Calophyllum brasiliensi Cambess (*Clusiaceae*) extracts showed antimicrobial activity and cytotoxicity, in vitro

*Extratos de jacareúba (C*alophyllum brasiliensi Cambess; Clusiaceae*) apresentaram atividade antibacteriana e citotóxica em modelos experimentais in vitro*

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Abstract

Objective – The aim of the study was to examine the antimicrobial activity, as well as the cytotoxicity of jacareuba plant extracts and verify if the traditional use of the plant is scientifically justified. *Jacareuba* plant extracts were found to be biologically active, in a high-throughput assay. As it was noticed, the plant is known as to be used in traditional medicine by communities of the Amazon Rain Forest, as well as other Amerindian communities. Although the plant is known to have biological activity, there is a lack of scientific information regarding the use of the plant against cancer cell lines and bacteria, here described. **Methods** – Six organic and aqueous extracts obtained from leaves, stem and fruits of *Calophyllum brasiliense* were tested against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria using disk diffusion assays and microdilution broth assays. Also, the extracts were tested against breast, prostate, colon, lung and central nervous system solid tumors and against leukaemia, using the SRB colorimetric assay. **Results** – Cytotoxic activity was observed against breast and colon cancers and leukaemia cell lines and against both Gram positive bacteria and against *Pseudomonas aeruginosae*. **Conclusions** – Biological activity observed supports the popular uses of traditional *jacareuba* remedies against diarrhea, oral diseases, or other infectious diseases, as well as against tumors, but further studies related to pharmacology, chemical and toxicology of the extracts are needed to support the rational use of medicinal plants.

Descriptors: Products with antimicrobial action; *Clusiaceae*; Vegetal extract

Resumo

Objetivo – Examinar a atividade antibacteriana e citotóxica de extratos vegetais obtidos de jacareúba e embasar cientificamente sua utilização popular. Extratos vegetais obtidos de jacareúba demonstraram atividade biológica, em modelo de triagem em grande escala. A planta é conhecida pelo seu uso tradicional por comunidades da Amazônia e das Américas. Embora a planta seja conhecida pelo seu uso tradicional, poucas informações científicas relacionadas a seu uso contra células tumorais e micro-organismos patogênicos são encontradas na literatura. **Métodos** – Seis extratos orgânicos e aquosos obtidos de folhas, caule e frutos de *Calophyllum brasiliense* foram testados contra bactérias Gram-positivas (*Staphylococcus aureus* e *Enterococcus faecalis*) e Gram-negativas (*Escherichia coli* e *Pseudomonas aeruginosa*) usando os ensaios da disco-difusão em Agar e microdiluição em caldo. Os extratos também foram testados contra células de tumor de mama, próstata, cólon, pulmão, sistema nervoso central e leucemia, usando o ensaio colorimétrico da sulforrodamina B (SRB). **Resultados** – Atividade citotóxica foi observada contra células de tumor de mama, colon e leucemia e contra ambas as bactérias Gram-positivas e contra *Pseudomonas aeruginosa*. **Conclusões** – Atividade biológica observada para os extratos suporta o uso popular de remédios tradicionais feitos com jacareúba contra diarreia, doenças orais, e outras, como doenças tumorais. Porém, existe a necessidade de se estabelecer parâmetros biológicos, químicos, farmacológicos e toxicológicos para que o uso racional de fitoterápicos ou de medicamentos feitos com compostos isolados de jacareúba possam ser comercializados ou usados no tratamento de doenças infecto-contagiosas e tumorais.

Descritores: Produtos com ação antimicrobiana; *Clusiaceae*; Extrato vegetal

Introdução

Calophyllum brasiliense Cambess., popularly known in the Brazilian Amazon rain forest as "jacareuba", is a member of the Clusiaceae family. Trees are up to 30 m tall; barks are deeply fissured. They can be found in the evergreen lowlands to *montana* forests, gallery forests, mangroves, in *Mauritia* palm swamps, and in seasonally flooded riverbanks[1]. Its wood is used in civil construction and in shipbuilding[2], as well as to treat some medical conditions like diarrhea, diabetes, worms, against herpes and rheumatism. It is also used as fuel[3-4]. The yellow resin found in the plant and the stem bark is used to treat chronic gastric ulcers and other ailments[5].

