

Full Length Research Paper

Antibacterial and antifungal activities of indican (indoxyl β -D-glucoside)

Ivanise Brito da Silva^{1*}, Izabela Lima Rangel¹, Roberta Maria de Leite Lima¹, Edeltrudes Oliveira Lima², Paloma Lys de Medeiros¹ and Sônia Pereira Leite¹

¹Laboratório de Cultura de Células, Departamento de Histologia e Embriologia, Universidade Federal de Pernambuco, Recife, Brasil.

²Laboratório de Micologia, Departamento de Ciências Farmacêuticas, Universidade Federal da Paraíba, João Pessoa, Brasil.

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Indican (Indoxyl β -d-glucoside), plant pigments found in true Indigo, are present in Indigofera genus plants, fungi and human urine. This study evaluated the antibacterial and antifungal activities of this pigment using the microdilution method. Antibacterial and antifungal activities were performed against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporium gypseum*. Minimum inhibitory concentrations (MICs) were measured according to the broth microdilution protocols by the Clinical and Laboratory Standards Institute (CLSI). The Indican showed only antifungal activity. It had MIC values of 128 μ g/mL for yeasts and 512 μ g/mL for most of the dermatophytes. The minimum fungicidal concentration (MFC) values of the Indican ranged between 512 μ g/mL: *C. tropicalis* (LM-6) and *C. krusei* (LM-6 and LM-8) and 1,024 μ g/mL: *C. albicans* (ATCC-76645, LM-108, LM-P20). Further studies are needed to clarify the potential fungicidal activity of the Indican and the possibility for topical applications.

Key words: Antibacterial activity, antifungal activity, indican.

INTRODUCTION

Brazilian medicinal plants have been used by the local population in the treatment of tropical diseases such leishmaniasis, malaria, schistosomiasis, fungal and bacterial infections (Duarte et al., 2005). In this context, the study of products from plant or synthetic substances with antimicrobial activity is gaining high prospects in the medical and pharmaceutical fields (Menezes and Lima,

2013). Plants have several chemical compound classes, including alkaloids. Indigo alkaloids belong to the bis-indole alkaloid class, being natural products used by mankind in dyes production and for medicinal purposes, in addition to being widely used in traditional Chinese medicine (Calvo, 2007). These pigments are present in Indigofera genus plants, fungi and human urine (Santos

*Corresponding author. E-mail: Ivanisebrito1@gmail.com. Tel: (81) 996617625.

and Torres, 2012). Indican is the precursor of indigo pigments (indigo and indirubin), thus being starting material of these metabolites biosynthesis (Maugard et al., 2002).

Superficial fungal infections affect approximately 20 to 25% of the world population and they are classified into inflammatory and non-inflammatory infections. Disease control precautions involve hygiene and proper treatment, but drugs used to treat these diseases are toxic, and some microorganisms are already resistant to them. Drugs based on natural compounds, isolated or synthetic, appear as a possible solution to cope with these microorganisms resistance to conventional treatments (Oliveira and Silva, 2008; Filho et al., 2010; Dias et al., 2013).

The indigo biosynthesis pathway varies among plant species, but has indican (indoxyl β -D-glucoside) as a common precursor. This compound is mainly located in young leaves and can be extracted into aqueous solution. Indican undergoes hydrolysis through β -glucosidase enzyme action, leading to indoxyl (Sandoval et al., 2011).

Indigo is used in popular medicine, having anti-inflammatory, antipyretic, antiviral and antimicrobial activities, and recent studies have proved its anticancer property and efficacy in psoriasis treatment (Chiang et al., 2013). Studies showed that the indirubin is one potent cyclin-dependent kinases (CDKs) with activities such as anti-inflammatory, immunomodulatory, antifungal and antileukemic, in addition to inhibiting several tumoral cell lines (Suzuki et al., 2005). Considering the vast potentiality of plants as sources for antimicrobial drugs, the objective of this study was to assess the antibacterial and antifungal activities of the main precursor of indigo, the indican (indoxyl β -D-glucoside).

