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Calophyllum brasiliense Extracts Induced Apoptosis in Human Breast Adenocarcinoma Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Authors IBS, AMAS, ECPH, FTCK' designed the study, author IBS performed the statistical analysis and wrote the protocol, authors MSFC and IBS wrote the first draft of the manuscript. Authors MSFC, SAF and MLBP managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Apoptosis, or programmed cell death, is linked to several mechanisms of cell growth control. The present work aimed at evaluating the induction of apoptosis in MCF-7 human breast adenocarcinoma cell by *Calophyllum brasiliense*.
Study design: The tests were performed in triplicates in the apoptosis assays and sextuplicates in the cytotoxic assays, to each group, and the data expressed the mean +/- standard deviations. The cytotoxicity IC50s were obtained based on nonlinear regression curve fit. Two-way ANOVA and Tukey's tests were applied in the apoptosis analyses.
Place and duration of study: The work was done at the Center for Research in Biodiversity (Cell Culture Laboratory, and Phytochemistry Laboratory), and Research Center (Molecular Biology Laboratory), Paulista University, between Jan 2019 and Dec 2019.

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Methodology: Two aqueous extracts, obtained from the stem (STE) and from the leaves (LFE) of *Calophyllum brasiliense* by a 24-h maceration, were submitted to a cytotoxic assay against MCF-7 breast cancer cell lines at the concentrations of 0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml, 10 µg/ml and 100 µg/ml. They were also subjected to the evaluation of apoptosis and necrosis cell death induction at concentrations of 50, 100 and 200 µg/ml, after 6 h, 12 h and 24 h. Curcumin was used as a reference drug for both cytotoxic (50 mM, 5.0 mM, 0.5 mM, 0.05 mM and 0.005 mM) and apoptosis/necrosis (12.5 µM, 25 µM and 50 µM / 6 h, 12 h and 24 h) assays. Apoptosis and necrosis were accessed by the use of annexin V and 7-AAD, in a two-channel flow cytometer.
Results: In terms of the cytotoxic activity, STE (IC50 7.86 µg/ml) was more toxic than LFE (IC50 74.35 µg/ml), and curcumin IC50 was 0.00159 µg/ml. STE induced 21.19 % and LFE, 20.63 %, in comparison to 13.4% of apoptosis induction by curcumin. The results of apoptosis induction in the cancer cells were achieved at 24 h, extract concentrations at 100 µg/ml.
Conclusion: Both the extracts, STE and LFE, were cytotoxic against MCF-7 breast cancer cell line, and induced more apoptosis in MCF-7 cells than curcumin, suggesting that they are high potential sources of natural product-inducing apoptosis agents to be used against adenocarcinoma breast cells.

Keywords: Annexin V; 7-AAD; cytotoxicity; clusiaceae; curcumin; flow cytometry; natural products.

1. INTRODUCTION

Cancer is characterized by the disordered growth of cells in any type of tissue. Breast cancer is the second most common type of neoplasia among women, although rare, it also occurs in men [1]. The therapeutic protocols are based on chemotherapy, radiation therapy and / or surgery [2].

Tumor cells acquire specific characteristics and are resistant to homeostasis signals [3]. The resistance signals include a lack of cell recognition of inhibiting growth factors, resisting to the apoptosis, allowing the activation of replicative immortality, angiogenesis induction and activation of tissue invasion and metastasis [4]. Apoptosis is a distinct type of cell death that is involved in the elimination of cells in normal tissues, as in embryogenesis, that also occurs in specific pathological contexts. It is characterized by nuclear aggregation and margination, condensation of the cytoplasm, and convolution of nuclear and cellular contours. At a later stage, nucleus fragments and protuberances formed on the surface of the cell separate the apoptotic bodies that are phagocytized by nearby phagocytes and degraded cells in lysosomes [5].

The induction of apoptosis is one of the most pursued mechanisms in an attempt to understand the way that tumor cells escape from their main site and double cross the immune system. Apoptosis induction mechanism is also employed as a target in the search for active anticancer compounds with a reduced level of side effects [6]. Apoptosis is the physiological programmed cell death that the organism uses to eliminate defective cells with compromised DNA, so as to eliminate old cells and to control homeostasis in an organized way without compromising the cell membrane and without causing inflammation in the tissue, since the apoptotic bodies are phagocytized by local cells [5]. The phagocytosis occurs by the exposition of phosphatidylserine from its membrane, which triggers the information coming from a cell that needs to be eliminated by natural reasons by the immune system [7]. Apoptosis proceeds unlike necrosis, which is an unprogrammed cell death process characterized by the disruption of the cell membrane and by the exposition of the intracellular compounds, being recognized by the cells of the immune system, causing inflammation [3,4]. As tumor cells are potentially resistant to the cell death induced by apoptosis and show an exacerbated growth in relation to the healthy tissue, cancer treatments that induce cell death via apoptosis are of great importance once it is supposed to have minimized side effects and could work as adjuvant in cancer therapies [8,9].

