

Antibiofilm Activity of *Equisetum Arvense*, Phytochemical Aspects, and Interference on the Activity of Antimicrobial Drugs

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Received: 4.08.2024; Accepted: 15.07.2025; Published: 30.09.2025

Abstract: Bacterial resistance to antimicrobial drugs is a critical issue in public health. Natural products represent an interesting source of new treatments. *Equisetum arvense* leaf extract (EALE) is widely used as a diuretic and anti-inflammatory in traditional medicine. The antimicrobial potential of EALE, however, is poorly explored. We performed antimicrobial and antibiofilm assays against clinical isolates of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. EALE was combined with clinically relevant antimicrobial drugs (AD) to investigate drug interactions *in vitro*. We used qualitative phytochemical assays, UPLC and GC-MS analysis, and an antioxidant test to characterize EALE. EALE was active against all tested species as planktonic cells and biofilms, and displayed ~87% of antioxidant efficiency. EALE increased the activity of AD against the Gram-negative pathogens but decreased its activity against *S. aureus*. EALE presented flavonoids, tannins, alkaloids, and saponins. The presence of flavonoids was confirmed by UPLC, and other antimicrobial compounds were found by GC-MS. EALE was effective against the planktonic cells and biofilms of the bacterial isolates and increased the activity of AD against Gram-negative strains. Our data open doors for the development of antimicrobial formulations for further *in vivo* tests.

Keywords: antimicrobial; *Equisetum arvense*; bacterial biofilms.

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1. Introduction

Infectious diseases caused by pathogenic bacteria are responsible for relevant clinical and economic problems such as increased mortality, prolonged hospitalizations, and high costs of patient care [1]. The growing scenario of bacterial resistance, a major public health problem caused mostly by the inadequate prescription and use of antimicrobial drugs (AD), makes effective pharmacotherapy a challenging endeavor. The exposure of bacteria to AD, even in adequate treatments, causes genomic alterations that lead to resistance mechanisms. Such mechanisms include expression of efflux pumps and lytic enzymes, biochemical modification of drug targets, and biofilm formation, which allow the survival of subpopulations in the presence of AD, preventing the cure of the disease [2, 3].

Biofilms are complex networks of microorganisms that develop on extracellular polymeric substances (EPS), which embed and shield bacteria against factors such as the scarcity of water and nutrients, extremes of temperature and pH, ultraviolet light, and antimicrobial compounds [4]. EPS can comprise carbohydrates, proteins, lipids, and nucleic acids, and their concentration and variety are influenced by the availability of nutrients and diversity of microorganisms that are on the biofilm [4, 5]. Biofilm formation is an important mechanism of bacterial resistance to AD: it works as a physical and chemical shield to drug diffusion, avoiding proper action over the microorganisms [2-5]. It is quite difficult for the currently available AD to overcome the protective effect of biofilms on bacteria, as well as other resistance mechanisms [2, 3]. Thus, developing new effective therapeutic options is of paramount importance.

Natural products (NP) are relevant sources of bioactive molecules that are useful for several purposes, including the treatment of infectious diseases [6]. The traditional use of extracts of herbal species for respiratory, skin, and urinary infections has been largely investigated. *Equisetum arvense*, also known as horse tail, is traditionally explored in several countries for its diuretic and anti-inflammatory effects [7, 8]. Its antimicrobial potential, however, is poorly explored. Here, we provide evidence on the antimicrobial and antibiofilm potential of *E. arvense* leaf extract (EALE) against clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. We also investigated the antioxidant potential of EALE. To the best of our knowledge, this is the first evidence of the effectiveness of EALE against biofilms of these species.

2. Materials and Methods

2.1. Preparation of the extract.

EALE was purchased from Florien (São Paulo, Brazil) as a 70% hydroalcoholic extract. An authenticity report provided by the manufacturer confirmed the botanical identity of the plants used for preparing the product. After visual and documentary inspection, the extract was filtered in sterile Whatman paper #10, frozen at -80°C, and freeze-dried. The dried matter was then weighed and stored at 4°C until used. Aliquots of the extract were prepared at a concentration of 4 mg/mL using sterile 0.9% saline and were frozen until used. As required by Brazilian law, access to genetic resources was registered in the National System for the management of Genetic Heritage (SisGen Process A55026D).

2.2. Preparation of microorganisms.

We used clinical isolates of *S. aureus* (from hemodialysis catheter tips), *E. coli* (uropathogenic isolates), and *P. aeruginosa* (tracheal secretion), 10 strains of each species, all from the collection of Anhanguera College. The isolates were grown in sterile BHI broth (Difco) and had their identity confirmed using the VITEK 2 system (version R04.02, bioMérieux, Marcy-l'Étoile, France), following the manufacturer's instructions.