MATERIALS AND METHODS

Microorganisms

Strains used in the antimicrobial assay were obtained from the archival collection of the Federal University of Paraíba Laboratory of Mycology (LM). They include *Staphylococcus aureus* (ATCC-6538, LM-17, LM-197), *Staphylococcus epidermidis* (ATCC-1288), *Pseudomonas aeruginosa* (ATCC-9027, ATCC-25853), *P. aeruginosa* (LM-606), *Candida albicans* (ATCC-76645, LM-108, LM-P20), *Candida tropicalis* (ATCC-13803, LM-6), *Candida krusei* (LM-6, LM-08), *Trichophyton rubrum* (LM-640, LM-600), *Trichophyton mentagrophytes* (LM-02, LM-202) and *Microsporium gypseum* (ATCC-189).

Suspension was standardized by the 0.5 Mc Farland scale tube, set through spectrophotometric reading (Leitz-Photometer 340-800) to 90% T (530 nm), corresponding to approximately 10^6 colony-forming units (UFC) per mL. The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber (Sahin et al., 2004).

Chemistry

Indican (Indoxyl- β -D glucoside), Cloranfenicol, Nistatin and Fluconazole were obtained from Sigma Aldrich, Brazil. The drugs

were dissolved in dimethylsulfoxide (DMSO), and sterile distilled water was used to obtain solutions of 1,024 μ g/mL for each. The concentration of DMSO did not exceed 0.5% in the assays.

Culture media

To test the biological activity of the products, the strains were maintained in appropriate culture media: Nutrient agar (NA) (Sigma-Aldrich, São Paulo, SP, Brazil) for bacteria, Roswell Park Memorial Institute (RPMI-1640) for yeasts, Sabouraud Dextrose Agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA) for filamentous. They were prepared and used according to the manufacturers' instructions.

Minimum inhibitory concentration (MIC)

MICs were measured according to the broth microdilution protocols by the Clinical and Laboratory Standards Institute (CLSI, 2008). The determination of MIC was performed in duplicate using 96 "U" bottomed well microplates. In each plate orifice, 100 μ L of double concentrated nutrient broth (CN) was added for bacteria, with 100 μ L solubilized Indican subsequent addition, which was also double concentrated. Through serial dilution, concentrations of 1.024 to 8 μ g/mL were obtained, and plates were sealed and incubated at 35°C for 24 h. Bacterial growth control was done with chloramphenicol (100 μ g/mL).

In antifungal activity was carried out by wells microdilution technique. Microplates were sealed and incubated at 35°C for 24-72 h for yeasts and at 28°C room temperature for 7-10 days for filamentous fungi. Negative controls (without drugs) were used to confirm strains viability, and sensitivity controls (for DMSO) were also included in the studies.

The minimum fungicidal concentration (MFC) was determined as the lowest concentration capable to inhibit total growth or promote less than three UFC, resulting in 99.9% fungicidal activity (Ernst et al., 1996; Espinel-Ingroff et al., 2002). The antimicrobial activity of the products was interpreted and considered active or not, according to the criteria proposed by Morales et al (2008): strong/good activity (MIC: <100 μ g/mL); moderate activity (MIC: 100-500 μ g/mL); weak activity (MIC: 500-1000 μ g/mL); and inactive product/no antimicrobial effect (MIC: >1000 μ g/mL).

To determine the MFC, the authors subcultured 1 μ L aliquots of MIC, MIC \times 2, and MIC \times 4 of Indican, Fluconazole, and Nistatin the control yeast growth onto Petri dishes containing SDA. After 24-48 h of incubation at 35°C, a reading was to to evaluate the MFC, based on the growth of the controls. The MFC was defined as the lowest product concentration that inhibited growth of the yeast or permitted less than three CFUs to occur, resulting thus in 99.9% fungicidal activity (Espinel-Ingroff et al., 2002). Biological activity assays were performed in duplicate, and the results were expressed as the arithmetic mean of the MIC and MFC.

RESULTS AND DISCUSSION

Bacterial resistance is currently one of the most important problems. In clinics, this problem is related to inadequate treatment and indiscriminate antibiotics use. This causes high mortality rates, especially when infections are caused by etiological agents, such as multidrug resistant *S. aureus*, *P. aeruginosa* and *Acinetobacter baumannii* (Gómez et al., 2008).

