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Original Research Article

Study of the antileukemic activity of *Mimosa caesalpiniifolia* Benth. ethanolic extract and fractions

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ABSTRACT

Mimosa caesalpiniifolia Benth. is a native plant to northeastern Brazil, traditionally used in folk medicine, with several pharmacological activities reported including antibacterial, anti-inflammatory, and antitumor. The present study evaluated the antileukemic potential of *M. caesalpiniifolia* Benth. ethanolic extract (EtOH) and its *n*-hexanic (HexF) and dichloromethane (DCMF) fractions. Previous analysis by our team revealed the constituents of high relative abundance in EtOH, HexF, and DCMF, like phytol (11.7%), lupeol (14.7%), and betulinic acid (70.3%), respectively. In the MTT cell viability test, EtOH, HexF, and DCMF induced dose-dependent cytotoxicity in human chronic myeloid cells (K562), with IC_{50} of 153.6 ± 0.1, 118.40 ± 0.2, and 40.0 ± 0.1 µg/mL, respectively ($p<0.05$). Additionally, DCMF (6-800 µg/mL) presented minor toxicity against normal human erythrocytes and murine macrophage cells. DCMF induced similar antileukemic effects (IC_{50}=64.2 ± 5.0 µg/mL) against human acute myeloid cells (HL-60). However, it did not exert antitumor activity on murine sarcoma (S180) cells ($p>0.05$).

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1. Introduction

Leukemia is a type of disease that originates in the bone marrow and corresponds to around 2.5% of all cases of cancer. The type of leukemia depends on the cell type of origin, such as lymphoblastic leukemia (Terwilliger and Abdul-Hay, 2017). Regarding the development time, leukemia can occur acutely or chronically, whereas with respect to the cell type affected, leukemia may be classified as lymphoid or myeloid (Leonard et al., 2017). The diagnosis and treatment of leukemia presented a considerable progress during the recent decades, although new therapeutic strategies with less side effects and higher selectivity are still required (Reckzeh et al., 2012; Coulidiati et al., 2015).

The therapeutic potential of medicinal plants and some of their secondary metabolites with several pharmacological properties such as flavonoids, alkaloids, terpenes, tannins and lignans has been the subject of incessant studies involving the prospection of new drug candidates (Mohammadhosseini et al., 2016; Aidi Wannes et al., 2017; Mohammadhosseini, 2017a; Nunes and Miguel, 2017). These substances are found as bioproducts of essential oils and plant extracts, possessing great potential for different therapeutic/medicinal purposes (Camilo et al., 2017; Ganesan and Xu, 2017; Mohammadhosseini, 2017b; Mohammadhosseini et al., 2017; Pavunraj et al., 2017). The growing interest with medicinal plants has been stimulating researchers and international pharmaceutical industries to invest in

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Fig. 1. *M. caesalpiniifolia* Benth. photographs of flowers (A), leaves (B) and stem (C).

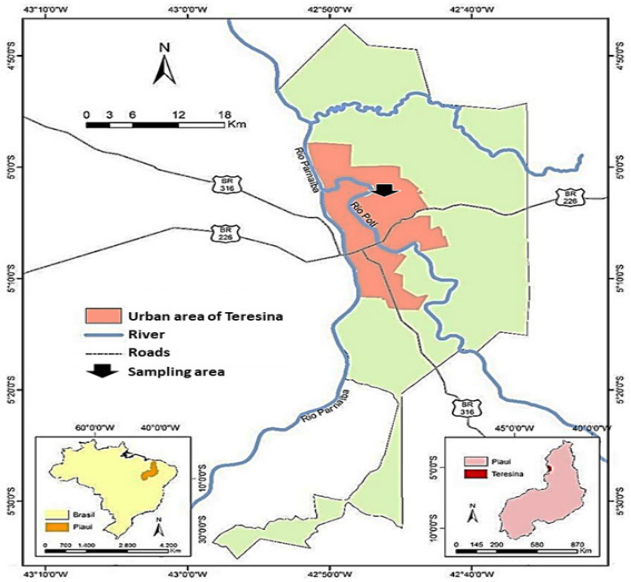

Fig. 2. Geographical map of the sampling area. Source: Messias (2015).

the research of new drugs.

