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Article

Plantago major leaf extract against bacteria and fungi of medical importance

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Antimicrobial drug resistance is a challenge to public health. Various microorganisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, carbapenemase-producing *Klebsiella pneumoniae*, *Pythium insidiosum,* and *Candida auris*, have developed resistance to commonly used antimicrobials in infection disease treatments. Consequently, there is an urgent need to explore and develop novel therapeutic drugs. Natural products, particularly medicinal plants, have received attention in the quest for innovative treatments for various diseases. *Plantago major* is a plant endowed with several biological properties, such as antibacterial, antifungal, and healing actions. This study aimed to investigate the antimicrobial activities of the methanolic extract obtained from the leaves of *P. majo*r. Microdilution assays were conducted to determine the minimum inhibitory concentration and minimum bactericidal and fungicidal concentration. Additionally, synergism with antimicrobial drugs was assessed using a time-kill curve analysis. A synergistic bactericidal interaction between the extract and imipenem was observed against carbapenemase-producing *K. pneumoniae*. For MRSA, a bacteriostatic synergism was noted in combinations of the extract with cephalotin and oxacillin. For *C. auris*, a fungistatic interaction was observed between the extract and amphotericin B. These results suggest the presence of bioactive compounds within the extract with therapeutic potential for combating infections caused by these microorganisms.

Keywords: Synergistic interaction. Natural products. Multi-drug resistant microorganisms. Antimicrobial drugs. Extract chemical composition. Mass spectrometry.

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INTRODUCTION

Antimicrobial resistance poses a grave public health concern, leading to increased mortality rates and escalating healthcare costs. More than 1.2 million deaths were attributable to antimicrobial resistance, and almost 5 million deaths are related to resistance (GLASS, 2022). Methicillin-resistant *Staphylococcus aureus* (MRSA), is characterized by multidrug resistance, and is particularly prevalent in healthcare settings (Kock, Becker, Cookson, 2010). *Pseudomonas aeruginosa*, known for its high propensity to develop resistance, is also associated with hospital environments and mechanical ventilation (Lister, Wolter, Hanson, 2009).

Klebsiella pneumoniae demonstrates resistance to aminoglycosides, carbapenems, and fluoroquinolones (Ramachadran *et al.*, 2020). Fungal resistance is also a significant concern, and is notably observed in *Candida auris*; which exhibits high rates of resistance to available antifungals (Chowdhary, Sharma, Meis, 2017). The treatment for sporotrichosis caused by *Sporothrix schenkii*, *S. globosa*, and *S. brasiliensis* has become increasingly challenging due to elevated resistance to azoles, amphotericin B, and echinocandins (Brilhante *et al.*, 2018). *Pythium insidiosum*, which causes invasive infections, presents unique challenges in treatment. Being a pseudo-fungus or oomycete, it shares characteristics and infection patterns with fungi. However, due to the absence of ergosterol in the plasma membrane, traditional antifungal treatments are ineffective, often necessitating surgical removal of infected areas (Griffith, Davis, Grant, 1992).

Natural products, including medicinal plants, harbor molecules endowed with various biological activities, such as antibacterial, antiviral, and antitumoral properties (Samuelsen, 2000) (Metiner, Ozkan, Ak, 2012) (Chiang *et al.*, 2002) (Shirley *et al.*, 2017). Medicinal plants are recognized as a contemporary therapeutic approach due to their widespread availability, lower toxicity, and the diverse array of chemical compounds they contain (Adom *et al*., 2017). Additionally, there is therapeutic promise in exploring the combination of antimicrobials with plant metabolites (Abreu *et al.*, 2015).

Plantago major, a plant with a broad geographic distribution, has been traditionally used for treating infections, cancer, and diarrhea; due to its anti-inflammatory, antioxidant, and healing properties. It contains five classes of biologically active compounds: benzoic compounds, flavonoids, iridoid glycosides, phenolic compounds, and triterpenes; along with carbohydrates, vitamins, and lipids (Samuelsen, 2000) (Adom *et al.*, 2017). The extract derived from *P. major* leaves, contain aucubin and baicalein - compounds inherent to the plant - exhibits

inhibitory effects on the growth of *Candida albicans*, indicating fungicidal activities (Shirley *et al.,* 2017). Furthermore, the antibacterial activities of *P. major* extract have already been documented against various strains, including *Escherichia coli, Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *S. aureus*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, and *Proteus mirabilis*. Notably, it has also demonstrated antibacterial effects against other Gram-positive and Gram-negative bacteria species (Metiner, Ozkan, Ak, 2012).

The objective of this study was to assess the antibacterial and antifungal activity of the methanolic extract from *Plantago major* leaves against methicillin-susceptible (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida auris*, *Pythium insidiosum*, *Sporothrix schenckii*, *S. globosa*, and *S. brasiliensis*. Additionally, the study aimed to investigate the synergistic effects of this extract when combined with commonly used antimicrobials against the aforementioned bacteria and fungi.