2.3. Antimicrobial activity assays.

We performed minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and minimal biofilm eradication concentration (MBEC) assays as

described in detail by our group [9]. Overnight-formed biofilms were prepared the day before the MBEC test in BHI broth.

The dried extract of EALE was prepared as a stock solution in sterile distilled water at 10 mg/mL and was diluted to reach concentrations ranging from 1024 to 8 µg/mL in 96-well sterile polystyrene plates. The isolates were cultured at 37°C in sterile BHI broth for 18 h before the tests. Resazurine staining (0.1 g/L) from blue to pink was used as described [9].

2.4. Qualitative phytochemical analyses of the extract.

We performed classical qualitative phytochemical analysis of the extract for flavonoids, alkaloids, saponins, and tannins [10]. The dried extract was resuspended in room temperature sterile distilled water to be used in these tests. Following, we analyzed the extract for volatile compounds by gas chromatography coupled to mass spectrometry (GC-MS) and for total polyphenolics by ultra-performance liquid chromatography (UPLC).

2.5. Analysis of volatile components by GC-MS.

GC-MS assay was performed as follows: EALE was mixed vigorously with HPLC-grade dichloromethane (1:1 v/v) using a vortex at maximum speed for 1 min, and the system was left at room temperature for phase separation. The dichloromethane supernatant was collected and injected into a QP 5050A GC-MS (Shimadzu, Japan) equipped with a PTE-5-Supelco column, using helium as carrier gas. The program used for analysis was described in detail by our group [11]. The results were recorded and processed using the software of the equipment and compared to its database. Detected substances were considered relevant if the average peak relative area (A%) was greater than 0.1% and similarity scores were equal to or greater than 80%.

2.6. Detection of flavonoids by UPLC.

UPLC was conducted as follows: the dried matter obtained by freeze-drying was used to prepare a solution at 5 mg/mL in HPLC-grade methanol, which was serially diluted in this same solvent to reach a concentration of 1 mg/mL. A 20 µL aliquot of this solution was then injected into an Acquity H-Class Bio UPLC/DAD (Waters, Germany) and analyzed at 254 nm. The program used for analysis was described in detail by our group [11].

2.7. Antioxidant assays.

The antioxidant potential of EALE was assessed using the β-carotene bleaching assay. The system was submitted to different exposure times, from 0 to 90 minutes, as previously described [12].

2.8. Interference with the activity of antimicrobial drugs.

The potential interaction between EALE and AD activity was evaluated in duplicate following the protocol established by Dias-Souza [13]. The antimicrobial agents employed in this test are in Table 1. Synergistic effects were defined when the average diameter of the inhibition zones produced by the test disks exceeded that of the control disks by at least 2 mm. Conversely, an antagonistic effect was inferred when the mean inhibition zone was reduced by 2 mm or more relative to the control. In cases where the inhibition zone diameters were greater

or smaller than the control, but lacked statistical significance, the outcome was interpreted as a trend toward synergism or antagonism, respectively.

Table 1. Antimicrobial disks are used in interference assays according to each species.

<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Gentamycin 10 µg/disk	Ampicillin 10 µg/disk	Ampicillin 10 µg/disk
Ciprofloxacin 5 µg/disk	Cefalexin 30 µg/disk	Cefalexin 30 µg/disk
Chloramphenicol 30 µg/disk	Meropenem 10 µg/disk	Nitrofurantoin 300 µg/disk

2.9. Statistics.

Normality of data was assessed through the Shapiro-Wilk test, and homoscedasticity was assessed through Bartlett's test. Mean diameters of the inhibition zones with and without the addition of EALE were analyzed using two-way ANOVA followed by a Bonferroni post hoc test. The means of the antioxidant activity photometric readings were analyzed using two-way ANOVA, followed by the Tukey test. The significance level was set at $p < 0.05$ and $p < 0.01$ for a highly significant level. All analyses were carried out in Bioestat 5.0 for Windows.

3. Results and Discussion

3.1. Antimicrobial activity of EALE.

MIC values of EALE were the same for all species. *S. aureus* strains were more sensitive to the extract in comparison to the Gram-negative isolates (Table 2), especially considering the results of the extracts in biofilms.

Table 2. Antimicrobial activity of EALE against microbial pathogens.

Parameter	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
MIC	8	8	8
MBC	64	64	32
MBEC	512	512	128

Data is presented as concentrations in µg/mL for all strains of each species. MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration; MBEC: minimal biofilm eradication concentration.