Brazilian biodiversity has been an extremely important source in the search of new therapeutic tools to be used as medicines. Approximately 20% of the world's biodiversity can be found in the Brazilian territory, particularly in the Amazon and the in the Atlantic Forests. Due to the huge biodiversity and the fact that 60% of all medicines come from natural sources [10], systematic screenings aiming at the identification of biologically active plant extracts is done as those focused on antitumor activity

against prostate and breast cancer cell lines [11-12]. The importance of antitumor natural products for therapeutics relies on molecules as paclitaxel, docetaxel and the vinca alkaloids, which are been used in cancer therapies for decades [12], as mitotic-cycle agents inhibiting the growth of tumor cells through different mechanisms of action [13].

The genus *Calophyllum* (Clusiaceae) is composed of several species that are used in traditional medicine for the treatment of gastric ulcers, pain, inflammatory processes, and others [14]. The genus is rich in plants containing bioactive compounds, such as xanthones, flavonoids, coumarins and calanolides, especially the isolated β-benzo-tripyranone molecule capable of inhibiting HIV-1 virus reverse transcriptase [15,16]. *Calophyllum brasiliense*, popularly known as "guanandi", is found in the equatorial and tropical forests from Brazil, as the Amazon rain forest and the Atlantic Forest, and is also traditionally used as anti-inflammatory, as analgesic and against gastrointestinal problems [14,17]. A previous *in vitro* study with Amazon plant extracts reported the cytotoxic activity against the breast cancer cell line MCF-7 for a *Calophyllum brasiliense* organic extract [12], but mechanistic studies are yet to be done.

Natural products are a potential source for new medicines to be used in cancer therapy [18-20]. Curcumin, a compound extracted from turmeric (*Curcuma longa*), has been studied for its antiproliferative or cytotoxic activities [21,22], as well as is extensively reported as an anti-inflammatory [23] and antioxidant agent [22,24,25]. In the present work, curcumin was used as a reference natural product compound having antiproliferative and anti-apoptotic activities [24,26,27].

The present work aimed at the evaluation of the cytotoxicity and the apoptosis-induction potential of the extracts obtained from the leaves and from the stem of *Calophyllum brasiliense* that previously have shown cytotoxic activity against human adenocarcinoma cells.

2. MATERIALS AND METHODS

2.1 Plant Material and Curcumin Preparation

2.1.1 Collection of plant material and extract preparation for the biological analyses

The stem and the leaves of *C. brasiliense* were collected in a region near Manaus (AM), under license from the Brazilian Ministry of the Environment, from *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (IBAMA), number 12A/2008. A voucher of the collected plant was deposited at the UNIP Herbarium [PSCamara 187; UNIP 102)], where it was taxonomically identified. The plant material was cleaned by removing contaminants (fragments of other organs, dust, sand, insect), dried in an air-circulating oven at 40 °C, then ground in a hammer mill (Holmes 201X) [28]. The extracts were obtained by a 24-h maceration with dichloromethane and methanol (1:1). Solvents were removed by rotavapor and the dry organic extracts from the stem (STE) and from the leaves (LFE) were kept under freeze at – 70 ^{o}C, up to use kin the biological assays.

2.1.2 Curcumin and doxorubicin preparation for the biological analyses

Curcumin (Sigma C7727-500MG) was used as the reference apoptosis-inducing substance for the assays [26]. The stock solution concentration of curcumin was 50 mM [22] and it was diluted with 50 % dimethylsulfoxide (DMSO50), in water (DMSO: water, 1:1). The final concentrations of curcumin in the 24-well plates were 12.5 μM, 25 μM and 50 μM. The stock solution of doxorubicin diluted in water a standard drug, was 25 mM, and the final concentration in the 96-well plates was 62.5 μM, used in the cytotoxic assay.

The stock concentration of the plant extracts STE and LFE was 40 mg/ml. The aqueous extract was diluted in Milli-Q grade water. The final concentrations of the extracts in the 24-well plates were 50 μg/ml, 100 μg/ml and 200 μg/ml.

2.2 Cell Culture

2.2.1 Cell culture management

The tumor cell line MCF-7 (estrogen receptor positive breast adenocarcinoma) was cultured in tissue culture flasks (Costar), supplemented with RPMI-1640 medium (Lonza), plus 5% fetal bovine serum (FBS) (Cambras), 1 % glutamine (Sigma) and 1 % gentamicin sulfate (Hypolabor), and maintained in an incubator (Thermo Fisher) at 37 ºC with 5 % CO_2 and 100 % relative humidity. Cells were submitted to weekly passages (trypsin-EDTA, Cambras). Cell densities were obtained through a cell counting chamber using the Trypan blue exclusion method. The tests were run in 24-well plates with a density of 1.5 x 10^4 cells per well and were

grown for 24 h to achieve 100 % confluency and a cell density of 3.0 x 10^5 cells per well, and thereafter the extracts were added. After that, end points were obtained by the sulforhodamine B (SRB) assay.