MATERIAL AND METHODS

Plant material and methanolic crude extract (ME)

The *P. major* samples were collected in 2020 from Monte Castelo, São Paulo State, Brazil; located at coordinates 21°17'55.75"S and 51°34'05.78"W. Voucher specimen (number 34828) was deposited at the Herbarium of the Institute of Biosciences of Botucatu, UNESP. The leaves were subjected to drying at 40 °C and subsequently triturated using a mortar, following the procedure outlined by Betoni *et al* (2006). The moisture content of different leaf samples was determined by weight before and after drying; yielding an average moisture content of 85%. For regulatory and ethical compliance, this research was registered with the number A5D6C13 in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge of the Ministry of the Environment of Brazil.

The plant material, after being dried, underwent maceration in 70% methanol under refrigeration and was filtered after 48 hours. The residual plant material was subjected to re-extraction with the addition of 70% methanol (Synth), and after 24 hours, it was filtered again, following the protocol outlined by Betoni *et al.* (2006). The combined filtrates were concentrated using a rotary evaporator set at 50 °C (Fisatom rotary evaporator) to eliminate methanol. The resulting extract (ME) was then stored in sterile bottles under refrigerated conditions until needed. The ME was sterilized by filtration using a filter membrane with a pore size of 0.22 μm. Subsequently, the dry weight of the sterilized ME was determined and a stock solution was prepared in a concentration of 120 mg mL^{-1}.

Flavonoid content

The flavonoid content was performed according to Quaresma *et al.* (2020). In a tube, 2.0 mL of a ME was combined with 1.0 mL of an aluminum chloride (Vetec) solution (5% m v^{-1}) and 2.0 mL of methanol (Synth) and mixtures were kept at room temperature for 30 minutes and the absorbance was measured using a Scientific brand Genesys 10S UV-Vis spectrophotometer at 425 nm. The flavonoid content was expressed as milligrams (mg) of quercetin equivalent (QE) per gram of ME and determined using prepared analytical concentration curves (5 to 40 μg mL^{-1}) of methanolic quercetin solution; the quercetin standard was acquired from Sigma-Aldrich.

Total phenolic content

The total phenolic content was measured according to Quaresma *et al.* (2020). In a tube, 0.5 mL of a ME, 2.0 mL of an aqueous calcium carbonate (Neon) solution (7.5% m v^{-1}), and 2.5 mL of aqueous Folin-Ciocalteu reagent solution (Dinâmica) (10% v v^{-1}) was heated in a water bath for 5 minutes at 50 °C, and the absorbance was measured at 760 nm. The total phenol content was expressed in mg of gallic acid equivalent (GAE) per gram of extract, and for this purpose, at different concentrations (5 to 60 μg mL^{-1}).

DPPH radical scavenging

The DPPH radical scavenging was obtained according to Quaresma *et al.* (2020), with some modifications. DPPH (Sigma-Aldrich) solution was prepared at a concentration of 35 μg ml^{-1} and its absorbance was measured at 517 nm and adjusted to 0.990. To obtain the IC_{50}, an analytical curve was constructed varying the concentration of the extract (5 to 33.33 μg ml^{-1}). To obtain the curve, 0.2 mL of ME was added with 2.8 mL of DPPH solution and the resulting mixtures were rested in the dark for one hour, after which the absorbance was measured again. For the control, the sample was replaced with methanol, and for the blank, DPPH was replaced with methanol, and ascorbic acid was used as a positive control.

Mass spectrometry

The ME was submitted to mass spectrometry analysis, to identify the compounds present in the extract. HPLC (Agilent model Infinity 1260) was used, using an Agilent model Zorbax C18 column (100.0 × 3.0 mm, 2.7 μm) coupled to a spectrometer Q-TOF pastes brand Agilent model 6520 B; and the ionization mode used was by electrospray. The samples were prepared in methanol with a concentration of 5 mg mL^{-1} and the chromatographic parameters were: mobile phase (A) composed of water acidified with formic acid (0.1% (v v^{-1}) and mobile phase (B) methanol (Carlo erba) with the gradient starting at 10% B (0 min), 98% B (0 – 15 min), 100% B (15 - 17 min); injection volume was 0.4 mL min^{-1}. The ionization parameters were: nebulizer pressure of 58 psi, drying gas at 8 L min^{-1} at a temperature of 220 ºC, and an energy of 4.5 KVA was applied to the capillary. For compound identification, errors in ppm (0 to ± 5) and spectra in high resolution, as well as fragmentation spectra, were compared to spectra published in the literature (De Andrade *et al.*, 2018) (Gao *et al.*, 2022) (Li *et al.*, 2014) (Mazzutti *et al.*, 2017a).

Microorganism strains

The bacterial strains studied included *Staphylococcus aureus* (MSSA) ATCC 2593, a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, a clinical isolate of *Pseudomonas aeruginosa* ATCC 27852, and a clinical isolate of carbapenemase-producing *Klebsiella pneumoniae*. Additionally, the fungal isolates comprised of clinical isolates of *Sporothrix brasiliensis*, *S. globosa*, and *S. schenkii*. Ten isolates of *Pythium insidiosum* from horses, along with one from dogs, were also included in the study. All strains were stored in the Department of Chemical and Biological Sciences at IBB, São Paulo State University. Furthermore, *Candida auris* CDC B11903 and a clinical isolate were obtained from the Instituto Adolfo Lutz of São Paulo State.