Jacareuba was collected and processed some years ago, together with other hundreds of plants, as part of a large biological screening program established by our group in late 1996[6-7]. From all these plants, 1,220 aqueous and organic extracts were made and tested against four strains of bacteria[8] and six human cancer cell lines[9-11]. Chemical substances have been isolated from Clusiaceae species, especially those derived from xanthones, flavonoids and coumarins. Previous reports relate the antibacterial and cytotoxic effects of some Clusiaceae species[12-13] and reinforce the importance of the family to Medicine and traditional medicine. In the present study, six scientifically unknown extracts obtained from different parts of *jacareuba* were tested for their antibacterial and cytotoxic potential.

The present study aims the identification of the antibacterial activity and cytotoxic activity of the extracts ob-

tained from jacareuba against bacteria and tumors that affects both human and veterinarian patients, and we intend to confirm that biological screening succeeded as a strategy to identify active plant extracts in a high-throughput screening methodology in a short period of time.

Methods

Plant collection

The stems, fruits and leaves of *C. brasiliense* were collected in May/1997, in an *igapó* forest nearby Manaus, AM, Brazil. Collection was made with authorizations numbers 053/99 and 038/99 by IBAMA (Brazilian Institute of the Environment and Renewable Natural Resources) and CGen (Genetic Patrimony Management Council). The plant was identified by Dr. Mateus B. Paciencia, and the voucher is deposited at Herbarium UNIP (Herbarium register number UNIP102, collector's number PS 187).

Preparation of extracts

Plant parts were collected according to the biomass availability, specifically leaves, stems and fruits. Plant material was dried in an air-circulating stove (Fanem) at 40°C and was ground in a hammer mill (Holmes) before being submitted to 24-hour maceration with methanol:dichloromethane (1: 1) (Merck). After the organic extraction, plant material was dried and a 24-hour maceration with water (Milli-Q) was done[13]. Extracts were lyophilized (Virtis) and stored under -20 °C (Revco) until use.

Extracts were prepared at 20 times the final test concentration, in water or dimethylsulfoxide 50% (DMSO 50) solution in water, for the antibacterial assays. Extracts were prepared at 400 times the final test concentration, in water or DMSO 50, as mentioned above, for the cytotoxicity assays.

Antimicrobial assay

Microdilution broth assay (MBA) was used to test the plant extracts in 96-well microplates. The inoculum was done at the concentration of 0.5 McFarland, or 1.5 x 10^8 UFC/mL, prepared from fresh colonies of bacteria as described below[8] and diluted to a final concentration of 3 x 10^2 UFC/mL.

Staphylococcus aureus ATCC 29213 (Sau), *Escherichia coli* ATCC 25922 (Ecol), *Enterococcus faecalis* ATCC 29212 (Efae) and *Pseudomonas aeruginosa* ATCC 27853 (Psa) were obtained (Oxoid) and seeded in Müeller-Hinton agar (Oxoid), in order to obtain a mother-plaque, to be used up to one month, if kept under refrigeration. The bacteria inocula of each ATCC strain were weekly obtained from mother-plaques and fresh colonies were left to grow in incubator at 36 °C, for 24 h to be used in the assay. A saline suspension was made with each bacterium in order to obtain a concentration of 1.5 x 10^8 CFU/mL (0.5 MacFarland). Then, the inoculum was diluted to 3.0 x 10^2 CFU/mL. One hundred-ninety microliters of the bacteria suspension were transferred to each microplate well. Ten microliters of each extract solution were added to the microplate wells and incubated at 36º C for 24 h. Results were firstly visually analyzed and classified according to the following patterns: X = culture flocks in the bottom of the well, C = turbidity with culture flocks being deposited, + = light turbidity, and L = total growth inhibition. Extracts that showed inhibitory activity at this concentration were submitted to a subculture in Müeller-Hinton Agar (Oxoid), in order to evaluate bacterial growth.