Mimosa caesalpiniifolia Benth. is a native plant to northeastern Brazil (Fig. 1), from the genus *Mimosa* L. (Fabaceae), and popularly known as "unha-de-gato", "sabiá" or "sansão do campo" (Sousa et al., 2013). The bark of this species is widely used in traditional medicine, as a bleeding stancher, wound washing, antibacterial and anti-inflammatory agents (Silva et al., 2016). In addition, the bark infusion of *M. caesalpiniifolia* Benth. is used internally as a tonic solution as also for the treatment of bronchitis (Monção et al., 2014). The vapor obtained from the flowers of *M. caesalpiniifolia* Benth. is also used by the native people to treat hypertension (Monção et al., 2015; Silva et al., 2016).

Among the pharmacological properties of *M. caesalpiniifolia* Benth. previously demonstrated are antioxidant (Lin et al., 2011; Aguiar et al., 2012), antimicrobial (Mohan et al., 2011), antiulcerogenic (Vinothapooshan and Sundar, 2010), antidiabetic (Ahmed et al., 2012), anti-inflammatory (Rakotomala et al., 2013), antinociceptive and antitumor (Silva et al., 2016). However, the antileukemic effects of *M. caesalpiniifolia* Benth. have not been reported so far. Therefore, the present study aimed to evaluate the antitumor potential of *M. caesalpiniifolia* Benth. ethanolic extract and fractions against human leukemia cell lines.

2. Experimental

2.1. Chemicals and plant material

Doxorubicin, etoposide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), trypan blue, and RPMI 1640 were purchased from Sigma-Aldrich (USA). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco-BRL (USA).

The stem bark of *M. caesalpiniifolia* Benth. was collected from native forest of Federal University of Piauí (UFPI), Teresina, Brazil (5º02'32.6"S 42º46'59.8"W) in May 2010 (Fig. 2). The plant specimen was identified and deposited in the Graziela Barroso Herbarium and a voucher specimen with number TEPB 26,824.

2.2. Extraction and isolation of chemical constituents

The extracts and fractions of *M. caesalpiniifolia* Benth. used in this study were previously obtained and reported by Monção et al. (2014, 2015). In brief, the stem bark was dried in air at room temperature, pulverized and later extracted exhaustively with ethanol at 1:4 (w/v) plant material/solvent. The filtered and combined ethanolic extracts were concentrated under reduced pressure on a rotary evaporator and lyophilized, yielding 36.7 g (4.2%) of dried ethanolic extract (EtOH). The stem bark ethanol extract (30.0 g) was suspended in MeOH-H_2O (2:1, v/v) and subjected to successive partitioning, resulting in the *n*-hexane (HexF) and dichloromethane (DCMF) fractions, yielding 2.7 g (7.4%) and 3.9 g (10.6%), respectively (Monção et al., 2014, 2015).

For the biological activity evaluation, all tested drugs were initially diluted in DMSO, forming a stock solution of 80 mg/mL that was kept at -20 ºC, and further diluted to the desired concentrations immediately before the experiments. The total amount of DMSO used in all procedures did not exceed 0.1% (vehicle).

2.3. Animals

Around 4 weeks old male BALB/c Swiss mice were used in this study (25 g), maintained at the Medicinal Plants Research Center (NPPM/UFPI) *bioterium*, and kept at a controlled temperature (24 ± 1 ºC), in a 12 h light/dark cycle, with water/food *ad libidum*. All protocols were approved by the Animal Research Ethics Committee (CEEA/UFPI: 053/2015, 08/2015).

2.4. Cell culture

Human leukemia cell lines HL-60 (acute myeloid) and K562 (chronic myeloid), were acquired from Banco de Células do Rio de Janeiro (BCRJ, Brazil). Murine sarcoma (S180) cell line was also acquired from BCRJ. All cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin and placed in humidified air at 37 ºC with 5% CO_2 atmosphere (Coulidiati et al., 2015).

