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Artigo

Histochemical, phytochemical and biological screening of *Plinia cauliflora* (DC.) Kausel, Myrtaceae, leaves

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RESUMO: "Rastreamento histoquímico, fitoquímico e biológico de folhas de *Plinia cauliflora* (DC.) Kausel (Myrtaceae)". No presente trabalho, foram investigadas a composição química e atividades biológicas de extratos brutos obtidos com etanol 50%, etanol 70%, acetona:água (7:3; v/v) e clorofórmio das folhas de *Plinia cauliflora* (DC.) Kausel, Myrtaceae, uma árvore nativa de várias regiões do Brasil. Os rastreamentos histoquímico e fitoquímico foram feitos de acordo com reações de caracterização e cromatografia em camada delgada. Para auxiliar na padronização dos extratos foram realizadas determinações do teor de fenóis totais e de flavonoides totais, espectrofotometricamente. A atividade antioxidante foi analisada pela porcentagem de sequestro de radicais livres usando solução de DPPH. A atividade antimicrobiana foi avaliada frente a bactérias patogênicas Gram-positivas, Gram-negativas e espécies de *Candida* utilizando os métodos de difusão em ágar e determinação da concentração inibitória mínima (MIC) de acordo com métodos padronizados. As folhas apresentaram lipídeos nas cavidades secretoras e fenóis, principalmente taninos, nas nervuras e parênquima paliçádico. Os extratos polares apresentaram flavonoides, taninos, alto teor de fenóis totais e de flavonoides totais. Os extratos mostraram elevada atividade antioxidante e a atividade antimicrobiana foi melhor contra as espécies de *Candida* do que contra as bactérias.

Unitermos: *Plinia cauliflora*, Myrtaceae, rastreamento químico, atividade antioxidante, atividade antimicrobiana.

ABSTRACT: In this work, chemical and biological activities of crude extracts obtained with 50% ethanol, 70% ethanol, acetone:water (7:3; v/v) and chloroform of *Plinia cauliflora* (DC.) Kausel, Myrtaceae, leaves, a native tree from several regions of Brazil, was investigated. Histochemical and phytochemical screenings were done according to characterization reactions and thin layer chromatography. To assist in extracts standardization, total phenol and flavonoids content spectrophotometric was performed. Antioxidant activity was analyzed by percentage of radical scavenging using DPPH solution. Antimicrobial activity was evaluated against Gram-positive, Gram-negative pathogenic bacteria and species of *Candida* using agar diffusion and minimal inhibitory concentration (MIC) determination methods according to standard methods. The leaves presented lipids at secretory cavity and phenols, mainly tannins, in nervures and palisade parenchyma. Polar extracts showed flavonoids, tannins and high content of phenols and flavonoids. The extracts showed great antioxidant activity and antimicrobial activity was better against *Candida* species than against bacteria**.**

Keywords: *Plinia cauliflora*, chemical screening, antioxidant activity, antimicrobial activity.

INTRODUCTION

Plinia cauliflora (DC.) Kausel is a plant from the family Myrtaceae, which has one hundred twenty nine genders. The several species from this family are source of essential oils, condiments, food and many of them are used in traditional medicine (Stasi & Hiruma-Lima, 2002). *Myrciaria cauliflora* (Mart.) O. Berg, *Myrtus cauliflora* Mart., *Eugenia cauliflora* DC. *Myrciaria jaboticaba* (Vell.) O. Berg, *Myrciaria tenella* (DC.) O. Berg and *Myrciaria trunciflora* O. Berg are scientific synonims of *P. cauliflora*. In Brazil, it is called popularly as "jabuticabeira" or "jaboticaba" (Lorenzi, 2000).

Morphologically, it is a tree about ten-fifteen

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meters of height, which bark scales every year, that has simple leaves, with flowers and fruits together the stem, feature by what this plant has been named. This tree occurs, preferentially, at flood plain and open forests being find at forests from Brazil, Argentine and Paraguay. *P. cauliflora* produces lots of comestible and delicious fruits twice or more times a year (Lorenzi, 2000; Barros et al., 1996). The bark of *P. cauliflora* is astringent and used by Brazilian people to treat diarrhoea and skin irritations (Lorenzi, 2000).

It has not been scientifically reported the chemical composition and biological properties of the leaves of *P. cauliflora* so that, the purpose of this study was to characterize histochemically and phytochemically the composition and to investigate antioxidant and antimicrobial activities of different crude extracts of these leaves.

