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# Actividad antimicrobiana de hongos endofíticos aislados de *Brugmansia suaveolens* Bercht. & J. Presl

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**Abstract**
Natural products produced from endophyte fungi have a broad spectrum of biological activity and can be grouped into several categories. Antimicrobials constitute an important group of therapeutic agents, which can be produced and obtained from living organisms. The main objective of this study is to investigate the endophytic fungi and the leaves of *Brugmansia suaveolens* Bercht. & J. Presl as elicitors, aiming at the production of bioactive substances with antimicrobial properties. The extracts were prepared from 19 fungi isolated from the leaves of *B. suaveolens* Bercht. & J. Presl, used as a biotic elicitor. The extracts were obtained by fermentation in a submerged medium with the addition of leaves from its dry leaves, and the antimicrobial activity of its extracts was evaluated using the agar diffusion method. The results showed that 57% of the endophytic fungi showed antimicrobial activity after fermentation with the elicitor. It was observed that the antimicrobial activity was increased compared to control cultures. The leaves of *B. suaveolens* Bercht. & J. Presl presented bioactives that stimulated the production of a substance with antimicrobial activity by endophytic fungi, the study of such compounds showed promise to clarify the application potential of these bioactives.
**Keywords:** Endophytic fungi; Elicitation; *Brugmansia suaveolens* Bercht. & J. Presl.

**Resumo**
Os produtos naturais produzidos a partir de fungos endófitos têm um amplo espectro de atividade biológica e podem ser agrupados em várias categorias. Os antimicrobianos constituem um grupo importante de agentes terapêuticos, os quais podem ser produzidos e obtidos a partir de organismos vivos. O principal objetivo desse estudo consiste na investigação dos fungos endofíticos e as folhas da *Brugmansia suaveolens* Bercht. & J. Presl como eliciadores, visando à produção de substâncias bioativas com propriedades antimicrobianas. Os extratos foram preparados a partir de 19 fungos isolados a partir das folhas da *B. suaveolens* Bercht. & J. Presl, utilizadas como eliciador biótico. Os extratos foram obtidos através de fermentação em meio submerso acrescido de folhas das suas folhas secas e avaliado a atividade antimicrobiana dos seus extratos através do método de difusão em ágar. Os resultados mostraram que 57

% dos fungos endofíticos apresentaram atividade antimicrobiana após a fermentação com o eliciador. Observou-se que a atividade antimicrobiana foi aumentada em comparação com as culturas controle. As folhas de *B. suaveolens* Bercht. & J. Presl apresentaram bioativos que estimularam a produção de substância com atividade antimicrobiana por fungos endofíticos, o estudo de tais compostos se mostrou promissor para o esclarecimento do potencial de aplicação destes bioativos.
**Palavras-chave:** Fungos endofíticos; Elicitacion; *Brugmansia suaveolens* Bercht. & J. Presl.

**Resumen**
Los productos naturales producidos a partir de hongos endófitos tienen un amplio espectro de actividad biológica y pueden agruparse en varias categorías. Los antimicrobianos constituyen un grupo importante de agentes terapéuticos, que pueden producirse y obtenerse de organismos vivos. El objetivo principal de este estudio es investigar los hongos endofíticos y las hojas de *Brugmansia suaveolens* Bercht. & J. Presl como inductores, con el objetivo de la producción de sustancias bioactivas con propiedades antimicrobianas. Los extractos se prepararon a partir de 19 hongos aislados de las hojas de *B. suaveolens* Bercht. & J. Presl, utilizados como elicitor biótico. Los extractos se obtuvieron por fermentación en medio sumergido con la adición de hojas de sus hojas secas, y se evaluó la actividad antimicrobiana de sus extractos mediante el método de difusión en agar. Los resultados mostraron que el 57% de los hongos endofíticos mostraron actividad antimicrobiana después de la fermentación con el elicitor. Se observó que la actividad antimicrobiana se incrementó en comparación con los cultivos de control. Las hojas de *B. suaveolens Bercht. & J. Presl* presentaron bioactivos que estimularon la producción de una sustancia con actividad antimicrobiana por hongos endofíticos, el estudio de dichos compuestos mostró ser prometedor para esclarecer el potencial de aplicación de estos bioactivos.
**Palabras clave:** Hongos endofíticos; Eliciação; *Brugmansia suaveolens* Bercht. & J. Presl.

