_{21}H_{20}O_{12}$	464	4987.6
	Quercetin	7.44	$C_{15}H_{10}O_7$	301	1527.6
Hexanic	1,4-dimethyl Benzene	3.60	C_8H_{10}	106	2373.8
	2,4-bis (1,1-dimethylethyl) Phenol	24.38	$C_{14}H_{22}O$	206	624.2

Table 2. Total phenol and flavonoid contents present in organic extracts of *C. pulcherrima* leaves.

Extract	Total phenolic content (mg GAE/g)	Flavonoid content (mg QE/g)
Hexanic	47.47 ± 5.29	34.02 ± 0.05
Ethyl acetate	58.53 ± 0.18	45.05 ± 0.97
Ethanolic	56.85 ± 1.77	36.83 ± 2.43

Antoxidant compounds are known by their free radical scavenge ability, promoting a regulation on the Reactive Oxygent Species (ROS). Our results shows that *C. pulcherrima* extracts present antioxidant properties in diferent models, the results of the antioxidants assay are disposed in table 3.

The ethyl acetate extract presented a total antioxidant activity of 49.33%, as for the DPPH and ABTS assays, the same extract showed an IC_{50} of 342.89 and 215.53 µg/mL respectively, as also, it was able to inhibit the lipid peroxidation in 32.64%. As for the ethanolic extract, the obtained results were slightly better when compared to the ethyl acetate extract, with a total antioxidant activity of 59.89%, and IC_{50} of 393.50 µg/mL in DPPH assay, and 299.28 µg/mL in the ABTS assay, as for the lipid peroxidation inhibition assay, the extract was able to achieve 51.03% of inhibition.

As for the hexane extract, it induced low total antioxidant activity and low lipid peroxidation inhibition, as also no activity was detected for the DPPH scavenging assay, still, is worth to note that in the ABTS model, the extract presented a eficiency of 61%, with a IC_{50} of 104.65 µg/mL, being the highest among the tested extracts. According to Romero-Correa et al. (2014) showed that the 2,4-bis (1,1-dimethylethyl) Phenol, a molecule present in the hexanic extract, is produced in plants as a response to pathogens, and it regulates the concentrations of H_2O_2.

Comparing with previous studies, Phuse and Khan (2018) observed that the ethyl acetate extract, obtained from leaves and flowers of *C. pulcherrima*, present a strong antioxidant activity, with a IC_{50} of 33.05 and 39.48 µg/mL respectively, in a superoxide radical assay. In another study Torre et al. (2017), related the levels of flavonoids in *C. pulcherrima* with the high antioxidant activity in extracts obtained form leaves, flowers and seeds, in different antioxidant models, like DPPH (IC_{50} = 10.46 µg/mL), hydrogen peroxide scavenging activity (IC_{50} = 26.44 µg/mL) and Reducing power assay (IC_{50} = 113 µg/mL).

Table 3. Antioxidant activities promoted by organic extracts of *C. pulcherrima* leaves.

Extract	TAA	DPPH		ABTS		Lipid Peroxidation inhibition
	(%)	IC50 (µg/mL)	% (0.5 µg/mL)	IC50 (µg/mL)	% (0.5 µg/mL)	(%)
Hexanic	27.06	*ND*	*ND*	104.65	61.97 ± 1.80	3.33 ± 0.02
Ethyl acetate	49.33	342.89	73.85 ± 0.52	215.53	40.62 ± 1.61	32.64 ± 0.02
Ethanolic	59.89	393.50	93.01 ± 0.11	299.28	42.49 ± 1.12	51.03 ± 0.19
BHT	85.15	87.10	73.96 ± 1.17	7.67	95.55 ± 0.07	50 ± 0.006
Ascorbic Acid	100	90.00	72.80 ± 1.17	90.93	92.16 ± 0.25	71.70 ± 0.004

TAA – Total antioxidant activity; DPPH – 2,2-diphenyl-1-picrylhydrazyl radical; ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline)- 6-sulfonic acid radical; BHT – butylated hydroxytoluene

In general, the extracts showed inferior antioxidant activities when compared to the BHT and ascorbic acid, however the ethanolic extract showed a moderate activity in the TAA and lipid peroxidation models, as well the hexanic extract showed a IC_{50} value next to the ascorbic acid in the ABTS model. It's important to highlight that, when a plant extract is obtained, it also presents small concentrations of macro and micronutrients that modulates the observed activity results, making it stronger or weakest, with that in mind, is it possible that, the molecules presents in the extract, once purified, or higher concentrations, may present a stronger antioxidant activity (Cataldi et al., 2003).

Antimicrobial activity

Antimicrobial resistance in several microorganism is a concern phenomenon that have driven research to prospect potential new antibiotics from various sources, with plants products being one of those sources. Plants possess several compounds that are able to inhibit bacteria and fungi growth (Aldholmi et al., 2019). On our study, the extracts did not induce antibacterial activity on the tested strains. However, it showed antifungal activity against some species of *Aspergillus*, *Candida* and *Cryptococcus*, as seen in table 4. The lowest MIC detected was from the ethanolic extract against *C. neoformans* HC43, at concentration of 7.81 µg/mL, while the hexanic and ethyl acetate extracts demanded twice the concentration. As for the strains *C. neoformans* HC44 and HC47, the MIC value for all extracts was of 62.6 µg/mL.

