



## Antifungal activity evaluation of *Aloe arborescens* dry extract against *trichosporon* genus yeasts

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**ABSTRACT.** The objective of this study was to investigate the antifungal activity of *Aloe arborescens* dry extract against *Trichosporon* genus yeast species. Extraction was carried out by means of a longitudinal incision in fresh leaves, which were collected on a vat, and the total volume was frozen and subsequently lyophilized. Then, 40 mg of the dry extract was dissolved in DMSO by gentle inversion in order to obtain a solution whose concentration was 4000  $\mu\text{g mL}^{-1}$ . This solution became limpid and slightly yellowish because the pigment of the latex was attenuated. It was performed serial dilutions from 2,000 to 15.625  $\mu\text{g mL}^{-1}$  with RPMI-1640 broth. There was already no pigment in the first dilution of 2000  $\mu\text{g mL}^{-1}$ . It was analyzed fifteen strains of *Trichosporon* spp., and *Candida albicans* ATCC 10231 was used as control strain. We carried out the reading of microplates in the ELISA reader device at a wavelength of 530 nm, after incubation for 24 and 48 hours, and it was determined the Minimum Inhibitory Concentration (MIC). The MIC<sub>50</sub> value obtained for all *Trichosporon* species and for *C. albicans* was 500  $\mu\text{g mL}^{-1}$ . As a result, we concluded that *Aloe arborescens* dry extract has antifungal activity against *Trichosporon* yeasts.

**Keywords:** medicinal plants, susceptibility test, opportunistic pathogens.

## Avaliação da atividade antifúngica do extrato seco de *Aloe arborescens* sobre leveduras do gênero *trichosporon*

**RESUMO.** Este trabalho teve por objetivo investigar a atividade antifúngica do extrato seco de *Aloe arborescens* frente a espécies de leveduras do gênero *Trichosporon*. A extração foi realizada por meio de uma incisão longitudinal em folhas frescas, e o volume total coletado foi congelado e, posteriormente, liofilizado. A seguir, 40 mg do extrato seco foram dissolvidos em 10 mL de DMSO por inversão delicada para obtenção da solução-mãe a uma concentração de 4000  $\mu\text{g mL}^{-1}$ . Tal solução tornou-se límpida e levemente amarelada, pois o pigmento do látex foi atenuado. A seguir, foram efetuadas diluições seriadas de 2.000 até 15,625  $\mu\text{g mL}^{-1}$  em caldo RPMI-1640. Na primeira diluição de 2.000  $\mu\text{g mL}^{-1}$  já não havia mais pigmento. Quinze cepas de *Trichosporon* spp. foram analisadas, e *Candida albicans* ATCC 10231 foi empregada como cepa controle. Após incubação por 24 e 48h, foi realizada a leitura das microplacas em um aparelho leitor de ELISA no comprimento de onda de 530 nm e determinada a Concentração Inibitória Mínima (CIM). O valor de CIM<sub>50</sub> encontrado para todas as espécies de *Trichosporon* analisadas e para *C. albicans* foi de 500  $\mu\text{g mL}^{-1}$ . Concluiu-se que o extrato seco de *Aloe arborescens* apresenta atividade antifúngica sobre leveduras do gênero *Trichosporon*.

**Palavras-chave:** plantas medicinais, perfil de susceptibilidade, patógenos oportunistas.

### Introduction

The empirical knowledge accumulation about medicinal plants usage has been handed down from ancient civilizations to the present day (DORIGONI et al., 2001). Consequently, phytotherapy is considered the most remote resource used for both prevention and treatment of diseases (MARTINS et al., 2005). This therapy has shown remarkable increment in recent years (YUNES et al., 2001). According to the World

Health Organization (WHO, 2011), 85% of the world population use medicinal plants to treat diseases. Allopathic medicine, in contrast, has been a therapeutic approach for just few people of developing countries, like Brazil, where there still are inhabitants without access to essential medicines nowadays (NOLLA; SEVERO, 2005).

The WHO recommends for countries with high plant biodiversity, such as Brazil, which is home to about 23% of the 250,000 plant species on the

planet, to increase the use of herbal medicines (WOLF et al., 2001). As a result, nowadays, medicinal plants have been added in some health services as a therapeutic resource for possible use in primary medical care, particularly in countries where access to therapies that combine quality treatment with low cost is a fundamental premise (NOLLA; SEVERO, 2005).