Susceptibility tests

Susceptibility tests for bacterial strains were carried out using the Resazurin Microtiter Assay (REMA), a method adapted from Martin, Camacho, Portaels, (2003) in 96-well microplates (Kasvi). Treatments were prepared in broth Brain Heart Infusion (BHI) (Kasvi) with concentrations ranging from 50 to 1.25 mg mL^{-1}. Inoculum were standardized using the McFarland 0.5 scale, and the microplates were incubated at 37 ºC/24 h. Controls included negative (BHI without bacteria), positive (BHI with bacteria), and ME sterility (BHI with extract). The Minimum Inhibitory Concentration (MIC), defined as the lowest concentration capable of inhibiting microbial growth, was determined by visualizing color changes after the addition of 50 μL of resazurin (0.5%) (ACS Científica). Bacterial growth was indicated by a color change from blue to pink. To determine the Minimum Bactericidal Concentration (MBC), subcultures of the tested concentrations were performed through microdilution on BHI agar plates, followed by incubation at 37 ºC for 24 hours.

To determine the Minimum Inhibitory Concentration (MIC) of the methanolic extract (ME) on *C. auris*, the microdilution methodology described in the M27 standard (2017) was employed. In this procedure, 100 μL of RPMI-

1640 medium (Sigma-Aldrich), buffered with 0.165 M MOPS (3-(N-morpholino) propanesulfonic acid) (LGC Biotecnologia), was added to the wells of a 96-well microplate (Kasvi) containing varying concentrations of the ME, ranging from 50 mg mL^{-1} to 10 mg mL^{-1}. Inoculum were standardized using the 0.5 McFarland scale, and the plates were then incubated at 37 °C/24 h. After the incubation period, subcultures of the well contents were performed on Sabouraud-dextrose agar (Kasvi) to determine the CFM, with plates being further incubated at 37 °C for an additional 24 hours. This method allowed for the determination of the MIC, which represents the lowest concentration of the ME inhibiting the growth of *Candida auris*.

Susceptibility tests for *Sporothrix schenckii*, *S. globosa*, and *S. brasiliensis* were conducted following the M38-A standard (2008). In each well, 100 µL of the inoculum and ME concentrations ranging from 10 mg mL^{-1} to 50 mg mL^{-1} were inoculated. The plates were then incubated at 35 °C for 48 hours, and the results were observed with the assistance of a mirror. Subcultures of the well contents were performed on Sabouraud-dextrose agar (Kasvi) to determine the Minimum Fungicidal Concentration (MFC), with plates being incubated at 27ºC/72 h following the protocol by Waller *et al.* (2017). For susceptibility tests with *Pythium insidiosum*, isolates were initially cultivated on Sabouraud-dextrose agar (Kasvi) for 5 days at 35 ºC. Subsequently, 5-millimeter discs of *P. insidiosum* were added to microtubes containing Sabouraud broth (Kasvi) and varying concentrations of *P. major* extract, ranging from 10 mg/mL to 50 mg mL^{-1} in a final volume of 1 mL. After the incubation period, the fragments were individually transferred to plates containing Sabouraud-dextrose agar (Kasvi) and cultured for 7 days at 35 ºC. The susceptibility of *P. insidiosum* to *P. major* extract was assessed by measuring the diameter (mm) of the mycelium at 24, 48, and 168 hours. The Minimum Fungicidal Concentration (MFC) was considered when there was no growth throughout the period, and when there was no growth in the first 24 hours but resumed after 48 hours, the action was deemed fungistatic (Araújo, Bosco, Sforcin, 2016). Considering that 10 isolates of *P. insidiosum* were tested, the MIC value at 90% inhibition will be calculated.

Synergistic interactions by time-kill curve

Following the determination of MIC values, assays for synergistic interactions by time-kill curve were conducted with the methanolic extract (ME) and antimicrobials. The objective was to reduce the amounts of antimicrobials used while maintaining efficacy against carbapenemase-producing *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27852, clinical isolates, and MRSA ATCC 33591, as well as clinical isolates. For each isolate, concentrations equivalent to 1/4 of the MIC of ME and 1/4 of the MIC of the respective antimicrobial were utilized. The extract was combined with tetracycline, cephalothin, and oxacillin (Sigma-Aldrich) for MRSA; with imipenem for *Klebsiella pneumoniae*, and with tetracycline and polymyxin B (Sigma-Aldrich) for *Pseudomonas aeruginosa*. After inoculation, the plates were incubated in a microplate reader (Biotech Epoch-2, Gen5 software) at 37 ºC/24 h with readings at 600 nm taken every two hours. Simultaneously, a 24-well plate was incubated at 37 ºC for 24 hours. Aliquots were taken at 0, 2, 4, 8, and 24 hours, followed by subcultures on BHI agar (Kasvi), which were then incubated at 37 ºC/24 h, following the CLSI guidelines (2015). The same procedures were applied to determine the interaction of the extract with amphotericin B (Sigma-Aldrich) for *Candida auris* CDC B11903 and a clinical isolate. Results were used to create charts on GraphPad Prism 5.00. These isolates were incubated in RPMI-1640 (Sigma-Aldrich), and subcultures were subsequently performed on Sabouraud-Dextrose agar (Kasvi) at 37 °C/24 h.

