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Antimicrobial and Antibiofilm Potentials of *Rhynchostegium riparioides* (Hedw.) Cardot Extracts

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Abstract

In this study, antimicrobial and antibiofilm activities of extracts obtained from the moss *Rhynchostegium riparioides* using ethanol, methanol, n-hexane, and water solvents were evaluated. Standard strains, food isolates, and clinical isolates, including multidrug-resistant strains, were utilized in the tests. The efficacy of the extracts was determined using disk diffusion and minimum inhibitory concentration (MIC) methods, and their phytochemical profiles were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The n-hexane extract showed the highest antimicrobial activity, demonstrating strong effects even at the lowest tested concentrations. This extract was predominantly composed (79.29%) of tris(2,4-di-tert-butylphenyl) phosphate. The methanol extract exhibited broad-spectrum activity, notably effective against *Enterococcus faecalis* and *Escherichia coli*, and major compounds identified included neophytadiene (21.55%), palmitic acid (18.65%), and linolenic acid (10.45%). In the antibiofilm assays, a strong inhibition of up to 95% was observed, particularly against *Listeria innocua*. These findings highlight the potential of the n-hexane extract for development as a natural antibiofilm agent. The findings underscore the potential for developing the n-hexane extracts as natural antibiofilm agents.

Keywords: Biofilm inhibation, antibacterial effect, Gas Chromatography-Mass Spectrometry (GC-MS).

Rhyncostegium riparioides (Hedw.) Cardot Ekstraktlarının Antimikrobiyal ve Antibiyofilm Potansiyelleri

Öz

Bu çalışmada, *Rhynchostegium riparioides* karayosunundan etanol, metanol, n-hekzan ve su çözücüleri ile elde edilen ekstraktların antimikrobiyal ve antibiyofilm aktiviteleri değerlendirilmiştir. Testlerde standart suşlar, gıda izolatı ve çoklu ilaç direncine sahip suşların da bulunduğu klinik izolatlar kullanılmıştır. Ekstraktların etkinliği disk difüzyon ve minimum inhibitör konsantrasyon (MİK) yöntemleriyle belirlenmiş, fitokimyasal profilleri ise Gaz Kromatografisi-Kütle Spektrometrisi (GC-MS) ile analiz edilmiştir. En yüksek antimikrobiyal aktiviteyi, test edilen en düşük konsantrasyonlarda bile güçlü etki gösteren ve içeriğinin %79,29'unu tris(2,4-di-tert-bütilfenil) fosfat bileşiğinin oluşturduğu n-hekzan ekstraktı sergilemiştir. Metanol ekstraktı geniş spektrumlu bir etki göstererek özellikle *Enterococcus faecalis* ve *Escherichia coli* üzerinde etkili bulunmuş, ekstraktta neofitadien (%21,55), palmitik asit (%18,65) ve linolenik asit (%10,45) gibi majör bileşikler tanımlanmıştır. Antibiofilm testlerinde özellikle *Listeria innocua* üzerinde %95'e varan güçlü bir inhibisyon gözlenmiştir. Bulgular, özellikle n-hekzan ekstraktının doğal antibiyofilm ajanı olarak geliştirilmesi açısından önem taşımaktadır.

Anahtar kelimeler: Biyofilm inhibisyonu, antibakteriyel etki, Gaz Kromatografisi-Kütle Spektrometresi (GC-MS).

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1. Introduction

The World Health Organization (WHO) and numerous scientific institutions predict that antimicrobial resistance will become one of the greatest threats to the global healthcare system in the near future (Abebe, 2020). Currently, approximately 700.000 people die annually due to antimicrobial resistance (O'Neill, 2016). Furthermore, if the rate of resistance acquisition continues at this pace, it is estimated that by 2050, 10 million people will die each year due to antimicrobial resistance (TEPAV, 2017). Multidrug-resistant (MDR) bacteria, due to their resistance to existing antibiotics, particularly act as opportunistic pathogens in hospital settings, causing increasing mortality rates and posing a serious threat to public health (Aslam et al., 2021). These examples clearly demonstrate the significant risk posed by pathogens resistant to current antibiotics to public health, explaining why antimicrobial resistance is considered one of the most critical global health challenges of the 21st century.

Biofilms are another resistance mechanism, which are microbial communities where bacteria organize themselves within self-produced extracellular polymeric matrices, adhering to surfaces (Samastı, 2021). This structure protects bacteria from antimicrobial agents and complicates infection treatment (Temel & Eraç, 2018). Detecting pathogenic microorganisms that form biofilms and determining their antibiotic resistance is crucial for disease management (Kartal, 2021). Current antibiotics used for treating biofilm-associated infections are often insufficient, highlighting the need to develop new antibiofilm agents.

As an alternative to bacteria resistant to existing antibiotics, exploring natural sources whose chemical compositions have not yet been fully elucidated offers significant advantages for discovering new antimicrobial agents. Foremost among these advantages is the rich phytochemical content developed over evolutionary processes. These natural compounds may contain unique functional groups and stereoisomeric configurations that are difficult to synthesize in laboratory settings (Newman & Cragg, 2016). Moreover, the constant interaction of these organisms with bacterial pathogens in their natural environments allows them to produce biological compounds with high specificity and efficacy against certain biological targets (Cowan, 1999). The synergistic effects of complex compound mixtures in natural extracts make it more difficult for pathogens to develop resistance compared to single compounds (Rios & Recio, 2005). Additionally, natural compounds tend to exhibit lower toxicity compared to synthetic ones, making them more attractive as potential therapeutic agents (Fabricant & Farnsworth, 2001).

Bryophytes, a group of non-vascular plants, although understudied, stand out due to their richness in secondary metabolites and bioactive compounds with therapeutic potential (Aslanbaba et al., 2017). Considering bryophytes' pioneering role in the transition and adaptation of plants from aquatic to terrestrial environments, their potential gains even greater significance (Wickett et al., 2014; Ursavaş and Ediş, 2024). It should also be noted that terrestrial ecosystems encountered by plants were already dominated by microorganisms; bryophytes, which emerged approximately 470–500 million years ago, have survived through evolutionary competition with microorganisms by developing compounds that allowed their persistence (Kenrick & Crane, 1997). This millions-of-years-long competition is a key factor contributing to the unique and rich chemical composition of bryophytes, making them attractive as therapeutic agents.

Mosses have been used ethnopharmacologically for many years across different regions such as India, China, and the Americas. This traditional use is based on the preference of local populations for mosses due to their wound healing, anti-inflammatory, and infection-preventing properties. Modern studies have reported that mosses exhibit antimicrobial, antiviral, antifungal, and neuroprotective effects (Motti et al., 2023; Şahin & Aslan, 2023). Reliable data derived from traditional use indicate a favorable toxicological profile of the plant and strengthen its potential for the development of new therapeutic agents in modern pharmaceutical research.

The plant group known as bryophytes is classified into mosses (Bryophyta), liverworts (Marchantiophyta), and hornworts (Anthocerotophyta) (Benek, 2024)[1]. The subject of the study, *Rhynchostegium riparioides* (Hedw.) Cardot, is a robust moss species exhibiting colors ranging from bright green to brownish tones, sometimes with darkish lower parts. Its shoots, which can grow up to 15 cm, are pendulous or prostrate and often leafless at the lower sections. It is typically found along stream on moist stone surfaces, acquiring a metallic sheen when dry (Smith, 2006)[1].

The species *R. riparioides* is noted for its ecological resilience. Its size and coverage vary depending on the quality and purity of the water it

inhabits; it tends to grow larger and spread more extensively in clean, cold waters (Haziri, 2018). Due to these characteristics, *R. riparioides* can be used as a biological indicator to assess the cleanliness of aquatic ecosystems (Smith, 2006; Rimac et al., 2022). Studies suggest that extracts of *R. riparioides* may exhibit antimicrobial effects against various pathogenic microorganisms (Basile et al., 1998). However, its efficacy against drug-resistant bacteria and its potential to prevent biofilm-related infections have yet to be extensively explored.

This study aims to investigate the antimicrobial and antibiofilm activities of *R. riparioides* extracts prepared using different solvents against various pathogens. The findings will contribute to identifying the antimicrobial and antibiofilm components of this moss and highlight its potential applications in pharmaceutical and medical fields. Additionally, the biochemical compositions of *R. riparioides* extracts prepared with different solvents (ethanol, methanol, and n-hexane) were elucidated through gas chromatography-mass spectrometry (GC-MS) analyses. Comparative analysis of their chemical compositions and biological activities is important for providing a solid scientific foundation for future studies.

2. Material and Methods

2.1. Moss samples

The moss *R. riparioides* was collected from Kardeşler/Zonguldak (41° 25' 07.2''N & 31° 43' 05.1''E) and identified by Ayşe Dilek Unan. The moss sample was placed in a sample bag and transported to the laboratory. Following natural air-drying under ambient conditions, tit was stored at the Fauna and Flora Research and Application Center (FAMER) of Dokuz Eylül University, located in Buca, Izmir, Turkey, under the herbarium code "FFDEU-MEB2," where it was preserved until experimental procedures were initiated.

2.2. Extraction procedure

Extraction was performed according to the method described by Canlı et al. (2015). To avoid potential toxic effects of the solvents, extracts intended for Minimum Inhibitory Concentration (MIC) and antibiofilm activity tests were freed from these solvents; the resulting dry residues were dissolved in 1% dimethyl sulfoxide (DMSO) to obtain water-based extracts. These extracts were prepared in volumes of 15 mL, with the contained substance amounts as follows: 0.661 g for the ethanol extract, 0.193 g for the methanol extract, and 0.016 g for the n-hexane extract. The aqueous extract was directly prepared in 15 mL of distilled water with a substance amount of 1.266 g.