Determination of Minimal Inhibitory Concentration and Minimal Bactericidal Concentration

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for extracts that showed a total growth inhibition using the same protocol described above. The minimal concentration at which there was no visual bacterial growth was taken as MIC, and the minimal concentration at which there was no bacterial growth after inoculation in the subculture was taken as MBC[8].

Cytotoxic assay

Cell culture technique

Tumor cell lines (MCF-7 breast adenocarcinoma; KM-12 colon adenocarcinoma; RPMI-8226 multiple myeloma; PC-3 prostate carcinoma and NCI-H460 lung large cell carcinoma) were cultivated in tissue-culture flasks (Coastar), supplemented with RPMI-1640 plus 5% fetal bovine serum (both Cambras) and 1% glutamine (Sigma), kept in an incubator (Forma) at

Table 1. Antibacterial and antitumor activity of organic and aqueous extracts obtained from leaves, stems and fruits of *Calophyllum brasiliense*

	Staphylococcus aureus		*Pseudomonas aeruginosa*		*Escherichia coli*		*Enterococcus faecalis*		Cytotoxicity [GI_{50} µg/mL]				
Extracts	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MCF-7	PC-3	NCI-H460	KM-12	RPMI-8226
OL	400	500	250	400	430	490	510	570	69.72	>100	95.18	5.50	>100
AL	400	900	>1000	>1000	>1000	>1000	600	840	>100	>100	15.69	4.00	0.56
OS	220	250	190	400	400	450	490	520	–	>100	>100	1.74	>100
AS	280	590	>1000	>1000	>1000	>1000	300	300	0.60	49.07	12.14	1.17	>100
OF	>1000	>1000	500	550	430	450	450	810	>100	>100	>100	>100	>100
AF	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>100	>100	>100	>100	>100

GI50= growth inhibition 50% (n=6); MIC=minimal inhibitory concentration given in µg/mL; MBC=minimal bactericidal concentration given in µg/mL; OL=organic extract from the leaves; AL=aqueous extract from the leaves; OS=organic extract from stems; AS=aqueous extract from leaves; OF=organic extract from fruits; AF=aqueous extract from fruits.

37ºC with 5% CO_2 and 100% relative humidity. Adherent cell lines were weekly passaged (Trypsin-EDTA, Cambras) and so were the non-adherent cell lines[14]. Cell densities were obtained with a hemocytometer chamber, using the trypan blue exclusion method. The same conditions were maintained during the assay and cell densities per well varied according to the cell line, as follows: MCF-7 (10,000), PC-3 (7,500); KM-12 (15,000); NCI-H460 (7,500); and RPMI-8226 (20,000). Cells are incubated for 24h before the drug/extract was added, and the drug/extract remained in contact with the cells for 48 h, in the microculture assay. After that, the end points are obtained by the SRB assay.

Doxorubicin (DOXO) and 5-fluorouracil (5-FU) were used as standard drugs in the assay. The highest DOXO concentration in the test was 2.5 x 10-5 M and the highest 5-FU concentration was 1.86 x 10-5 M for 5-FU. Five ten-fold dilutions were made, and the dose X response curves obtained show the cytotoxic trend observed for each extract.

SRB assay

Viable cells were fixed to the 96-microplates with cold trichloroacetic acid solutions (50µL/well of 50% TCA for adherent cells and 80% TCA for non-adherent cells). Microplates were washed with water five times until complete the removal of non-viable cells. A hundred □L of Sulforhodamine B 0.4% in acetic acid were added to each well and were kept in contact to the cells for 10 minutes. After that, unbound Sulforhodamine B (SRB binds to proteins of viable cells) was removed from plate by washing four times the wells with 0,1% acetic acid solution. The remaining stain was then ressuspended with the addition of 100µL of Trisma Buffer. The variation in the amount of remaining proteins bound to the stain was measured by obtaining the optical densities of the wells in a spectrophotometer reader at 515 nm[15]. The growth inhibition 50% (GI50) was obtained.