Statistical Analyses

The statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by Tukey's test in GraphPad Prism 5.00, considering $p < 0.05$ for statistical significance.

RESULTS

Flavonoid content, total phenolic content, and DPPH

Table I displays the flavonoid content, total phenolic content, and DPPH radical scavenging activity. The flavonoid content was measured at 0.90 ± 0.01 mg QE g^{-1}, while the total phenolic content registered at 75.98 ± 1.66 mg GAE g^{-1}. Notably, the DPPH radical scavenging activity was found to be 26.82 ± 0.40 µg mL^{-1}.

TABLE I - Total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity from methanolic extract of *P. major*

Sample	TPC	TFC	DPPH
	mgGAE g^{-1} extract	mgQE extract (mg g^{-1})	IC_{50} (µg.mL^{-1})
Ethanolic extract	75.98 ± 1.66	0.90 ± 0.01	26.82 ± 0.40
Ascorbic acid	--	--	3.95 ± 0.08

Note: GAE gallic acid equivalent; QE quercetin equivalent.

Mass spectrometry

Table II outlines a methodology for the identification of compounds in the ME (methanol extract) of *P. major* utilizing mass spectrometry. The compounds identified include verbasoside, plantamajoside, verbascoside, isoverbascoside, and martynoside. The molecular structures of these identified compounds are illustrated in Figure 1.

TABLE II - Chemical analysis of methanolic extract of *Plantago major* leaves by HPLC-(–)-ESI-MS/MS

Rt (min)	$[M - H]^-$	Exact Mass	Error (ppm)	Fragment ions (*m/z*) MS/MS	Molecular formula	Tentative identifiifcation	Reference
3.98	455.0853	--	--	20 eV: **96**	--	NI	
4.68	461.1653	461.1664	– 2.39	15 eV: 315, 161, 135, **113**,85, 71, 59	$C_{20}H_{30}O_{12}$	Verbasoside (Decaffeoyl-acteoside)	(Gao et al., 2022); Pubchem[a]
6.85	639.1908	639.1931	– 3.6	20 eV: 477, 394, 315, **161**	$C_{29}H_{36}O_{16}$	Plantamajoside	(de Andrade et al., 2018; Li et al., 2014)
7.16	623.1965	623.1981	– 2.57	20 eV: 461, 315, **161**, 113	$C_{29}H_{36}O_{15}$	Verbascoside	(Mazzutti et al., 2017); Pubchem[b]
7.51	623.1924	623.1981	– 2.73	30 eV: 461, 315, **161**	$C_{29}H_{36}O_{15}$	Isoverbascoside	(Mazzutti et al., 2017); Pubchem[b]
8.41	651.2277	651.2294	– 2.61	--	$C_{31}H_{40}O_{15}$	Martynoside	(Mazzutti et al., 2017)

Note: NI: no identify; ahttps://pubchem.ncbi.nlm.nih.gov/compound/Verbasoside#section=Other-MS; bhttps://pubchem.ncbi.nlm.nih.gov/compound/Verbascoside#section=Mass-Spectrometry