## 1. Introduction

Natural products produced from endophyte fungi have a broad spectrum of biological activity and can be grouped into several categories. Endophytic fungi are a poorly investigated group of microorganisms, representing an abundant source of new bioactive compounds (Zhang, Song, Tan, 2006).

The term endophyte is applied to organisms, including fungi that live within the tissues of plants for all or part of their life cycle without causing apparent infections, that is, endophytes are synergistic with their host (Strobel et al., 2004).

Many viable strategies can be adapted to efficiently increase the production of bioactive compounds during fermentation processes. Elicitation are an effective tool to promote the expression of biosynthetic pathways responsible for producing secondary metabolites (Liu et al., 2014). An elicitor is defined as a substance that, when introduced in small concentrations, initiates or improves the biosynthesis of specific compounds. These strategies mainly include precursor feeding, adding biotic and abiotic elicitors, using enzymes and other substances (Otero, Nielsen, 2010).

Antimicrobial resistance has become a global health problem. The indiscriminate use of these drugs has led to the selection of resistant strains that are difficult to treat therapeutically. The search for new biomolecules with antimicrobial capacity has intensified in recent years. Antimicrobials constitute an important group of therapeutic agents, which can be produced and obtained from living organisms (Ribeiro, Ribeiro, 2008).

*Brugmansia suaveolens* Bercht. & J. Presl belongs to the Solanaceae family, popularly known as trobeteira and is used as an anti-inflammatory, antimicrobial and analgesic, the leaves and flowers being used in the form of ointment or tincture (Wu, Tanksley, 2010).

From a phytotherapeutic point of view, the trumpet is a plant with anticholinergic properties, having as its main active substance scopolamine, atropine and several tropic alkaloids (Oliveira, Pires, Costa, 2003; Simões et al., 2008). Tropanic alkaloids are bicyclic nitrogenous organic compounds, called tropane 8-methyl-8-azabicyclo (2,3.1octane). The tropane ring is formally made up of the pyrrolidine and piperidine rings. The esterification of the hydroxyl group with aromatic acids originates the most important alkaloids in the pharmaceutical field and can be found in the Solonaceae families. These alkaloids are important in traditional medicine and have historically contributed to the elaboration and construction of

prototypes from which synthetic analogues were developed, mainly from the classes of anticholinergic drugs and local anesthetics (Bracjet et al., 1999; Cusido et al., 1999).

The main objective of this work was to investigate the elicitation potential of *B. suaveolens* Bercht. & J. Presl leaves and, in this way, to increase the production of secondary metabolites and to evaluate the activity of these metabolites in inhibiting the growth of different microorganisms.

## 2. Methodology

The leaves of *Brugmansia suaveolens* Bercht. & J. Presl were collected in Teresópolis, Rio de Janeiro, Brazil, with geographic coordinates altitude of 895 m; - 22º26'09.38"S; - 42º58'33.22" during the months of April to December of the year 2019. The plant material was dried in an oven at 40 ºC for a period of 5 days and then submitted to crushing in a blender, then the processed dry extract was sterilized in a 365 nm UV light camera (HQ® - Model: SP930-35) and used in the elicitation process.