For use in disk diffusion tests and GC-MS (Gas Chromatography-Mass Spectrometry) analyses, extracts were prepared as formulations in 25 mL solvent volumes with substance amounts of 0.452 g for ethanol, 0.682 g for methanol, and 0.062 g for n-hexane. The contents of the extracts prepared for different tests are presented in the table below.

Table 1. Extract informations.

Test type	Solvent	Amount of substance (mg/ml)
MIC & Antibiofilm tests	Ethanol	44.066
	Methanol	12.866
	n-Hexane	1.066
	Water	84.4
Disc difusion & GC-MS analysis	Ethanol	18.08
	Methanol	27.28
	n-Hexane	2.48

2.3. Microorganisms

In this study, a total of 27 strains were analyzed, comprising 7 Food Isolates (FI), 12 Standard Isolates (ST)—including one yeast strain—and 13 Clinical Isolates (CI), of which two were yeast strains. These microorganisms were obtained from the microbiology laboratory of the Department of Biology, Faculty of Science, Dokuz Eylül University (Table 3-5).

2.4. Inoculum preparation

Bacterial strains were pre-cultured at 37°C for 24 hours, while the yeast strain was incubated at 28°C for 48 hours. To standardize microbial density, all cultures were adjusted to a turbidity of 0.5 McFarland using sterile saline (0.9% NaCl). This process resulted in approximate concentrations of 10^8 CFU/mL for bacterial suspensions and 10^7 CFU/mL for *Candida albicans*. The standardized inocula were prepared for use in all experimental studies (Gül et al., 2025).

2.5. Disc diffusion method

The antimicrobial activity of *R. riparioides* extracts was evaluated using the disk diffusion method, as described by Andrews (2003). Each solvent was tested at an equivalent volume (150 µL) and loaded onto 6 mm antimicrobial susceptibility test discs. Rather than applying the entire volume at once, these volumes were incrementally loaded in 10 µL steps. To allow for the evaporation of residual solvents, the disks were left to dry overnight, yielding disks containing 2,71 mg of substance for the ethanol extract, 4,09

mg for the methanol extract, and 0,37 mg for the n-hexane extract. Subsequently, pre-prepared microorganisms, suspended in sterile saline solution, were inoculated onto the surface of Petri dishes to ensure uniform coverage. Following inoculation, the extract-loaded disks were placed onto the agar surface, and the plates were incubated. The diameters of the resulting inhibition zones were measured in millimeters (mm) using a caliper and recorded.

Sterile blank disks and the extraction solvent (ethanol) served as negative controls, while Gentamicin and Tobramycin antibiotic disks were utilized as positive controls. All tests were performed in triplicate, and the results are presented as the mean with standard errors (Baldas & Altuner, 2018).

2.6. Minimum inhibitory concentration test

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an extract that inhibits visible bacterial growth. For MIC determination, the serial dilution method recommended by Benek et al. (2024) was employed, yielding the following concentration ranges: 14,68 – 0,11 mg/ml for ethanol, 4,28 – 0,03 mg/ml for methanol, 0,35 – 0,01 mg/ml for n-hexane, and 28,13 – 0.21 mg/ml for water. Wells containing no extract served as positive controls, while wells without bacteria were used as negative controls. All assays were conducted in triplicate.

2.7. Antibiofilm activity

In this study, the antibiofilm assays were conducted by adapting the method described in Tunca-Pinarlı (2023). The experimental procedure consisted of two main stages: determination of optimal biofilm formation conditions and evaluation of antibiofilm activity. In the first stage, incubation times and culture media conditions were optimized to achieve maximal biofilm formation by the microorganisms. In the second stage, under these optimized conditions, the biofilm inhibition potential of the extracts was assessed.

2.8. Gas chromatography-mass spectrometry (GC-MC) analysis

GC-MS Analysies were carried out with adaptations from the method described by Bozkurt et al. (2024). The experiments utilized an Agilent GC 8890 coupled with an Agilent GC/MSD 5977B system (Agilent Technologies Inc., Santa Clara, CA, USA), featuring an HP5-MS capillary column measuring 30 m in length, 0.25 mm in internal diameter, and 0.25 µm film thickness. The analytical parameters were set as follows: injector temperature maintained at 350°C, helium served as the carrier gas with a flow rate of 1 ml/min. The injection was performed in split mode with a split ratio of 10:1, using an injection volume of 1 µl of the ethanol extract. The oven temperature program started at 40°C and was ramped up to 350°C at 4°C per minute, followed by a 10-minute hold at 350°C. For mass spectrometry, the transfer line and interface temperatures were both maintained at 280°C, while the ion source temperature was set to 230°C. Identification of compounds was based on matching retention times with the Wiley-NIST MS library database.

2.9. Statistics

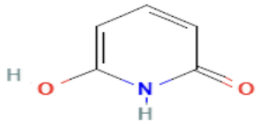
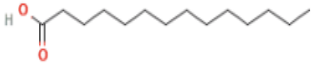
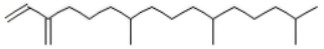
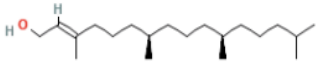
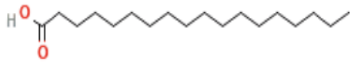
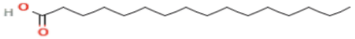
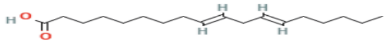
The results obtained from three independent repetitions for each activity are expressed as mean ± standard deviation (SD). Following statistical analysis of the data, values were determined using Four-Parameter Logistic Regression with a 95% confidence interval. Data were analyzed using One-Way ANOVA (Analysis of Variance) and Pearson correlation tests in R Studio (Version: 2025.05.01+513). The level of statistical significance was set at $p \leq 0.05$ (Dyakov et al., 2011).

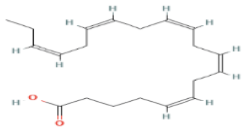
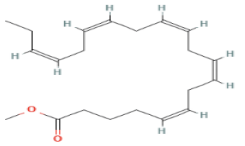
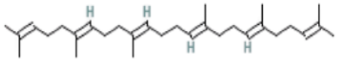
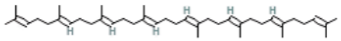
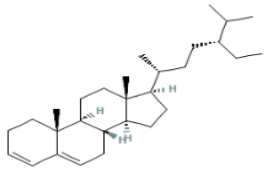
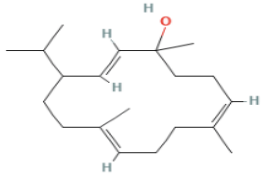
3. Results

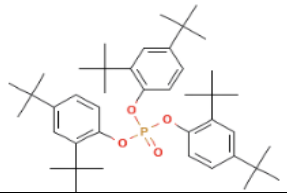
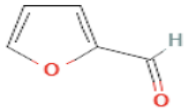
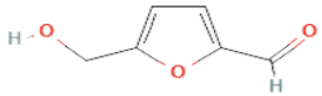
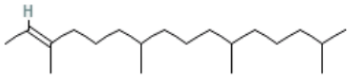
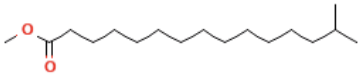
3.1. Biochemical compounds in extracts

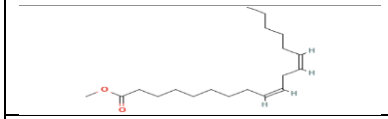
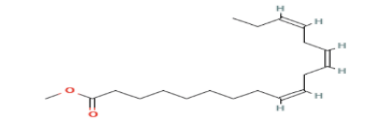
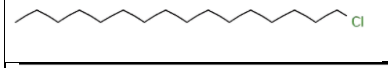
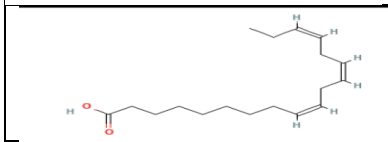
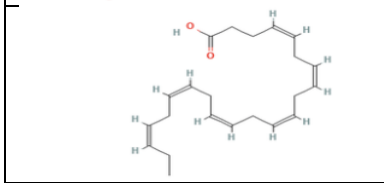
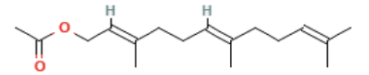
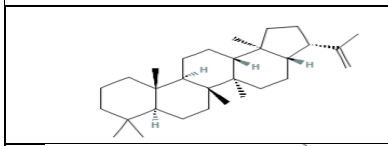
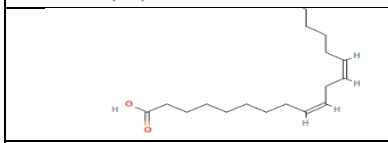
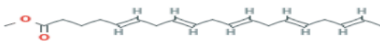
The area percentages of the compounds identified by GC-MS analysis are presented in Table 2.

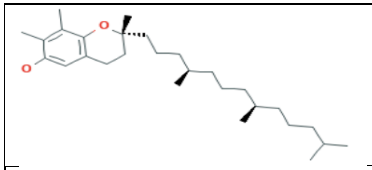
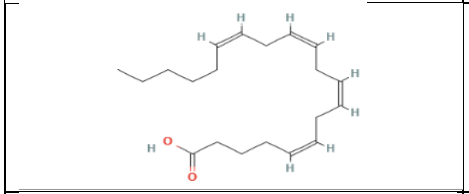
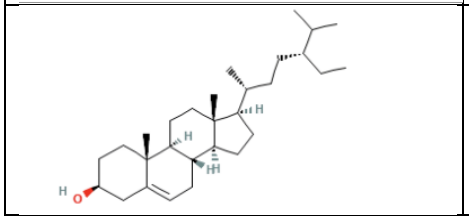
Table 2. Biochemical profiling of R. riparioides extracts.