In the contemporary world, medicine has become more welcoming to the use of antimicrobial substances derived from plants since many microorganisms have developed resistance to antimicrobials commonly used these days. Therefore, the need to find new substances with antimicrobial properties is a challenge that must be faced through research studies (PEREIRA et al., 2004).

'Babosa' is the popular name given to an African plant that belongs to the Liliaceae family and the *Aloe* genus, which comprises more than 300 species. Many of them are used in several countries, including Brazil, for medicinal and cosmetic purposes. Among the existing species, the best known are: *Aloe socotrina*, *A. arborescens*, *A. chinensis*, *A. ferox* and *A. vera*, which is the most studied by the food, pharmaceutical, cosmetic and herbal industries. This last species is also known as *A. barbadensis* because it grows spontaneously and abundantly in the Barbados Island (CREA, 1995).

It is estimated that over two hundred active substances are part of *Aloe* spp. extracts. Indeed, the use of plants as therapeutic resources is typically not linked to just one substance, called active principle, which cannot act effectively when isolated despite being the main component for a specific function. This phenomenon, called synergism between substances, must be taken into account (HIRATA; SUGA, 1977).

According to Spoerke and Smolinske (1990), the dry mass obtained from *A. arborescens* leaves has approximately 2.0% of anthraquinones, a class of compounds with antimicrobial potential represented by the substances aloë-emodin, A and B aloin, barbaloin and isobarbaloin (KOSHIOKA et al., 1982).

Phytochemical studies have demonstrated the presence of various compounds with pharmacological interest derived from primary and secondary metabolism of *A. vera*. In 1997, Atherton (1997) found the following components: enzymes, amino acids, vitamins, mono and polysaccharides, anthraquinones, saponins, salicylates, steroids and lignin. In the contemporary world, these compounds have been assigned to a myriad of

biological activities, such as antitumor, immunostimulant (LEE et al., 2001), antimicrobial (REIDER et al., 2005), analgesic (HIRATA; SUGA, 1977), anti-inflammatory, antioxidant (INAN et al., 2007), anti-ulcer (BLITZ et al., 1963) and cellular regeneration (SEYGER et al., 1998). These findings validate the utility of this medicinal plant in the rehabilitation of human health (SMITH et al., 2008).

Although the majority of infections caused by yeasts are due to *Candida* (PFALLER; DIEKEMA, 2004; PFALLER et al., 2007), there are other yeast genera that can be considered opportunists (e.g. *Trichosporon*). Indeed, such yeasts have taken advantage of immunocompromised condition and indwelling devices in addition to the irrational use of broad-spectrum antimicrobials to colonize and infect high risk patients (NUCCI; MARR, 2005; PFALLER; DIEKEMA, 2004; PFALLER et al., 2007). In this context, life-threatening infections caused by *Trichosporon* are very difficult issues to manage (PFALLER et al., 2007).

Over the last fifteen years, the incidence of infections caused by fungi has significantly increased. These diseases have been occurring as hospital contaminations and affecting individuals with impaired immune systems (TORTORA et al., 2005).

Even though the infection caused by *Trichosporon* yeasts is considered very rare, a growing number of cases have been mainly reported in immunosuppressed patients with hematological malignancies and neutropenia (FAGUNDES JR. et al., 2008). However, serious infections caused by *Trichosporon asahii*, once described as limited to immunocompromised, oncologic and hematologic patients, have been frequently reported in elderly patients with severe heart failure, thereby leading to an increase in the death rate of such patients hospitalized at ICU (LEMES et al., 2010). Consequently, the increasing morbidity and mortality by fungal infections, particularly those caused by *Trichosporon* genus yeasts, has led to the interest of developing more efficient antimycotic therapies. In this context, the objective of the current study was to investigate the antifungal activity of *A. arborescens* dry extract against *Trichosporon* genus yeast species.

## Material and methods

### Place of performance

The experiments were performed at the Laboratory of Medicinal Plants and Herbal Medicines and the Laboratory of Clinical Microbiology of the Faculty of Pharmaceutical Sciences of the Federal University of

Alfenas (Unifal, Minas Gerais State) located in Alfenas, Minas Gerais State, Brazil.