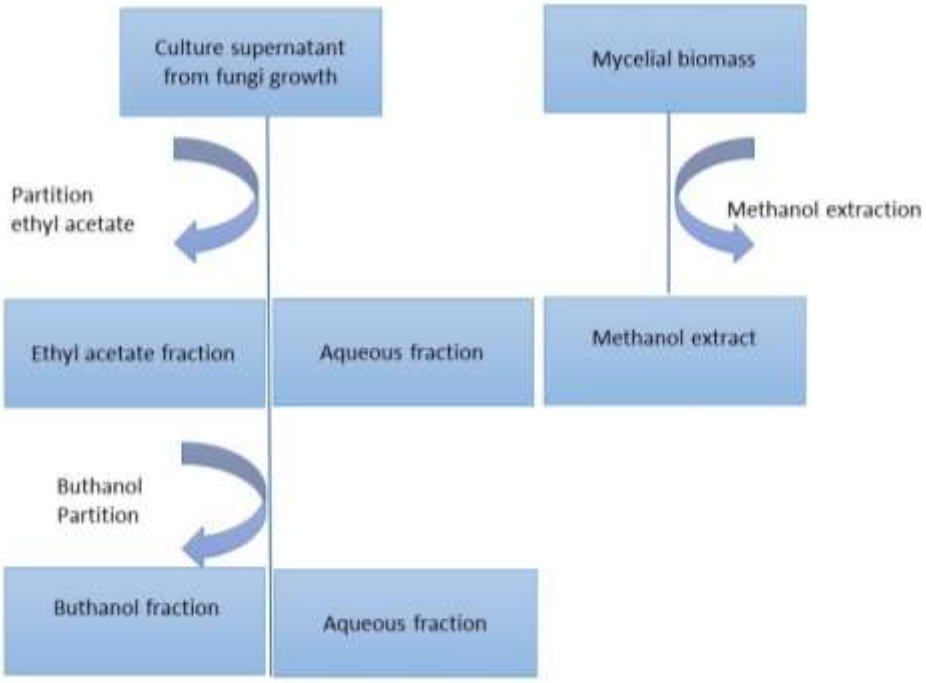
In the process of isolation of endophytic fungi, fresh leaves were used, which were disinfected with water and 2% chlorhexidine and then treated by immersion in 70% ethyl alcohol for 30 secund, 2.5% NaCL for 3 minute and sterile distilled water for 5 minute. Afterwards, the samples were macerated and inoculated 20µL of the macerated in a sterile manner in Petri dishes containing PDA culture medium (20% Potato Infusion, 20% Glucose, 1.7% Agar - KASVI® - Model: K25-610102) added of 100µL terramycin (1mg /mL) to inhibit bacterial growth in the isolation of fungi, incubated at 28 ºC for 48 h. After the growth of the microorganisms, they were isolated, through successive replications in solid PDA culture medium until obtaining isolated colonies, then they were stored at 4 ºC and numerically cataloged (Guimarães et al., 2010; Azevedo, Costa, 1973).

After the isolation of the endophytes, the purification was used the Technique of Tween or by successive repetitions (Azevedo, Costa, 1973). Fungal isolates were stored in triplicate, according to the methodology of Castellani (1939) and stored at 4 ºC. The endophyte frequencies were calculated through the number of colonies obtained divided by the number of incubated fragments.

The endophytic fungi were sterilely inoculated 20µL in 25 mL of malt extract fermentation medium (Nitrogen 0.55%, Protein 4.4%, Ash 3.8%, Sodium chloride 0.7% - HIMEDIA® - Model: RM004B-500G) for 48 h under stirring (200 rpm) at 28°C. The mycelium was filtered and inoculated into 50 mL of Czapek culture medium (3.0% sucrose; 0.2% NaNO3; 0.1% K2HPO4; 0.05% MgSO4.7H2O; 0.05% KCL; FeSO4.7H2O 0.001%) added 20 mg of dried leaves as elicitors under agitation (200 rpm) for 15 days, in parallel, fungi were also cultivated without the addition of biotic elicitor as negative control of experiment (Guimarães et al., 2010; Gao, Yong, Dai, 2011).

After the elicitation process, the mycelia were separated by filtration. The fungal fluid was partitioned with ethyl acetate and butanol and concentrated in a rotary evaporator. The mycelial biomass extract was obtained from infusion in methanol for 7 days (Figure 1). Subsequently, each extract obtained was submitted to antimicrobial tests in order to assess its inhibitory capacity (Guimarães et al., 2010; Gao, Yong, Dai, 2011).