2.2.2 Sulforhodamine B assay

The viable cells were fixed in the 96-microplates with cold trichloroacetic acid (TCA) solutions (50 µL/well of 50 % TCA). Microplates were washed with water five times until non-viable cells were totally removed. Plates were left to air-dry for 24 h. A hundred µl of sulforhodamine B (SRB) per well was added, and the dye was left to react for 10 minutes. After that period, plates were washed five times with 1 % acetic acid until the complete removal of unbound SRB. Plates were left to air-dry for 24 h. The stain was resuspended with 100 µl of Trisma Buffer. The amounts of viable cells were measured by obtaining the optical densities of the wells in a microplate spectrophotometer reader (Biotek 408x) at 515 nm. The percentage of cell lethality was obtained from the formula 100 X [(T-T0)/(C-T0)], which is the comparison between the control (untreated cells) and test (cells treated with drug/extract) cell growth and time zero growth (which is the cell growth until addition of extract). Also, the IC50 was obtained, as previously described [29].

2.2.3 Cytotoxicity assay

In order to obtain the inhibition concentration 50 % (IC50), the extracts were tested at final concentrations of 0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml, 10 µg/ml and 100 µg/ml, curcumin (CUR; Sigma), a natural product that shows apoptotic induction activity, which was used as control in the assays, was tested at final concentrations of 0.0000125 mM, 0.000125 mM, 0.001255 mM, 0.0125 mM and 0.125 mM, and doxorubicin, an antitumor drug, was used as standard drug and was tested at the final concentrations of 0.000625 nm, 0.00625, 0.625 nm, 6.25 nm and 62.5 nM. Results were calculated based on nonlinear regression curve fit (GraphPad Prism® 7.0).

2.3 Apoptosis/Necrosis Assay

Flow cytometry was performed in order to identify the type and percentage of death occurring in MCF-7 cells. Apoptosis (Annexin + / 7-AAD-), late apoptosis / necrosis (Annexin + / 7-AAD +) and necrosis (Annexin - / 7-AAD +) were detected using apoptosis Annexin V and 7-AAD (Becton Dickinson - BD). The living cells do not present positivity for both markers. For calibration and validation of the markers, heat-dead MCF-7 cells labeled with Annexin V or 7-AAD and with both markers were used. The technique was performed using the template provided by the manufacturer (Becton Dickinson - BD) for Annexin V conjugated to phycoerythrin (PE) and 7-AAD. The acquisition of 30 seconds for each sample. Tests were performed at 6 h, 12 h 24 h of cell treatment.

2.4 Thin Layer Chromatography of LFE and STE

In order to evaluate the presence of coumarins in STE and LFE, a thin layer chromatography (TLC) analysis was performed. The extracts were prepared at a concentration of 40 mg/ml. The analyses conducted as described by Wagner and Bladt [30] to coumarins. The flavonoid rutin (Sigma) was used as a reference compound and were diluted to 3.5 mg/ml. The TLC system was composed by a 0.2 mm pre-coated TLC aluminum sheet Silica Gel G/UV_{254} as stationary phase (Macherey-Nagel; cat. nº 818333, lot 802046), two solvent systems were used as mobile phase: toluene: ethyl ether (1:1) saturated with 10% acetic acid, and ethyl acetate: formic acid: acetic acid: water (100:11:11:26). Visualization was made under U.V. 254 nm and 365 nm, before and after reaction with 10% KOH diluted in ethanol.

2.5 Statistical Analyses

The tests were performed in triplicates in the apoptosis assays and sextuplicates in the cytotoxic assays, to each group, and the data expressed the mean +/- standard deviations. Shapiro Wilk normality test was applied (P = .31, P = .34, P = .05 for STE 6 h, 12 h and 24 h, respectively, and P = .34, P = .56, P = .34 for LFE 6 h, 12 h and 24 h, respectively). Cytotoxicity IC50 values were obtained based on nonlinear regression curve fit (GraphPad Prism® 7.0). A simple two-way ANOVA parametric test was used for the statistical calculations, followed by the Tukey post-test, considering time and concentration as the two variables. Significances for all analyses were considered at the level of $\alpha < 0.05$.

3. RESULTS AND DISCUSSION

In the present study, the presence of apoptotic cells was verified by labeling phosphatidylserine (PS) exposed in the inner surface of the cytosol-

bound lipid bilayer membrane with Annexin V. Annexin V is a protein that easily binds to phospholipids, and that consequently shows high affinity to PS. At the beginning of apoptosis, the cell membrane remains intact, but undergoes a disorganization, and the PS, which is translocated to the outer surface of the bilayer membrane, gets exposed and available to the interaction with Annexin V before the loss of cell membrane integrity [7] 7-AAD is a nuclear marker that intercalates with any DNA fragment, as long as the cell membrane is permeable and allows its penetration into the cell. The use of both Annexin V and 7-AAD in the analyses enables the discrimination between apoptotic or necrotic cell death.