Results

Two extracts resulted from each plant material: organic (OL) and aqueous (AL) extract of the leaves – yields

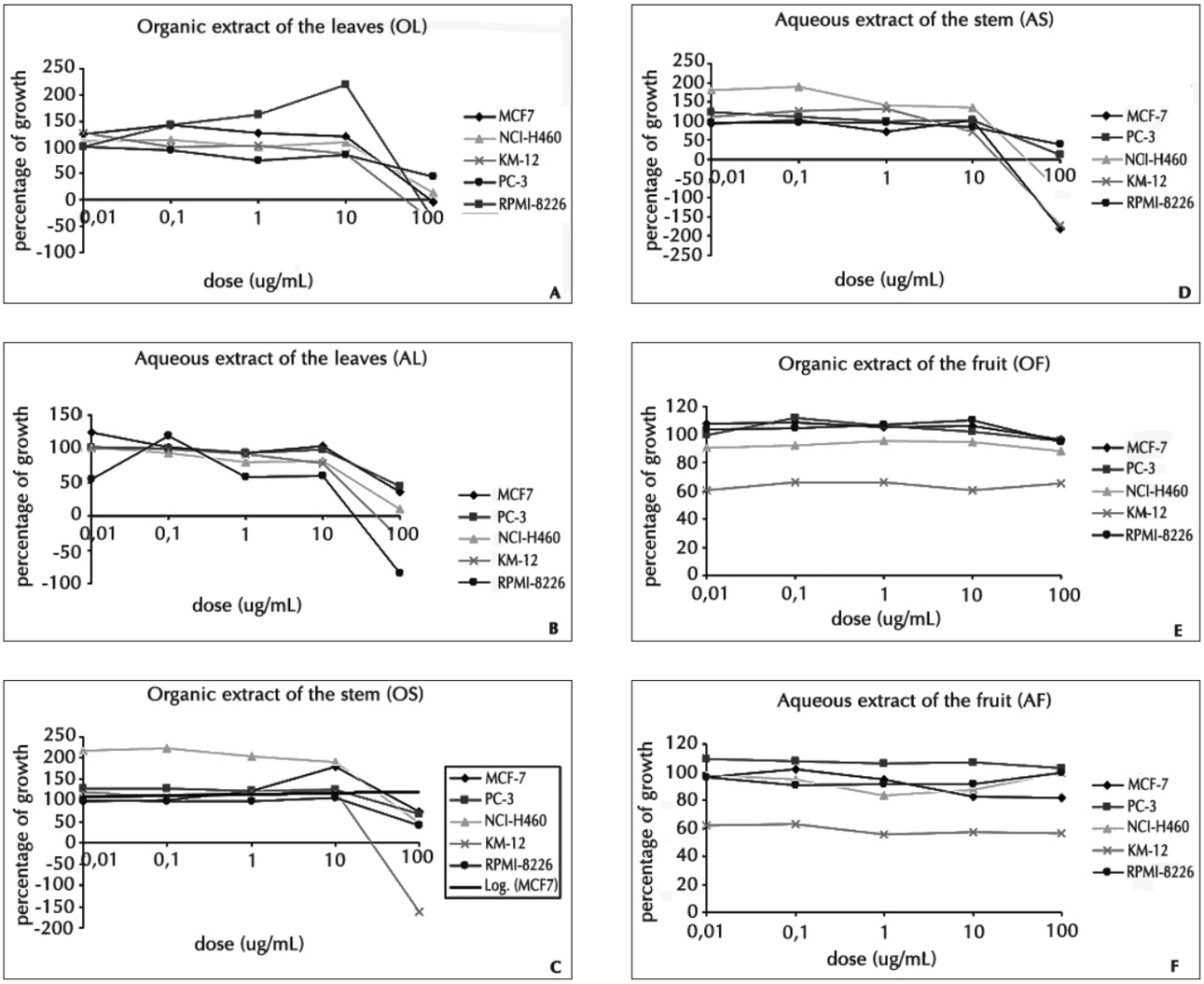

Figure 1. Graphics corresponding to the percentage of growth of human tumor cell lines after treatment with the following extracts: organic (A) and aqueous (B) extracts of the leaves; organic (C) and aqueous (D) extract of the stem; and organic (E) and aqueous (F) extracts of the fruits. Graphic made with mean of sextuplicates.