In vitro antimicrobial activity of indican synthetic

Table 1. Indican compound antibacterial activity.

Compounds	<i>S. aureus</i> ATCC-6538	<i>S. aureus</i> LM-17	<i>S. aureus</i> LM-197	<i>S. epidermidis</i> ATCC-12228	<i>P. aeruginosa</i> ATCC- 9027	<i>P. aeruginosa</i> LM-606
Indican	*	*	*	*	*	*
Chloramphenicol	*	*	**	**	*	**
Sensitivity control	*	*	*	*	*	*

*: Microorganism Growth; **: growth inhibition of the microorganism.

Table 2. Indican compound antifungal activity.

Compounds	<i>Candida albicans</i> ATCC-7664	<i>Candida albicans</i> LM-108	<i>Candida albicans</i> LM-P20	<i>Candida tropicalis</i> ATCC-13803	<i>Candida tropicalis</i> LM- 6	<i>Candida krusei</i> LM-6	<i>Candida krusei</i> LM-08
Indican	**	**	**	*	**	**	**
Nystatin	**	**	**	**	*	**	*
Fungal strain	*	*	*	*	*	*	*

*: Microorganism growth; **: growth inhibition of the microorganism.

Table 3. Indican MIC ($\mu\text{g/ml}$) on *Candida* genus.

Microbial strain	*MIC ($\mu\text{g/mL}$)	Nystatin	Fungal strain
<i>C. albicans</i> ATCC-76645	128	**	*
<i>C. albicans</i> LM – 108	256	**	*
<i>C. albicans</i> LM – P20	256	**	*
<i>C. tropicalis</i> LM- 6	512	NI	*
<i>C. krusei</i> LM-13	128	**	*
<i>C. krusei</i> LM-08	128	NI	*

*Microorganism Growth. **: Growth inhibition of the microorganism; NI: no inhibition.

compound was determined in this study. Indican showed no inhibition, although having growth on Gram-positive and negative strains (Table 1). In this test, it was possible to observe strains of bacterial growth in indican and Cloranfenicol standard antibiotic, demonstrating these microorganisms resistance to the compounds.

Bacteria use several mechanisms to ensure their growth in the presence of antibiotics, among them are: enzymatic inactivation, cell wall permeability changes, cell wall precursor changes, among others (Calvo et al., 2006). The difficulty in finding active drugs has led to a search for more potent drugs (Castro et al., 2006). Multidrug resistant microorganism infections complicates treatment, reduces the number of effective antibiotics, and creates the need for broad activity spectrum drugs use, configuring as a public health problem (Dutra, 2015).

I. suffruticosa leaf extracts (aqueous, methanolic, ethyl acetate and hexane) phytochemical screening showed alkaloid, steroid, triterpene, flavonoids, carbohydrates and coumarins presence. The aqueous extract showed antimicrobial activity against the strains *S. aureus*, *T. rubrum* (LM-09, LM-13) and *M. cani* (Leite et al., 2006).

Indican, indoxyl β -D-glucoside, is the main indigo and indirubin formation precursor, which is located in Indigofera genus plant young leaves, and is also obtained by synthesis. This study data related to synthetic Indican antifungal activity with *Candida* genus yeast-formed strains are shown in Table 2.

The compound, indican inhibited *C. albicans* (ATCC-7664; LM-108; P20), *C. tropicalis* (LM-6) and *C. krusei* (LM-6; LM-08), and did not inhibit *C. tropicalis* (ATCC-13803). In the study, it was observed that the *C. tropicalis* (LM-6) and *C. krusei* (LM-08) yeasts strains were resistant to Nistatina and sensitive to Indican.

Antifungal resistance is a challenge to clinical practice, with infectious agent isolation and MIC of potential drugs used for its treatment being suggested when possible (Colombo, 2003). The determination of MIC for Indican is shown in Table 3. Beginning with 128 $\mu\text{g/mL}$ concentration, there was inhibition of growth and with the 256 $\mu\text{g/mL}$ concentration, there was inhibition of most microorganisms.