Chemical Structure	Compound name	RT	Formula	MW (g/mol)	RR Ethanol extract	RR Methanol extract	RR n-Hexane extract	Known Activity
	2,6-Dihydroxypyridine	13.658	$C_5H_5NO_2$	111.1	1.30	-	-	-
	Tetradecanoic acid	31.101	$C_{14}H_{28}O_2$	228.37	0.81	0.62	-	Anti-inflammatory activity (Najda et al., 2021)
	Neophytadiene	32.398	$C_{20}H_{38}$	278.5	41.16	21.55	-	Antibacterial and Antifungal activity (Ceyhan-Güvensen & Keskin, 2016)
	Phytol	32.242	$C_{20}H_{40}O$	296.5	2.89	1.56	-	Antioxidant and Antimicrobial activity (Islam et al., 2018)
	Stearic acid	40.450	$C_{18}H_{36}O_2$	284.5	6.07	0.88	0.87	Antifungal and Anti-inflammatory activity (Guimarães & Venâncio, 2022; Miao et al., 2015)
	Palmitic acid	36.003	$C_{16}H_{32}O_2$	256.42	6.97	18.65	0.73	Anticancer activity (Yu et al., 2023)
	Linoelaidic acid	40.768	$C_{18}H_{32}O_2$	280.4	1.55	-	-	Carcinogenesis and Proliferative activity (Ip et al., 1994)

	Eicosapentaenoic acid	45.238	$C_{20}H_{30}O_2$	302.5	2.86	-	-	Antimicrobial activity (Chanda et al., 2018)
	Icosapent methyl	44.261	$C_{21}H_{32}O_2$	316.5	-	1.09	-	-
	Squalene	59.590	$C_{30}H_{50}$	410.7	1.39	1.07	-	Antioxidant, anti-inflammatory, and anticancer activity (Lou-Bonafonte et al., 2018)
	Lycopersene	62.543	$C_{40}H_{66}$	547	2.24	-	-	Antioxidant, Anti-inflammatory, and Anticancer activity (Lou-Bonafonte et al., 2018)
	Stigmasta-3,5-diene	66.218	$C_{29}H_{48}$	396.7	1.63	-	-	Antistaphylococcal, Antihypertensive, Antiulcer, and Antidiabetic activity (Tabassum et al., 2022)
	Thunbergol	74.875	$C_{20}H_{34}O$	290.5	1.02	-	-	Antioxidant and Anti-inflammatory activity (Shah et al., 2023)
	Nonacosane	45.207	$C_{29}H_{60}$	408.8	-	-	0.66	-
	Docosane	47.891	$C_{22}H_{46}$	310.6	-	-	1.46	Antioxidant activity (Salem et al., 2016)

	Pentacosane	50.689	$C_{25}H_{52}$	352.7	-	-	2.48	-
	Hexacosane	53.591	$C_{26}H_{54}$	366.7	-	-	2.52	Antioxidant activity (Marrufo et al., 2013)
	Heneicosane	56.580	$C_{21}H_{44}$	296.6	-	-	2.16	Antibacterial activity (Wijayanti & Dewi, 2022)
	Octacosane	59.611	$C_{28}H_{58}$	394.8	-	-	4.43	Antimicrobial activity (Khatua et al., 2016)
	Heptacosane	66.056	$C_{27}H_{56}$	380.7	-	-	0.75	Antioxidant activity (Akpuaka et al., 2013)
	tris(2,4-di-tert-butylphenyl) phosphate	73.546	$C_{42}H_{63}O_4P$	662.9	-	-	79.29	Anti-enterococcal, Antioxidant activities (AlRaddadi et al., 2024)
	Furfural	6.493	$C_5H_4O_2$	96.08	-	0.71	-	Antimicrobial activity (Chai et al., 2013)
	5-Hydroxymethyl-furfural	6.493	$C_6H_6O_3$	126.11	-	9.70	-	Anti-inflammatory activity (Kong et al., 2019)
	Phytene-2	32.672	$C_{20}H_{40}$	280.5	-	1.51	-	-
	Methyl Palmitate	34.901	$C_{17}H_{34}O_2$	270.5	-	1.99	-	Anti-inflammatory activity (El-Demerdash, 2011)

	Methyl Linoleate	39.546	$C_{19}H_{34}O_2$	294.5	-	0.74	-	Antifungal activity (Pinto et al,2017)
	Methyl Linolenate	39.763	$C_{19}H_{32}O_2$	292.5	-	1.13	-	-
	1-Chlorohexadecane	39.987	$C_{16}H_{33}Cl$	260.899	-	0.82	-	-
	Linolenic acid	41.728	$C_{18}H_{30}O_2$	278.4	4.60	10.45	-	Anti-inflammatory and Antibacterial activity (Yan et al.,2024)
	Docosahexaenoic acid	46.568	$C_{22}H_{32}O_2$	328.5	-	4.38	-	Anticancer activity (Jiao et al., 2017)
	Farnesyl acetate	63.596	$C_{17}H_{28}O_2$	264.4	-	1.20	-	Antioxidant, Antibacterial Anticancer activity (Tian et al., 2022)
	Diploptene	72.803	$C_{30}H_5O$	410.7	4.69	2.81	-	-
	Linoleic acid	40.953	$C_{18}H_{32}O_2$	280.4	3.26	-	-	-
	Methyl eicosa-5,8,11,14,17-pentaenoate	46.123	$C_{21}H_{32}O_2$	316.5	2.89	2.21	-	Antifungal activity (Pinto et al., 2017)

	Vitamin E	68.229	$C_{29}H_{50}O_2$	430.7	1.59	-	-	Antioxidant and Anti-inflammatory activities (Aboubakr et al., 2023)
	Arachidonic acid	44.066	$C_{20}H_{32}O_2$	304.5	-	1.20	-	Anti-inflammatory activities (Das, 2018)
	Beta sitosterol	72.083	$C_{29}H_{50}O$	414.7	-	5.71	-	Anti-inflammatory activities (Mattioli et al., 2013)

3.2. Disk diffusion test results

All extracts were tested at a volume of 150 microliters, with three replicates performed. Strains for which no antimicrobial activity was observed in the disk diffusion test are indicated with a '-' symbol.

Table 3. The antimicrobial activity of *R. riparioides* against standard microorganisms (Inhibition zones in mm).

Standard Isolated Microorganisms	Ethanol	Methanol	n-Hexane	Gentamicin (10 µg)	Tobramycin (10 µg)
Bacillus subtilis DSMZ 1971	-	-	-	30	26
Candida albicans DSMZ 1386	-	-	-	12	13
Enterobacter aerogenes ATCC 13048	-	-	-	24	18
Enterococcus faecalis ATCC 29212	7.00 ± 0.00	7.00 ± 0.00	-	12	8
Escherichia coli ATCC 25922	-	-	-	22	20
Listeria monocytogenes ATCC 7644	-	-	-	28	24
Pseudomonas aeruginosa DSMZ 5071	-	-	-	15	22
Pseudomonas fluorescens P1	-	-	-	13	12
Salmonella enteritidis ATCC 13076	-	-	-	21	-
Salmonella typhimurium SL 1344	-	-	-	24	15
Staphylococcus aureus ATCC 25923	-	-	-	21	14
Staphylococcus epidermidis DSMZ 20044	-	-	7.00 ± 0.00	22	20

Table 4. The antimicrobial activity of *R. riparioides* against foodborne microorganisms (Inhibition zones in mm).

Food Isolated Microorganisms	Ethanol	Methanol	n-Hexane	Gentamicin (10 µg)	Tobramycin (10 µg)
Enterococcus durans	-	-	-	30	26
Enterococcus faecium	-	-	-	12	13
Klebsiella pneumoniae	-	-	7.00 ± 0.00	24	18
Listeria innocua	7.00 ± 0.00	-	-	12	8
Salmonella infantis	-	-	-	22	20
Salmonella kentucky	-	-	-	28	24
Escherichia coli	-	7.00 ± 0.00	-	15	22

Table 5. The antimicrobial activity of *R. riparioides* against clinical isolated microorganisms (Inhibition zones in mm).

Clinic Isolated Microorganisms	Ethanol	Methanol	n-Hexane	Gentamicin (10 µg)	Tobramycin (10 µg)
Staphylococcus aureus	-	-	-	30	26
Streptococcus mutans	-	-	-	12	13
Staphylococcus hominis	-	-	-	24	18
Staphylococcus haemolyticus	-	-	-	12	8
Staphylococcus lugdunensis	-	-	7.00 ± 0.00	22	20
Shigella boydi	-	-	-	28	24
Acinetobacter baumannii	-	-	-	15	22
Shigella flexneri	-	-	-	13	12
Staphylococcus aureus	-	-	-	21	-
Enterococcus faecalis	-	-	-	24	15
Klebsiella pneumoniae	-	-	-	21	14
Candida tropicalis	-	-	-	22	20
Candida glabrata	-	-	7.00 ± 0.00	7	8

3.4 MIC & MBC tests results

The MIC values represent the minimum concentrations of the extracts required to inhibit visible microbial growth. MBC (Minimum Bactericidal Concentration) and MFC (Minimum Fungicidal Concentration) values indicate the lowest concentrations at which the extracts exert bactericidal or fungicidal effects, respectively, by completely eliminating the tested microorganisms.