#### Plant material collection and extract preparation

The *Aloe arborescens* species was collected on farms surrounding the country area of Alfenas in June of 2011. Healthy leaves of *A. arborescens* were sliced, packed in plastic bags and then sent to the Laboratory of Medicinal Plants and Herbal Medicines of Unifal, Minas Gerais State. The species was identified by the co-advisor of this study by analyzing its inflorescences, and a voucher specimen was deposited at Ual (Herbarium of Federal University of Alfenas), located in Alfenas. The registration number of this voucher specimen is 2272. Subsequently, the processes of extraction and drying were carried out.

We have extracted the compounds by a process that obtains the pool of the two fractions (gel or mucilage and latex). This process is done manually and consists of a longitudinal incision in the base of fresh and fleshy leaves of *A. arborescens*. Mucilage and latex were collected on a vat by scraping, using a spatula. Then, the extract was frozen, dried and stabilized by a lyophilizer at a temperature of  $-40^{\circ}\text{C}$  for a timing-dependent way based on the amount of water present in the leaves collected. At the end of this process, the extract was packed in a sterile container and stored in an area free of humidity and daylight (BRUNETON, 2001).

#### Dry extract dilution

We have weighed 40 mg of *Aloe arborescens* dry extract, and it was used 10 mL of DMSO (dimethylsulfoxide) to dissolve this material by gentle inversion, thereby obtaining a solution of  $4\text{ mg mL}^{-1}$  ( $4000\text{ }\mu\text{g mL}^{-1}$ ), which became limpid and slightly yellowish because the pigment of the latex (one of the two fractions) was attenuated. Then, it was performed serial dilutions of the extract at concentrations from 2,000 to  $15.625\text{ }\mu\text{g mL}^{-1}$  with RPMI-1640 broth (INLAB, São Paulo State, Brazil) containing L-glutamine and 2% of glucose but no sodium bicarbonate. This broth was buffered with 0.165 M MOPS (Ludwig, Rio Grande do Sul State, Brazil) to a final pH of 7.0. There was already no pigment in the first dilution of  $2,000\text{ }\mu\text{g mL}^{-1}$ . In order to check the possible interference of DMSO in the results of microorganism susceptibility to the extract, we have carried out a test in which it was added 100  $\mu\text{L}$  of DMSO with RPMI and 100  $\mu\text{L}$  of the yeast suspension into microplates. As a result, there was found no inhibitory effect on microbial growth.

#### Samples from *Trichosporon* genus yeasts

The 15 *Trichosporon* strains used in this experiment were kept in a refrigerator in the Laboratory of Clinical Microbiology of Unifal. We have worked with 12 *T. asahii* strains isolated from different human sites, one *T. asahii* CBS 2479 strain, one *T. ovoides* CBS 7556 strain and one *T. inkin* CBS 5585 strain. *Candida albicans* ATCC 10231 was used as standard strain in all tests.

#### Yeast reactivation and preparation of microbial suspensions

The inoculum was prepared with pure fungal colonies that were subcultured on Sabouraud dextrose agar, containing 300 mg of chloramphenicol, and incubated in microbial incubator at  $30^{\circ}\text{C}$  for 48 hours. The fungal colonies were suspended in 5 mL of 0.85% sterile saline, and the suspension was stirred during 15 s using vortex machine. The cellular density was adjusted in conformity with the tube 0.5 of McFarland scale and the Wicherham card in order to obtain from  $1 \times 10^6$  to  $5 \times 10^6\text{ CFU mL}^{-1}$ . Then, this suspension was diluted twice in RPMI-1640 to obtain a final solution concentration from  $5 \times 10^2$  to  $2.5 \times 10^3\text{ UFC mL}^{-1}$ .

#### Antimicrobial activity evaluation - broth dilution method

The broth dilution method provides quantitative results and is not influenced by the microorganism growth rate. Two methods may be employed: macrodilution or microdilution, which was chosen to develop this study. The antimicrobial activity evaluation was conducted through the  $\text{MIC}_{50}$  (minimum inhibitory concentration required to inhibit the growth of 50% of samples) obtained from *A. arborescens* dry extract.