**Figure 1:** Protocol for obtaining fungal extracts.




Source: Authors.

The antimicrobial activity of the elicited extracts was evaluated through adaptations of the agar diffusion method, described by Kirby and Bauer (1996). We used strains of Gram negative bacteria *Klebsiella pneumoniae* and *Proteus mirabilis*, isolated from clinical urine samples, kindly provided by the São Luis Laboratory, located in the city of Teresópolis, RJ, Brazil. The microorganism colonies were suspended in sterile distilled water until obtaining 0.5 degree Mac Farland turbidity; obtaining an inoculum of $10^8$ bacteria/mL of suspension.

Then, the cells were inoculated in Mueller-Hinton agar and distributed on the culture medium with swab in all directions, trying to cover the entire surface, with the aid of an automatic pipette and sterile tip, 10µL of the extract was dripped onto paper. of filter and this was added to the center of the plate (Test Plate). Plates 28 were incubated for 24 h at 37ºC, all tests were performed in triplicate.

Then, the cells were inoculated on Mueller-Hinton agar and distributed on the culture medium with swab covering the entire surface.

The extracts were diluted in sterile water until reaching the final concentration of 250 µg/mL and 10 µL of the extract was dropped onto filter paper and added to the center of the plate. Then, the plates were incubated for 24 h at 37 ºC. The following were used as control of the experiment: the extract without the elicitor, sterile distilled water and standardized antimicrobial discs: AMP = (ampicillin 10 mg), SFT = (sulfazotrim 25 mg), TET = (tetracycline 30 mg), GEN = (gentamicin 10 mg), CIP = (ciprofloxacin 5 mg), LVX = (levofloxacin 5 mg), CFL = (cephalothin 30 mg) and IPM = (imipinem 10 mg) from the LABORCLIN® brand. The experiments were carried out in triplicate.

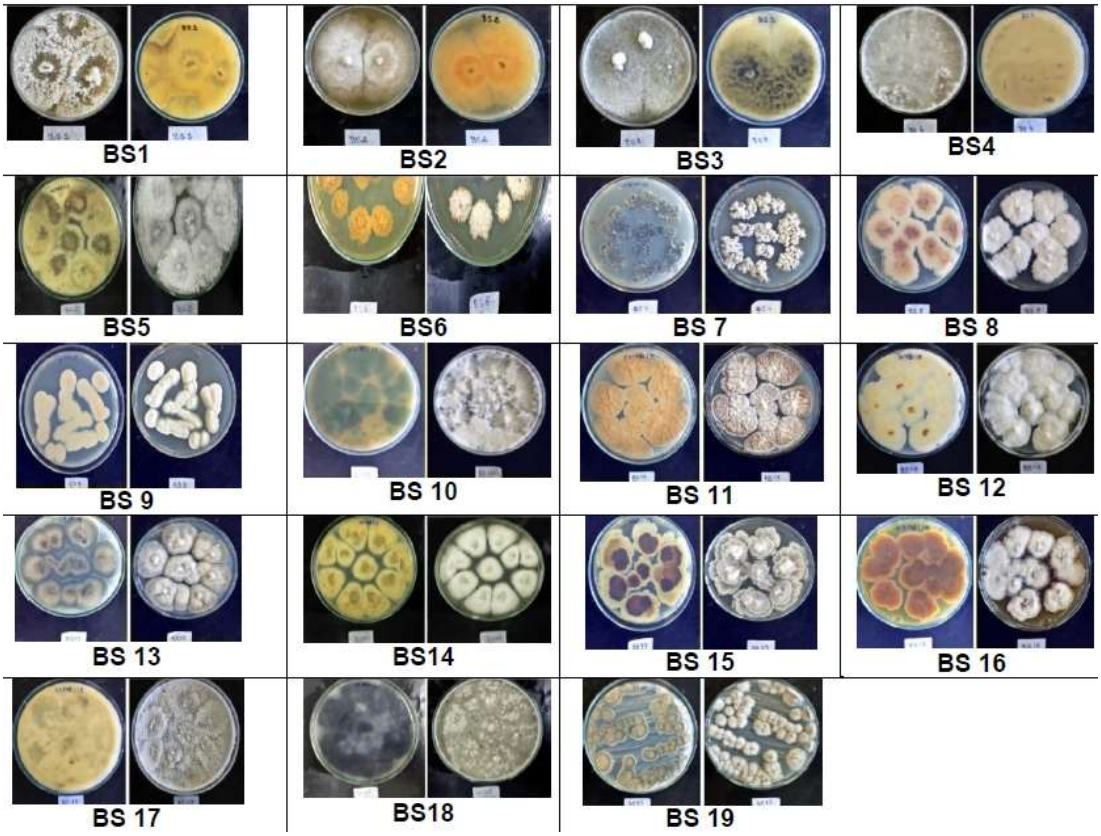
The growth of the strains tested to the different extracts was semi-quantified by measuring the halo of inhibition, in a millimeter scale and compared with the control plates. The analysis of the inhibition efficiency of the extract was performed using the percentage of inhibition parameter. This parameter was obtained through the following equation: % I = Hextract – Hsolvent / Hextract x 100. Where: Hextract - is the average of three measurements of the inhibition halo of the extract; Solvent