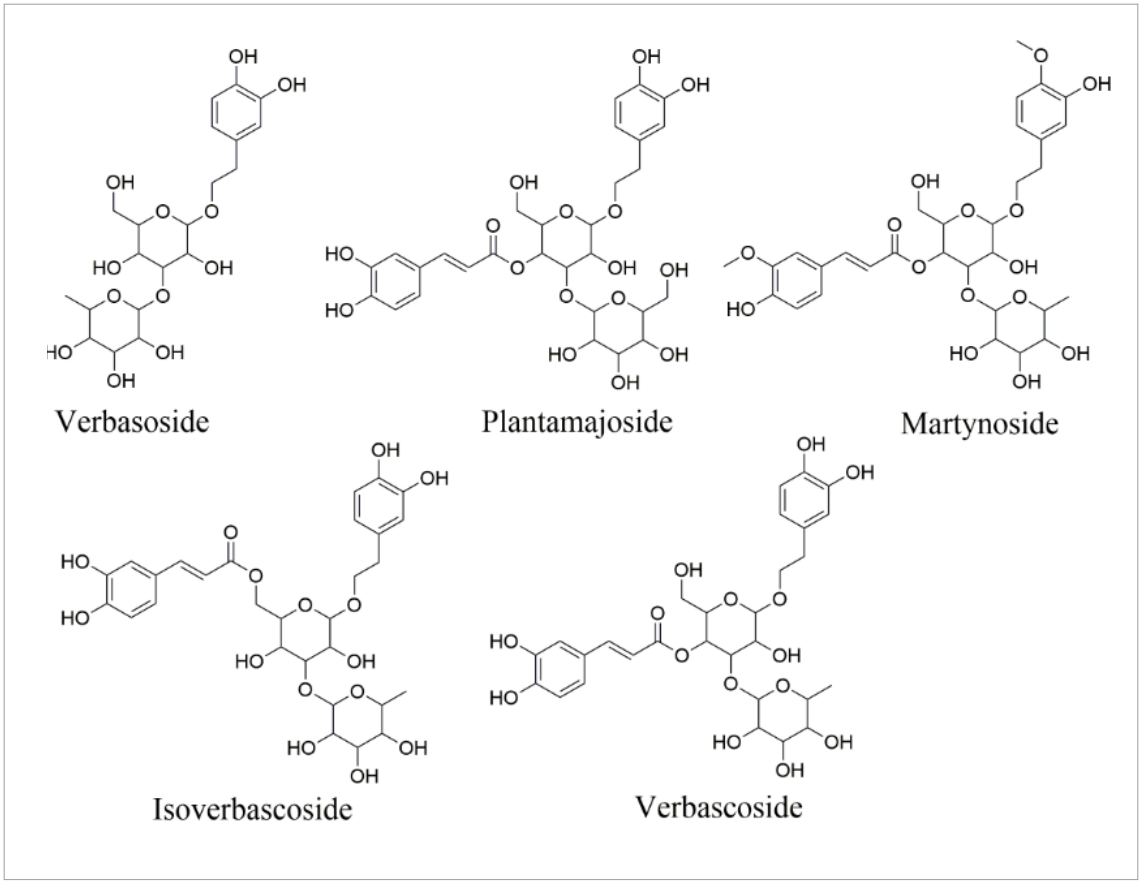

FIGURE 1 - Compounds identified in the extract of *P. major* by mass spectrometry.

Susceptibility tests

The susceptibility test results for bacterial isolates are presented in Table III. The minimum inhibitory concentration (MIC) values were 35 mg mL^{-1} for MRSA ATCC 33591 and 2.5 mg mL^{-1} for the clinical isolate. Against *P. aeruginosa*, the MIC was 35 mg mL^{-1} for ATCC 27852 and 45 mg mL^{-1} for the clinical isolate, while for MSSA, the MIC was 2.5 mg mL^{-1} for the clinical isolate. Bactericidal action was observed at these concentrations. However, KPC and MSSA ATCC 2593 were not inhibited at the tested concentrations. Table IV displays the susceptibility test results for fungal isolates. The MIC for *C. auris* CDC B11903 and the clinical isolate, as well as for *S. schenckii*, *S. globosa*, and *S. brasiliensis*, was 10 mg mL^{-1}. The extract exhibited fungicidal activity against *Sporothrix species* at the same concentrations, but for *C. auris*, it demonstrated only fungistatic activity at the tested concentrations. Additionally, the sensitivity test results for *P. insidiosum* are included in Table IV, revealing a 90% MIC of 45 mg mL^{-1} for the extract against *P. insidiosum* isolates, indicating oomicidal activity.

TABLE III - Minimum inhibitory concentrations and minimum bactericidal concentrations against bacterial strain

	MIC (mg mL^{-1})	MBC (mg mL^{-1})
MRSA ATCC 33591	35[a]	35[a]
MRSA clinical isolate	2.5[a]	2.5[a]
P. aeruginosa ATCC 27852	35[a]	35[a]
P. aeruginosa clinical isolate	45[a]	45[a]
KPC clinical isolate	>50[b]	>50[b]
MSSA ATCC 2593	>50[a]	>50[a]
MSSA clinical isolate	2.5[a]	2.5[a]

Different letters on the same column represent significant differences in antimicrobial activity in differents microorganisms: $p \leq 0.05$.

TABLE IV - Minimum inhibitory concentrations and minimum fungicide concentrations against fungi strains

	MIC (mg mL^{-1})	MFC (mg mL^{-1})
C. auris CDC B11903	10[a]	>50[a]
C. auris clinical isolate	10[a]	>50[a]
S. schenkii	10[a]	10[a]
S. globosa	10[a]	10[a]
S. brasiliensis	10[a]	10[a]
*P. insidiosum**	45[a]	45[a]

* For *P. insidiosum*, the 90% MIC was performed with the eleven clinical isolates.
Different letters on the same column represent significant differences in antimicrobial activity in differents microorganisms: $p \leq 0.05$.

Synergistic interactions

The time-kill curve illustrates a synergistic interaction between the *P. major* extract and antimicrobials, showcasing fungistatic, bacteriostatic, and bactericidal effects. In Figure 2 (A), a reduction in log CFU/mL is observed in the curves corresponding to combinations of the extract with cephalothin and oxacillin, indicating synergism with a bacteriostatic effect. In Figure 2 (B), a bacteriostatic interaction is evident between ME and cephalothin, along with an association of ME and oxacillin. In Figure 2 (C), a 5-unit reduction in log CFU/mL indicates bactericidal synergism between ME and imipenem. However, there was no observed synergistic interaction between the extract and tetracycline, as well as between the extract and polymyxin B for *P. aeruginosa* ATCC 27852 (D) and the clinical isolate (Figure 2-E). Figure 2 (F) reveals fungistatic synergism between amphotericin B and the extract, while Figure 2 (G) depicts fungistatic synergism between the extract and amphotericin B.