#### Microdilution

The microdilution method uses microplates with 96 wells and volumes up to 0.2 mL (200  $\mu\text{L}$ ). An aliquot of 100  $\mu\text{L}$  of RPMI-1640 liquid culture medium was transferred to all wells of the penultimate row (negative control), and, hereafter, 100  $\mu\text{L}$  of culture medium and 100  $\mu\text{L}$  of inoculum (yeast suspension) were added into each well of 9 out of 11 rows used. The inoculum was prepared by adding 100  $\mu\text{L}$  of microbial suspension at concentration from  $5 \times 10^2$  to  $2.5 \times 10^3\text{ UFC mL}^{-1}$  to tubes containing 5 mL of 0.85% sterile saline. The concentrations of extract used in the microdilution method were 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and  $15.625\text{ }\mu\text{g mL}^{-1}$ . In the first row, it was added 100  $\mu\text{L}$  of *A. arborescens* dry extract initially

prepared at a concentration of 2,000  $\mu\text{g mL}^{-1}$ . Thereupon, in the subsequent rows, it was added the concentrations from 1,000 to 15.625  $\mu\text{g mL}^{-1}$ . However, it is necessary to clarify that the extract concentrations were reduced to half in order to calculate the MIC<sub>50</sub> (MIC required to inhibit the growth of 50% of samples) due to the prior addition of 100  $\mu\text{L}$  of microbial suspension in each well.

In order to have a comparative parameter, 100  $\mu\text{L}$  of fluconazole at a concentration of 1  $\mu\text{g mL}^{-1}$  (GUO et al., 2009) and 100  $\mu\text{L}$  of inoculum were added into the last row. The inoculum and RPMI-1640 broth, without the extract, were applied in each well of the 9<sup>th</sup> row of the microplates as a positive control. Subsequently, the plates were capped and incubated in microbiological incubator at 35°C for 24 and 48 hours. After incubation, the microplates were analyzed in the ELISA reader device (Anthos Zenyth 200rt), which indicates microbial growth from different absorbance values, at a wavelength of 530 nm. We have carried out the tests in duplicate. Ultimately, the values of MIC and MIC<sub>50</sub> (MIC required to inhibit the growth of 50% of samples) were determined.

## Results and discussion

MIC values after 24 and 48 hours of incubation and MIC<sub>50</sub> value were calculated at several concentrations of *Aloe arborescens* dry extract. For this purpose, it was used the broth microdilution method in conformity with CLSI, and the readings were performed by Anthos Zenyth 200rt Microplate Reader with filter at a wavelength of 530 nm (LEMES et al., 2010). As a result, two *T. asahii* strains have exhibited MIC of 250  $\mu\text{g mL}^{-1}$  in 24 hours, while the remaining samples, including *T. asahii* CBS 2479, *T. ovoides* CBS 7556, *T. inkin* CBS 5585 and *C. albicans* ATCC 10231 standard strain, have exhibited MIC of 500  $\mu\text{g mL}^{-1}$ . In addition, all samples have exhibited MIC of 500  $\mu\text{g mL}^{-1}$  in 48 hours, so the MIC<sub>50</sub> for all the 15 strains was 500  $\mu\text{g mL}^{-1}$ .

MIC values obtained for *C. albicans* ATCC 10231 in this study (500  $\mu\text{g mL}^{-1}$ ) were similar to those values found by Asamenew et al. (2011) with the same standard strain (400  $\mu\text{g mL}^{-1}$ ); in addition, these scientists also observed the antimicrobial action of *A. harlana* latex on several bacteria and some fungi.

In 2010, Cardoso et al. (2010) conducted an analysis of seasonal antimicrobial potential of *A. arborescens* extracts. These scientists determined the minimum inhibitory concentration (MIC) of *A. arborescens* ethanolic and chloroformic extracts

during the four annual seasons. At the concentrations tested, all extracts caused growth inhibition of *B. subtilis*, *E. coli*, *E. faecalis*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *S. typhimurium*, *S. aureus* and *C. albicans* strains. In this context, the MIC of the chloroformic extract ranged from 128  $\mu\text{g mL}^{-1}$  to 1024  $\mu\text{g mL}^{-1}$ , whereas the MIC of the ethanolic extract ranged from 256  $\mu\text{g mL}^{-1}$  to 1024  $\mu\text{g mL}^{-1}$ . All chloroformic extracts showed MIC of 512  $\mu\text{g mL}^{-1}$  on *P. mirabilis*, and four ethanolic extracts inhibited the *P. aeruginosa* growth at a concentration of 512  $\mu\text{g mL}^{-1}$ . Moreover, *C. albicans* yeasts did not grow when they were exposed to the chloroformic extract at a concentration of 512  $\mu\text{g mL}^{-1}$ . This inhibitory concentration was very close to the one obtained in the present study against the same yeast (500  $\mu\text{g mL}^{-1}$ ).