- is the average of three measurements of the solvent inhibition halo.

## 3. Results and Discussion

Nineteen endophytic fungi were isolated, which were separated through macroscopic analysis of their colonial morphology and were duly cataloged (Figure 2). The isolates were grown in Petri dishes containing the PDA culture medium, added with terramycin, suggesting that they are resistant species to this antibiotic. It was not possible to identify the fungi in the present study, due to the taxonomic complexity of the different species, however, in the studies by El-Hawary et al. (2016), endophytic fungi of the species *Solanum nigrum* L., also belonging to the Solanaceae family, were isolated, identifying the fungi: *Aspergillus flavus*, *Aspergillus* sp, *Fusarium avenaceum* strain, *Aspergillus oryzae* strai, *Nectria rigidiuscula*, *Fusarium* sp, *Microdiplodia* sp, *Paraconiothyrium* sp in *Eurothiomycetes* sp.

**Figure 2**: Isolated endophytic fungi from leaves of *B. suaveolens* Bercht. & J. Presl. Endophytic fungi isolated from the leaves. Nineteen different endophytic fungi were isolated and coded as BS 1 to BS 19.




Source: Authors.

The frequency of isolation of endophytic fungi of *Brugmansia suaveolens* Bercht. & J. Presl (Table 1) was mainly detected in the leaves of the plant, which suggests that after the entry of microorganisms through the leaves, there is a migration to the different tissues of the plant, due to the stomata, likely ports of entry for endophytes.

The frequency of isolation of endophytic fungi was also described for the leaves of tropical plants Pueraria phaseoloides (72.5%) and Theobroma grandiflorum (58.7%) (Galvão, 1998).

**Table 1:** Frequency of endophytic fungi isolated from *Brugmansia suaveolens* Bercht. & J. Presl leaves.

| Host plant | Type of plant tissue | | Total frequency (%) |
|---|---|---|---|
| | Leaf | | |
| | Isolated quantity | Incubated fragments | |
| *Brugmansia suaveolens* Bercht. & J. Presl | 19 | 5 | 38,00% |

Source: Authors.

Fungi BS4 (11.35%), BS11 (10.92%), BS12 (11.50%) and BS16 (12.87%) had higher average yields compared to other isolated fungi (Table 2). Microbial extracts when produced on a small scale enable a reduced cost in in vitro assays. It is possible to show that microbial transformation is an effective and versatile tool for the discovery of new bioactive compounds with antimicrobial activity