Curcumin is a natural compound isolated from the *Curcuma longa* (Zingiberaceae) rhizome [20] that has previously shown antitumor activity [31], besides its ability of inducing apoptosis in MCF-7 breast tumor cells [32,33] and lead tumor cells to apoptosis [21]. Curcumin was extensively studied for its anti-inflammatory, antiangiogenic, antioxidant and antitumor effects [26]. Recent studies showed that curcumin alone or in combination with other antitumor agent is able to effectively induce apoptosis in breast tumor cells [21]. An example of this combination is the association of curcumin with paclitaxel. Curcumin alone or in combination with the drug, which is already used in cancer therapy protocols, has the ability to induce apoptosis in breast tumor cells, improving the amount of pro-apoptotic proteins such as p53, Bid, caspase 3, caspase 8 and Bax [32]. Curcumin is also a chemopreventive compound, as it suppresses, retards or reverses carcinogenesis, besides being a very promising agent that reduces cancer morbidity and mortality by delaying the carcinogenesis process [14]. Due to its known properties, curcumin was selected as the reference apoptosis-inducing substance, and the apoptosis-inducing percentage was used as a reference and determinant limit to compare the apoptosis-inducing indices obtained for the plant extracts tested in the present analysis.

Table 1 reports IC50 for the extracts and controls. The cytotoxicity of the plant extracts used in the present analysis against MCF-7cells, reported as IC50, was 7.86 µg/ml for STE, 74.35 µg/ml for LFE, 5.38 µM for curcumin, and 0.66 µM for doxorubicin. Present findings suggest the significant cytotoxic activity of both doxorubicin and curcumin, in relation to the aqueous extracts, STE and LFE. In comparison to other reports, the fruit peel, fruit pulp and fruit seed extracts obtained from the fruits of *Garcinia dulcis*, known as "Mundu" in Malaysia, were tested against liver tumor cells and showed IC50 of 46.33 µg/ml, 38.33 µg/ml and 7.5 µg/ml, respectively [34]. A study done with the bark extract from *G. schomburgkiana*, found in Thailand, reported that two isolated xanthones named schomburgones A and B showed IC50 ranging from 1.45 µM to 9.46 µM [35]. As a reference from isolated natural products, taxanes as paclitaxel, isolated from *Taxus brevifolia*, showed IC50 of 33.4 nM against MCF-7 cells [36], while curcumin showed IC50 of 40 µM against MCF-7 cells [37]. In terms of the species, the organic extracts from the leaves of *C. brasiliense* showed antitumor activity *in vitro* against prostate tumor cells, melanoma and renal carcinoma [38], which is in accordance to the present findings.

After the standardization and validation of the controls, it was possible to determine the percentage of death by apoptosis (Annexin + / 7-AAD-), by necrosis (Annexin - / 7-AAD +), dead cells with double labeling (late apoptosis and necrosis, Annexin + / 7-AA-D +) and live cells, as shown in Table 1 and in Fig. 2.

The extracts STE and LFE showed apoptotic percentage of 21.19 % and 20.63 %, respectively, in 24 h of treatment. A study carried out with extracts from *Ziziphus spina* Christi (Rhamnaceae), showed an apoptotic percentage of 3.2% against MCF-7 cells, in a 48-hour trial, and also showed inhibition of the mitotic cycle by arresting at G1 phase [39]. The paclitaxel and docetaxel (semi-synthetic compound from *Taxus baccata*) also act in the mitotic cycle, and consequently, can lead the cells to death by apoptosis [21,40]. After 24 h of treatment and assay with Annexin V and PI, paclitaxel showed a percentage of cells in the initial phase of apoptosis of 25.7% [36]. In the present work, results related to the percentage of apoptosis induction by extracts STE and LFE can be considered extremely promising, when compared to the literature.

Figs. 2 and 3 show the results obtained by the two-way ANOVA test made for curcumin, STE and LFE, considering variations of concentration and time. Fig. 2A shows the analysis made with curcumin. According to the analysis, the interaction of the variables showed significance ($F(4,18)=3.55$; $P = .026$) and corresponded for 30.31% of the variance, the time showed significance ($F(2,18)=4.66$; $P = .024$) and

corresponded for 19.86% of the variance, while the concentration was not considered significant (*P* = .05). Fig. 2B shows the results obtained by the two-way ANOVA test made for STE, and the interaction of the variables showed significance (F(4,18)=5.76; *P* = .004) and corresponded for 46.05% of the variance, the time and concentration did not show significance (*P* = .05). Fig. 2C shows the analysis made with LFE. The interaction of the variables showed extreme significance (F(4,16)=13.98; *P* < .001) and corresponded for 53.90% of the variance, the concentration was also considered extremely significant (F(2,16)=15.71; *P* < .001) and corresponded for 30.28% of the variance, while the time was not significant (*P* = .05).

