Cordia verbenacea DC. (Boraginaceae): quality control parameters, phytochemical screening and evaluation of anti-*Candida* activity

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ABSTRACT: *Cordia verbenacea* occurs all along the Brazilian coast and it is widely used to treat various inflammatory processes. In the present study we aimed to describe a physical-chemical profile and pharmacognostic vegetal drug as well as to draw a chromatographic profile of the crude extract and its fractions. We have observed the occurrence of secondary metabolites such as flavonoids, terpenes and tannins. The study of anti-*Candida* activity of extracts indicated the best results for *C. glabrata* (9.77-36.06 µg.mL^{-1}), intermediate values for *C. krusei* (312.5-625 µg.mL^{-1}), *C. tropicalis* (312.5-625µg.mL^{-1}) and high values for *C. albicans* (1250>2500 µg.mL^{-1}) and *C. parapsilosis* 625 - 2500 µg.mL^{-1}).

Keywords: *Cordia,* phytochemical, anti-*Candida*

RESUMO: *Cordia verbenacea*: parâmetros físico-químicos, fitoquímico da droga vegetal e atividade anti-*Candida* do extrato hidroetanólico e suas frações. A *Cordia verbenacea* ocorre ao longo de todo litoral brasileiro e é amplamente usada para tratar vários processos inflamatórios. O presente trabalho teve como objetivos descrever um perfil físico-químico e farmacognóstico da droga vegetal, bem como traçar um perfil cromatográfico do extrato bruto e suas frações. Em nossos resultados pôde-se verificar a ocorrência de metabólitos secundários, tais como flavonóides, terpenos e taninos. O estudo da atividade anti-*Candida* indicaram ótimos valores para de CIM para *C. glabrata* (9,77 - 36,06 ug.mL^{-1}), valores intermediários para *C. krusei* (312,5 - 625 mg.mL-1) e C. tropicalis (312,5 – 625 µg.mL^{-1}) e valores altos para *C. albicans* (1250 – 2500 µg.mL^{-1}) e *C. parapsilosis* (625-2500 mg.ml^{-1}).

Palavras-chave: *Cordia*, fitoquímica, anti-*Candida*

INTRODUCTION

The World Health Organization (WHO) points since the 90's that 65-80% of the population in developing countries use medicinal plants as therapeutic agents. In Brazil, many plants are used for medicinal purposes with little or no scientific evidence (Veiga Jr. *et al*., 2005).

Herbal drugs in order to be used as raw material for production of herbal medicines should match the quality specifications, to ensure the efficacy and safety of the product. For this purpose, it is necessary to realize the physicochemical characterization of plant raw material and to identify and to quantify the secondary metabolites responsible for the biological activities. If the bioactive chemical markers are not determined for the herbal drug it is necessary to perform a preliminary phytochemical screening for the determination of the major classes of metabolites present in the plant material.

Boraginaceae family includes approximately 100 genera with over 2,000 species distributed around the world and some of these species were studied regarding antimicrobial activity (Barroso *et al*., 2002). For example, Zavaleta *et al*. (2011) reported that the methanolic extract of (aerial parts) from *Cordia globosa* (Jack.) Kunth presented antimicrobial activity against Gram-positive bacteria with a minimum inhibitory concentration range of 125-1000 µg.mL^{-1}.

Cordia verbenacea DC. is a shrub, perennial, evergreen, with leaves vigorous, dull and crinkly surface, flowers small, white, showy corollas and aerial parts with strong odor given off by easily handling. The species occurs throughout the entire Brazilian coast and in the Atlantic Forest. Popular names in Brazil include *maria-preta*, *maria-milagrosa*

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and *catinga-de-barão*, being the most common *erva-baleeira* or *baleeira* (Lorenzi & Matos, 2002; Ladeira, 2002).

Hydroethanolic extracts of aerial parts from *C. verbenacea* are largely employed to treat several topical inflammatory processes. The plant is used for arthritis, rheumatism and spinal disorders, administered orally as an infusion or decoction. The identified active compounds of the plant are essential oil components and flavonoids, mainly artemetin that exhibited anti-inflammatory and antibacterial activity (Matias *et al*., 2010).

