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In vitro antimicrobial activity of the crude extract of the endophytic bacterium *Pseudomonas aeruginosa* (SS93) isolated from *Sapindus saponaria* L.

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ABSTRACT. Endophytes colonize the interior of plant tissues without causing any damage to their hosts. The plant *Sapindus saponaria* L., popularly known as 'sabão-de-soldado', presents a diversified endophytic microbiota and also medicinal properties. Endophytic microorganisms may produce secondary metabolites with different biotechnological properties. The present study aimed to evaluate the *in vitro* antibacterial and antifungal capacity of the crude extract of secondary metabolites produced by the endophytic bacteria *P. aeruginosa* SS93 isolated from *S. saponaria* leaves. The metabolites extract was obtained using the organic solvent ethyl acetate, and the antimicrobial activities were tested against six pathogenic bacteria (*Enterococcus faecalis* [ATCC 29212], *Pseudomonas aeruginosa* [ATCC 27853], *Shigella flexneri* [ATCC 12022], *Salmonella enterica* [CCCD a016], *Escherichia coli* [ATCC 25922], and *Staphylococcus aureus* [ATCC 25923]), and pathogenic fungi (*Fusarium oxysporum*, *Glomerella* sp., *Sphaceloma* sp., *Fusarium solani*, *Maniliophtora perniciosa*, and *Sclerotinia sclerotiorum*), by agar diffusion method. In the antibacterial assay, the best results were obtained against *E. faecalis* and *S. aureus,* where the formation of inhibition halos was observed in all tested concentrations, especially at 500 and 700 μg mL^{-1}. Positive inhibitory activity against phytopathogenic fungi was observed, with the highest inhibition recorded against *F. oxysporum* (61.1%), followed by *Sphaceloma* sp. (55.7%), *M. perniciosa* (35.6%), *F. solani* (34.4%), and *Glomerella* sp. (30.4%).

Keywords: agar diffusion method; endophytes; metabolites; antifungal activity.

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Introduction

Endophytic microorganisms are defined as fungi or bacteria that colonize the interior of plant tissues. They can colonize the host without causing any damage or disease (Sridhar, 2019; Tiwari; Kang, & Bae, 2022). Furthermore, endophytes are important sources of new compounds with a wide range of biological activities of industrial interest (Strobel, 2003; Schulz & Boyle, 2005; El-Bondkly, El-Bondkly, & El-Bondkly, 2021; Santos et al., 2022). These microbes can be used to produce antimicrobials that inhibit the development of human pathogenic bacteria and phytopathogenic fungi (Phongpaichit, Rungjindamai, Rukachaisirikul, & Sakayaroj, 2006; Weber, Kappe, Paululat, Mösker, & Anke, 2007; Ghate, Shastry, & Rekha, 2021; Kapoor, Ntemafack, Chouhan, & Gandhi, 2022).

The use of endophytic microorganisms and their secondary metabolites with biotechnological properties to control pathogens from plants and humans has been increased (Santra, Maity, & Banerjee, 2022). The genus *Pseudomonas* comprises a heterogeneous group of bacteria with more than 60 species. These microorganisms can be isolated from many different niches, and can use a wide range of organic compounds both as an energy source and for the production of secondary metabolites (Gross & Loper, 2009; Verhagen, Trotel-Aziz, Couderchet, Höfte, & Aziz, 2010; Raio, & Puopolo, 2021; Elbehiry et al., 2022; Saati-Santamaría, Selem-Mojica, Peral-Aranega, Rivas, & García-Fraile, 2022).

Several species of *Pseudomonas* are well known for their ability to control plant and human pathogens by producing different compounds with antifungal (Liu et al., 2021; Salem, Ali, Reyad, Abd-Elsalam, & Hashem,

2022; Khan, Gao, Zhang, Xue, & Zhang, 2022) and antibacterial activities (Cardozo et al., 2013; Jayammal & Sivakumar, 2013; Yan et al., 2018; Kafantaris et al., 2021).

The medicinal properties of certain plants may be related to the metabolites that are produced by endophytic microorganisms (Azevedo et al., 2002). The plant *Sapindus saponaria* L., belonging to the Sapindaceae family and popularly known as 'sabão-de-soldado', presents a diversified endophytic microbiota and also medicinal properties (Garcia et al., 2012; Ataides et al., 2018; Santos et al., 2019). Some endophytes isolated from *S. saponaria* leaves have already shown important biological activities such as the production of extracellular enzymes, control of phytopathogens (Santos et al., 2019), and the production of secondary metabolites with antioxidant properties (Polli et al., 2020).