2.5. MTT assay of cell viability

To assess the cytotoxic effects of *M. caesalpiniifolia* Benth. EtOH and fractions (HexF, DCMF), the MTT assay was performed as previously reported by Mosmann (1983) with minor adjustments. Briefly, $5x10^4$ cells were seeded in 96-well plates and incubated with the drugs at concentrations ranging from 0.5 to 500 µg/mL, for 24 h at 37 ºC and 5% CO_2 atmosphere. Etoposide (ETO, 50 µg/mL) and doxorubicin (DOXO, 50 µg/mL) were used as the known cytotoxicity drug reference materials. Afterwards, MTT (5 mg/mL) was added and incubated for 4 h in the same conditions as described above. Formazan crystals were dissolved in DMSO for additional 30 min, and the optical density was recorded in a microplate spectrophotometer (BioTek, Winooski-VT, USA) at 550 nm (Gonçalves et al., 2016).

2.6. Determination of drug toxicity in macrophages

Murine macrophages were collected from the peritoneal cavities of mice and plated in 96-well culture plates, at $2x10^6$ cells/mL concentration (Alves et al., 2017). Cells were cultured in RPMI 1640 supplemented with 10% inactivated FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Culture plates were incubated at 37 ºC and 5% of CO_2 during 3 h for cell adhesion. After adhesion period, macrophages were incubated with *M. caesalpiniifolia* Benth. EtOH and fractions (HexF, DCMF), at concentrations ranging from 6 to 800 µg/mL, for 24 h. After this period, the cytotoxicity was determined by the MTT assay as described in methods.

2.7. Red blood cell lysis assay

The hemolytic activity was evaluated by mixing 80 µL of a 5% suspension of fresh human red blood cells (O+) in PBS, with 20 µL of *M. caesalpiniifolia* Benth. ethanolic extract and fractions (EtOH, HexF and DCMF), at concentrations ranging from 25 to 800 µg/mL, for 1 h at 37 ºC and 5% of CO_2. The reaction was slowed by adding 200 mL of PBS and the suspension was centrifuged (1000 g for 10 min). The supernatant was then transferred to a 96-well plate and cell lysis was quantified by spectrophotometrical measurement of absorbance at 540 nm. The maximal lysis and blank control were obtained by replacing the extract sample with an equal volume of PBS or distilled water, respectively. The hemolysis percentage was determined as previously described (Rodrigues et al., 2015).

2.8. *Ex vivo* assay of sarcoma 180 in mice

Initially, S180 cells were kept in the peritoneal cavities

Table 1

Assessment of the main chemical compounds (%) from the ethanolic stem bark extract (EtOH) of *Mimosa caesalpiniifolia*, compared with its hexane (HexF) and dichloromethane (DCMF) fractions.

Compound	Molecular formula	EtOH (%)	HexF (%)	DCMF (%)
Lactic acid	$C_{29}H_{22}O_3$	9.2		
Methyl palmitate	$C_{17}H_{34}O_2$		6.1	
Neophytadiene	$C_{20}H_{38}$	4.0		
Methyl linoleate	$C_{19}H_{34}O_2$		8.1	
Methyl oleate	$C_{19}H_{36}O_2$		5.1	
Hexadecanoic acid	$C_{19}H_{40}O_2$	6.4		
Methyl docosanoate	$C_{23}H_{46}O_2$		3.9	
Octadec-9-enoic acid	$C_{18}H_{34}O_2$	4.1		
Phytol	$C_{20}H_{40}O$	11.7		
Methyl tetracosanoate	$C_{25}H_{50}O_2$		5.0	
Methyl hexacosanoate	$C_{27}H_{54}O_2$		3.3	3.1
Sigmasterol	$C_{29}H_{48}O$		4.8	
3,4,5-Trihydroxy benzoate, ethyl	$C_{19}H_{34}O_5$	6.1		
Lupenone	$C_{30}H_{48}O$		5.4	
Lupeol	$C_{30}H_{50}O$		14.7	3.3
Methyl octacosanoate	$C_{29}H_{58}O_2$			3.2
Betulinic acid	$C_{31}H_{50}O_3$		4.3	70.3
β-Sitosterol	$C_{29}H_{50}O$	6.8	3.0	
α-Tocopherol	$C_{32}H_{56}O_2$	7.3		
1-Triacontanol	$C_{33}H_{70}O$	6.5		