The *Candida* genus, which is responsible for local and systemic infections, showed *C. tropicalis*, *C. parapsilosis*,

Table 4. Minimum fungicides concentrations (MFC, µg/mL) of indican on *Candida* genus

MFC of Indican (µg/mL)	Species of the genus <i>Candida</i>
512	<i>C. tropicalis</i> (LM-6)
	<i>C. krusei</i> (LM-6)
	<i>C. krusei</i> (LM-08)
1,024	<i>C. albicans</i> (ATCC-76645)
	<i>C. albicans</i> (LM-108)
	<i>C. albicans</i> (LM-P20)

Table 5. Minimum inhibitory concentration (MIC, µg/mL) of Indican on dermatophytes.

Microbial strain	MIC (µg/mL)*	Fluconazole	Fungal strain
<i>T. rubrum</i> (LM-640)	512	**	*
<i>T. rubrum</i> (LM-600)	1,024	**	*
<i>T. mentagrophytes</i> (LM-02)	512	**	*
<i>T. mentagrophytes</i> LM-202	512	**	*
<i>M. gypseun</i> ATCC-189	512	**	*

*Microorganism growth; **: growth inhibition of the microorganism.

C. glabrata, *C. krusei*, *C. lusitaniae* and *C. guilliermondii* species as the most pathogenic agents. These microorganisms have also developed antifungal agent resistance. Taking into account mutations or increased genes expression, these pathogens have become resistant to azole agents (Menezes et al., 2009).

The minimum fungicides concentrations (MFC) are shown in Table 4. This study showed that from 512 µg/mL concentration, *C. tropicalis* LM-6 and *C. krusei* (LM-13;LM-08) strains were totally inhibited, and 1,024 µg/mL concentration inhibited *C. albicans* (ATCC-7664; LM-108; LM-P20) too.

C. tropicalis and *C. krusei* yeasts are opportunistic pathogens, having the ability to colonize different body parts (skin, gastrointestinal and genitourinary tract and respiratory system), and being resistant to several drugs (Pappas et al., 2009; Praneenararat, 2014). *C. albicans* exhibit polymorphism that makes species capable of changing its shape according to environment conditions, in addition to favoring different tissues colonization (Modrzeswska and Kurnatowski, 2013).

There are few available drugs for candidiasis treatment. In addition to low supply, the drugs used have high toxicity and are not too efficient due to microorganisms acquired resistance (Wong et al., 2014).

Indican MIC determination on *Trichophyton* and *Microsporum* genus dermatophytes is shown in Table 5. At the 512 µg/mL concentration, only *T. rubrum* LM-600 was not inhibited. The 1,024 µg/mL concentration showed all strains growth inhibition (*T. rubrum* LM-640; 600; *T. mentagrophytes* LM-02; LM-202 and *M. gypseun*

ATCC-189).

Antifungal agents frequent use combined with inadequate treatment are responsible for promoting these pathogens resistance to drugs used in medical routine. The difficulty in finding active drugs has led to a search for more potent drugs (Castro et al., 2006). Researches involving synthetic or natural (extracted from medicinal plants) compounds that have active ingredients against multidrug resistant strains are necessary (Gonçalves et al., 2009).

Results showed Indican antifungal potential on yeasts and dermatophytes. According to Sartoratto et al. (2004) and Houghton et al. (2007), Indican MIC can be considered to have good biological activity against *C. albicans*, *C. krusei*, *T. rubrum*, *T. mentagrophyte* and *M. gypseun*. *S. aureus*, *S. epidermidis* and *P. aeruginosa* bacteria and *C. tropicalis* ATCC-13803 yeast showed resistance to the compound.

Chiang et al. (2013) proved indigo naturalis antimicrobial activity, which comes from *Strobilanthes formosanus* Moore, on *Staphylococcus* spp. strains and non-dermatophytes onychomycosis. *Isatis costata* indole alkaloids also showed antifungal activity over ten strains, showing best results on *Trichophyton simii* and *Trichophyton Schoen leinii* dermatophytes, having 80% growth inhibition, while *Candida albicans* yeast had approximately 69% growth inhibition (Fatima et al., 2007). *I. suffruticosa* leaves aqueous extract showed growth inhibition against dermatophytes and bacteria (Leite et al., 2006).