In this work, we evaluated the antibacterial and antifungal capacity of the crude ethyl acetate extract containing secondary metabolites produced by the endophytic bacteria *P. aeruginosa* SS93 isolated from *S. saponaria* leaves, using the agar diffusion method.

Material and methods

Microorganisms

The bacterium *P. aeruginosa* (SS93) isolated as endophyte from healthy leaves of *S. saponaria* L. (Sapindaceae) was retrieved from the Collection of Endophytic and Environmental Microorganisms (CMEA) of the Laboratory of Microbial Biotechnology (LBIOMIC) of the *Universidade Estadual de Maringá* (UEM), state of Paraná – Brazil. The bacterium was grown in Trypticase Soy Broth medium (TSB) for 48 hours at 28ºC.

The six pathogenic bacteria (*Enterococcus faecalis* [ATCC 29212], *Pseudomonas aeruginosa* [ATCC 27853], *Shigella flexneri* [ATCC 12022], *Salmonella enterica* [CCCD a016], *Escherichia coli* [ATCC 25922], and *Staphylococcus aureus* [ATCC 25923]) and the pathogenic fungi (*Fusarium oxysporum*, *Glomerella* sp., *Sphaceloma* sp., *Fusarium solani*, *Maniliophtora perniciosa*, and *Sclerotinia sclerotiorum*) also belong to the CMEA. The pathogenic bacteria were also grown in TSB medium (Tryptic Soy Broth) (24 hours at 37ºC), while the fungi were cultured on Potato Dextrose Agar (PDA) at 28ºC for 7 days.

Obtaining crude ethyl acetate extract

A total of 4 liters of culture medium was distributed into 16 Erlenmeyer flasks, each with a capacity of 500 ml, containing 250 ml of the medium in each flask. An aliquot of 500 µL of the adjusted endophytic bacterial solution at 0.880 (600 nm), measured using a spectrophotometer, was inoculated into Erlenmeyer flasks containing 250 mL of TSB medium (Tryptic Soy Broth). Then, the flasks were incubated at 28°C for 72 hours with 110 rpm orbital shaking. Subsequently, the cultures were centrifuged at 2,600 g for 15 min. The pellet formed was discarded and the supernatant was used for the extraction of the metabolites.

The supernatant was partitioned using the organic solvent ethyl acetate (3 × 70 mL) in a separating funnel. The organic fraction was concentrated under reduced pressure in a rotary evaporator, as described by Polonio et al. (2015).

Antibacterial activity

The pathogenic bacteria were grown for 24 hours in TSB medium and subsequently adjusted to a concentration of 1.5 x 10^8 colony-forming units mL^{-1}, using the McFarland 0.5 scale. The bacterial suspension (100 µL) was then evenly spread on Petri dishes containing TSA medium (Trypticase Soy Agar), using a Drigalsky's handle. Subsequently, 5 discs of Whatman filter paper No. 4 (Ø 5mm) were inserted in each plate, which were then impregnated with the crude extract of the metabolites previously diluted in Methanol (MeOH) at different concentrations (0, 100, 300, 500, and 700 µg mL^{-1}).

For the positive control, we used discs inoculated with 20 µg of TETREX® tetracycline (Bristol-Myers-Squibb), and the negative control was performed only with 10 µL of MeOH for comparison with the treatments. The plates were incubated at 37ºC for 24 hours. The bactericidal activity was evaluated by the formation of inhibition halos, measuring the distance of the halos (mm) from the edge of the filter paper discs to the edge of the halos. The results were then compared to the controls, consisting of tetracycline and MeOH. The experiment was conducted in triplicate, and the average halo sizes in millimeters for each treatment were subjected to Analysis of Variance. Subsequently, a statistical comparison was performed using the Tukey test with a significance level of $p<0.05$. The software Sisvar 5.3 was used for these analyses (Ferreira, 2011).

Antifungal activity

The pathogenic fungi were previously grown on PDA for 7 days at 28°C. Subsequently, disks (Ø 6mm) of the mycelium from these colonies were inoculated on fresh PDA plates, and a sterile filter paper disc (Ø 5mm) with 10 µL of the metabolic extract at 700 µg mL^{-1} was inoculated at the opposite pole, at a distance of 4 cm from each other. The tests were performed in triplicate, along with the positive control containing the commercial fungicide Cernonil WP (IHARA), 700 µg and Frowncide 500 SC (IHARA), with a dilution of 10^{-1} (Control 1), and the negative control, only with MeOH (Control 2).