Compound identification in the GC-qMS analysis was performed using retention time and interpretation of the mass spectra (molecular ion [M+-], base peak and main fragments) in comparison with mass spectra of isolated compounds, computational libraries and literature data. The compounds with area (%) less than 3.0 are not shown (Monção et al. 2014, 2015).

of mice in the Laboratory of Experimental Cancerology (UFPI, Brazil). Before experimentation, ascites were induced in the animals by i.p. injection of $5x10^4$ cells (Ferreira et al., 2011). After 7-10 days, the animals were euthanized by cervical dislocation and the ascitic fluid immediately removed by aspiration. Then, the cells were centrifuged (1500 rpm/5 min), washed twice in 10 mL sterile PBS and counted in a Neubauer chamber using Trypan Blue (0.4%; 1:1). The cell concentration was adjusted to $1x10^6$ cells/mL in supplemented RPMI 1640 medium, plated in a 96-well plate, and the test drugs (EtOH, HexF and DCMF) were added to each well (0.5-500 µg/mL) for 72 h and further proceeded to the MTT viability test, as mentioned above.

2.9. Statistics

Statistical difference was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test and significance was considered when $p<0.05$. The IC_{50} values were acquired by plotting normalized data to the Hill equation (Eqn.1):

$$f=Min+(Max-Min)/(1+(IC_{50}/[drug]^n) \quad \text{(Eqn.1)}$$

Where, Max and Min represent the maximum and minimum values, respectively; IC_{50} the half-maximal effective concentration of the drug tested, and n the Hill coefficient (Graphpad Prism, USA).

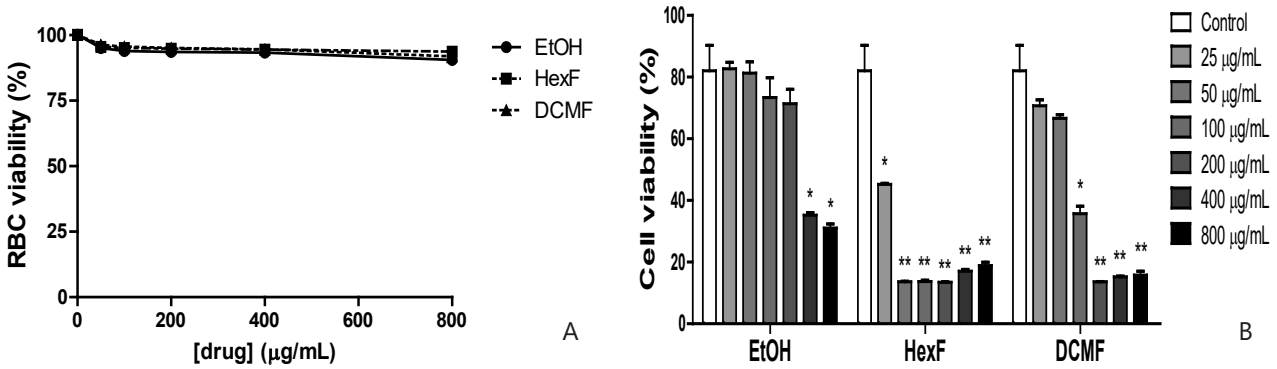

Fig. 3. Toxicity evaluation of *M. caesalpiniifolia* Benth. extract and fractions against normal human and animal cells. Effects of EtOH, HexF, and DCMF (25-800 µg/mL) against human erythrocytes (A) and murine macrophage cells (B). Macrophage viability was quantified by the MTT assay and data are displayed as mean ± SEM, obtained from two independent (n=2) experiments in triplicate. ANOVA: Dunnett's test, *p<0.05; **p<0.01 (from control).