Table 6. MIC and MBC/MFC concentrations of the ethanol extract of *R. riparioides*.

Microorganisms	MIC	MBC/MFC
E. faecalis ATCC 29212	7340 µl/ml	14680 µl/ml
L. innocua (FI)	7340 µl/ml	14680 µl/ml

Table 7. MIC and MBC/MFC concentrations of the methanol extract of *R. riparioides*.

Microorganisms	MIC	MBC/MFC
E. faecalis ATCC 29212	2140 µl/ml	4280 µl/ml
E. coli (FI)	4280 µl/ml	4280 µl/ml

Table 8.MIC and MBC/MFC concentrations of the n-Hekzan extract of *R. riparioides*.

Microorganisms	MIC	MBC/MFC
S. epidermidis DSMZ 20044	350 µl/ml	350 µl/ml
K. pneumoniae (FI)	350 µl/ml	350 µl/ml
S. lugdunensis (CI)	350 µl/ml	350 µl/ml
C. glabrata (CI)	350 µl/ml	350 µl/ml

3.4.1. Results of the MIC test conducted for biofilm

Table 9. MIC and MBC/MFC concentrations of the ethanol extract of *R. riparioides*.

Microorganisms	MIC
Bacillus subtilis DSMZ 1971	14680 µl/ml
Listeria innocua (FI)	3670 µl/ml

Tablo 10. MIC and MBC/MFC concentrations of the methanol extract of *R. riparioides*.

Microorganisms	MIC
Listeria innocua (FI)	1070 µl/ml

3.4 Antibiofilm effect results

The antibiofilm activities of the extracts against the tested strains are presented in the graphs between Figure 1 and Figure 21. OD_{550} refers to the optical density measured at 550 nm, which was used for the spectrophotometric quantification of biofilm mass stained with crystal violet. The percentage of inhibition values presented in the figures were calculated relative to the positive control using the following formula: % Inhibition = [(ODcontrol – ODsamples) / ODcontrol] × 100.

In the *E. coli* strain, treatments with the ethanol extract of *R. riparioides* were found to reduce biofilm formation at all tested concentrations. The highest reduction was recorded at a concentration of 7.3450 µg/mL, with a biofilm inhibition rate of 70.02%. At other concentrations, inhibition rates of 68.72% at 14.6900 µg/mL, 67.53% at 1.8362 µg/mL, 66.07% at 0.9181 µg/mL, 64.92% at 0.4591 µg/mL, and 60.07% at 3.6725 µg/mL were observed.

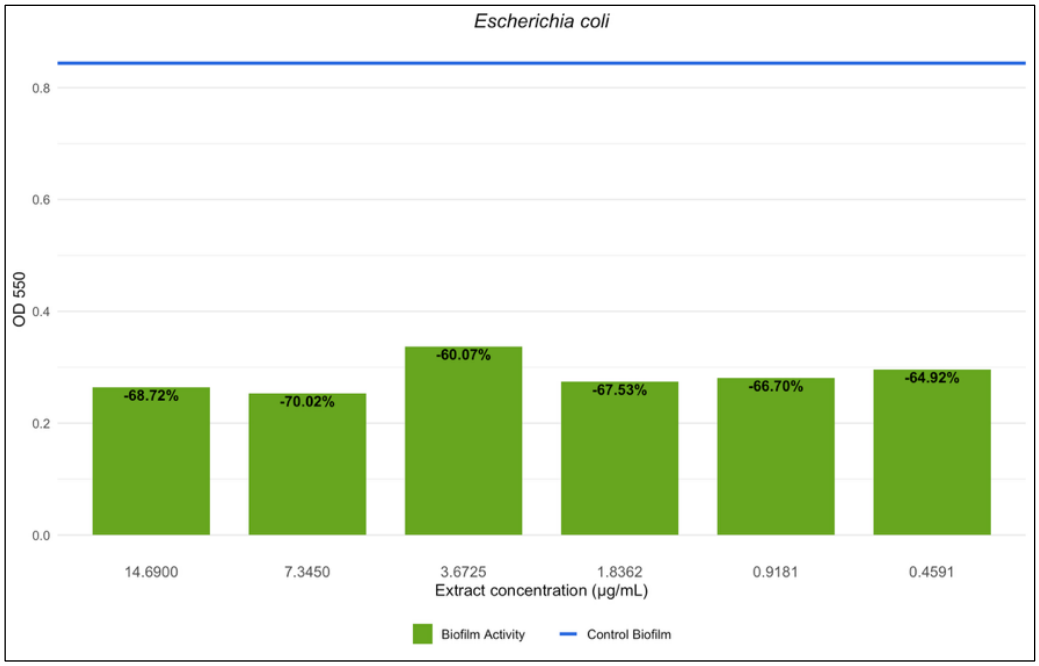


Figure 1: Graph showing the antibiofilm effect of *Rhynchostegium riparioides* ethanol extract against *Escherichia coli*

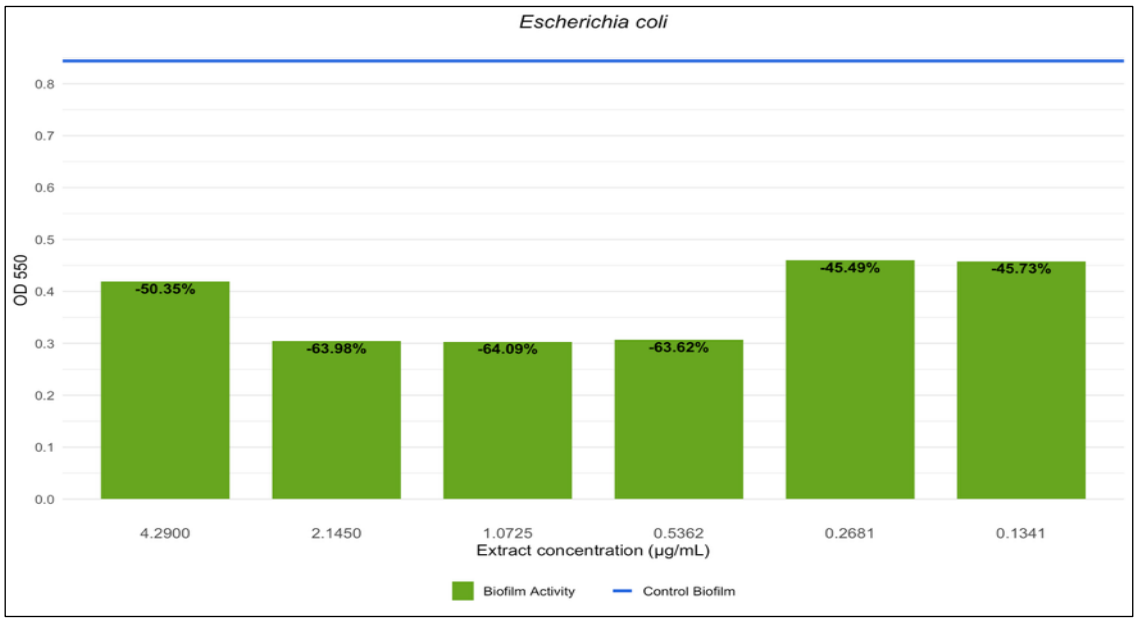


Figure 2: Graph showing the antibiofilm effect of *Rhynchostegium riparioides* methanol extract against *Escherichia coli*

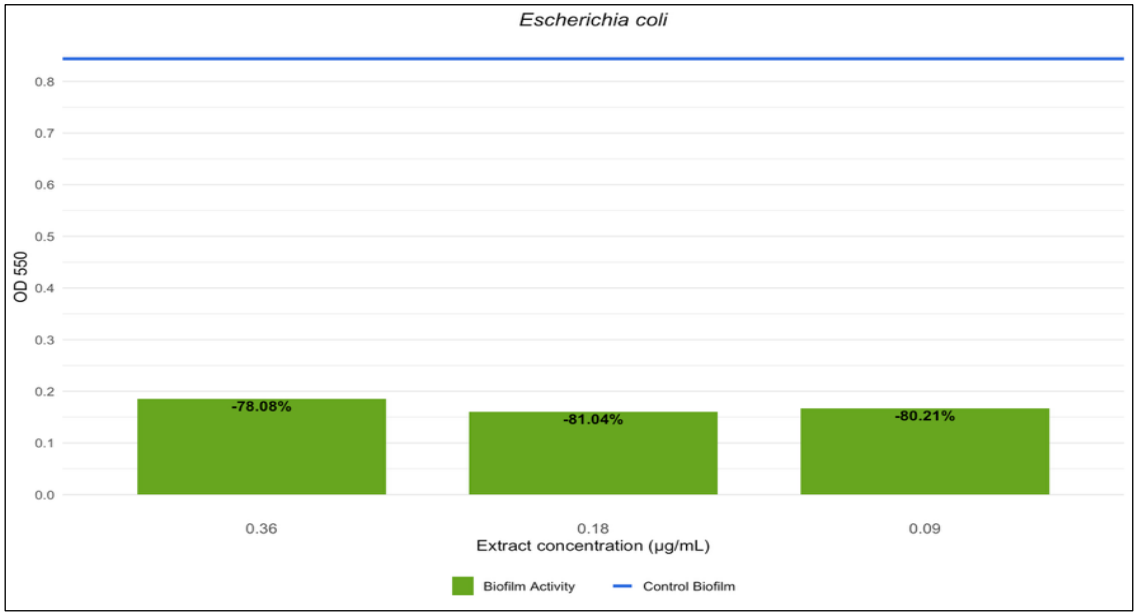


Figure 3: Graph showing the antibiofilm effect of *Rhynchostegium riparioides* n-hexane extract against *Escherichia coli*

In the *E. coli* strain, treatments with the methanol extract of *R. riparioides* were found to reduce biofilm production at all tested concentrations. The highest reduction was observed at a concentration of 1.0725 µg/mL, with a biofilm inhibition rate of 64.09%. At the other concentrations, inhibition rates of 63.62% at 0.5362 µg/mL, 63.98% at 2.1450 µg/mL, 60.35% at 4.2900 µg/mL, 45.73% at 0.1341 µg/mL, and 45.49% at 0.2681 µg/mL were recorded.