The results obtained in the current study suggested the fungistatic action of *A. arborescens* dry extract, evident in the decreased proliferation and impaired development of several *Trichosporon* genus yeast species. According to Young et al. (2003), a fungistatic drug allows concurrent use of other drugs, thereby increasing therapeutic efficacy. Furthermore, the antifungal activity observed in this study may also represent a mode of action distinct from those ones observed in drugs already available in the market, such as azole derivatives.

Another Aloe extract, obtained from *A. vera*, has been tested against other microorganisms, such as viruses and bacteria, and has shown satisfactory effects. Zandi et al. (2007) demonstrated the antiviral effects of *A. vera* glycerin gel against herpes simplex virus type 2, which causes genital herpes and complications such as encephalitis, meningitis and wounds. Moreover, in conformity with Pandey and Mishra (2010), the ethanolic extract of *Aloe vera* exhibited significant inhibitory activity against two species of Gram positive bacteria (*Staphylococcus aureus* and *Enterococcus bovis*) and six different Gram negative bacteria (*E. coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Morganella morganii*). The MIC values obtained for *Enterococcus bovis*, *Staphylococcus aureus*, *Proteus vulgaris* and *Proteus mirabilis* were equivalent to those ones described in this experiment (500  $\mu\text{g mL}^{-1}$ ).

Rosca-Casian et al. (2007) reported antifungal activity of the hydroethanolic extract obtained from *A. barbadensis* leaves against *Botrytis gladiolorum*, *Fusarium oxysporum* f. sp. *gladioli*, *Heterosporium prunetti* and *Penicillium gladioli*. The inhibition of these phytopathogenic fungi of ornamental plants is due to the presence of the main chemical of the extract, aloin, a heterosidic anthraquinone present in all species of *Aloe*.

According to Pandey and Mishra (2010), *A. barbadensis* antimicrobial activity has been related to two main phytochemicals present in this vegetable: acemannan, an acetylated mannose, and anthraquinones. Acemannan forms a protective mucilaginous layer around the urogenital tract of victims of opportunistic pathogens, but this occurs only when the *Aloe* juice is consumed orally. Nevertheless, recently, the marketing of foods and beverages based on *A. barbadensis* was banned by Anvisa because there is no proof of safety or registration for this purpose (MARTINS et al., 2005). Regarding anthraquinones, it has been described that these substances have the same mechanism of action of tetracyclines, which inhibit bacterial protein synthesis by blocking a ribosomal subunit of bacteria (PANDEY; MISHRA, 2010). However, studies about antifungal mechanisms of action involving anthraquinones have not yet been performed.

The present research corroborates the literature findings about the inhibitory activity of *A. arborescens* dry extract against opportunistic microorganisms. Nonetheless, there are no previous studies involving *Trichosporon* species to enable comparative analyses. Thus, it is suggested that further research studies be undertaken in order to reaffirm the antifungal activity of *A. arborescens* dry extract against *Trichosporon* yeasts.

## Conclusion

It was proven the antifungal activity of *A. arborescens* dry extract against *Trichosporon* genus yeast species through determination of MIC<sub>50</sub>, which was 500 µg mL<sup>-1</sup>. Therefore, horizons were expanded for the development of new herbal medicines with fungistatic action, and there was an incentive to the study of the antimicrobial potential of plant species belonging to the Brazilian flora. In this context, future experiments should direct themselves towards assays in vivo in order to allow a better understanding of the mechanism of action as well as the possible toxic effects associated with new drugs originated from plants.

## Acknowledgements

We would like to thank to Professors Edson Jorge de Souza, Amanda Latércia Tranches Dias, Luiz Cosme Cotta Malaquias, to technicians Elenice Rodrigues dos Santos and Vanderlei Flausino and to Maria Lopes Rezende for supplying the samples of *A. arborescens*.

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Received on October 17, 2012.

Accepted on May 26, 2014.

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