The compounds present on out extracts are associated with antifungal activity in the literature. The gallic acid, present in ethyl acetate and ethanolic extracts, have showed antifungal activity *in vitro* and *in vivo* models, Li et al. (2017) associated gallic acid's antifungal effect to the capacity of inhibiting the ergosterol biosynthesis pathway, which is a molecule present in fungi's cell membrane, leading to a decrease on cell viability. Another molecule present in both extracts is the isoquercetin, which according to Kim et al. (2019), this molecule induce an accumulation of ROS and compromising the cell membrane permeability, and its effect is increase when combined another antifungical drugs. Comparing with the literature, the methalonic extract from *C. pucherrima* have showed antifungical activity against *C. neoformans* in the agar well diffusion assay (Vivek et al., 2013). As well, Aguiar et al. (2019) showed that *C. pulcherrima* saline extract present antifungical activity against different *Candida* species.

Taken together, the effectiveness from our extracts compared to those in the literature is probably because the difference in the chemical composition, as well the concentrations of each compound present on the extract, a higher variety of compounds can promote a strong antifungical response due to the probability of those compounds

interact in different targets, as well, a small variety of compound can provide a stronger response in one single site, therefore, the intensity of antifungical activity varies according to the composition and compounds concentration (Chouhan et al., 2017).

Table 4: Minimum inhibitory concentrations of organic extracts obtained of *C. pulcherrima* leaves

Species	Identification	Extracts of *C. pulcherrima* leaves (µg/mL)		
		Hexanic	Ethyl acetate	Ethanolic
Candida tropicalis	URM1150	325	750	750
Candida guilliermondii	URM6558	*ND*	750	*ND*
Candida albicans	URM4990	*ND*	750	*ND*
Candida krusei	URM4263	750	750	*ND*
	ATCC6258	187.5	93.75	750
Cryptococcus neoformans	HC43	15.6	15.6	7.81
	HC44	62.5	62.5	62.5
	HC47	62.5	62.5	62.5
Aspergillus terreu	URM4347	187.5	93.75	*ND*
Aspergillus tamarii	URM4348	*ND*	750	*ND*
Candida parapsilosis	URM22019	*325*	750	*ND*

ND = Not detected or above 750 µg/mL

Cytotoxicity and immunostimulatory effect

Comparing to the control group, all extracts didn't induced toxic effects at concentrations equal or superior to 25 µg, as for the concentration of 50 µg, the extract induced a decrease in cell viability (figure 1). Few cytotoxicity studies of *C. pulcherrima* extracts have been published so far, Aguiar et al. (2019) didn't detect cytotoxicity from *C. pulcherrima* saline extract, at concentrations of 50, 25, 12.5 and 3 µg/mL, against balb/c mice splenocytes.

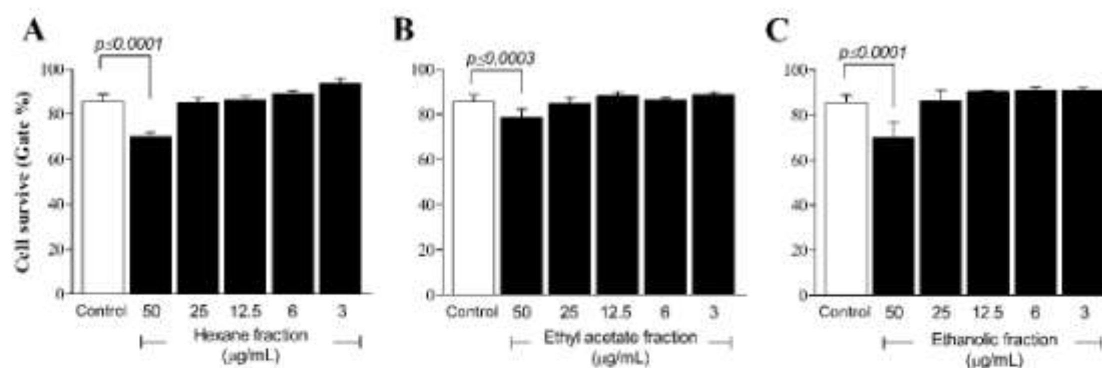

Figure 1. Cytotoxicity assay using splenocytes from Balb/c mice treated with of *C. pulcherrima* in different concentrations. White vertical bars represent control group and black vertical bars represent cells treated with different concentrations of the extracts. Vertical bars represent the average of two independent experiments performed in triplicate. *#p = 0.002.*

Based on cytotoxicity data, the immunostimulatory assay was performed using concentrations of 12.5 µg. As seen in figure 2, the hexanic was able to induce the production of all analyzed cytokines, when compared to control and other extracts. The ethyl acetate extract was able to promote the production of TNF-α and IL-17, while the ethanolic extract only induced IL-6 production.