In the *E. coli* strain, treatments with the hexane extract of *R. riparioides* were found to significantly reduce biofilm formation at all tested concentrations. The highest reduction was recorded at a concentration of 0.18 µg/mL, with a biofilm inhibition rate of 81.04%. At the other concentrations, inhibition rates of 80.21% at 0.09 µg/mL and 76.08% at 0.36 µg/mL were observed.

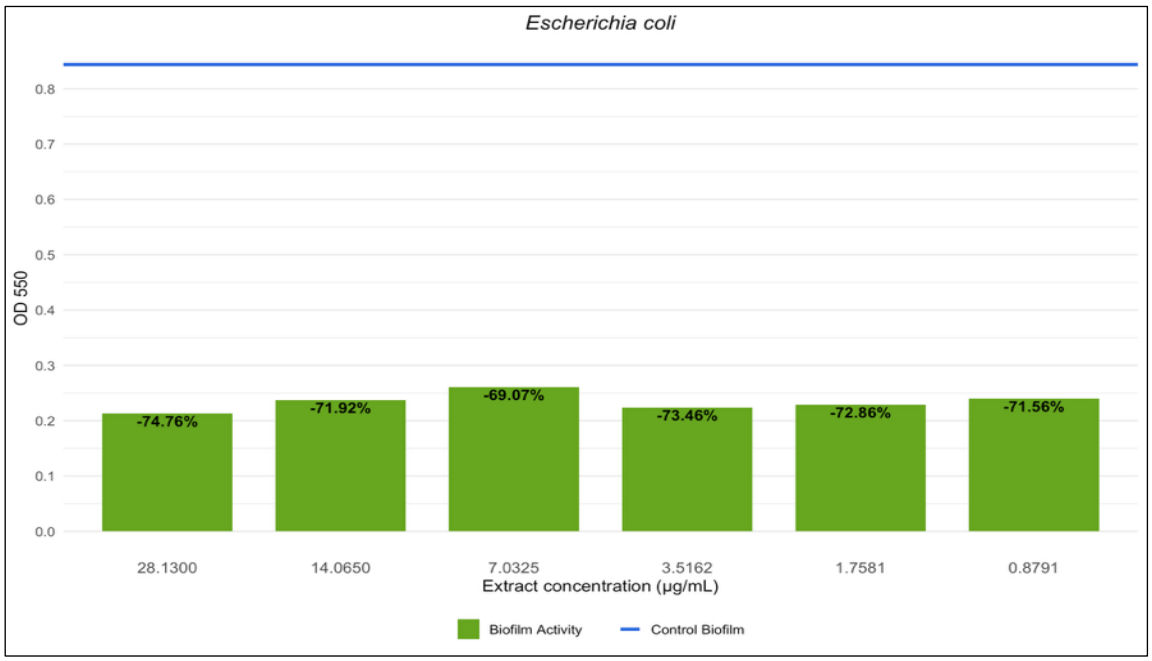


Figure 4: Graph showing the antibiofilm effect of *Rhynchostegium riparioides* water extract against *Escherichia coli*

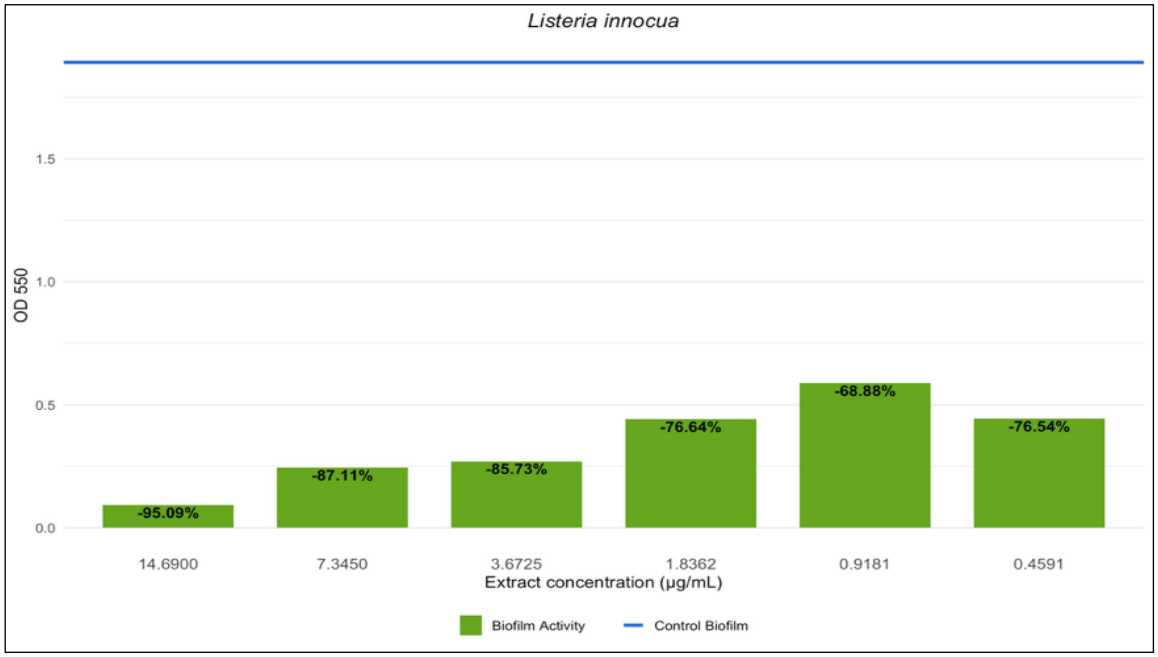


Figure 5: Antibiofilm effect of *Rhynchostegium riparioides* ethanol extract against *Listeria innocua*

In the *E. coli* strain, treatments with the water extract of *R. riparioides* were found to markedly reduce biofilm production at all tested concentrations. The highest reduction was observed at a concentration of 28.1300 µg/mL, with a biofilm inhibition rate of 74.76%. At the other concentrations, inhibition rates of 73.46% at 3.5162 µg/mL, 72.86% at 1.7581 µg/mL, 71.92% at 14.0650 µg/mL, 71.56% at 0.8791 µg/mL, and 69.07% at 7.0325 µg/mL were recorded.

In the *L. innocua* strain, treatments with the ethanol extract of *R. riparioides* were found to strongly reduce biofilm formation at all tested concentrations. The highest reduction was recorded at a concentration of 14.6900 µg/mL, with a biofilm inhibition rate of 95.09%. At the other concentrations, inhibition rates of 87.11% at 7.3450 µg/mL, 85.73% at 3.6725 µg/mL, 76.64% at 1.8362 µg/mL, 76.54% at 0.4591 µg/mL, and 68.88% at 0.9181 µg/mL were observed.

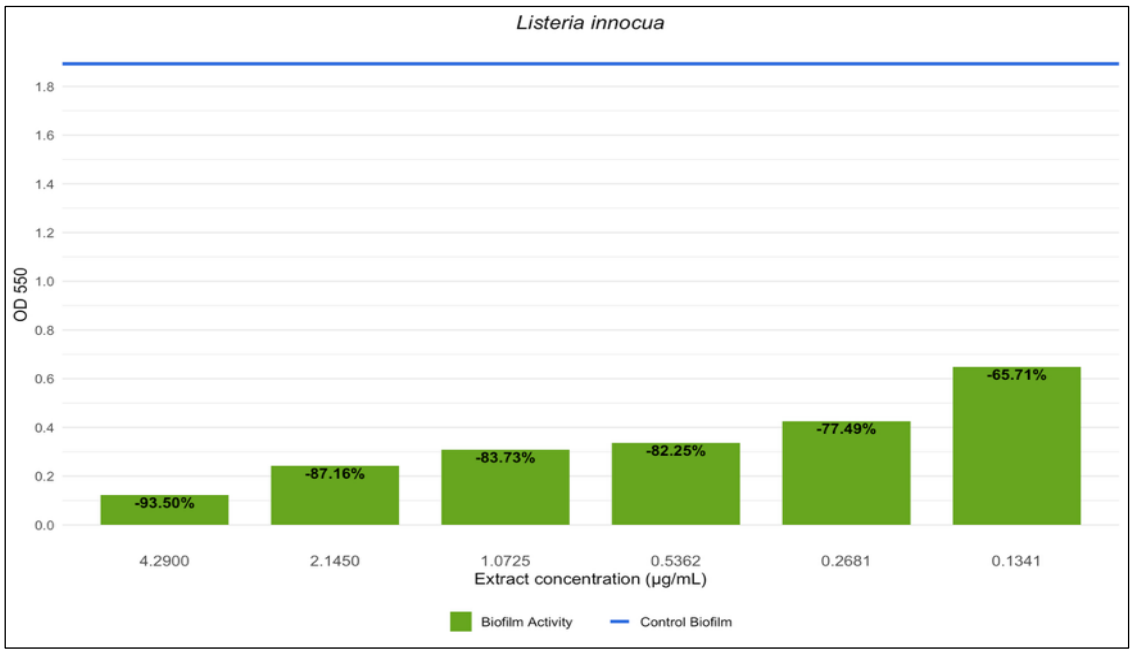


Figure 6: Graph illustrating the antibiofilm effect of the methanol extract of *Rhynchostegium riparioides* against *Listeria innocua*