MATERIAL AND METHODS

Plant material and preparation of extracts

The leaves of *Plinia cauliflora* (DC.) Kausel (Myrtaceae), collected from São Carlos, Brazil, (22º01'16.6"S; 47º53'57.0"W) in 2004, were identified by Marcos Sobral and deposited at Herbário da Escola Superior de Agricultura "Luiz de Queiroz", ESALQ, USP-Piracicaba, Brazil (voucher ESA nº 96038). The leaves were dried at 40 ºC for 4 d, comminuted and percolated individually with 50% ethanol (50 EtOH), 70% ethanol (70 EtOH), acetone:water (7:3; v/v) (Ac:H_2O) and chloroform ($CHCl_3$). The extracts were concentrated under reduced pressure and lyophilization. The yield of each extract was 35%, 30%, 20% and 2%, respectively.

Bacterial and fungal strains

Tests were performed on reference strains obtained from American Type Culture Collection (ATCC): *Bacillus subtilis* (ATCC 9362); *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228); *Escherichia coli* (ATCC 10536); *Candida albicans* (ATCC 64548); *Candida parapsilosis* (ATCC 22019); *Candida tropicalis* (ATCC 750). After overnight growth at 37 ºC the inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standard in order to achieve the suitable suspension of 1.5 x 10^8 bacteria/mL and 1.0 x 10^6 to 5.0 x 10^6 yeast/mL (NCCLS, 2002; NCCLS, 2003a).

Histochemical screening

Thin transverse sections of the midvein were handmade with a stainless steel razor. Some sections were treated with 2% sodium hypochloride solution, submitted to ethanol-cetone-hexane sequence, rehydrated, washed with distilled water and used as negative control. Anatomical identification was done with 0.05% Toluidin Blue in buffer at pH 6.8 (Kraus & Arduin, 1997). Identification of hydrophilic substances used: ferric chloride for phenolic compounds (Johansen, 1940), vanillin in HCl (9%) solution for tannins (Valette et al., 1998) and picric acid for alkaloids. Identification of lipophilic substances used: Sudan red III for total lipids (Johansen, 1940), Nile Blue sulphate for acid and neutral lipids (Cain, 1947), osmium tetroxide for unsaturated lipids (Ganter & Jollés, 1969-1970), Nadi staining for essential oil and oleoresin (David & Carde, 1964), antimony trichloride for steroids (Hardman & Sofowora, 1972), sulphuric acid for sesquiterpene lactones (Geissman & Griffin, 1971) and 2,4-dinitrophenylhydrazine for terpenoids (Ganter & Jollés, 1969-1970).

Preliminary phytochemical screening and thin-layer chromatography (TLC) profile

Powdered leaves were extracted appropriately to perform characterization reactions of secondary metabolites classes according to Henriques et al. (2004), Zuanazzi and Montanha (2004), Schenkel et al. (2004) and Santos and Mello (2004). Dragendorf's, Bouchardat's, Mayer's and Bertrand's reagents were used to indicate the presence of alkaloids by precipitate formation. Flavonoids were characterized by fluorescence by complexation with aluminum chloride visualized at 235 and 355 nm. The formation and persistence of foam after 20 min of previous agitation and addition of chloridric acid 2 N was used to characterized saponins. For tannins, it was done precipitation reactions with gelatin, ferric chloride to detect condensed and/or hydrolyzed tannins and lead acetate to detect only hydrolyzed tannin. One-dimensional TLC silica gel analysis of the four extracts was performed in chloroform:methanol:n-propanol:water (5:6:1:4; v/v) and visualized by spraying anisaldehyde/H_2SO4 and heating with UV irradiation at 235 nm and by spraying 1% ferric chloride in methanol (Wagner et al., 1984).