**Table 2**: Yield of fungal extracts.

| Fugu cod. | AcOEt (%) | BuOH (%) | MeOH (%) | Aqueous (%) | Average yield (%) |
|---|---|---|---|---|---|
| BS1 | 9,8% | 10,9% | 9,3% | 12,0% | 10,5% |
| BS2 | 4,5% | 7,6% | 8,7% | 7,8% | 7,15% |
| BS3 | 10,2% | 6,9% | 5,5% | 9,8% | 8,10% |
| BS4 | 9,9% | 8,7% | 7,6% | 19,2% | 11,35% |
| BS5 | 10,0% | 9,8% | 6,3% | 10,2% | 9,07% |
| BS6 | 6,9% | 5,5% | 4,7% | 10,2% | 6,82% |
| BS7 | 7,6% | 10,5% | 8,7% | 9,7% | 9,12% |
| BS8 | 6,5% | 6,7% | 7,6% | 7,1% | 6,97% |
| BS9 | 10,0% | 7,6% | 10,9% | 8,7% | 9,35% |
| BS10 | 9,9% | 9,6% | 10,6% | 6,9% | 9,25% |
| BS11 | 9,7% | 4,9% | 15,9% | 11,2% | 10,92% |
| BS12 | 10,9% | 9,9% | 15,6% | 9,6% | 11,50% |
| BS13 | 6,8% | 5,7% | 4,9% | 7,9% | 6,32% |
| BS14 | 7,7% | 4,3% | 9,7% | 9,3% | 7,75% |
| BS15 | 10,0% | 4,5% | 7,7% | 9,1% | 7,82% |
| BS16 | 6,9% | 10,8% | 19,7% | 10,1% | 11,87% |
| BS17 | 11,1% | 9,3% | 12,3% | 7,7% | 7,25% |
| BS18 | 7,8% | 5,6% | 8,9% | 6,7% | 7,25% |
| BS19 | 6,7% | 7,7% | 8,9% | 9,2% | 8,12% |

Source: Authors.

Microorganisms have the ability to produce a wide variety of both primary and secondary metabolites, including enzymes, amino acids, vitamins, pigments, immune response modulating agents, toxins, anti-tumor agents, plant growth factors and antimicrobial substances (Li, et al., 2001; Li, Strobel, 2001; Stamford, Araújo, Stamford, 1994; Kleinkauf, Von Dohren, 1990; Alexopoulos, Mims, 1996, Demain, 1992, Trilli, Michilini, Montovani, et al., 1978; Bach, Kimati, 1999; Wang et al., 2000; Rodrigues, Hesse, Werner, 2000; El-Hawary, 2016).

The extracts from fungi BS9 and BS17 showed the best antimicrobial activity against the gram negative bacterium *Proteus mirabilis*, extracted both with BuOH and AcOEt solvents. As well as the fungi BS1, BS9 and BS17, showed better activity against the bacteria *Klebsiella pneumoniae*, extracted with BuOH and AcOEt. The fractions that showed the highest activity were those of BuOH origin (Table 3). The results demonstrate that the leaves function as natural elicitors for these fungi, stimulating the production of molecules with antimicrobial activity.

**Table 3:** Antimicrobial activity of fungal extracts against *P. mirabilis* and *K. pneumoniae* extracted from dry leaves. Bacterial growth positive (+); H2O = 00, 00% (not showed activity); white (no elicitor) = 00.00% (showed no inhibition).