Fig. 3 shows the comparison of the apoptosis-induction related to extracts STE and LFE. In terms of the statistical findings, it can be observed that interaction accounts for 50.79% of the total variance and was considered extremely significant (F(10,33)=6.34; p<0.0001). It means that the differences that were observed depended on both extracts and their concentrations together. The extracts, which represent two different cell treatments, account for 11.02% of the total variance and was considered significant (F(5,33)=2.74; *P* = .035); and extract concentration accounts for 16.57% of the total variance and was considered extremely significant (F(2,33)=10.35; *P* < .001), which means that the differences that were observed also depended on the variation of the concentrations. LFE was more constantly effective than STE.

C. brasiliense extracts and isolated compounds have been previously studied, as well as reports on different biological activities against neglected disease pathogens as *Tripanosoma cruzi* [41-45] and against *Leishmania* sp. [46-49], and against bacteria [50], especially against neglected disease pathogen *Mycobacterium tuberculosis* [51,52] and *Helicobacter pylori* [53,54,55], involved in ulcer and stomach cancer. The studies reporting the cytotoxic effects of extract or isolated compounds is also done, as the anticancer activity of coumarins [51,56-61] and xanthones [62,63] and the toxicogenomic activity of coumarins [17,64]. Cancer chemoprevention of coumarins was also accessed [56], as well as the antioxidative effects of coumarins [65] and of xanthones [66]. Antinociceptive, anti-inflammatory and central nervous system effects of coumarins [67] and xanthones [18]. The anti-HIV activity of coumarins was accessed [68], and their potential of flavonoids to impair with gastric H+,K+-ATPase activity [69] and with calcium entry in mast cells [70]. Finally, Ito et al. [57] reported the apoptosis-induction potentiality of coumarins against leukemia cell line HL-60 Fig. 4 shows the TLC analyses made with LFE and STE, using rutin as a reference substance.

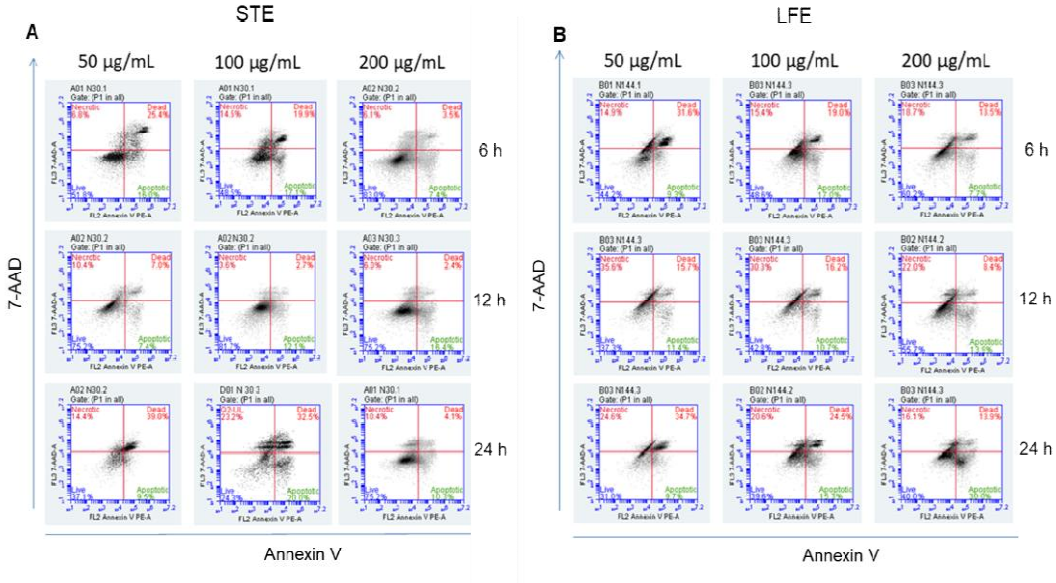
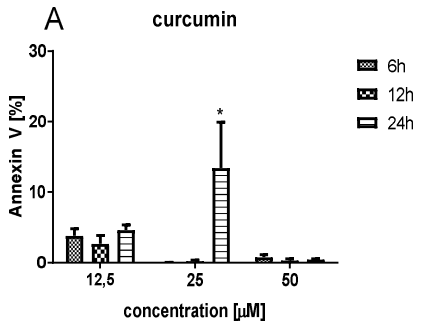


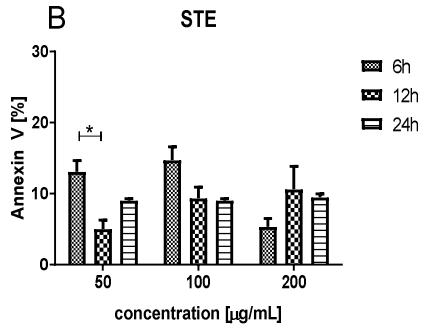
Fig. 1. Plots of flow cytometry representing the percentages of apoptosis of cytotoxic aqueous extracts STE (A) and LFE (B) obtained from stems and leaves of *Calophyllum brasiliense*