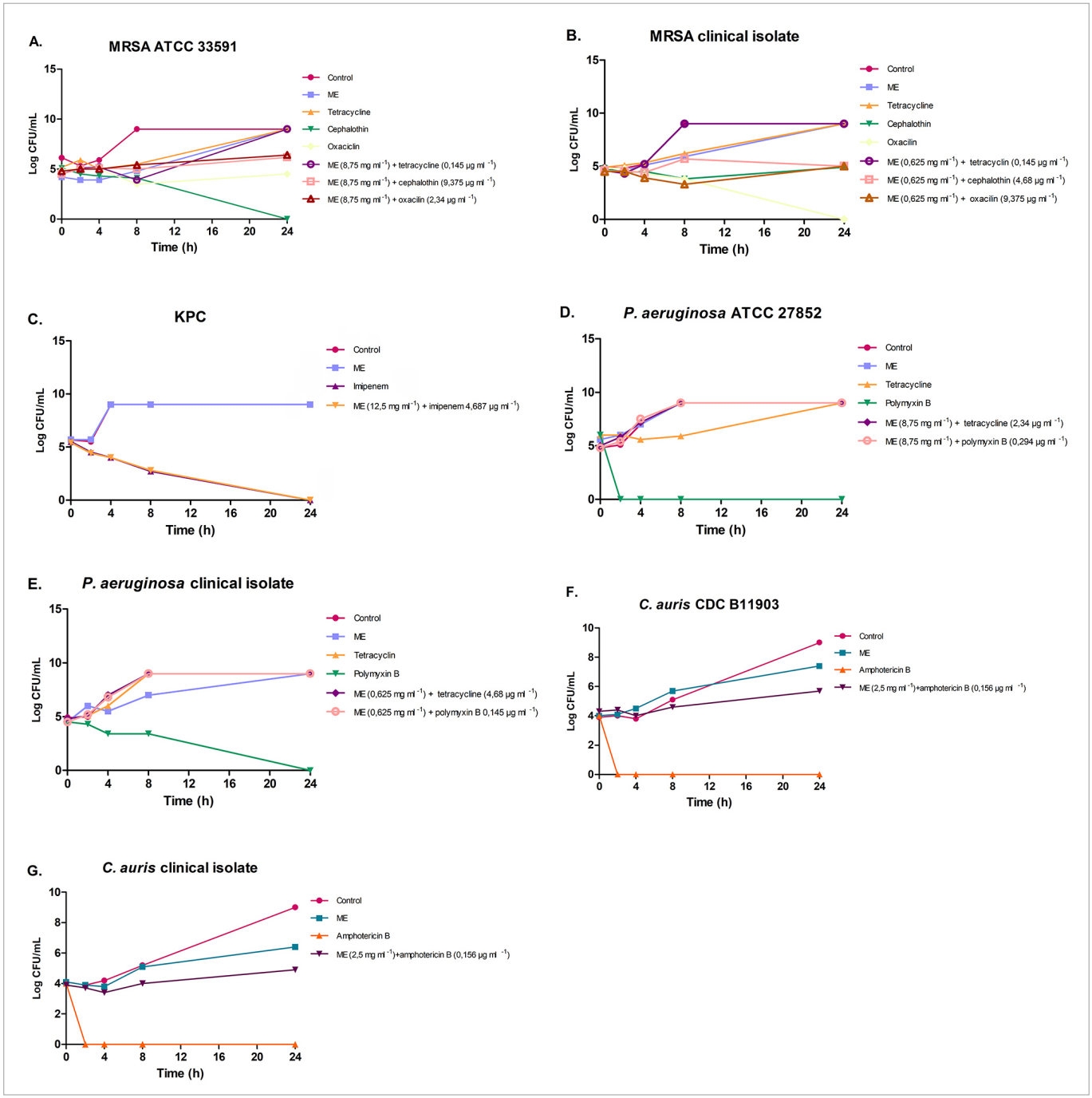

FIGURE 2 - Time kill curve to verify synergistic interaction between the extract, tetracycline, cephalothin, and oxacillin against: methicillin-resistant *S. aureus* (MRSA) ATCC 3359 (A), against methicillin-resistant *S. aureus* (MRSA) clinical isolate (B), interaction between the extract and imipenem against clinically isolated carbapenemase-producing *Klebsiella pneumoniae* (C), interaction between the extract and tetracycline and polymyxin B against *Pseudomonas aeruginosa* ATCC 27852 (D) and clinical isolate (E), interaction between the extract and amphotericin B against *Candida auris* CDC B11903 (F) and interaction between the extract and amphotericin B against the clinical isolate *Candida auris* (G).

DISCUSSION

The total phenolic content identified in the methanolic extract of *P. major*, amounting to 75.98 mg GAE g^{-1}, corroborates with the findings of Mazzutti *et al.* (2017a); who reported values ranging from 35.30 to 113.20 mg GAE g^{-1} in extracts obtained through subcritical water extraction and microwave-assisted extraction at various temperatures. In contrast, the total flavonoid content was determined to be 0.90 mg QE g^{-1}, differing from a study by Beara *et al*. (2009); who reported 5.31 mg QE g^{-1} in an extract obtained from *P. major* in Serbia using methanol. This variation in total flavonoid values could be attributed to the geographic locations where the plants were collected, as flavonoids and phenolics, the primary compounds, play a pivotal role in the antioxidant activities of the *Plantago* genus (Beara *et al.*, 2009).