3.2. Phytochemical aspects of EALE.

Qualitative assays were positive for flavonoids, alkaloids, saponins, and tannins. UPLC-DAD analysis further confirmed the presence of polyphenolics in EALE (Figure 1).

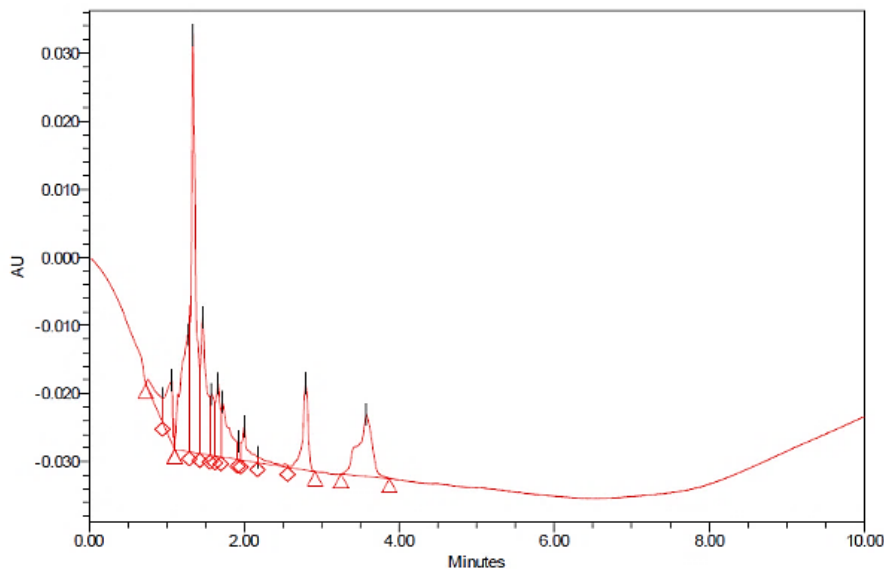


Figure 1. UPLC analysis of EALE. AU: arbitrary units.

Retention times of the prominent peaks are suggestive of apigenin (~ 1 min), quercetin (between 1 and 1.5 min), kaempferol (~ 1.5 min), protocatechuic acid (~ 2 min), vanillic acid (~ 2.5 min), and caffeic acid (~ 3.5 min) [14-18].

GC-MS analysis of volatile compounds present at EALE indicated the presence of several substances, and nine of them met the criteria of A% superior to 0.1% and similarity score equal to or superior to 80%. The results are shown in Table 3.

Table 3. Volatile compounds found in the extract using GC-MS

RT	A%	Name	Similarity score (%)
2.055	6.35	L-5-Propylthiomethylhydantoin	86
2.271	11.24	Hydrazine, 1,2-dimethyl-	82
2.331	4.62	Propanoic acid, ethyl ester	86
2.525	39.92	Pentanoic acid, 3-methyl-4-oxo-	81
2.693	1.18	Isobutyl acetate	96
3.02	0.86	2-Ethoxytetrahydrofuran	96
3.45	0.49	1,2-Diacetylhydrazine	87
3.546	0.24	1,2-Propanediol, 2-acetate	97
5.942	11.91	1,4-Butanediol, diacetate	95

RT: retention time (in minutes); A%: average peak relative area percentile.

3.3. Antioxidant potentials of EALE.

EALE displayed 86.77% protection (Figure 2) and was superior to the control ($p < 0.05$).

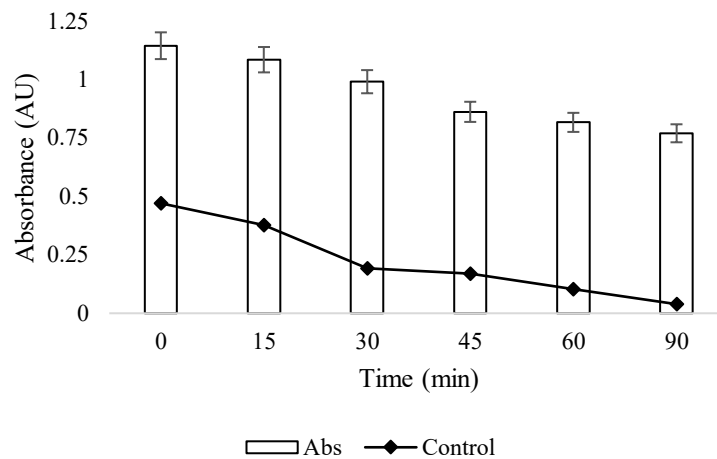


Figure 2. Results of the antioxidant test performed by the β -carotene bleaching method. The black line represents the control, bars represent EALE (with standard deviation).