C. verbenacea essential oil have been extensively studied in terms of chemical composition and presented anti-inflammatory action and antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Pinho *et al.,* 2011; Rodrigues *et al.,* 2012). However, there are few phytochemical studies of non-volatile components of its extracts and their antifungal activity was not evaluated.

The search for medicinal plants as a source of active antimicrobial occupies a large group of researches, since the occurrence of resistant strains to existing antibiotics is increasing. Thus, a study of the anti-Candida activity of the hydroethanolic extract of leaves from *C. verbenacea* and its fractions (aqueous, *n*-butanol, ethyl acetate and hexane) was here performed, besides a pharmacognostic characterization of herbal drug and chemical analysis of herbal drug and extract.

MATERIAL AND METHODS

Plant material

Cordia verbenacea DC. leaves were collected in the experimental field of Centro Pluridisciplinar de Ciências Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas (CPQBA-UNICAMP), Betel district, Paulínia city, São Paulo State, Brazil (22°47'15.91"S 47°06'42.87"O). A voucher specimen was deposited at Herbarium of São José do Rio Preto under the reference number SJRP 31235. The plant material was dried in an oven (40°C) with air circulation. To evaluate the loss of water and volatiles compounds, three samples of 10 g were weighed diary until to obtain constant mass of the material. The dried material was ground in a knife mill.

Pharmacognostic quality control assays

The plant drug pharmacognostic assays were performed according to pharmacopeic methods or other validated methods, including: determination of particle size distribution (Ansel *et al*., 2000), determination of loss on drying (Brasil, 2010), extractable matter (method 1; WHO, 2001), determination of total and acid-insoluble ash (WHO, 2001) and pH.

Phytochemical screening in the plant drug

The phytochemical screening was realized according to Costa (2001) to determine the secondary metabolites classes present in the plant drug (saponins, tannins, flavonoids, anthraquinones, coumarins, alkaloids and methylxanthines). The tests were performed in triplicate.

Extract obtainment

The hydroethanolic extract (70 %) was prepared as described: 100 g of plant drug were extracted with 900 mL of ethanol 70% during 7 days (maceration); the extractive solution was filtered and concentrated under reduced pressure and dried in an oven at 40°C to yield the hydroethanolic dried extract.

Chromatographic analysis of hydroethanolic dried extract

TLC analysis was performed to obtain chromatographic profiles of the extract and to confirm the results of the phytochemical screening in the plant drug. TLC was developed in silica gel plate aluminum backed using the following conditions (Wagner *et al*., 1996): a. flavonoids - *n*-butanol: acetic acid: water 60:37:03 (v/v) as eluents and Natural Products reagent plus polyethylene glycol as spray reagent; b. alkaloids – chloroform: ethyl acetate 60:40 (v/v) and Dragendorff reagent as spray reagent; c. terpenes – hexane: ethyl acetate: isopropanol 70:28:02 (v/v) as eluent and sulfuric anisaldehyde as spray reagent; d. tannins - ethyl acetate: formic acid: water 80:10:10 and 90:05:05 (v/v) and ferric chloride 1 % (ethanol) as spray reagent.

For HPLC analysis, 10.0 mg of dried extract were dissolved in 1.0 mL of methanol: water (95:05, v/v) and submitted to further clean up by reversed phase solid phase extraction in a SampliQ® column (C18 45 µm; 500 mg; 6 mL) that was activated by methanol and conditioned with methanol: water (95:05, v/v). Following application of sample, the column was eluted with 4.0 mL of methanol: water (95:05, v/v). The eluated was dried in a desiccator with silica gel under reduced pressure, dissolved in methanol (1.0 mL), and filtered through PVDF membranes (0.45 µm) prior to HPLC analysis. Aliquot of 20 µL was injected onto Hypersil Gold® C18 column (250 × 4.6 mm, 5 µm), which were eluted with a mixture of methanol and water, initially at 95:05 (v/v), changing by linear gradient to 100% methanol over 38 min. The solvent flow rate was 1.0 mL/min. Detection was at 250 nm.