To assess the initial percentage growth inhibition index (Im%), the mycelial growth area (in cm^2) of the phytopathogens was measured using ImageJ software v1.46r. The mycelial growth area values of the pathogens in each treatment and control (in triplicate) were used to calculate the Im% using the formula Im% = (1 - MT/MC) x 100, where MT represents the area of each triplicate treatment in cm^2, and MC represents the average area of the triplicate control in cm^2 (Oliveira et al., 2020). Statistical analysis was performed by comparing the mean mycelial growth areas of the treatments with the controls using the Skott-Knott test ($p>0.05$), with the assistance of the statistical software Sisvar v.5.3 (Ferreira, 2011).

Results and discussions

The production of metabolites by microorganisms has long been known and explored (Strobel, 2003; Schulz & Boyle, 2005; Brader et al., 2014; Oliveira et al. 2020). Most of the compounds produced by fungi or bacteria act by inhibiting the growth of other microorganisms. The production of these metabolites may be influenced by biotic and abiotic factors and could be related to the host's physiological situation (Azevedo et al., 2002).

The antibacterial activity using the crude extract of secondary metabolites obtained from the endophytic strain *P. aeruginosa* (SS93) diluted in methanol (MeOH) against six pathogenic bacteria showed positive results against *E. faecalis*, *S. flexneri*, *S. enterica,* and *S. aureus* (Table 1). For *E. faecalis* and *S. aureus*, the formation of inhibition halos was observed in all tested concentrations, especially at 500 and 700 µg mL^{-1} (Figure 1). Against *S. flexneri* and *S. enterica*, the formation of halos was observed with statistically significant values, however, smaller than the controls with antibiotic. There was no formation of halos against *E. coli* and *P. aeruginosa*.

Table 1. Activity of the crude extract of secondary metabolites of the endophytic strain SS93 (*Pseudomonas aeruginosa*) against different pathogenic bacteria.

Treatment	Pathogenic bacteria (Inhibition halo diameter in mm)					
	E. faecalis	*P. aeruginosa*	*S. flexneri*	*S. enterica*	*E. coli*	*S. aureus*
100 µg mL^{-1}	4.0±0.0^{b}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	8.3±0.6^{b}
300 µg mL^{-1}	5.0±0.0^{bc}	0.0±0.0^{a}	0.0±0.0^{a}	4.6±0.6^{b}	0.0±0.0^{a}	11.0±1.0^{bc}
500 µg mL^{-1}	7.0±1.0^{de}	0.0±0.0^{a}	0.0±0.0^{a}	5.6±1.2^{b}	0.0±0.0^{a}	12.3±1.5^{cd}
700 µg mL^{-1}	8.0±0.0^{e}	0.0±0.0^{a}	7.0±1.7^{b}	5.6±2.1^{b}	0.0±0.0^{a}	12.0±2.0^{cd}
Controls						
Methanol	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
Antibiotic	6.0±0.0^{cd}	3.0±1.0^{b}	10.0±0.0^{c}	15.0±0.0^{c}	8.6±1.2^{b}	15.0±0.0^{d}

Same lowercase letters in the same column do not differ statistically according to the Skott-Knott test ($p<0.05$).

The antibacterial activity of metabolite extracts from bacteria of the genus *Pseudomonas* isolated from different environments has already been described (Compant et al., 2013; Alvin, Miller, & Neilan, 2014; Spago et al., 2014), and it might be related to several classes of metabolic compounds such as polyketides, indole derivatives, peptides, glycolipids, lipids, aliphatic compounds, and phenazines like pyrrolnitrine (Leisinger & Margraff, 1979; Tomar, Lai, Khan, Singh, & Sharma, 2019; Jones et al., 2021; Uzma, Iqbal, & Hasnain, 2022).

Cardozo et al. (2013) tested metabolites obtained from *P. aeruginosa* isolated from orange leaves (*Citrus sinensis* cv. Valencia) and reported positive inhibitory results against the pathogenic bacterium *S. aureus*. In another study, Lee et al. (2013) corroborated the results found by Cardozo et al. (2013) and also described antibacterial activity by the metabolites produced by strains of *Pseudomonas* sp., especially against the pathogenic bacterium *S. aureus*.