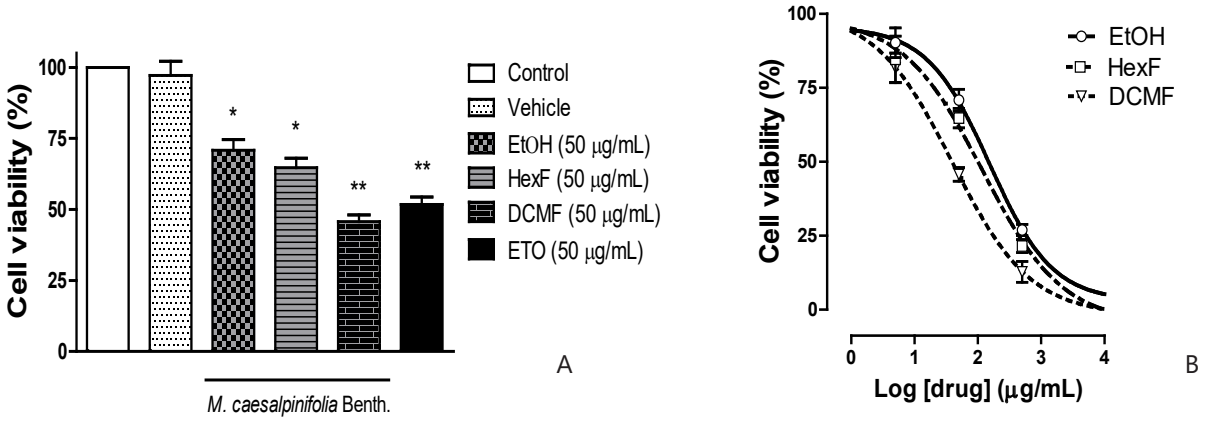

Fig. 4. Effects of *M. caesalpiniifolia* Benth. extract and fractions against K562 cells. (A) Cell viability (%) of K562 cells after 24 h incubation with EtOH, HexF, and DCMF from *M. caesalpiniifolia* Benth. stem bark. Vehicle (DMSO 0.1%) and etoposide (ETO) were used as negative and positive control, respectively. (B) Dose-response curves of EtOH, HexF, and DCMF after 24 h incubation (5-500 µg/mL) in K562 cells. Cell viability was quantified by the MTT assay and data are displayed as mean ± SEM, obtained from three independent (n=3) experiments in triplicate. ANOVA: Dunnett's test, *p<0.05; **p<0.01 (from control).

3. Results and Discussion

3.1. *M. caesalpiniifolia* Benth. EtOH, HexF and DCMF: major constituents and cytotoxicity

The genus *Mimosa* is known to have a high concentration of polyphenols, such as flavonoids, lignans, alkaloids, terpenoids, steroids and saponins (Monção et al., 2015). As previously reported by our team, the relative abundance (%) and the molecular formula of the major compounds identified in the *M. caesalpiniifolia* Benth. stem bark are presented in Table 1. The constituents of high relative abundance in the EtOH extract were phytol (11.7%), lactic acid (9.2%), α-tocopherol (7.3%) and sitosterol (6.8%). In the HexF and DCMF extracts, the major components identified were lupeol (14.7%) and betulinic acid (70.3%), respectively (Table 1).

Thus, lupeol and betulinic acid appear to be the major components and presumably responsible for the cytotoxic effects reported in this study. In fact, betulinic acid has been extensively studied for its antitumor and antileukemic properties. Ehrhardt and coworkers (2004), demonstrated that betulinic acid was more potent than 9 out of 10 standard therapeutics and especially efficient in tumor relapse, which acts by triggering the mitochondrial pathway of apoptosis in cancer cells. In contrast to its cytotoxicity against a variety of cancer types, normal cells and tissue are relatively resistant to betulinic acid, suggesting a strong therapeutic potential (Fulda, 2008). It was also shown that betulinic acid has cytotoxic activity against HL-60 and K562 human leukemic lines, with IC_{50} values of approximately 21 µg/mL (Faujan et al., 2010; Wu et al., 2010).

The second most important drug identified in *M. caesalpiniifolia* Benth. stem bark, lupeol, is also a highly studied compound with established antitumor and antileukemic effects (Aratanechemuge et al., 2004; Saleem, 2009). In its mechanism of action, lupeol is associated to a modulatory signaling pathway of nuclear factors such as kappa-B (NFκB) and the phosphatidylinositol 3-kinase PI3K/Akt (Saleem et al., 2004). Against leukemic K562 cells, lupeol exhibited an IC_{50} of about 100 µg/mL (Lin et al., 2001), and therefore, considerable less potent than betulinic acid.