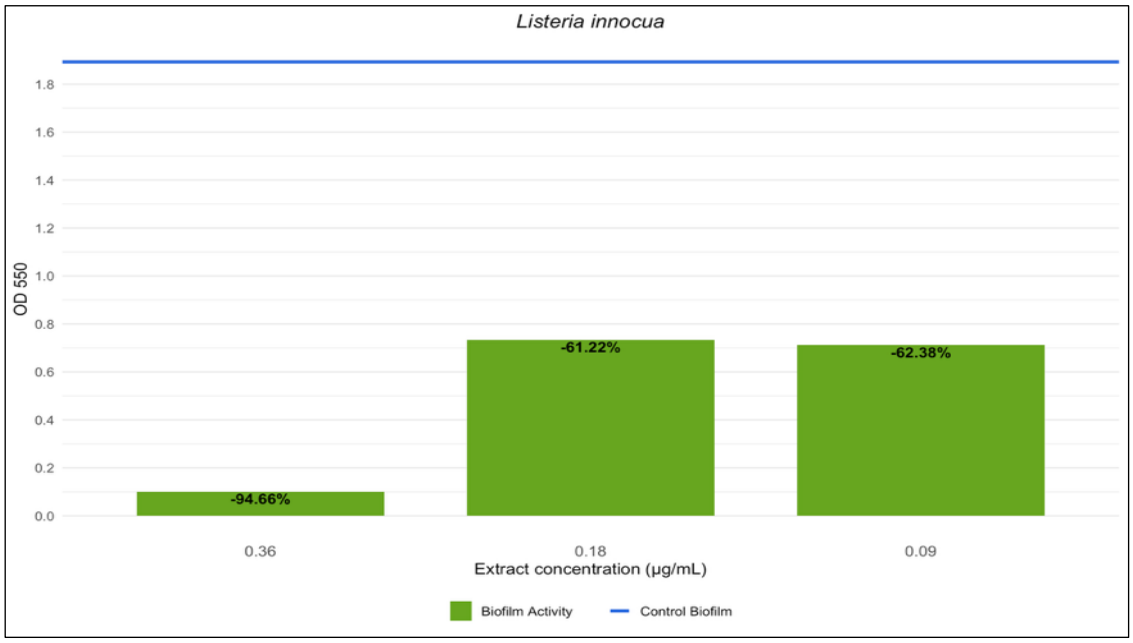


Figure 7: Antibiofilm activity graph of *Rhynchostegium riparioides* n-hexane extract against *Listeria innocua*

In the *L. innocua* strain, treatments with the methanol extract of *R. riparioides* were found to significantly reduce biofilm formation at all tested concentrations. The highest reduction was observed at a concentration of 4.2900 µg/mL, with a biofilm inhibition rate of 93.50%. At the other concentrations, inhibition rates of 87.16% at 2.1450 µg/mL, 83.73% at 1.0725 µg/mL, 82.25% at 0.5362 µg/mL, 77.49% at 0.2681 µg/mL, and 65.71% at 0.1341 µg/mL were recorded.

In the *L. innocua* strain, treatments with the hexane extract of *R. riparioides* were found to reduce biofilm formation at all tested concentrations. The highest reduction was recorded at a concentration of 0.36 µg/mL, with a biofilm inhibition rate of 94.66%. At the other concentrations, inhibition rates of 61.22% at 0.18 µg/mL and 62.38% at0.09 µg/mL were observed.

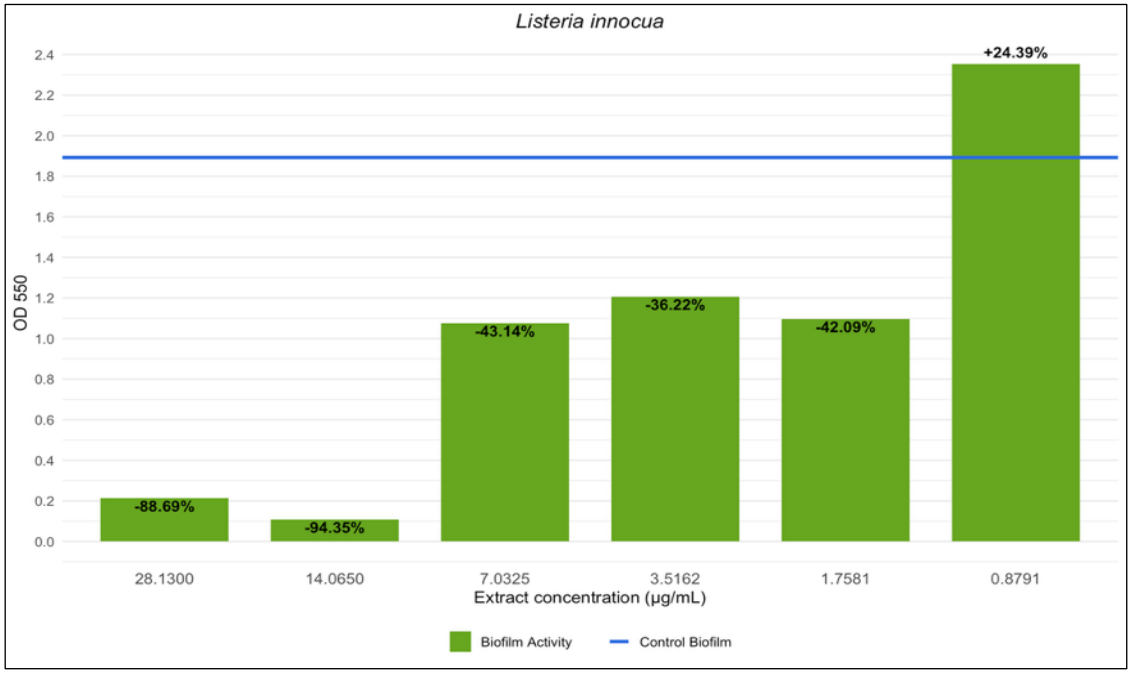


Figure 8: Graph illustrating the antibiofilm effect of the water extract of *Rhynchostegium riparioides* against *Listeria innocua*

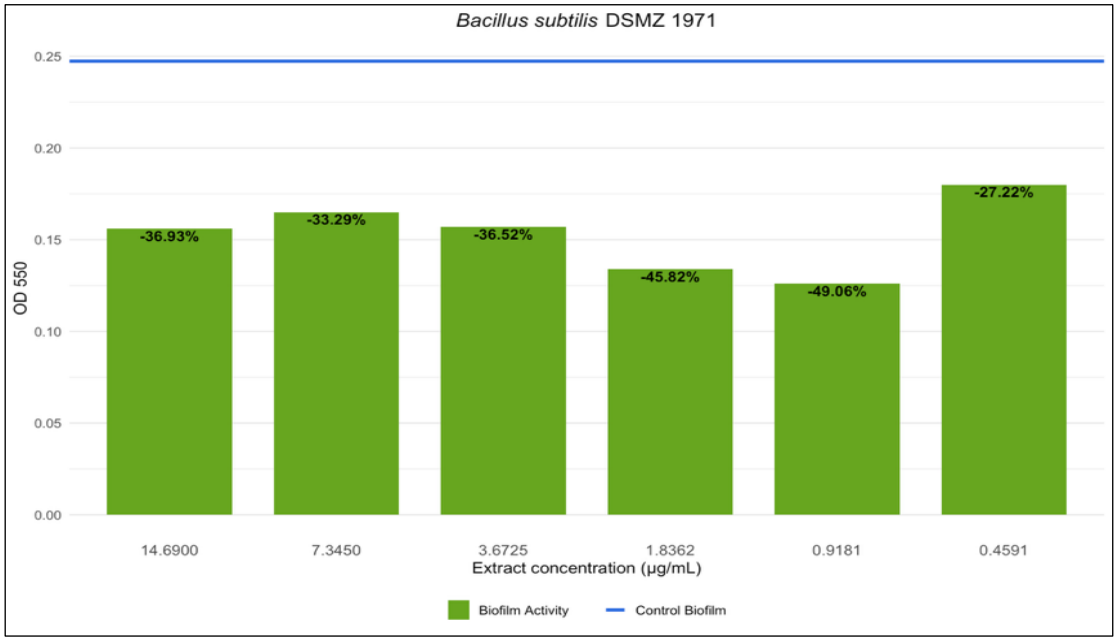


Figure 9: Graph illustrating the antibiofilm effect of the ethanol extract of *Rhynchostegium riparioides* against *Bacillus subtilis* DSMZ 1971

In the *L. innocua* strain, treatments with the water extract of *R. riparioides* resulted in both a decrease and an increase in biofilm formation depending on the concentration. The highest reduction was observed at a concentration of 14.0650 µg/mL, with a biofilm inhibition rate of 94.35%. At the other concentrations, inhibition rates of 88.69% at 28.1300 µg/mL, 43.14% at 7.0325 µg/mL, 42.09% at 1.7581 µg/mL, and 36.22% at 3.5162 µg/mL were recorded. In contrast, at a concentration of 0.8791 µg/mL, a 24.39% increase in biofilm formation was observed.