Many bacteria species, including *P. aeruginosa*, can produce molecules that may be used as control substances for pathogenic fungi and bacteria (Raaijmakers, Vlami, & Souza, 2002; El-Sheshtawy & Doheim, 2014). Cultivated plants are subject to attack by several phytopathogenic microorganisms impairing their final yield. As an alternative to reduce the use of chemical compounds in agriculture, the use of endophytes as biological control agents may contribute to more eco-friendly agricultural practices (Oliveira et al., 2020).

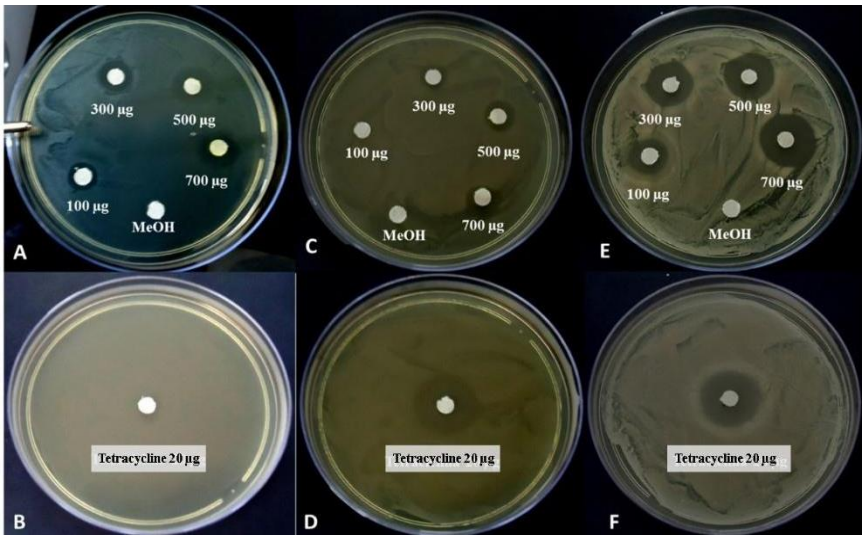

Figure 1. Antimicrobial activity of the crude extract of secondary metabolites from the endophytic strain SS93 (*Pseudomonas aeruginosa*) against pathogenic bacteria. (A), (B) *Enterococcus faecalis* against the SS93 metabolite and antibiotic Tetracycline respectively; (C), (D) *Salmonella enterica* against the SS93 metabolite and antibiotic Tetracycline respectively; (E), (F) *Staphylococcus aureus* against the SS93 metabolite and antibiotic Tetracycline respectively.

Pseudomonas aeruginosa produces various secondary metabolites with significant biological activity. Among these metabolites, pyocyanin (Abdelaziz, Kamer, & Al-Monofy, 2023; Kassob & Hummadi, 2023) and aeruginolysin exhibit potent antimicrobial activity against bacteria and fungi. Pyocyanin, a blue-green phenazine pigment, has been shown to inhibit the growth of a wide range of microorganism's species (Abdelaziz et al., 2023). Aeruginolysin, on the other hand, is a pore-forming toxin that can lyse target cells (Andrejko et al., 2019). These metabolites have been studied for their potential use in the development of new antibiotics. According to a study by Solecka, Zajko, Postek, & Rajnisz (2012), these secondary metabolites can be used as a basis for the development of novel antibiotic drugs.

In this work, we observed that the antifungal activity of the crude extract of the endophytic strain against phytopathogens fungi showed positive inhibitory activity against *F. oxysporum* (61.1%), followed by *Sphaceloma* sp. (55.7%), *M. perniciosa* (35.6%), *F. solani* (34.4%), and *Glomerella* sp. (30.4%). However, for *S. sclerotiorum,* the crude extract showed no statistically significant inhibitory activity (Table 2, Figure 2).

Ziedan and El-Mohamedy (2008) demonstrated the interaction between a *P. fluorescens* strain and the phytopathogen *F. oxysporum* by scanning electron microscopy. As a result, these authors found a deterioration on the cell wall of the pathogen, which might be related to the secondary metabolites produced by the bacterium. This mechanism of action may occur by antagonism. Some species of *Pseudomonas* can produce metabolites with antifungal activity, especially against *Fusarium* and *Sclerotinia* pathogens (Zhou, Zhao, & Dai, 2014).

Table 2. Inhibition index percentage of mycelial growth (Im%) of the crude metabolites extract (700 µg ml^{-1}) obtained from the endophytic bacterium *Pseudomonas aeruginosa* SS93 against *Fusarium oxysporum* (FO), *F. sonali* (FS), *Glomerella* sp. (GLO), *Sphaceloma* sp. (SPHA), *Maniliophthora perniciosa* (MP), and *Sclerotinia sclerotiorum* (SS).