Table 2. Biological activity of crude extracts and isolated compounds from *Calophyllum brasiliense*

Plant part	Extract/compound	Bilogical activity	References
Bark	Chromanones brasiliensophyllic acid, isobrasiliensophyllic acid, brasiliensophyllic acid B, isobrasiliensophyllic acid B, brasiliensophyllic acid C and isobrasiliensophyllic acid C	Antibacterial	16
Heart wood	Jacareubin, 6-deoxyjacareubin, 1,3,5,6-tetrahydroxy-2-(3-methyl-butenyl)xanthone, and 1,3,5,6-tetrahyroxy-2-(3-hydroxy-3-methyl-butyl)xanthone	Trypanocidal	18
Leaves	Hexane extract	Anti-HIV1	24
Leaves	Coumarins	Anti-K562, U251 and PC3	26
Leaves	Calanolides A and B and soulattrolide	Anti-HIV1	25
Leaves	Phenolic compounds hyperin, ectoflavone, quercetin, gallic acid and protocatechuic acid	Analgesic	27
Leaves, stem and fruits	Polar and non-polar extracts and protocatechuic acid	Anti-Sau (6538P)	21
Seed kernels	Trans and cis chromanone series (three homologous each series)	Antibacterial and cytotoxic activity	17
Bark and timber extracts	Xanthones	No	19
Bark resins	Brasiliensic and inophylloidic acids	No	28
Leaves	jacareubin and 1,3,5,6-tetrahydroxy-2-(3,3-dimethylallyl) xanthone	Anti-Sau	20
Leaves	Dichloromethane extract and hexane fraction	Leishmanicide	29
Leaves	(–) mammea A/BB	Leishmanicide	30
Leaves	(–) mammea A/BB natural and its synthetic and derivatives	Leishmanicide	31
Stem bark	Brasimarins A, B and C and 11 coumarins from calanolide or inophyllon groups	Epistein-Barr	32
Stem bark	Dichloromethane fraction from the hexane extract (maybe xanthones and flavonoids)	Gastroprotective	5
Stem bark	GUT-70	Antileukemic by activation of caspases 2, 3, 8 and 9	23
Stem bark	Hydroethanolic extract and dichloromethane fraction	Antiulcer related to anti-Helicobacter pilori activity	33
	Xanthones, mammea A/BA, mammea C/OA	Inhibition of H(+), K(+) ATPases from dog stomach	22
	Coumarins	Induction of apoptosis	34
	Methanolic extract	Antispasmodic	35
	Coumarins	Cytotoxic, antitumor and antiproliferative	36
	Angiotensin-converting enzyme inhibition	37	
Root wood	Dichloromethane extract	Antiyeast	38
Roots	Methanol extract	Imunostimulation	39
Roots, flowers and fruits	Friedelin, 1,5-dihydroxyxanthone	Antinociceptive	40

Sau = *Staphylococcus aureus*.

11.75% and 4.42%, respectively; organic (OS) and aqueous (AS) extracts of the stems – yields 12.31% and 0.59 %, respectively; organic (OF) and aqueous (AF) extracts of the fruits – yields 8.76% and 2.97 %, respectively.

Results can be seen in Table 1. OL showed to be more active against *P. aeruginosa* (MIC=250 µg/mL; MBC=400 µg/mL), although there can be seen activity against the other three bacteria. AL showed activity only against *S. aureus* (MIC=400 µg/mL; MBC=500 µg/mL) and against *E. faecalis* (MIC=600 µg/mL; MBC=800 µg/mL). OS was active against all four bacteria, and its best activity was observed against *P. aeruginosa* (MIC=190 µg/mL; MBC=400 µg/mL), followed by its activity against *S. aureus* (MIC=220 µg/mL; MBC=250 µg/mL), *E. coli* (MIC=400 µg/mL; MBC= 450 µg/mL) and *E. faecalis* (MIC=490 µg/mL; MBC=520 µg/mL). AS showed activity against both Gram positive bacteria, *S. aureus* (MIC=280 µg/mL; MBC=590 µg/mL) and E. faecalis (MIC=MBC=300 µg/mL). OF showed to

be more active against *E. coli* (MIC=430 µg/mL; MBC=450 µg/mL) although also active against *P. aeruginosa* and *E. faecalis*. AF did not show antibacterial activity. Considering bacterial activity for all six extracts, they can be classified as follows: AF<AS=AL<OF<OL=OS.