3.4. Interference of EALE on the activity of antimicrobial drugs.

The combination of EALE to gentamycin, ciprofloxacin, and chloramphenicol against *S. aureus* resulted in significant antagonism ($p < 0.05$). However, the combination of EALE to ampicillin, cefalexin, meropenem, and nitrofurantoin against the Gram-negative pathogens resulted mostly in significant synergism ($p < 0.05$). Significant antagonism was detected in some of the tested combinations against *E. coli* (Table 4).

This study describes the antimicrobial activity of *E. arvensis* extract against planktonic cells and biofilms of pathogenic bacterial species of clinical relevance. Such studies are scarce for this plant, which is traditionally used for kidney diseases.

Table 4. Results of the interference test of EALE on antimicrobial drugs.

Strain	GEN	GEN <u>+E</u>	CIP	CIP <u>+E</u>	CHLO	CHLO <u>+E</u>	Strain	AMP	AMP <u>+E</u>	CFE	CFE <u>+E</u>	NITRO	NITRO <u>+E</u>	Strain	AMP	AMP <u>+E</u>	CFE	CFE <u>+E</u>	MERO	MERO <u>+E</u>
S1	13	10Δ	24	16Δ	25	18Δ	E1	13	17♦	16	15	13	9Δ	P1	0	10♦	0	8♦	22	26♦
S2	17	11Δ	22	17Δ	26	18Δ	E2	11	15♦	10	18♦	7	5Δ	P2	11	14♦	0	10♦	21	28♦
S3	14	11Δ	23	17Δ	26	19Δ	E3	4	16♦	12	18♦	11	11	P3	9	11♦	0	8♦	20	25♦
S4	14	10Δ	22	15Δ	23	16Δ	E4	10	15♦	10	10	14	11Δ	P4	0	12♦	0	11♦	23	24
S5	15	12Δ	23	18Δ	24	15Δ	E5	10	17♦	17	18	10	8Δ	P5	10	14♦	0	9♦	20	27♦
S6	22	14Δ	24	15Δ	25	16Δ	E6	12	16♦	17	19♦	9	11♦	P6	0	10♦	0	18♦	22	24♦
S7	18	10Δ	26	16Δ	25	17Δ	E7	10	13♦	21	22	13	13	P7	11	14♦	0	9♦	23	32♦
S8	18	14Δ	21	15Δ	20	18Δ	E8	11	17♦	22	25♦	13	14	P8	0	17♦	0	15♦	23	27♦
S9	26	14Δ	23	15Δ	23	19Δ	E9	12	24♦	9	14♦	13	14	P9	0	8♦	0	14♦	20	23♦
S10	19	17Δ	22	16Δ	22	18Δ	E10	8	17♦	17	18	8	12♦	P10	15	17♦	0	16♦	22	26♦

Data is expressed as the size of inhibition zones in mm. S1-10: *S. aureus* strains from #1 to #10, E1-10: *E. coli* strains from #1 to #10, P1-10: *P. aeruginosa* strains from #1 to #10, GEN: gentamycin, CIP: ciprofloxacin, CHLO: chloramphenicol, AMP: ampicillin, CFE: cefalexin, NITRO: nitrofurantoin, MERO: meropenem, +E – addition of EALE at MBC to the antimicrobial disks. Δ: statistically significant antagonism, ♦: statistically significant synergism. Values in +E columns without symbols did not meet the criteria for synergism or antagonism (i.e., values > or < 2 mm compared to EALE-free disks) and are considered indifferent regarding the addition of EALE.

The qualitative tests indicated the presence of alkaloids, saponins, tannins, and flavonoids in EALE, and the presence of flavonoids was confirmed by UPLC analysis (Figure 1). Flavonoids and tannins are markedly relevant phytochemicals concerning the antioxidant potential of EALE. They are also associated with the antimicrobial potential of the extract. Possible related mechanisms include inhibition of cell wall synthesis, disruption of the cell membrane, and inhibition of enzymes involved in biosynthetic pathways [19]. Using GC-MS, we detected L-5-propylthiomethylhydantoin, pentanoic and propanoic acid, and other important antimicrobial compounds that are not chemically related to the mentioned phytochemicals. Endophytic and soil microorganisms can produce organic acids in plants, such as propanoic and pentanoic acids [20, 21]. Interestingly, we detected these acids using GC-MS in guava and passion fruit juice extracts, which presented antimicrobial activity [9].