Interleukins are proteins that play an important role in leukocytes signaling pathway, according to Akdis et al. (2016) while the IL-4, IL-6 and IL-17 are directly involved in proinflammatory systems, inducing the release of some cytokines and recruitment of neutrophils and monocytes, the IL-10 possess a immunosuppressive effect. While the TNF-α is cytokine with a role of inducing apoptosis in cancerous cells, and it is involved in auto immune diseases (Chu, 2013), as for the IFN-ϒ, it is involved in several immunogenic effects, among then, activation of genes involved in the anti-microbial activities (Kak et al., 2018).

In the last decades, several studies have been carried out with the aim of understanding the effects of natural products over the immune system, Hewage et al. (2019) evaluated the effect from S*argassum horneri* extract in the immunological system, and the authors observed an increase on immunologic cells activity, like the T-helper, cytotoxic T cells, macrophages and eosinophils, as also the cytokines TNF-α, IFN-ϒ, IL-4, IL-5, IL-13 and IL-6. Similar results were also find by Sousa et al. (2020), investigating the influence of *Passiflora edulis* over IL-4, IL-6, IL-10, IL-17, IFN-ϒ and TNF-α levels, the authors noticed an increase in all cytokines concentrations, still, the authors stands that, since IL-10 and IL-4 were present in higher

concentrations comparing to the others cytokines, *P. edulis* extracts present a predominant anti-inflammatory status. Different from our results, since only IL-10 was only present in higher concentration in the cells treated with hexanic extract, therefore the ethyl acetate and ethanolic extract induces more a pro-inflammatory effect, while the hexanic extract, due to the IL-10 induction present an anti-inflammatory status.

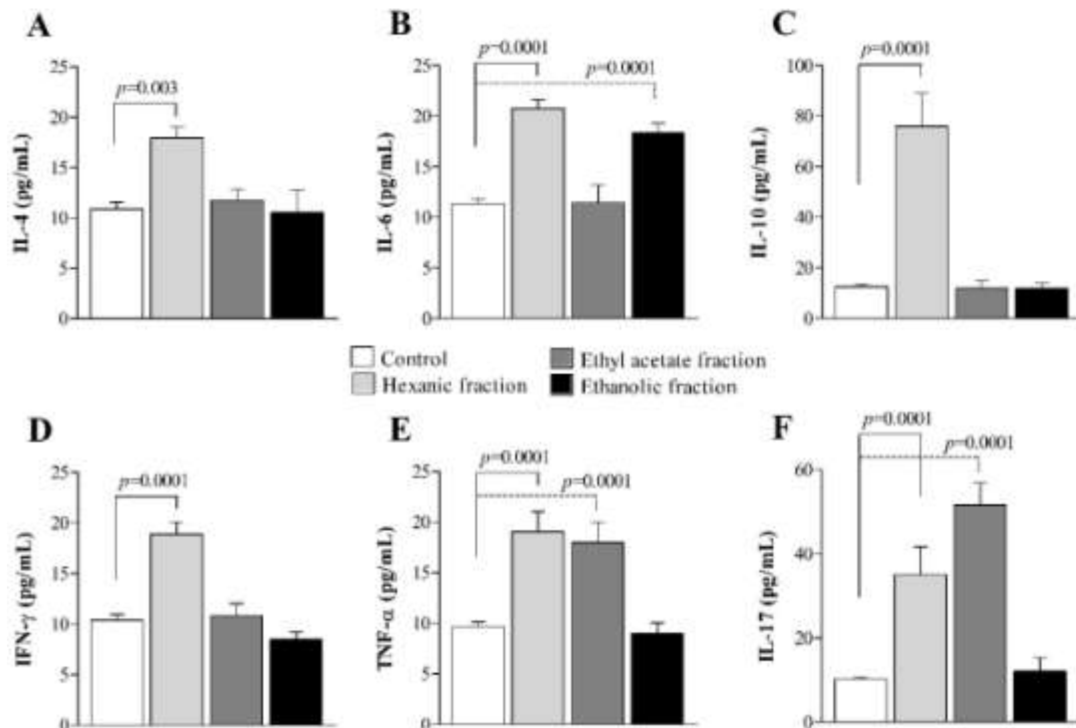

Figure 2. Profile of cytokine production in culture supernatants of splenocytes from Balb/c mice treated with organic extracts of *C. pulcherrima* leaves at a concentration of 12.5 µg/mL. Figure (A, B, C, D, E and F) can be observed levels of IL-4, IL-6, IL-10, IFN-γ, TNF-α and IL-17, respectively. Vertical bars are representing the medium of two independent experiments performed in triplicate..

Conclusion

The phytochemical characterization of *C. pulcherrima* leaves revealed the presence of several compounds, among then, the organic acids, flavonoids and phenols. A high antioxidant and antifungical potential have been identified in organic extracts obtained from leaves. Moreover, a significant immunostimulatory effect was promoted especially by hexanic extract, suggesting a possible wound healing potential by this plant.

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