Studies on Indican synthetic are scarce in the literature.

However, studies on indican isolated from *I. suffruticosa* leaves showed cell lines growth inhibition in mice with Sarcoma 180 (Maranhão, 2008). Research by Lima et al. (2014) demonstrated that indican has anticancer and hepatoprotective activities.

Conclusions

Indican showed antibacterial activity in the tested strains. However, this compound showed good antifungal activity against different species, genus, candida and moderate activity against *Trichophyton* and *Microsporium* genera filamentous fungi, thus suggesting that this compound could be used in antifungal topic agent development. However, further investigation would be necessary on the use for the treatment of superficial mycoses.

Conflict of interests

The authors have not declared any conflict of interest.

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REFERENCES

- Calvo TM (2007). Sustainable use of Brazilian biodiversity - chemical and pharmacological prospection in higher plants: *Alchornea glandulosa*, *Alchornea triplinervia* (Euphorbiaceae), *Indigofera truxillensis* and *Indigofera suffruticosa* (Fabaceae). Thesis (Doctorate in Chemistry) - Sao Paulo: Universidade Estadual Paulista. P 216.
- Castro TL, Coutinho HDM, Gedeon CC, Santos JM, Santana WJ, Souza LBS (2006). Mecanismos de resistência da *Candida* sp wwa antifúngicos. *Infarma*. 18:30-35.
- Chiang YR, Li A, Leu YL, Fang JY, Lin YK (2013). An In vitro study of the antimicrobial effects of *Indigo naturalis* prepared from *Strobilanthes formosanus* Moore. *Molecules* 18(11):14381-14396.
- CLSI -Clinical and Laboratory Standards Institute (2008). Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi: Approved standard - Second edition. CLSI document M38-A2. CLSI, Wayne.
- Colombo AL, Guimarães T (2003). Epidemiologia das infecções hematogênicas por *Candida* spp. *Rev. Soc. Bras. Med. Trop.* 36(5):599-607.
- Dias MFRG, Filho FB, Santos MVPQ, Amorim AGFA, Schechtman RC, Azulay DR (2013). Update on therapy for superficial mycoses: Review article part I. *Braz. Ann. Dermatol.* 88(5):764-774.
- Duarte MC, Figueira GM, Sartoratto A, Rehder VL, Delarmelina C (2005). Anti-*Candida* activity of Brazilian medicinal plants. *J. Ethnopharmacol.* 97(2):305-311.
- Dutra GG, Costa MP, Bosenbecker EO, Lima LM, Siqueira HCH, Cecagno D (2015). Controle da infecção hospitalar: Função do enfermeiro. *J. Res. Nurs. Found.* 7(1):2159-2168.
- Espinel-Ingroff A, Chaturvedi V, Fothergill A, Rinaldi MG (2002). Optimal testing conditions for determining MICs and Minimum Fungicidal Concentrations of new and established antifungal agents for uncommon molds: NCCLS collaborative study. *J. Clin. Microbiol.* 40(10):3776-3781.
- Fatima I, Anis I, Ahmad I, Malik A, Afza N (2007). A New Alkaloid from *Isatis costata*. *Turk. J. Chem.* 31(4):443-447.
- Filho RJS, Vilar FC, Mota LM, Leal CL, Pisi PCB (2010). Infecção do trato urinário to urinário. *Medicina (Ribeirão Preto)* 43:118-25.
- Gonçalves DCPS, Lima ABM, Leão LSNO, Filho JRC, Pimenta FC, Vieira JDG (2009). Detecção de metalo-beta-lactamase em *Pseudomonas aeruginosa* isoladas de pacientes hospitalizados em Goiânia, Estado de Goiás. *Rev. Soc. Bras. De Med. Trop.* 42(4): 411-414.
- Houghton PJ, Howes, MJ, Lee CC, Steventon G (2007). Uses and abuses of *in vitro* tests in ethnopharmacology: Visualizing an elephant. *J. Ethnopharmacol.* 110(3):391-400.