Evaluation of anti-*Candida* activity

Selection and cultivation of yeast strains

The strains ATCC (American Type Culture Collection) *Candida albicans* (90028), *C. krusei* (6258), *C. parapsilosis* (22012), *C. glabrata* (90030) and *C. tropicalis* (750) were used. The strains were grown on Sabouraud agar and incubated at 35 ° C for 24 - 48 hours.

Preparation of samples

Calculations were performed to obtain an initial concentration in the first well of the microplate 2500 µg.mL^{-1}. Thus, there was a solution of 10 mg.mL^{-1} for the extract and its fractions. In order to prepare the stock solution for diluting the crude extract, the aqueous fraction, hexane and n-butanol, distilled water was used, while for the ethyl acetate fraction initial dilution was performed in dimethylsulfoxide (DMSO) and subsequently further dilutions were prepared in water in order to obtain the stock solution of 10 mg.ml^{-1}.

Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The Minimum Inhibitory Concentration (MIC) against Candida species was performed using the microplate dilution method, according to protocol of Clinical and Laboratory Standards Institute (CLSI) M27-A3 (CLSI, 2008) with modifications. The culture medium used for cultivation of *Candida* sp. was the RPMI-1640 medium containing glutamine and phenol red, without sodium bicarbonate, supplemented 3-[N-morfin] propanesulfonic acid (MOPS) at a concentration of 0,165 mol.L-1, pH 7.0, sterilized by filtration. After the addition of 100 µL of RPMI 1640 to each well 100 uL of the diluted extract or fractions was added to the first well of each row microplate. Also 100 µL of positive control solution of amphotericin B and diluted negative control solutions of DMSO were added to microplate. Serial dilutions were performed and then were added 100 µL of yeast suspension previously prepared to all microplate wells (except those intended for sterility control of the medium). The final cell concentration was 2.5x10^{3} cells.mL^{-1}. Sample concentrations ranged from 2500 µg.mL^{-1} to 1.25 µg.mL^{-1} and 16 µg.mL^{-1} to 0.0078 µg.mL^{-1}. The microplates were incubated for 48 h at 35,5°C under constant agitation at 150 rpm. The inhibition of fungal growth was evidenced by the addition of 20µL of resazurin (0.1%) after incubation at 35°C for about 3 h.

RESULTS AND DISCUSSION

The mass of plant material was constant after 72 h in the drying process of the fresh plant material and the loss of water and volatiles after this time was calculated as 77,6 % (m/m). Table 1 summarizes the results of pharmacognostic analysis of plant drug.

TABLE 1. Quality control pharmacognostic parameters of plant drug.

Assays	Results
Pharmacopeial powder fineness*	moderately coarse
Average size particle (mm)	0.4293 ±0.008
pH	7.01 ±0.011
Loss on drying (%)	8.40 ±0.21
Total ash (%)	17.40 ±0.46
Acid-insoluble ash (%)	1.02 ±0.06
Extractable matter (%)	31.48 ±0.49

* BRASIL, 2010

The particle size evaluation of powdered plant material is an important specification to be established. Extraction process is affected by particle size and the smaller the particle size the greater the efficiency of extraction (Simões *et al*., 2007). The results of determination of particle size distribution are presented in Table 1 and in Figure 1. The average particle size obtained was 0.4293±0.0080 mm (Ansel *et al.,* 2000). Most of the particles presented size of 180-850 µm (Figure 1). According to the Brazilian Pharmacopoeia (ANVISA, 2010) this powder is classified as moderately coarse.

The result of loss on drying of the plant drug was 8.4 % (m/m) that is in agreement with recommended value of literature (less than 14 %) to avoid degradation of secondary metabolites and growth of microorganisms (Simões *et al*., 2007). The values obtained for total ash (physiological and non-physiological ash) and acid-insoluble ash (non-physiological ash) were 17.40% and 1.02%, respectively. Acid-insoluble ash measures the amount of silica present, especially as sand and siliceous earth.

The determination of extractable matter determines the amount of constituents extracted with a given extraction method from a determined amount of plant material. In this work we used the Method 1 of World Health Organization (WHO, 2001) and we obtained as result 31.48 % (mass of dried extract per mass of plant material x 100).