Cytotoxicity was observed in some extracts. GI50 values were obtained from the logarithmic regression curve and formula, and show the trend of the extract to be more or less cytotoxic (Table 1). OL showed expressive cytotoxicity against KM-12 (GI50 5.50µg/mL), MCF-7 (GI50 69.72µg/mL) and NCI-H460 (GI50 95,18 µg/mL) (Figure 1A) while AL was more active against RPMI-8226 (GI50 0,56 µg/mL), NCI-H460 (GI50 15.69 µg/mL) and KM-12 (GI50 4.00µg/mL) (Figure 1B). OS showed activity against the KM-12 cell line (GI50 1.74 µg/mL) (Figure 1C). AS showed activity against all four adherent cell lines, particularly against MCF-7 (GI50 0.60µg/mL) and KM-12 (GI50 1.17µg/mL) (Figure 1D). OF and AF did not show cytotoxic activity (Figures 1E and 1F).

Discussions

Antibacterial activity observed here within shows that the popular uses of traditional *jacareuba* remedies against diarrhea, oral diseases, or other infectious diseases can be partially supported considering infectious diseases caused by the pathogens tested in the present assay. Although the antibacterial activities of these extracts were slight against bacteria, it was observed that the extracts made from the stem and leaves showed a better inhibitory activity if compared to the extracts made with fruits, confirming the popular use of the stem bark and leaves infusions. In the present work, the extracts obtained from the stems showed to have a slight efficacy in killing bacteria over the extracts obtained from the leaves, and for that reason, the popular uses of the bark may be justified, but not indicated.

Extracts obtained from stem and leaves showed cytotoxicity, and the extracts obtained from fruits did not show to be cytotoxic. Extracts obtained from stem and leaves show to be more active against bacteria as well, and a diminished activity was observed for the extracts obtained from fruits. Organic extracts showed to be more efficient against bacteria, and aqueous extracts showed to be more efficient against cancer cell lines.

Previous reports (Table 2) relate the presence of chromanones in the bark[16] and in the seed kernels[17] of the plant, while xanthones were found in the heart wood[18-19], and in the stem bark[5,19], the organ used in traditional remedies against diarrhea. Jacareubin and other xanthones were related to leaves[20] and heartwood[18] of the plant. In terms of antibacterial activity, chromanones from bark[16] and seed kernels[17], polar and non-polar extracts and protocatechuic acid of leaves, stem and fruits[21] were reported to be active. Paradoxally, some chromanones isolated from seed kernels[17] were reported to show cytotoxicity, as well as coumarins isolated from the leaves[22] and the isolate from the stem bark named as GUT-70, or GUT-70, characterized as a tricyclic coumarin, 5-methoxy-2,2-dimethyl-6-(2-methyl-1-oxo-2-butenyl) -10-propyl-2H,8H-benzo[1,2-b;3,4-b']dipyran-8-one (C(23)H(26)O(5)), showed inhibitory activity against leukemia cell lines[23].

We identified *jacareuba* extracts with a slight antimicrobial (OS and OL) activity. Nonetheless, cytotoxicity was also observed in those extracts. Although further studies are needed in order to characterize the toxic in vivo implications of the bark tea intake, it is strongly recommended that teas, which are complex mixtures of compounds, made with *jacareuba* organs shall not be ingested without medical recommendation as antibacterial remedies, especially in children care. On the other hand, the significant cytotoxic results implicate in a new area of research of natural product anticancer therapy with isolates from these extracts. The present findings also highlight the efficacy of choosing biological screening as a method of bioprospection.

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