3.2. Assessment of toxicity in normal cells

During evaluation of *M. caesalpiniifolia* Benth. extract and fractions cytotoxicity against normal cells, it was possible to demonstrate that both EtOH, HexF and DCMF, when incubated at concentrations ranging from 25 to 800 µg/mL, were not able to promote any damage in human erythrocytes ($p>0.05$) (Fig. 3A). In contrast, HexF induced high toxicity in macrophages, reducing the cell viability from the concentration of 25 µg/mL ($p<0.05$), whereas DCMF induced cell damage only at higher concentrations ($p<0.05$) (Fig. 3B). EtOH promoted less toxicity on macrophages, only from 400 µg/mL ($p<0.05$). Therefore, the DCMF fraction was the less toxic drug tested against normal human and animal cells. Previous studies reported that the cytotoxicity effect of fruits extracts on murine macrophage cells revealed the presence of lupenone and lupeol only in the toxic extracts (Diop Ndiaye et al. 2016). This may explain the high toxicity presented by HexF in these cells, since lupenone and lupeol are among its main constituents (Table 1).

3.3. Evaluation of the antileukemic activity

Cytotoxicity assays performed by MTT method to evaluate the antitumor potential of *M. caesalpiniifolia* Benth. against human chronic myeloid leukemia cells

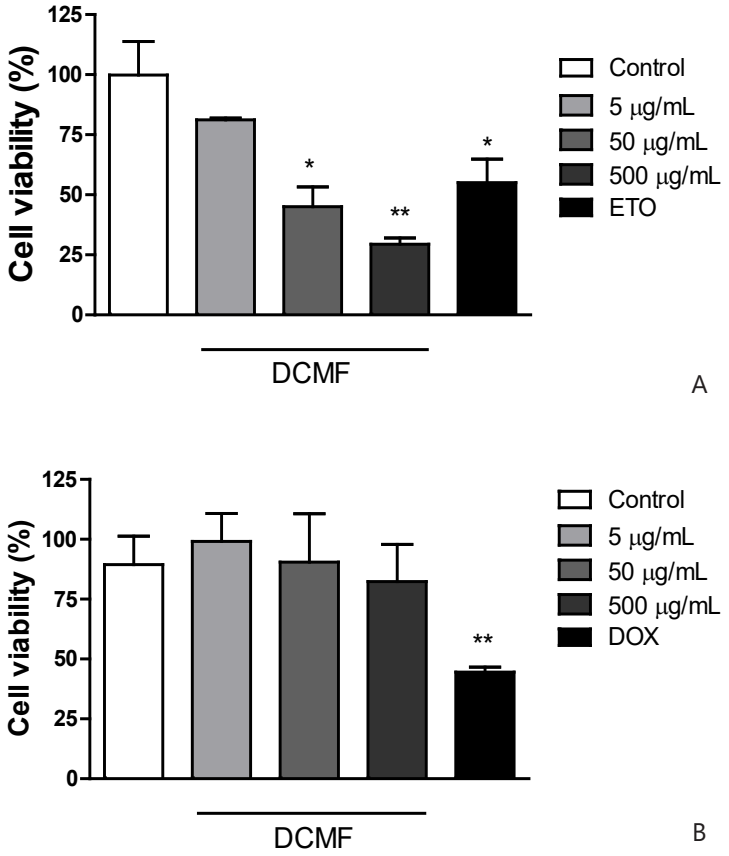

Fig. 5. Effects of DCMF against HL-60 and S180 cells. Cell viability (%) of HL-60 (A) and S180 (B) cells after 24 h incubation with DCMF (5-500 µg/mL) from *M. caesalpiniifolia* Benth. stem bark. Etoposide (ETO) and doxorubicin (DOXO) were used as positive control. Cell viability was quantified by the MTT assay and data are displayed as mean ± SEM, obtained from three independent (n=3) experiments in triplicate. ANOVA: Dunnett's test, *p<0.05; **p<0.01 (from control).