Table 1. Inhibitory concentration 50 % (IC50) and flow cytometry analysis labeled with Annexin V and 7-AAD to evaluate apoptosis and necrosis by stem (STE) and leaves (LFE) aqueous extracts (*Calophyllum brasiliense*) against MCF-7 cells in 24 h of treatment. CUR=curcumin; DOXO-doxorubicin

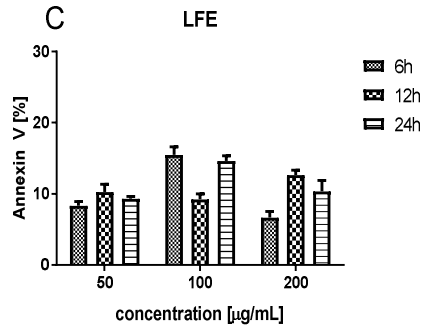
Extracts	IC50	7-AAD [%]	Annexin + 7-AAD [%]	Living cells [%]	Apoptotic cells [%]
CUR	5.38 µM	5.44	65.55	15.60	13.40
DOXO	0.66 µM	NT	NT	NT	NT
STE	7.86 µg/ml	21.49	35.72	21.60	21.19
LFE	74.35 µg/ml	17.85	32.84	28.68	20.63



ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	216.5	4	54.14	F (4, 18) = 3.553	P=0.0264
concentration	81.77	2	40.89	F (2, 18) = 2.684	P=0.0955
time	141.8	2	70.92	F (2, 18) = 4.655	P=0.0235
Residual	274.2	18	15.24		



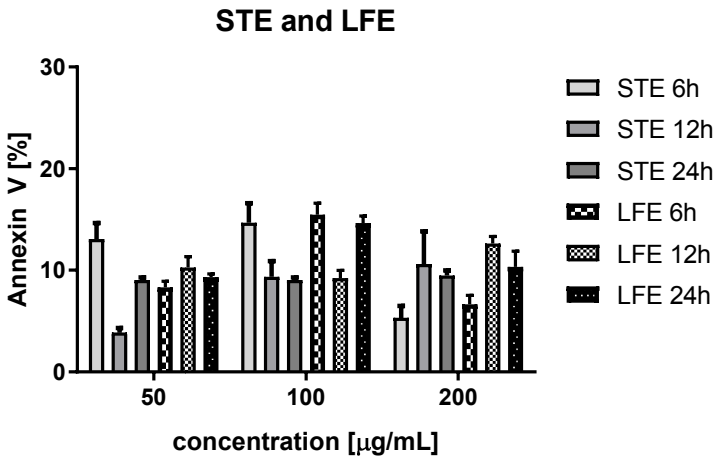
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	170	4	42.49	F (4, 18) = 5.756	P=0.0036
concentration	32.47	2	16.23	F (2, 18) = 2.199	P=0.1398
time	33.78	2	16.89	F (2, 18) = 2.288	P=0.1302
Residual	132.9	18	7.382		



ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	120.2	4	30.04	F (4, 16) = 13.98	P<0.0001
concentration	67.5	2	33.75	F (2, 16) = 15.71	P=0.0002
time	6.353	2	3.176	F (2, 16) = 1.479	P=0.2575
Residual	34.37	16	2.148		

Fig. 2. Percentage of apoptosis in variation of time (6 h, 12 h and 24 h) and concentration of treatments. (A) Curcumin; (B) Extract STE; (C) Extract LFE.

Curcumin final concentrations: 12.5 µM, 25 µM and 50 µM; Extract concentrations: 50 µg/ml, 100 µg/ml and 200 µg/ml; Two-way ANOVA followed by Tukey post-test, significance among means of α<0.05.



ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
Interaction	305.2	10	30.52	F (10, 33) = 6.342	P<0.0001
concentration	99.59	2	49.8	F (2, 33) = 10.35	P=0.0003
treatment	66.21	5	13.24	F (5, 33) = 2.752	P=0.0348
Residual	158.8	33	4.813		

Fig. 3. Percentage of apoptosis of extracts STE and LFE in variation of time (6 h, 12 h and 24 h) and concentration of treatments

Extract concentrations: 50 µg/ml, 100 µg/ml and 200 µg/ml; Two-way ANOVA followed by Tukey post-test, significance among means of α<0.05