- Leite SP, Vieira JRC, de Medeiros PL, Leite RMP, de Menezes Lima VL, Xavier HS, de Oliveira Lima E (2006). Antimicrobial activity of *Indigofera suffruticosa*. *Evid. Based Complement. Altern. Med.* 3(2):261-265.
- Lima IR, Vieira JR, Silva IB, Leite RM, Maia MB, Leite SP (2014). Indican from Añil (*Indigofera suffruticosa* Miller) as an herbal protective agent to the liver. *Anal. Quant. Cytol. Histol.* 36(1):41-45.
- Mann CM, Markham JL. (1998.) A new method for determining the minimum inhibitory concentration of essential oils. *J. Appl. Microbiol.* 84(4):538-544.
- Maranhão JB (2008). Indican, indigo and indirubina of *Indigofera suffruticosa* leaves on neoplastic cell lines. Thesis (MS in Pathology). Recife: Federal University of Pernambuco. Available at: http://repositorio.ufpe.br/xmlui/bitstream/handle/123456789/8868/arquivo4561_1.pdf?sequence=1&isAllowed=y
- Maugard EE, Sayette A, Choisy P, Legoy MD (2002). β -Glucosidase-Catalyzed Hydrolysis of Indican from Leaves of *Polygonum tinctorium*. *Biotechnol. Prog.* 18(5):1104-1108.
- Menezes CP, Lima EO (2013). Atividade antifúngica de óleos essenciais sobre cepas de *Cladosporium carrionii*. *Rev. Bras. Pharm.* 94:49-53.
- Menezes EA, Mendes LG, Cunha FA (2009). Resistência a antifúngicos de *Candida tropicalis* isoladas no Estado do Ceará. *J. Braz. Soc. Trop. Med.* 2:354-355.
- Modrzewska B, kurnatowski P (2013.) Selected pathogenic characteristics of fungi from the genus *Candida**. *Ann. Parasitol.* 59(2):57-66.
- Morales G, Paredes A, Sierra P, Loyola LA (2008). Antimicrobial activity of three *Baccharis* species used in the traditional medicine of Northern Chile. *Molecules* 13(4):790-794.
- Oliveira AC, Silva RS (2008). Desafios do cuidar em saúde frente à resistência bacteriana: Uma revisão. *Rev. Eletrônica de enfermagem* 10(1):189-197.
- Pappas PG, Kauffman CA, Andes D, Benjamin DK, Jr Calandra TF, Edwards JE, Jr Filler SG, Fisher JF, Kullberg BJ, Ostrosky ZL, Reboli AC, Rex JH, Walsh TJ, Sobel JD (2009). Clinical Practice Guidelines for the Management of Candidiasis: Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* 48(5):503-35.
- Praneenararat S (2014). The first reported case of colonic infection caused by *Candida tropicalis* and a review of the literature. *Case Rep. Gastroenterol.* 8(2):199-205.
- Sahin F, Gulluce M, Daferera D, Sökmen M, Polissiou M, Agar C, Özer H (2004). Biological activities of the essential oils and methanol extract of *Origanum vulgare* ssp. vulgare in the Eastern Anatolia region of Turkey. *Food Control* 15(7):549-557.
- Sandoval FS, Méndex CC, Cardador MA (2011.) Preliminary study of the indican production in tissue cultures of *Indigofera suffruticosa* Mill. e-Gnosis. 8:1-7.
- Santos AA, Torres JC (2012). Produtos naturais de origem vegetal no Brasil sob uma perspectiva histórica. *Perspect. Sci. Technol.* 4(1-2):33-42.
- Sartoratto A, Machado ALM, Delarmelina C, Figueira GM, Duarte MCT, Rehder VLG (2004) Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Bras. J. Microbiol.* 35:275-280.
- Souza EL, Stamford TLM, Lima EO, Trajano VN (2007). Effectiveness

of *Origanum vulgare* L. essential oil to inhibit the growth of food spoiling yeasts. Food Control 18(5):409-413.

Suzuki K, Adachi R, Hirayama A, Watanabe H, Otani S, Watanabe Y, Kasahara T (2005) Indirubin, a Chinese anti-leukaemia drug, promotes neutrophilic differentiation of human myelocytic leukaemia HL-60 cells. Br. J. Haematol. 130(5):681-690.