In the *B. subtilis* DSMZ 1971 strain, treatments with the ethanol extract of *R. riparioides* were found to reduce biofilm production at all tested concentrations. The highest reduction was recorded at a concentration of 0.9181 µg/mL, with a biofilm inhibition rate of 49.06%. At the other concentrations, inhibition rates of 45.82% at 1.8362 µg/mL, 36.93% at 14.6900 µg/mL, 36.52% at 3.6725 µg/mL, 33.29% at 7.3450 µg/mL, and 27.22% at 0.4591 µg/mL were observed.

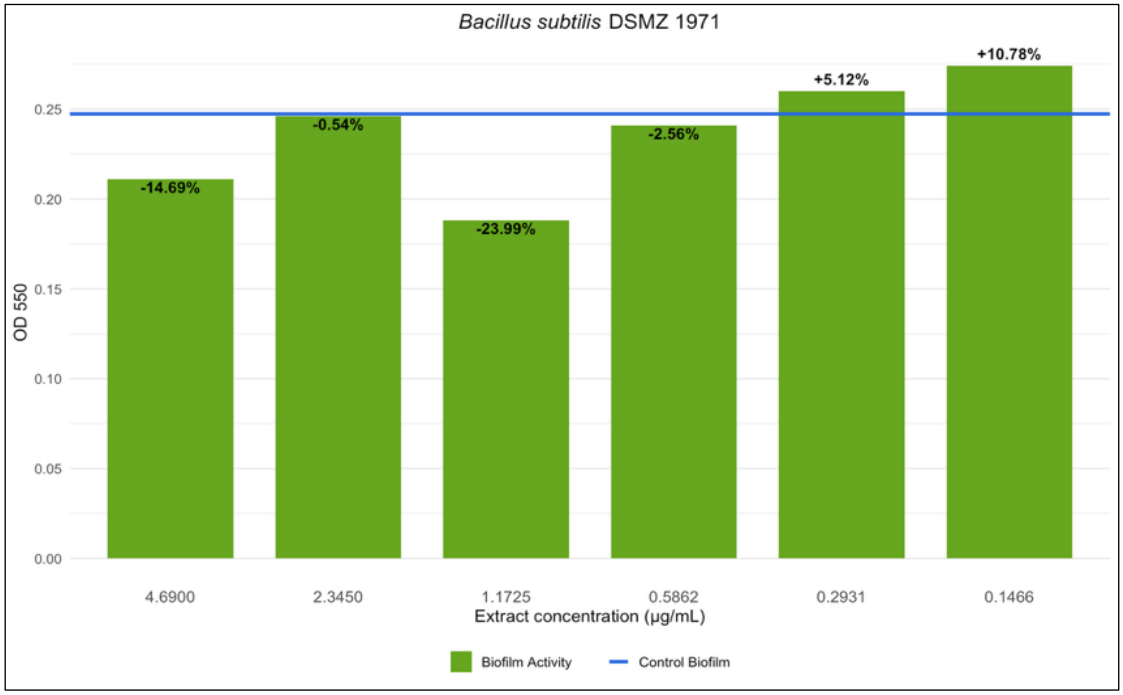


Figure 10: Graph illustrating the antibiofilm effect of the methanol extract of *Rhynchostegium riparioides* against *Bacillus subtilis* DSMZ 1971

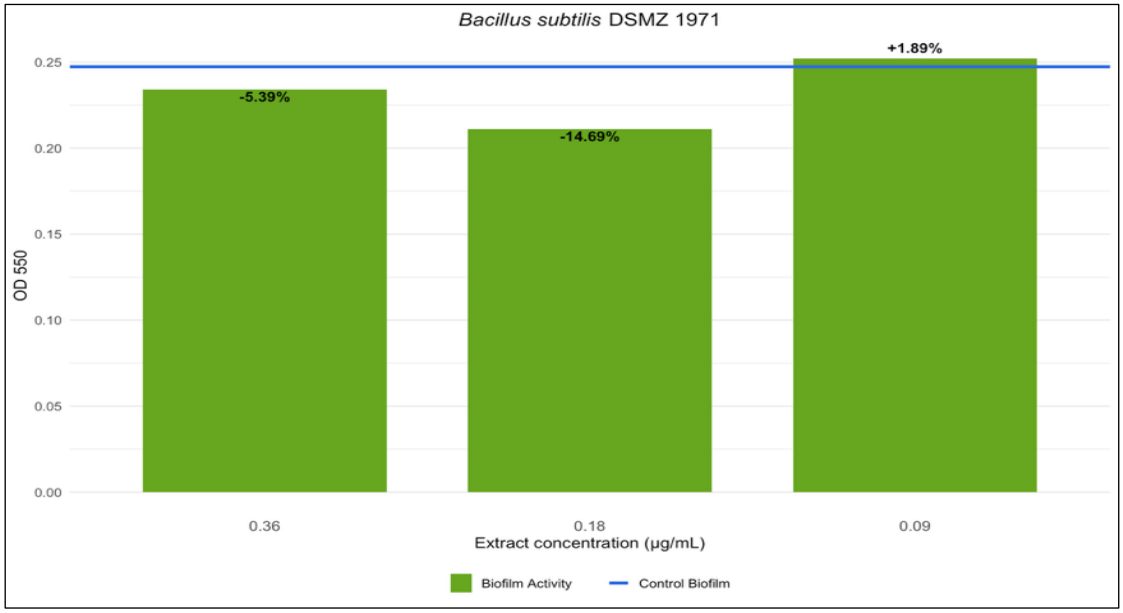


Figure 11: Graph illustrating the antibiofilm effect of the *n*-hexane extract of *Rhynchostegium riparioides* against *Bacillus subtilis* DSMZ 1971

In the *B. subtilis* DSMZ 1971 strain, treatments with the methanol extract of *R. riparioides* resulted in both increases and decreases in biofilm formation depending on the concentration. The highest increase was observed at a concentration of 0.1466 µg/mL, with a 10.78% rise in biofilm formation, followed by a 5.12% increase at 0.2931 µg/mL. In contrast, reductions in biofilm formation were recorded as 23.99% at 1.1725 µg/mL, 14.69% at 4.6900 µg/mL, 2.56% at 0.5862 µg/mL, and 0.54% at 2.3450 µg/mL.

In the *B. subtilis* DSMZ 1971 strain, treatments with the hexane extract of *R. riparioides* resulted in both decreases and increases in biofilm formation at lower concentrations. The highest reduction was recorded at a concentration of 0.18 µg/mL, with a biofilm inhibition rate of 14.69%. A 5.39% reduction was observed at 0.36 µg/mL, while a 1.89% increase in biofilm formation was detected at 0.09 µg/mL.

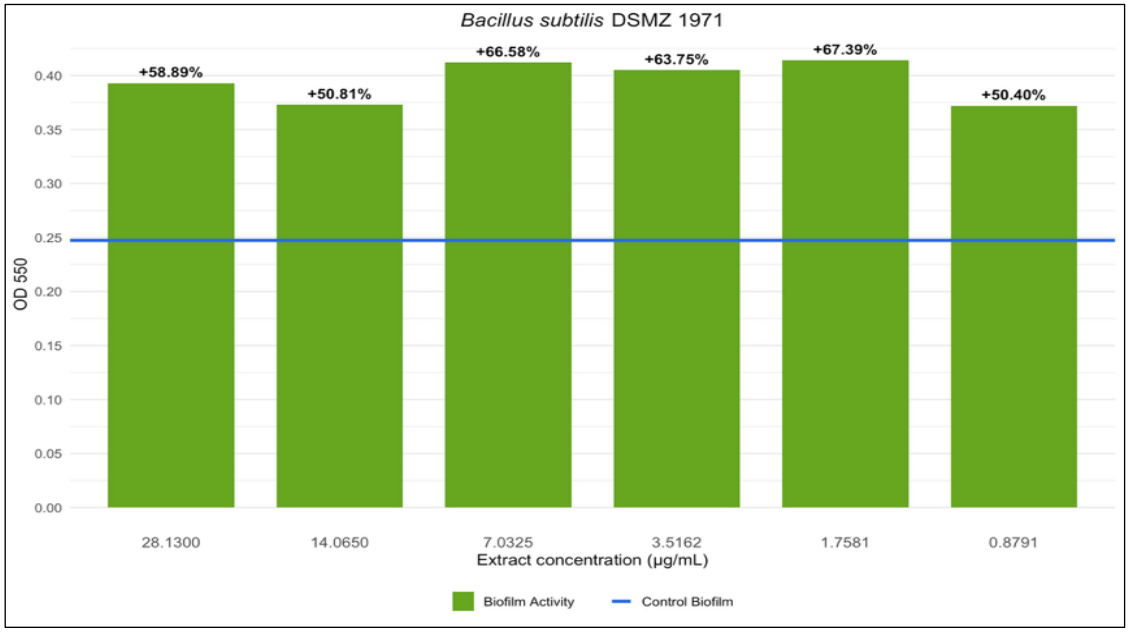


Figure 12: Graph illustrating the antibiofilm effect of the water extract of *Rhynchostegium riparioides* against *Bacillus subtilis* DSMZ 1971