| | *Proteus mirabilis* | | | | *Klebsiella pneumonia* | | | |
|---|---|---|---|---|---|---|---|---|
| Code Fungus | H2O (%) | BuOH (%) | AcOEt (%) | MeOH (%) | H2O (%) | BuOH (%) | AcOEt (%) | MeOH (%) |
| BS1 | 00,00% | 00,00% | 00,00% | 00,00% | 00,00% | 80,00% | 90,00% | 80,00% |
| BS2 | 00,00% | 00,00% | 00,00% | 00,00% | 00,00% | 85,71% | 90,00% | 33,33% |
| BS3 | 30,33% | 00,00% | 84,61% | 00,00% | 00,00% | 87,50% | 88,23% | 33,33% |
| BS4 | 23,33% | 90,00% | 87,50% | 00,00% | 00,00% | 86,66% | 88,23% | 60,00% |
| BS5 | 46,00% | 00,00% | 00,00% | 33,33% | 60,00% | 60,00% | 60,00% | 83,33% |
| BS6 | 00,00% | 60,00% | 60,00% | 33,33% | 33,33% | 60,00% | 60,00% | 00,00% |
| BS7 | 68,23% | 60,00% | 00,00% | 00,00% | 00,00% | 00,00% | 88,23% | 00,00% |
| BS8 | 57,50% | 00,00% | 00,00% | 00,00% | 00,00% | 00,00% | 60,00% | 00,00% |
| BS9 | 40,00% | 90,47% | 88,23% | 60,00% | 60,00% | 80,00% | 90,00% | 20,00% |
| BS10 | 30,00% | 00,00% | 00,00% | 00,00% | 00,00% | 00,00% | 87,50% | 00,00% |
| BS11 | 00,00% | 00,00% | 00,00% | 00,00% | 60,00% | 00,00% | 00,00% | 81,81% |
| BS12 | 10,00% | 00,00% | 00,00% | 00,00% | 33,33% | 00,00% | 00,00% | 20,00% |
| BS13 | 20,00% | 00,00% | 00,00% | 00,00% | 00,00% | 81,81% | 83,33% | 00,00% |
| BS14 | 10,00% | 00,00% | 60,00% | 33,33% | 80,00% | 33,33% | 00,00% | 33,33% |
| BS15 | 14,61% | 00,00% | 00,00% | 00,00% | 00,00% | 86,66% | 00,00% | 60,00% |
| BS16 | 40,00% | 33,33% | 00,00% | 00,00% | 00,00% | 20,00% | 80,00% | 60,00% |
| BS17 | 38,23% | 85,71% | 80,00% | 33,33% | 60,00% | 81,8% | 84,61% | 33,33% |
| BS18 | 60,00% | 00,00% | 60,00% | 81,81% | 33,33% | 81,81% | 00,00% | 83,33% |
| BS19 | 00,00% | 00,00% | 60,00% | 20,00% | 00,00% | 00,00% | 81,81% | 20,00% |
| White (no elicitor) | 00,00% | | | | | | | |
| H2O | 00,00% | | | | | | | |
| bacterial growth | + | | | | | | | |

Source: Authors.

The results of the antibiograms are shown in Table 4.

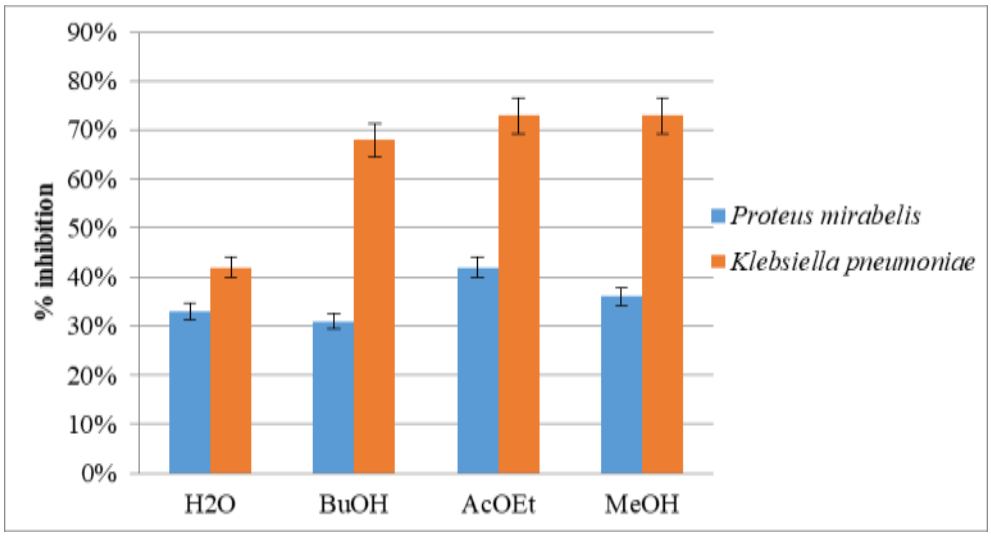
**Table 4:** Results of commercial antibiotic control tests for each microorganism. AMP = (ampicillin 10 mg), SFT = (sulfazotrim 25 mg), TET = (tetracycline 30 mg), GEN = (gentamicin 10 mg), CIP = (ciprofloxacin 5 mg), LVX = (levofloxacin 5 mg), CFL = (cephalothin 30 mg) and IPM = (imipinem 10 mg), variable according to the antibiotics used.