There are many different secondary metabolites in medicinal plants. Some of these substances have pharmacological activities and they are considered the chemical markers of the species. According to data in scientific literature, *C. verbenacea* leaves have as main secondary metabolites flavonoids such as artemetin (Matias *et al*., 2010). Phytochemical screenings are generally

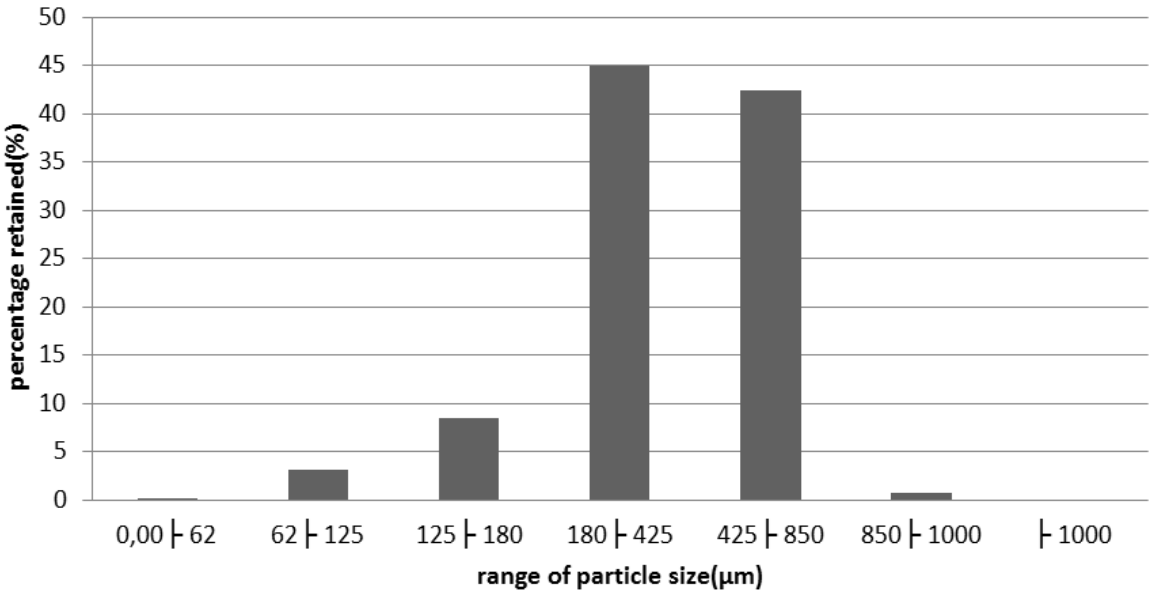

FIGURE 1. Determination of particle size distribution.

performed by using chromatographic techniques as TLC and chemical reactions for secondary metabolites class identification.

The results of phytochemical screening using chemical reactions for the plant drug (Table 2) suggest the presence of saponins, tannins, flavonoids and alkaloids in leaves of *C. verbenacea*. Regarding alkaloid reactions, the positive results were observed as a turbid solution (not a precipitate) only with Dragendorff and Bourchard reagents.

The TLC analyses interpretation were based on the use of selective spray reagents and mobile phases for metabolite secondary classes. Results indicated the presence in the extract (ethanol 70%) of flavonoids and terpenes (probably essential oil components), as previously described in the literature (De Carvalho *et al*., 2004; Matias *et al*., 2010), and tannins. Alkaloids were not detected in TLC and the results of chemical reactions for this secondary metabolite class were doubtful. Possibly, these compounds are present in minor concentration in *C. verbenaceae* (trace levels), but in other Boraginaceae species such as *Cordia sellowiana* Cham. and *Cordia myxa* L., alkaloids are the main components (Barroso & Oliveira, 2009).

The HPLC-UV profile of the extract at 250 nm is presented in a chromatogram in Figure 2. We developed an initial gradient method on reversed phase (C18) for this extract. The main peak observed in the chromatogram presented a retention time of 32.07 min, being one of the least polar compounds of the extract (total analysis time = 38 min). Other peaks were observed in the beginning (polar compounds) and in the middle (the most prominent between

TABLE 2. Results of the phytochemical screening of plant drug.