In concordance with Mazzutti *et al.* (2017b), DPPH free radical scavenging activity values ranging from 20.80 to 42.90 μg mL^{-1} were observed in extracts obtained through two distinct methods utilizing water as a solvent at different temperatures. This supports the findings of the present study, where a value of 26.82 μg mL^{-1} indicates that the extract is very active as a radical inhibitor; as extracts with an IC_{50} less than 50 ug mL^{-1} are considered very active (Reynertson, Basile, Kennelly, 2005). Another study reported a broader range of DPPH free radical scavenging activity, varying between 65.6 and 1296 μg mL^{-1}. However, these employed diverse extraction methods such as, soxhlet extraction, ultrasound-assisted extraction, and supercritical fluid extraction. The differences in free radical scavenging activity are likely attributed to the varied methods employed to obtain the extract and the use of different solvents (Mazzutti *et al.*, 2017a).

The mass spectrometry analysis revealed the presence of verbascoside and isoverbascoside, two isomeric phenylethanoid glycosides derived from caffeic acid, among other identified compounds. These compounds are potentially linked to the diverse biological activities of *P. major*, including antitumor, antimicrobial, antioxidant, anti-inflammatory, and anti-thrombotic properties (Mazzutti *et al.*, 2017b). Additionally, plantamajoside, a bioactive caffeic acid derivative associated with antimicrobial, antioxidant, and anti-inflammatory activities, was also detected (Mazzutti *et al.*, 2017a). Martynoside, another phenylethanoid compound identified, is known for its antiviral action (Ruchapowl *et al.*, 2021). Compounds belonging to the phenylethanoid class, including those found in *P. major*, exhibit a range of biological activities such as protection against ultraviolet radiation, anticancer effects, anti-inflammatory properties, and antimicrobial actions (Malarz, Yudina, Srojakowska, 2023).

Metiner, Ozkan, Ak, (2012) demonstrated that extracts of *P. major* obtained from acetone and ethanol exhibit antibacterial activities against both Gram-positive and Gram-negative species. The acetone extract displayed MIC values of 28.50 mg mL^{-1}, 14.25 mg mL^{-1}, 14.25 mg mL^{-1}, and 28.50 mg mL^{-1} for *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, respectively. However, the ethanol extract showed no activity against these species. It's worth noting that variations in inhibitory values against *S. aureus* between the present study and Metiner, Ozkan, Ak, (2012) could be attributed to factors such as temperature, pressure, extraction method, and the choice of solvent. These variables have the potential to influence the composition of the extract, and the differences in the compound composition may explain the variations in inhibitory efficacy against *S. aureus* observed between the studies

The ethanol extract of *P. major* exhibited activity against *Candida albicans*, as determined by the disk diffusion method. Following 24 hours of incubation, there was an inhibition zone measuring 10.6 mm, and after 72 hours, it decreased slightly to 8.8 mm. Notably, these results were comparable to the effects of itraconazole treatment, which demonstrated an inhibition zone of 10.6 mm and 8.6 mm after 24 and 72 hours, respectively (Sharma *et al.*, 2016).

Polyphenols derived from plants possess a range of biological properties, including antioxidant, anti-inflammatory, anti-platelet, and antimicrobial activities (Duda-Chodak, Tarko, 2023). The efficacy of caffeic

acid and caffeic acid phenethyl ester against *Candida auris* was investigated, revealing MIC values ranging between 4 and 128 μg mL^{-1} for caffeic acid and 1 to 64 μg mL^{-1} for caffeic acid phenethyl ester. Notably, the latter compound demonstrated fungicidal activity against one of the ten *C. auris* isolates assessed in this study (Rossato *et al.*, 2021). These findings underscore the potential of plant-derived polyphenols, particularly caffeic acid and its phenethyl ester, as effective agents with antimicrobial activity against *C. auris*.

Stropiglia *et al.* (2011) provided evidence that methanol extracts derived from species of the genus *Pterocaulon* exhibit activity against *Sporothrix schenckii*. *Pterocaulon polystachyum*, in particular, demonstrated promising results, with MIC values ranging between 156 and 312 μg mL^{-1} and a minimum fungicide concentration varying from 312 to 1250 μg mL^{-1}. Additionally, brown propolis produced by *Apis mellifera* was found to be active against *Sporothrix brasiliensis*, including strains resistant to itraconazole; with MIC values ranging from 0.19 to 1.56 mg mL^{-1} and minimum fungicidal concentration (CFM) ranging from 0.78 to 3.125 mg/mL (33). The essential oil extracted from *Rosmarinus officinalis* also exhibited efficacy against itraconazole-resistant *S. brasiliensis*. In a study involving Wistar rats with experimentally induced skin lesions, treatment with the essential oil led to lesion remission and delayed the spread of the fungus to other organs (Waller *et al.*, 2021). The outcomes of these studies underscore the potential of natural products as alternatives for developing innovative therapeutic approaches for infections caused by *Sporothrix spp*.