| Microrganism | Control Resistant | Control Sensitive * |
|---|---|---|
| *Klebsiella pneumoniae* | AMP, SFT, TET | GEN, CIP, LVX |
| *Proteus mirabilis* | AMP, CFL, TET | AMI, IPM, GEN. |

Source: Authors.

Of the extracts presented in this work, 53% of the elicited extracts showed antimicrobial activity against *Proteus mirabelis* and *Klebsiella pneumoniae*, being 33% extracted by H2O, 31% extracted by BuOH, 42% extracted by AcOEt and 36% extracted by MeOH, showing inhibition for strain of Proteus mirabelis. In contrast, 42% extracted by H2O, 68% extracted by BuOH, 73% extracted by AcOEt and 73% extracted by MeOH showed antimicrobial activity against the bacteria *Klebsiella pneumoniae* (Figure 2).

**Figure 2:** Percent inhibition of the respective fractions against *K. pneumoniae* and *P. mirabelis* strains.




Source: Authors.

In the studies by Zheng et al. (2011), 170 endophytic fungi derived from *Cymodocea serrulata, Ovalis halophila* and *Thalassia hemprichii* were isolated, which were evaluated for their ability to produce antimicrobial compounds against 10 human pathogenic microorganisms. About 69% of the isolates exhibited antimicrobial activity against at least one test strain. Among the active fungi, 7 isolates exhibited strong antimicrobial activity.

In a recent study, Ting et al. (2013) 11 investigated 39 endophyte fungi, 21 were able to produce substances in vitro while the others were not active. Furthermore, the most active broth of endophyte IV 403 was extracted with ethyl acetate and n-butanol, and comparisons of the antifungal activity of the extracts indicated that the main active metabolites were extracted by the ethyl acetate fraction.

Similar results can be observed in the present study, in which these solvents present effective extraction of metabolites. It is possible to show that elicitation is an effective and versatile tool for the discovery of new bioactive compounds with antimicrobial activity, being advantageous when compared to conventional synthetic methods. The elicitation of cell culture systems is promising, since they presented favorable results in the fermentation of antimicrobials and other fermented products; improving the secondary metabolism of plants or cells *in vitro* (Broecling, et al., 2004; Qian, et al., 2006; Yoshikawa, et al., 1993).

## 4. Conclusion

The results of this study reveal that biotic elicitation was successfully employed for the production of secondary metabolites from the endophytes of *Brugmansia suaveolens* Bercht. & J. Presl. This is the first study aimed at biotic elicitation using endophytic fungi and *B. suaveolens* Bercht. & J. Presl leaves themselves as elicitors in cultivation, demonstrating a promising antimicrobial source against gram negative bacteria.

This study reflects the potential of biotic elicitation of endophytes in the production of novel and bioactive substances and shows the urgent need to study the microbiota associated with plant species in the Brazilian flora, considering the risk of loss of endophytic biodiversity, due to the rapid reduction of tropical florests. A more detailed exploration is needed to elucidate the precise mechanism of secondary metabolite production induced by endophyte fungi.

Thus, it demonstrated that biotic elicitation is an effective tool for the production of secondary metabolites from the leaves of *B. suaveolens* Bercht. & J. Presl, strain producing antimicrobial compounds. The use of endophytic fungi proved to

be promising and evidenced the probable production of compounds not yet described in the literature, of an innovative nature, encouraging the continuation of future research by the group.

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