The MIC of EALE against Gram-positive and Gram-negative strains was the same, although the MBC for *S. aureus* was half the value for Gram-negative strains, as it was for MBEC values. To the best of our knowledge, this is the first description of such effectiveness for EALE, especially the antibiofilm effect. This is particularly relevant considering that MBEC values can be more than 1000 times superior to MIC values.

From the few existing studies on the antimicrobial activity of *E. arvensis*, it seems that extracts prepared with this plant may not be active for Gram-negative species, as it is against Gram-positive species, as observed for the MBC values (Table 2), except for the essential oil, which was active against Gram-positive and Gram-negative strains [22]. A 70% hydroethanolic extract of *E. arvensis* stems was active against ATCC strains of *S. aureus* (25 mg/mL), *Streptococcus pneumoniae*, and group A *Streptococcus pyogenes* (12.5 mg/mL). In the disk diffusion assay, the extract was active at 100 mg/mL. No antimicrobial activity was detected against Gram-negative pathogens [23]. Similarly, *E. arvensis* extract prepared with dried parts of the plant was poorly active against uropathogenic isolates of *E. coli* [24].

The stem extract of *E. arvensis* was active against Gram-positive oral pathogens [25]. An *E. arvensis* extract was also described to be active against maize *Aspergillus sp.* and *Fusarium sp.* phytopathogenic strains [26]. These studies described *E. arvensis* extracts being active at mg/mL scale, except for a study that demonstrated the antimicrobial activity of *E. arvensis* at µg/mL scale against an ATCC *S. aureus* strain [27]. More recently, silver nanoparticles prepared by green synthesis from *E. arvensis* extracts were effective in reducing the biofilm formation potential of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *E. coli* isolates [28]. We believe that a reasonable explanation for the remarkable differences between the mentioned studies and the present investigation is that, here, we used an extract prepared with fresh leaves, whereas the previous studies explored dried parts of the plant, mostly stems, and not leaves. Thus, phytochemical variations with an influence on pharmacological properties are expected. The efficiency of extraction procedures and susceptibility of the isolates also help to explain the differences between this study and the previously published data.

Drug-herbal interactions are one of the main interests of our group, and, to the best of our knowledge, interactions involving *E. arvensis* are described here for the first time. We found that combining EALE with antimicrobial drugs may produce both synergic and antagonistic effects (Table 4): synergism was more frequent in Gram-negative isolates, and antagonism was detected in *S. aureus* isolates. It is possible that phytochemicals present in EALE interacted with molecular targets in Gram-negative strains in a way that the antimicrobial drugs could have more effective interactions with their molecular targets [19]. Phytochemicals present at

EALE may have impaired the activity of the drugs tested against *S. aureus* by direct interaction with them [9, 19]. In this context, a study suggested that *E. arvensis* may impair the antiviral activity of drugs used in the treatment of AIDS [29]. Such interference effects are poorly predictable and require experimental evidence to be confirmed [30-32].

The antibiofilm effect of *E. arvensis* is poorly described. Here, the MBEC was 64 times higher than MIC for the Gram-negative isolates, whereas the MBEC for *S. aureus* isolates was 16 times higher. Biofilms are present in nearly all infectious diseases caused by bacterial strains, and as previously exposed, are a relevant mechanism of bacterial resistance to AD. A study described that a 0.1% ethyl acetate extract produced with *E. arvensis* shoots can also decrease biofilm biomass [33], but the bacterial viability of the remaining biofilm was not assessed.

4. Conclusions

EALE was effective against planktonic (free) cells and biofilms of Gram-positive and Gram-negative bacterial pathogenic species, possibly due to the combined effect of flavonoids, tannins, and organic acids. Furthermore, it presented a relevant antioxidant effect. The extract will be explored in further studies related to the development of formulations for the treatment of infectious diseases.

Author Contributions

Conceptualization, M.V.D.S.; methodology, M.V.D.S., R.M.D.S.; formal analysis, R.M.D.S., G.P.F., M.V.D.S.; investigation, W.S.L., G.P.F.; resources, R.M.D.S.; data curation, M.V.D.S., R.M.D.S.; writing—original draft preparation, W.S.L.; writing—review and editing, M.V.D.S., S.C.G.; visualization, M.V.D.S.; supervision, M.V.D.S.; project administration, M.V.D.S., R.M.D.S. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

No new data were created or analyzed in this study. Data sharing is not applicable.

Funding

This research received no external funding.

Acknowledgments

We are thankful to Dr Isabela Ceravolo (René Rachou Institute) for kindly providing suggestions on this paper.

Conflicts of Interest

The authors declare no conflict of interest.

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