Propolis and geopropolis extracts have demonstrated effectiveness against *Pythium insidiosum*, as reported by Araújo, Bosco, Sforcin, (2016). The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (CFM) of propolis varied from 1.0 to 3.4 mg mL^{-1}. On the other hand, geopropolis exhibited a fungistatic action, with concentrations ranging between 3.4 and 7 mg mL^{-1}. An alternative therapeutic approach for pythiosis involves combining antimicrobials with natural products. Jesus *et al.* (2015) reported that the combination of thymol, carvacrol, tigecycline, minocycline, azithromycin, clarithromycin, and itraconazole proved to be beneficial against *P. insidiosum* isolates. This suggests that the synergistic effects of such combinations may offer enhanced therapeutic outcomes for pythiosis treatment.

Plantago major is rich in a diverse array of bioactive compounds, encompassing flavonoids, alkaloids, terpenoids, phenolic compounds, iridoid glycosides, fatty acids, polysaccharides, and vitamins. These compounds are distributed across various plant parts, including seeds, leaves, flowers, and roots. The diverse biological activities attributed to *P. major* are a result of these chemical components. The plant extracts of *P. major* have been shown to induce the generation of reactive oxygen species, leading to oxidative stress in bacteria. Additionally, *P. major* exhibits the capability to reduce bacterial counts in *Streptococcus pneumoniae* infection in mice. Furthermore, it demonstrates antineoplastic activity, showing effectiveness against human renal and breast adenocarcinoma as well as melanoma cells (Adom *et al.*, 2017). These findings highlight the multifaceted biological potential of *P. major*, making it a subject of interest for various therapeutic applications.

A potential therapeutic strategy involves combining natural products with commonly used antimicrobials. The leaf extract of *Berberis microphylla* shows a synergistic effect with cephalothin against *S. aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, and *Staphylococcus epidermidis* ATCC 12228. Similarly, the stem extract demonstrates synergistic action with cephalothin against *S. aureus* and *Bacillus subtilis* ATCC 6633. Additionally, the root extract exhibits synergistic activity with the antibiotic against *B. cereus* and *S. epidermidis* (Manosalva *et al.*, 2016).

Mentha pulegium essential oil exhibits a synergistic effect with imipenem against imipenem-resistant *Acinetobacter baumannii*. Additionally, a synergistic interaction was observed between the essential oil of *Artemisia herba alba* and imipenem against *A. baumannii* (Bekka-Hadji *et al.*, 2022). Similar synergistic interactions between natural products and antibacterial drugs are also noted against fungal species. The ethanolic extract of *Ocimum basilicum* var. Maria Bonita demonstrates

synergistic activity with amphotericin B against *Cryptococcus neoformans*. The minimum inhibitory fraction index ranged from 0.187 to 0.75 μg ml^{-1}, indicating a reduction in MIC values for the combinations. Specifically, in combination, the MIC of amphotericin B decreased from 1.56 to 0.099 μg mL^{-1}, and the extract MIC decreased from 625 to 78 μg ml^{-1} (Cardoso *et al.*, 2017).

Several natural products have exhibited synergistic interactions with tetracycline against *Paenibacillus alvei*. Propolis extracts, when combined with the antibiotic, demonstrated bactericidal activity within two hours. Similarly, the combination of nisin and tetracycline exhibited bactericidal action within the same timeframe. Additionally, synergistic bactericidal effects were observed after four hours in combinations involving eugenol and tetracycline, as well as cinnamaldehyde and tetracycline. In the case of the combination of melittin and tetracycline, a synergistic action was noted, although the interaction was found to be bacteriostatic, as reported by Sani *et al.* (2022).

The essential oil of *Eucalyptus camaldulensis* has been found to exhibit synergistic action with polymyxin B, causing a decrease in the MIC of the antibiotic, as reported by Knezevic *et al.* (2016). In a separate study conducted by Arslan, Aygan, Kocabas (2018), the methanolic and ethanolic extracts of *P. major* demonstrated synergistic action with amoxicillin, clavulanic acid, and ceftriaxone against *A. baumanii*. This synergistic effect was evidenced by inhibitory zones of up to 28 mm in disk diffusion tests, highlighting the potential of these extracts in enhancing the effectiveness of commonly used antibiotics against *A. baumannii*.

CONCLUSION

The methanolic extract of *Plantago major* leaves exhibits inhibitory action against a range of microorganisms that include: *Staphylococcus aureus* (*S. aureus*), methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Sporothrix schenckii* (*S. schenkii*), *Sporothrix globosa* (*S. globosa*), *Sporothrix brasiliensis* (*S. brasiliensis*), *Candida auris* (*C. auris*), and *Pythium insidiosum* (*P. insidiosum*). Moreover, the extract from *P. major* leaves displays synergistic activities when combined with cephalothin, oxacillin, imipenem, and amphotericin B against MRSA, *Klebsiella pneumoniae* carbapenemase (KPC), and *C. auris*, respectively. The compounds identified in the methanolic extract of *P. major* leaves possess various biological activities, including antimicrobial effects. These findings underscore the potential of *Plantago major* as a valuable resource for the development of novel therapeutic treatments against infectious diseases caused by the microorganisms investigated in this research.

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STATEMENTS AND DECLARATIONS

Conflict Of Interest

The authors declare that they have no conflicts of interest.

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Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material

No new data were generated or analysed in support of this research.

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