Treatment	Pathogenic fungi					
	FO	FS	GLO	SPHA	MP	SS
	Im%	Im%	Im%	Im%	Im%	Im%
P. aeruginosa SS93	61.1±2.6^a	34.4±1.9^a	30.4±2.7^a	55.7±0.7^a	35.6±6.4^a	0.1±1.1^a
Control 1*	19.5±3.0^b	18.5±2.1^b	19.1±3.1^a	17.0±0.9^b	2.0±0.9^b	1.9±0.6^a
Control 2**	0.0±1.3^c	0.0±1.7^c	0.0±1.0^b	0.0±1.7^c	0.0±0.0^b	0.0±0.0^a

Same lowercase letters in the same column do not differ statistically according to the Skott-Knott ($p < 0.05$). *Control 1: phytopathogen against commercial fungicide; **Control 2: phytopathogen against methanol (10 µL).

Pseudomonas species may be isolated from different niches and hosts, which suggests great adaptability and a promising source of biologically active secondary metabolites (Bano & Musarrat, 2003; Anand & Kulothungan, 2010; Verhagen, Trotel-Aziz, Jeandet, Baillieul, & Aziz, 2011; Gupta, Panwar, & Jha, 2013; Janek, Łukaszewicz, & Krasowska, 2013; Zhou et al., 2014). Besides, the capacity of endophytes associated with *S. saponaria* to produce secondary metabolites with biotechnological properties of interest (Polli et al., 2020), reinforce the importance of studies that explore more endophytic isolates from this plant. This

approach aims to select strains with potential for application in different areas, such as in the health and agriculture fields, similar to the characteristics found in this work with the endophyte SS93.

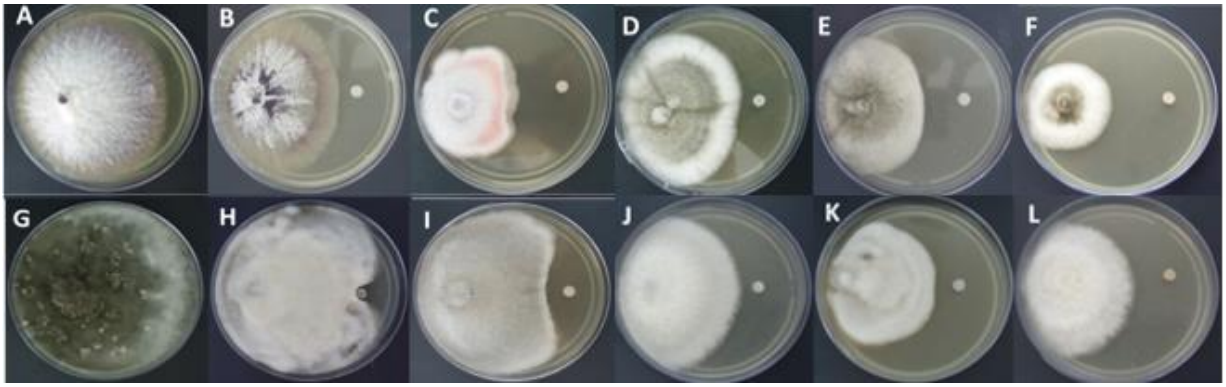

Figure 2. Agar diffusion assay of the crude extract of secondary metabolites of the endophytic strain *Pseudomonas aeruginosa* SS93 against the phytopathogens. (A), (B), (C) *Fusarium oxysporum* against methanol, commercial fungicide and metabolites from SS93 respectively; (D), (E), (F) *Sphaceloma* sp. against methanol, commercial fungicide, and metabolites from SS93 respectively; (G), (H), (I) *Maniliophthora perniciosa* against methanol, commercial fungicide and metabolites from SS93 respectively; (J), (K), (L) *Glomerella* sp. against methanol, commercial fungicide, and metabolites from SS93 respectively.

Conclusion

Our research findings suggest that the endophytic bacterium *P. aeruginosa* SS93 has the ability to produce antibacterial and antifungal compounds, making it a promising candidate for the isolation and production of pharmaceutical molecules with antibacterial properties. Specifically, these compounds show efficacy against *E. faecalis* and *S. aureus,* making them valuable for the pharmaceutical industry. Furthermore, they exhibit potential in agriculture for controlling phytopathogens such as *F. oxysporum* and *Sphaceloma* sp. It is important to continue studying these metabolites, not only to identify the chemical composition of the extract, but also to conduct isolated antimicrobial evaluations.

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