Determination of total phenolics and total flavonoids

Total phenolics in the extracts were determined with Folin-Denis reagent according to the method from AOAC (1984). Briefly, 100 µL of aqueous solutions (20 mg/mL) of extracts were diluted 1:200 and samples from 0.2 to 0.5 mL of these solutions were added to 7 mL of water and 0.5 mL of Folin-Denis reagent. After 3 min, it was added 1 mL of 35% Na_2CO_3 solution completing the volume to 10 mL with water. The absorbance was measured at 760 nm using Shimadzu UV-1603 spectrophotometer after 30 min. A standard curve was plotted using different concentrations of an aqueous solution of tannic acid. The percentage of total phenolics compound in the extracts was calculated using a linear equation based on the

standard curve. Total flavonoids content was determined by colorimetrical method described by Djeridane et al. (2006). The extracts (0.2 g) were dissolved in 20 mL of 80% methanol, extracted for 2 h at room temperature and centrifuged at 3000 g for 15 min. The volume of the supernatant was made up to 100 mL with 80% methanol. A portion of 2 mL was taken and added to 2 mL of 2% $AlCl_3$ methanol solution. The absorbance was measured at 430 nm after 15 min at room temperature. A standard curve was plotted using different concentrations of rutin solution in 80% methanol. The percentage of total flavonoids in the extracts was calculated using a linear equation based on the standard curve.

Antioxidant activity

Antioxidant potential of the extracts at 250 µg/mL in methanol was evaluated by radical scavenging capacity with 0.004% 2,2-diphenyl-1-picryl-hydrazyl (DPPH) in methanol using spectrophotometry at 517 nm. Gallic acid, rutin and vitamin C were used as positive control (250 µg/mL). It was added 2.50 mL of DPPH solution to 1.00 mL of samples, shaked and kept at dark place for 30 min. Blank solutions were done with 1.00 mL of extract solution added to 2.50 mL of methanol and negative control consisted of 1.00 mL of methanol added to 2.50 mL of DPPH solution. Anti-radical activity was calculated as discolour percentage of DPPH according to the equation: % = (DA-SA/DA).100; where: DA = DPPH absorbance; SA = sample absorbance (Falcão et al., 2006).

Antimicrobial assay of crude extracts

Stock solutions of 100 mg/mL of each extract were prepared in dimethylsulfoxide (DMSO) and were conveniently diluted in culture medium. All tests were performed according to standard methods and to Ostrosky et al. (2008) review applied for medicinal plants.

Agar diffusion method (NCCLS, 2003b)

The test was made with 1 x 10^6 CFU/mL in Petri dishes. Steel templates were placed on the solid medium and 50 µL of the extracts (50 mg/mL), 50 µL of DMSO:BHI (BHI) (1:1; v/v) and 50 µL of ampicillin solution (50 µg/mL) were separately added to each well. After 2 h at 4 °C the plates were incubated at 37 ºC for 24 h. Bacterial growth inhibition was determined by inhibition zones diameter around the wells.

Assay for antimicrobial activity

Minimal inhibitory concentration (MIC) values were determined by microdilution method (NCCLS, 2002; NCCLS, 2003a). Final dilutions ranging from 5 mg/mL to 0.078 mg/mL of crude extract were prepared in sterile 96-well microplates. The wells were filled with BHI for antibacterial approach; RPMI-1640 medium with 2% glucose and 0.165 mol/L 3-[N-morpholine] propanesulfonic acid (MOPS) at pH 7 for antifungal. Final bacterial inocula were 2.5 x 10^5 CFU/mL and incubated aerobically at 35 ºC for 24 h. Final yeast inocula were 2.5 x 10^3 CFU/mL incubated at 35 ºC for 48 h at 100 rpm. Ampicillin and amphotericin B were used as positive control to bacteria and yeast, respectively. Bacterial growth was indicated by addition of 0.01% resazurin aqueous solution and MIC values were identified as the lowest extract concentration in which no growth is visible showed by changing colour of resazurin from blue (abscence of growth) to pink (growth) (Gabrielson et al., 2002). Yeast growth was indicated by addition of 20 µL of 2% 2,3,5-triphenil tetrazolium chloride (TTC) aqueous solution (Eloff, 1998). Yeast MIC values were identified as the lowest extract concentration in which there was no growth and therefore TTC changed the colour from yellow (absence of growth) to red (growth). Minimal bactericidal concentration (MBC) and minimal fungal concentration (MFC) were determined by subculturing the microplates in Petri dishes with Müller-Hinton-agar for bacteria and Sabouraud-agar for yeast, incubated at 35 ºC for 24 h, and defined as the lowest extract concentration which there was no visible growth of colony.