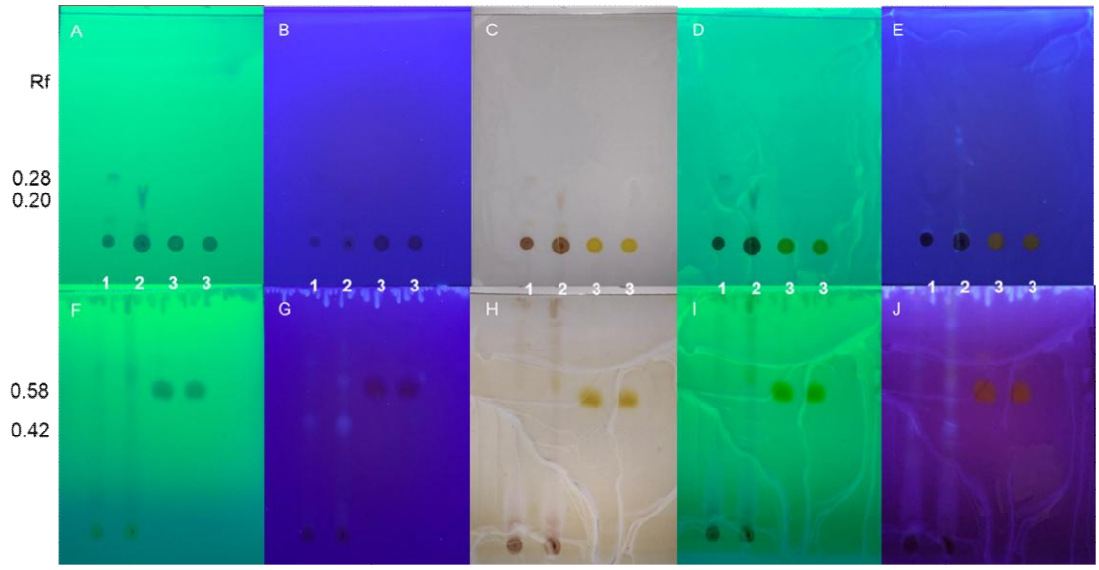


Fig. 4. Thin layer chromatograms for the identification of coumarins in the aqueous extract from the stem (STE) and from the leaves (lFE) of *Calophyllum brasiliense*

1=STE; 2=LFE; 3 and 4=rutin; A, B, C, D and E were run with toluene: ethyl ether (1:1) saturated with 10% acetic acid; F, G, H, I and J were run with ethyl acetate: formic acid: acetic acid: water (100:11:11:26); A, D, F and I were observed under U.V. 254 nm; B, E, G and J were observed under U.V. 365 nm; C and H were observed under fluorescent light; D, E, H, I and J were sprayed with 10% KOH in ethanol; Blue or pale-blue spots, as well as light browny spots indicate coumarins [30]

The chemical complexity of *C. brasiliense* has being explored for almost twenty years. The structures of some compounds that are already identified in the species as shown in Figs 5 and 6.

In the present study, the presence of coumarins in both STE and LFE extracts was reported. Mammea-type coumarins, [42,44,46-49,51,52,58,60,61,65]; calanolide-type coumarins [45,68,71] sulamarin [43] and soulattrolide [67,68] were identified in the leaves. From the barks, stem barks or heartwood, coumarins as calophyllolide [57] and brasimarins [56] were isolated, as well as mammea-type coumarins [57,69]. Xanthones like jacareubin are more likely to be reported to occur in heartwood [41,50,63,66,70], as well as other xanthone-type compounds [18,62] and eventually mammea-type coumarins [50]. Also, brasiliensic and isobrasiliensic acids occur in the stem bark [55,72] and eventually in other plant organs [54], and chromanone acids occur in the bark [53]. In terms of pharmacological potential, *Calophyllum brasiliense* shows a wide range of activities related to a variety of chemicals that must be further accessed.