Secondary metabolites	Assays	Results
saponins	Foaming index	+
tannins	Gelatin test	+
	Ferric chloride	+
flavonoids	Shinoda	-
	Taubock	+
	Pew	-
	Ferric chloride	+
	Aluminum chloride	+
anthraquinones (free state)	Borntrager	-
anthraquinones (glycosides)		-
alkaloids	Dragendorff	+
	Mayer	-
	Bertrand	-
	Bourchard	+
coumarins	UV detection	-
methylxanthines	Reaction with ammonia	-

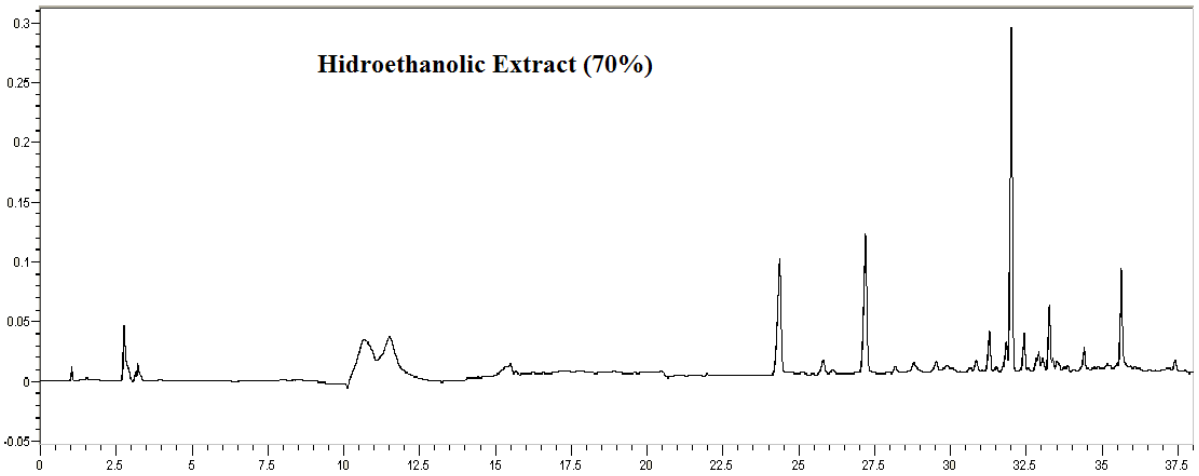

FIGURE 2. HPLC profile of *C. verbenacea* hydroalcoholic extract.

TABLE 3. Minimum Inhibitory Concentration (MIC) and minimum fungicidal concentration (MFC) (µg.mL^{-1}) of crude extract and fractions of *C. verbenacea*.

Extract/fraction (f.)	*C. albicans*		*C. parapsilosis*		*C. glabrata*		*C. tropicalis*		*C. krusei*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Extract	2500	>2500	625	>2500	9,77	>2500	312,5	312,5	312,5	2500
Aqueous f.	>2500	>2500	2500	>2500	9,77	>2500	625	>2500	312,5	625
Hexane f.	1250	>2500	1250	1250	39,06	>2500	625	625	312,5	625
n-butanol f.	>2500	>2500	1250	>2500	19,53	>2500	625	625	312,5	>2500
ethyl acetate f.	1250	>2500	1250	1250	19,53	>2500	625	1250	625	2500
amphotericin B	0,25	0,25	0,50	0,50	0,50	0,50	0,50	2,00	0,50	0,50

10 and 17 min) of the chromatogram. In general, the profile of the extract is simple in the evaluated wavelength.

In summary, so far this work showed results applicable as quality parameters for *C. verbenacea* plant drug and confirmed the presence of some classes of secondary metabolites described for the species.