RESULTS AND DISCUSSION

It was noted that the leaves of *Plinia cauliflora* (DC.) Kausel (Myrtaceae) has the same anatomical organization as Myrtaceae family, showing dorsiventral mesophyll emphasing secretory cavities and idioblasts containing calcium oxalate crystals (druseans and monocristals). It was verified the presence of phenols, tannins and alkaloids in midvein and smaller veins; phenols were also verified in palisade parenchyma. Lipids were verified mainly in secretory cavities and reactions were positive for acid and unsaturated lipids, steroids, sesquiterpene lactones and terpenoids; it was noted weak positive result for essential oil. Preliminary phytochemical analysis of the drug showed only the presence of hydrolysable tannins and flavonoids. TLC confirmed these results since three extracts showed two yellow fluorescent spots of flavonoids (R_f = 0.37 and 0.42) with anisaldehyde/H_2SO_4, heating and observation under UV light and one gray spot of tannin (R_f = 0.25). One strong blue spot of tannin (R_f = 0.25) was observed by spraying ferric chloride 1% in methanol. In contrary of histochemical reaction, TLC tests did not indicate the presence of alkaloids, which must be investigated. $CHCl_3$ extract showed only a weak blue spot around start point. Terpenoids were detected with anisaldehyde/H2SO4 with spot around R_f = 1.00. Except to $CHCl_3$ extract, the three extracts showed content of phenolic compounds higher than 45%. Total flavonoids content were not found in $CHCl_3$ extract and about 1% in polar extracts (Table 1).

Table 1. Total phenolic and flavonoid content in the extracts of the leaves of *P. cauliflora.*

Extract	Phenolic content (%)	Flavonoid content (%)
50 EtOH	45.84±1.05	1.36±0.01
70 EtOH	62.08±1.36	1.25±0.03
Ac:H_2O	60.25±3.60	1.53±0.01
$CHCl_3$	0.27±0.09	0.00

a) value is mean±standard deviation.

Polar extracts showed high antioxidant activity, beyond 90%, but significantly smaller than the controls gallic acid, rutin and vitamin C (Figure 1). Choi et al. (2006) suggested that many biological activities in plants are due to the content of total phenols like flavonoids and tannins. Plant phenols have potential antioxidant activity, mainly by acting as oxygen radical scavengers (Quettier-Deleu et al., 2000). Other studies (Souza et al., 2007; Tadhani et al., 2007) showed that extracts with high content of phenols have high antioxidant activity and that was seen in this study since chloroformic extract exhibited the smaller phenol content and antioxidant activity.

Agar diffusion method showed great inhibition zones (Table 2) for polar extracts. However, MIC and MBC determination of 50 EtOH, 70 EtOH and Ac:H_2O extracts have showed weak antibacterial activity whereas $CHCl_3$ had not any activity at the highest concentration tested (Table 2). The bacteria selected for this study are present in the human being daily as normal microbiota, e.g., *Staphylococcus aureus* and *Staphylococcus epidermidis*; as food contaminants, e.g., *Escherichia coli* or used to sterility control, e.g., *Bacillus subtilis* (Chomnawang et al., 2005; Beovic, 2006; Abdou et al., 2007; Pesavento et al., 2007). Polar extracts showed great activity against *Candida* species and *C. tropicalis* was more sensible to them since MFC values were the smallest. $CHCl_3$ extract did not showed activity at the highest concentration tested (Table 2). This work had interesting results against *Candida* species. *Candida albicans* is the yeast species more often isolated from biological samples being an usual skin and mucosa colonizing agent with many hosts in the oral cavity, and higher incidence rates among young children and patients infected with HIV, but is necessary to pay attention to the emergence of other *Candida* species as infecting agents (McCullough et al., 1996; Runyoro et al., 2006; Zhang et al., 2006). Resistance to commonly used agents, toxicity and costs impelled the search for new agents and it can be noted that in recent years the research for new active compounds from natural sources has been increasing (Duarte et al., 2005; Runyoro et al., 2006).

Histochemical and phytochemical results showed phenolic prevalence in the composition of the leaves and extracts of *P. cauliflora* and can contribute to the standardization of the extracts. Besides, it was possible to indicate that polar extracts of the leaves have biological activities as antioxidant and antimicrobial that can be due to their phenolic content. It should be emphasize the presence of flavonoids and tannins derivates in polar extracts and the activity against *Candida* species showed by them. It was noticed that $CHCl_3$ extract showed small composition of phenolic content, therefore it could explain the smallest antioxidant and antimicrobial activities.

This study is important since it is the first to indicate the leaves extracts of *P. cauliflora* as new source of antioxidant and antimicrobial molecules and because it was determined the composition of the leaves. These investigations showed a preliminary standardization of the leaves by histochemical and phytochemical methods.