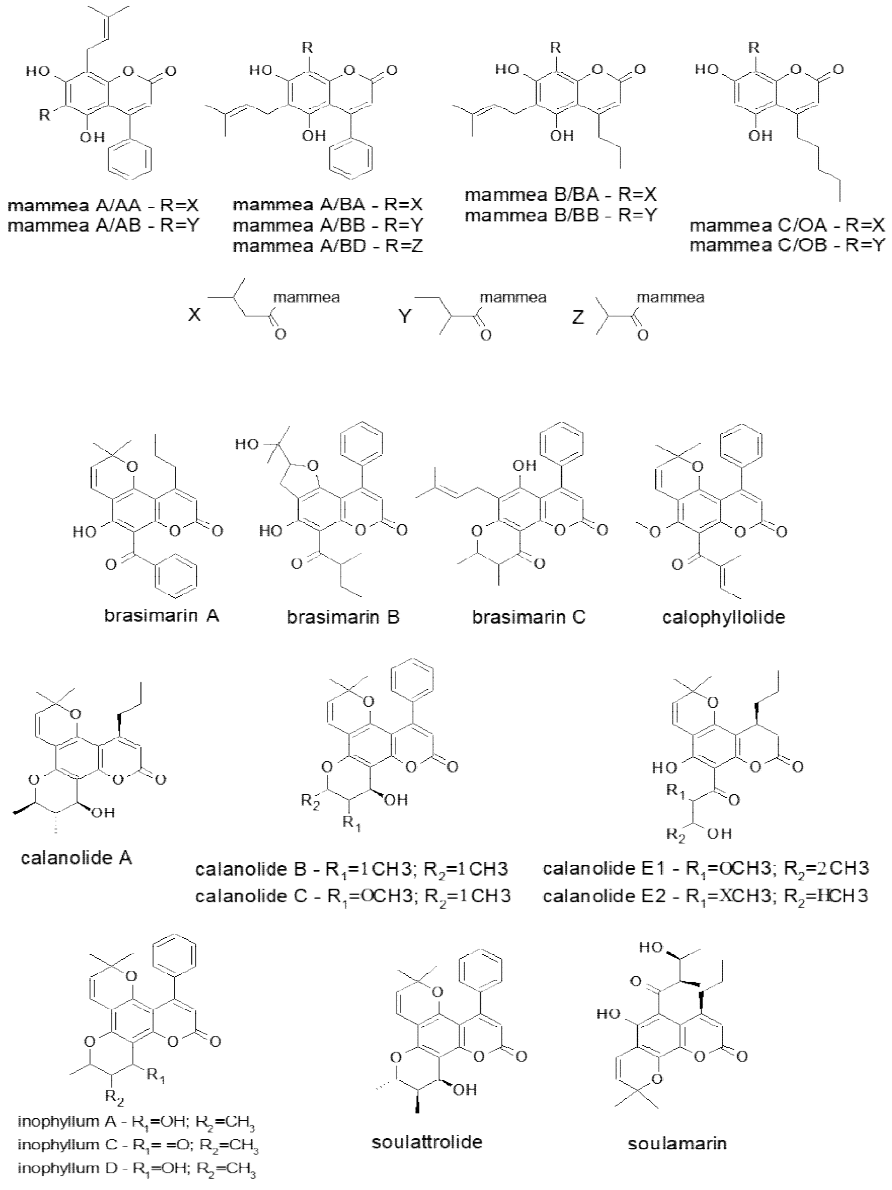


Fig. 5. Coumarins isolated from *Calophyllum brasiliense*

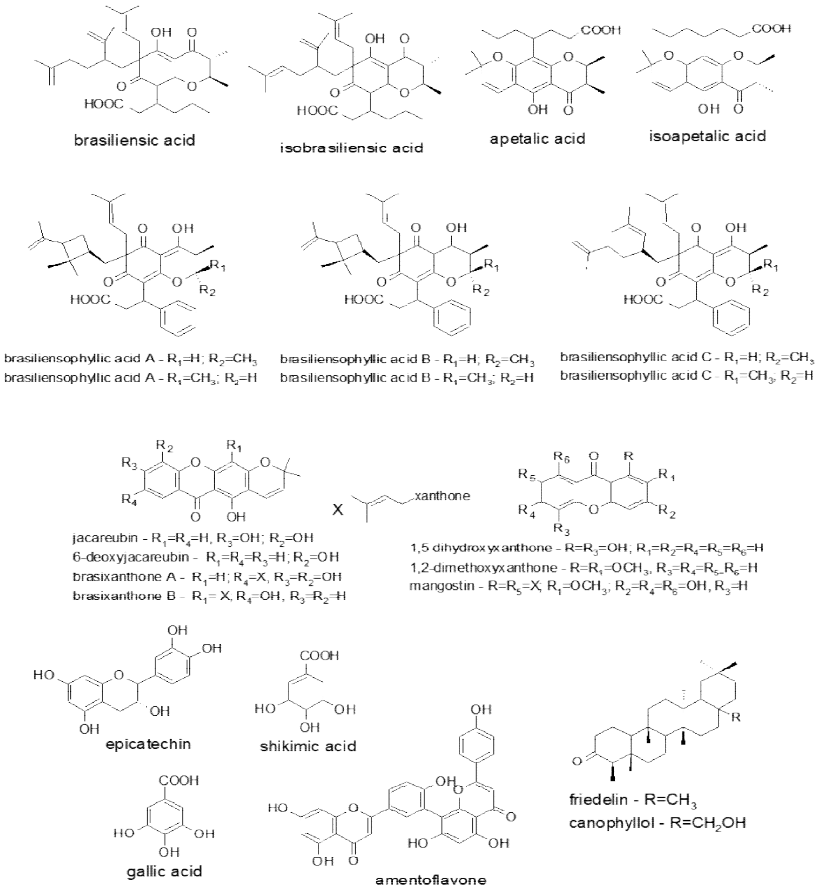


Fig. 6. Xanthones, triterpenes and phenolic compounds isolated from *Calophyllum brasiliense*

4. CONCLUSION

The present work evidenced that crude extracts obtained from the stem and from the leaves of *Calophyllum brasiliense* induced apoptosis in human breast adenocarcinoma cells, and that the apoptotic induction was higher than that observed for curcumin. Notwithstanding, the extracts presented high antitumor potential by means of their apoptosis-induction capacity.

CONSENT

It's not applicable.

ETHICAL APPROVAL

It's not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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