(K562), showed that EtOH, as well as HexF and DCMF, incubated at 50 µg/mL for 24 h reduced the cell viability from 100% (control) to 70.8 ± 3.7%, 64.7 ± 3.3% and 45.8 ± 2.3% (p<0.05), respectively (Fig. 4A). As expected, the positive control etoposide (50 µg/mL) reduced cell viability to 51.7 ± 2.6% from control (p<0.05), whereas the vehicle (DMSO 0.1%) was not able to induce any change when incubated under the same conditions. Although both drugs act by a concentration-dependent manner, DCMF was more potent, exhibiting an IC_{50} of 40.0 ± 0.1 µg/mL, while EtOH and HexF showed IC_{50} values of 153.6 ± 0.1 µg/mL and 118.40 ± 0.2 µg/mL, respectively (Fig. 4B).

The high concentration of betulinic acid in DCMF might explain why this fraction of *M. caesalpiniifolia* Benth. demonstrated an interesting antileukemic activity when compared to HexF and EtOH. These findings corroborate to previous studies describing the antileukemic potency of betulinic acid (Faujan et al., 2010; Wu et al., 2010). Likewise, a low antileukemic activity induced by HexF and EtOH can be attributed to the low concentrations of lupeol and phytol, their major constituents, respectively.

3.4. Antitumor selectivity of DCMF

Since the dichloromethane fraction of *M. caesalpiniifolia* Benth. exhibited a better antitumor potential and less toxicity against normal cells among the drugs tested, the DCMF was that only used for the posterior experiments involving an evaluation of the antitumor selectivity. For that reason, we investigated whether DCMF would also have an antitumor effect against human acute leukemia cell line like HL-60 as well as non-human cancer cells (S180). Our data showed that DCMF reduced cell viability from 100 ± 14% (control) to 81 ± 0.7%, 45 ± 8.2%, and 29 ± 2.6% (p<0.05), when the cells were incubated, respectively, with 5 µg/mL, 50 µg/mL, and 500 µg/mL (Fig. 5A), exhibiting an IC_{50} of 64.2 ± 5.0 µg/mL. As expected, etoposide (50 µg/mL) reduced cell viability to 55 ± 9.9% (p<0.05). These data are in accordance with recent findings by our group describing the antiproliferative potential of DCMF against different types of human solid tumors like HCT-116 (colon carcinoma), OVCAR-8 (ovarian carcinoma) and SF-295 (glioblastoma) (Monção et al., 2015). Meanwhile, DCMF was not able to induce any significant

cytotoxicity against murine sarcoma cells (S180), at the same conditions mentioned above (Fig. 5B), suggesting that this fraction seems to be more selective against human cancer cells. Although, additional experiments are necessary to confirm this finding.

4. Concluding remarks

The present study evaluated for the first time, the antileukemic potential of *M. caesalpiniifolia* Benth. ethanolic extract (EtOH) and its *n*-hexanic (HexF) and dichloromethane (DCMF) fractions obtained from the stem bark of this Brazilian native plant. The data obtained by our group demonstrated that the DCMF fraction presented antitumor potential against both acute (HL-60) and chronic (K562) human myeloid leukemia cell lines. These findings were probably due to the high concentration of betulinic-acid in its composition, a well-known naturally occurring triterpenoid with several pharmacological properties, including anticancer. Additionally, we have demonstrated that the DCMF fraction was not only the most potent among the tested drugs, but also the less toxic against normal human and animal cells. DCMF was also more selective to human cancer cells, since it did not induce significant cytotoxicity in murine sarcoma cells (S180).

Leukemia is increasingly a global health issue, and therefore, the development of new therapeutic strategies with less side effects and higher selectivity seems to be critical. In this way, our results were of great interest since the DCMF showed selectivity against human leukemic cells, and certainly will contribute to future research involving new anticancer drugs. However, additional investigations are necessary to evaluate the toxicity level of *M. caesalpiniifolia* Benth. extracts, with the aim of developing safe doses for its use *in vivo* and further as a final pharmaceutical product for the treatment of leukemias.

Conflict of interest

The authors declare that there is no conflict of interest.

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