Determination of anti-Candida activity

Aligianis and collaborators (2001) proposed a classification for plant materials based on the results of MIC, considering strong inhibition to MIC 500 µg.mL-1; moderate inhibition to MIC between 600 and 1500 µg.mL^{-1} and weak inhibition for MIC above 1600 µg.mL^{-1}. Other authors (Mbaveng *et al*., 2012; Rios & Recio, 2005) establish that a very interesting inhibitory concentration is 100 µg.mL^{-1} for crude extract but Mbaveng *et al.* (2012) still consider a moderate activity to MIC values ranging between 100-625 µg.mL^{-1}.

Table 3 shows the values of MIC and MFC for the crude extract of *C. verbenacea* as well as its fractions. Thus, the crude extract showed a weak activity against strains of *C. albicans*, a moderate activity against *C. parapsilosis*, and a strong activity against *C. tropicalis*, *C. krusei* and *C. glabrata*, with only fungicidal activity against *C. tropicalis* and *C.krusei.*

The aqueous and n-butanol fractions showed no activity up to 2500 µg.ml^{-1} against *C. albicans*; against *C. parapsilosis* n-butanol fraction obtained a moderate activity while the aqueous fraction obtained a weak activity, both fractions had moderate activity against *C. tropicalis* and strong activity against *C. glabrata* and *C. krusei.* The hexane and ethyl acetate fractions, showed moderate activity against strains of *C. albicans, C. parapsilosis* and *C. tropicalis* and strong activity against *C. glabrata* and *C. krusei.*

The crude extract and fractions showed better results against strains of *C. glabrata* only inhibiting the growth of this strain, but being unable to kill it. Moreover, fractions and crude extract were fungistatic, as verified in CFM of *C. glabrata.*

Thus, among all the results here obtained, the one which highlights and holds attention is the strong fungistatic against *C. glabrata* where the MIC

ranges from 9.77 to 39.06 µg.mL^{-1}, but it failed to show any fungicide activity. This variation may be related to the presence of tannins in the crude extract and in almost all fractions as found in the analysis by thin layer chromatography (Table 3), because tannins have fungistatic activity, in addition to other classes of secondary metabolites which were determined by the TLC and phytochemical screening tests. *C. glabrata* were more sensitive to extract and aqueous fraction that demonstrate a fungistatic activity than compared with other strains. This data is very useful due to *C. glabrata* is one of the non-*albicans* species that most affects the population in general, besides being resistant to azoles and other antifungals, such as fluconazole (Passos *et al*., 2002; Arif *et al*., 2009; Agha *et al*., 2011).

Studies of antifungal activity of essential oil of *C. verbenacea*, showed a high performance related to strains of *C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei*. Thus, comparing the results of this study with the results of the studies by Rodrigues and collaborators (2012), which tested the antimicrobial activity *of C. verbenacea* essencial oil obtaining MIC values 512 µg.mL^{-1} for *C. albicans* and *C. krusei*, the results presented in this work were more effective in the case of *C. krusei*, plus showing activity against other *Candida* species. It is noteworthy that the strains used in both studies are different, and there may be discrepancies in the comparisons of the results.

Thus, a possible extension of studies with *C. verbenacea* not just focused on the essential oil, but also in the production of extracts, would be an important strategy in the search for new active substances with antifungal activity.

CONCLUSION

The collection site of the branches was in the area of cultivation of excellence, but it should be useful to consider for the physical-chemical control of plant drug, the values of loss on drying, total ash and acid insoluble ash. These pharmacognostic parameters are important and can be used to establish specifications for the plant drug.

There are secondary metabolites that serve as chemical markers to verify the authenticity of plant drug as well as being the reason for future phytochemical studies, such as flavonoids, tannins and terpenes that have been highlighted in the preliminary phytochemical screening and thin layer chromatography.

The anti-Candida activity demonstrated promising activity for the crude extract and its fractions, depicting a portion of the biological activity of extracted metabolites, especially against non-*albicans* strains, *C. parapsilosis*, *C. glabrata* which was obtained best results for MICs, ranging from 9.77 to 39.06 µg.mL^{-1}), *C.krusei* and *C. tropicalis*.

We proposed that a possible expansion of the studies of *C. verbenacea* should not only focused on the essential oil, but also in the production of extracts, since the major strategies is to search for new active substances.

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