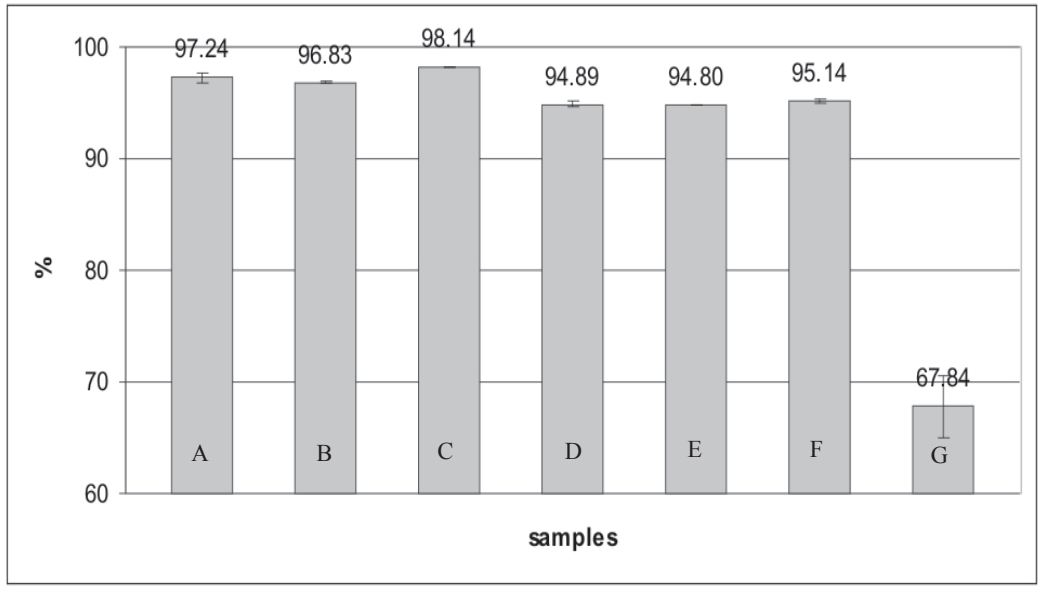

Figure 1. Radical scavenging capacity. Percentual ± standard deviation. A) gallic acid. B) rutin. C) vitamin C. D) 50 EtOH extract. E) 70 EtOH extract. F) Ac:H_2O extract. G) $CHCl_3$ extract.

Table 2. Antimicrobial activity of *P. cauliflora* leaves extracts.

Samples	Inhibition zone[a]				MIC/MBC[b]				MIC/MFC[c]		
	Sa	*Se*	*Bs*	*Ec*	*Sa**	*Se**	*Bs**	*Ec**	*Ca**	*Cp**	*Ct**
50 EtOH	18.1±1.6	19.3±2.0	19.1±1.2	19.0±1.7	5.0/5.0	5.0/5.0	>5.0/>5.0	5.0/5.0	0.62/>10	0.62/>10	0.62/1.25
70 EtOH	18.4±1.0	18.7±0.5	18.1±.2	18.1±2.0	5.0/5.0	5.0/5.0	>5.0/>5.0	5.0/>5.0	0.62/>10	0.62/>10	0.62/2.50
Ac:H_2O	18.7±1.2	20.1±1.5	19.5±0.3	19.6±1.7	5.0/5.0	5.0/5.0	>5.0/>5.0	5.0/>5.0	0.62/>10	0.62/>10	0.62/1.25
$CHCl_3$	-	-	-	-	>5.0/>5.0	>5.0/>5.0	>5.0/>5.0	>5.0/>5.0	>10/>10	>10/>10	>10/>10
amp**	15.0±0.8	14.7±0.6	15.1±0.2	15.0±0.6	3.1/3.1	1.6/1.6	1.6/1.6	1.6/1.6	n.e.	n.e.	n.e.
amphB**	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	0.25/0.25	0.25/0.25	0.25/0.25

Sa: Staphylococcus aureus; Se: Staphylococcus epidermidis; Bs: Bacillus subtilis; Ec: Escherichia coli; Ca: Candida albicans; Cp: *Candida parapsilosis*; *Ct: Candida tropicalis*. amp: ampicillin; amphB: amphotericin B; a) values expressed, in milimeters, as mean of three determinations ± standard deviation; b) MIC and MBC determination; c) MIC and MFC determination; -: no inhibition; n.e.: not evaluated; * values expressed in mg/mL; ** values expressed in μg/mL.

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