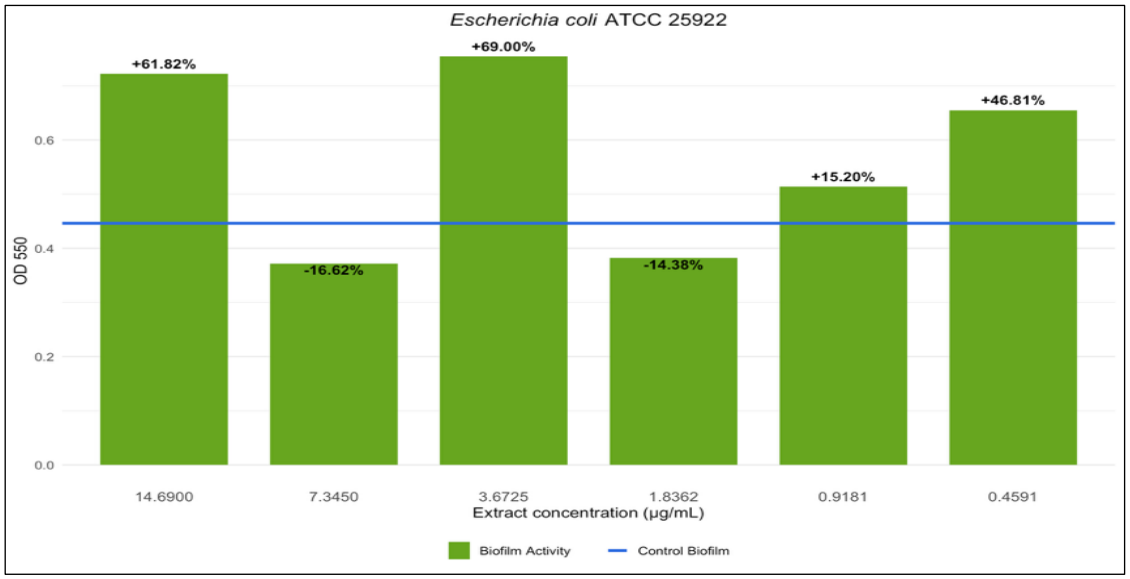


Figure 13: Graph illustrating the antibiofilm effect of the ethanol extract of *Rhynchostegium riparioides* against *Escherichia coli* ATCC 25922

Biofilm formation was found to increase at all tested concentrations. The highest increase was recorded at a concentration of 1.7581 µg/mL, with a 67.39% rise in biofilm formation. At the other concentrations, increases of 66.58% at 7.0325 µg/mL, 63.75% at 3.5162 µg/mL, 58.89% at 28.1300 µg/mL, 50.81% at 14.0650 µg/mL, and 50.40% at 0.8791 µg/mL were observed.

In the *E. coli* ATCC 25922 strain, treatments with the ethanol extract of *R. riparioides* resulted in concentration-dependent variations in biofilm formation levels. While an increase in biofilm production was observed at certain concentrations, a decrease was recorded at others. The highest increase was observed at a concentration of 3.6725 µg/mL, with a 69.00% rise in biofilm formation. At the other concentrations, increases of 61.82% at 14.6900 µg/mL, 46.81% at 0.4591 µg/mL, and 15.20% at 0.9181 µg/mL were recorded. In contrast, reductions of 16.62% at 7.3450 µg/mL and 14.38% at 1.8362 µg/mL were observed.

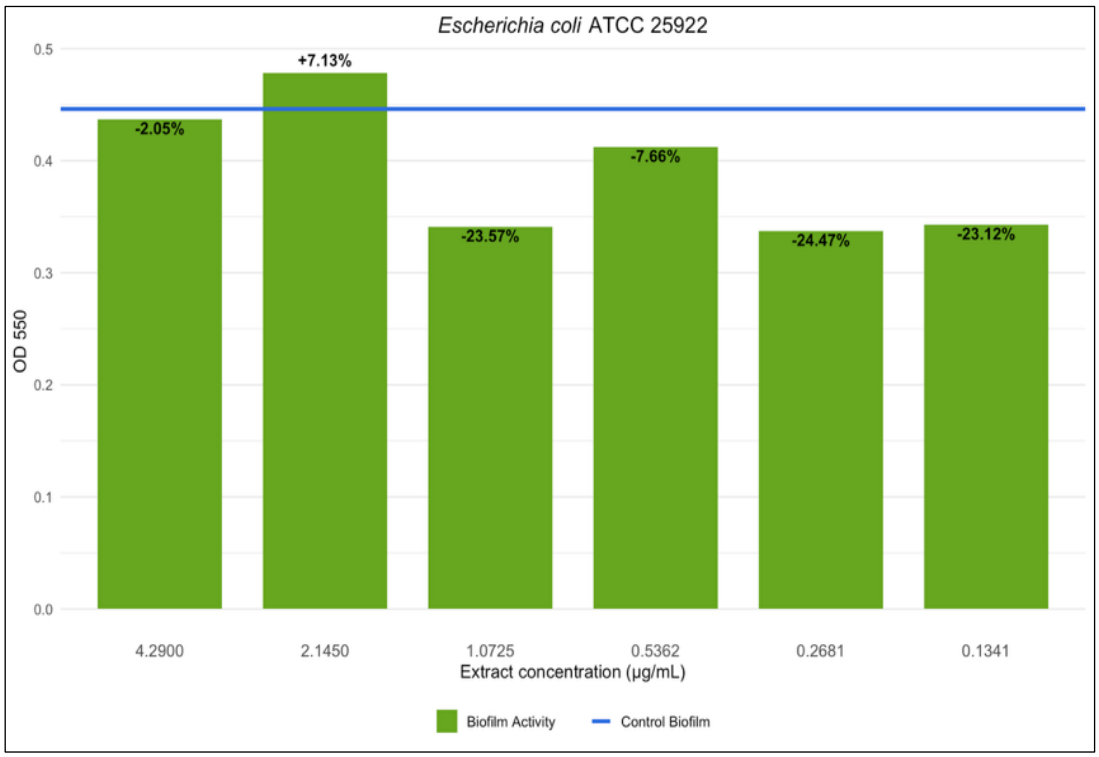


Figure 14: Graph illustrating the antibiofilm effect of the methanol extract of *Rhynchostegium riparioides* against *Escherichia coli* ATCC 25922

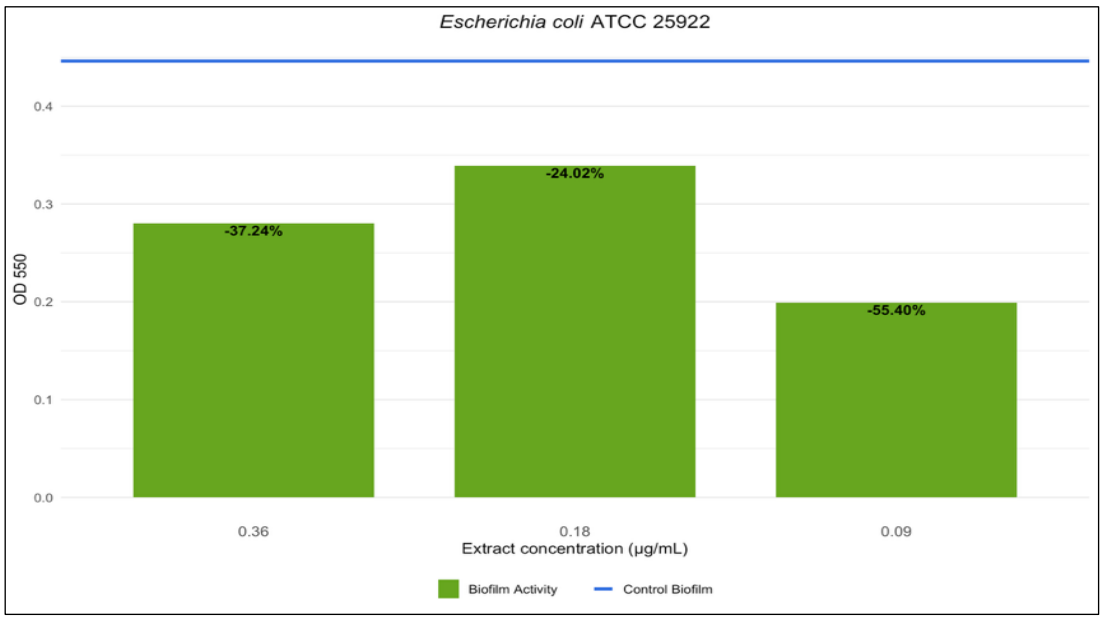


Figure 15: Graph illustrating the antibiofilm effect of the *n*-hexane extract of *Rhynchostegium riparioides* against *Escherichia coli* ATCC 25922

Both increases and decreases in biofilm formation were observed at different concentrations. The highest increase was recorded at a concentration of 2.1450 µg/mL, with a 7.13% rise in biofilm formation. In contrast, inhibition was detected at the other concentrations, with reductions of 2.05% at 4.2900 µg/mL, 23.57% at 1.0725 µg/mL, 7.68% at 0.5362 µg/mL, 24.47% at 0.2681 µg/mL, and 23.12% at 0.1341 µg/mL.

In the *E. coli* ATCC 25922 strain, treatments with the water extract of *R. riparioides* were found to reduce biofilm formation at all tested concentrations. The highest inhibition rate was recorded at a concentration of 0.09 µg/mL, with 55.40% inhibition. At the other concentrations, inhibition rates of 37.24% at 0.36 µg/mL and 24.02% at 0.18 µg/mL were observed.

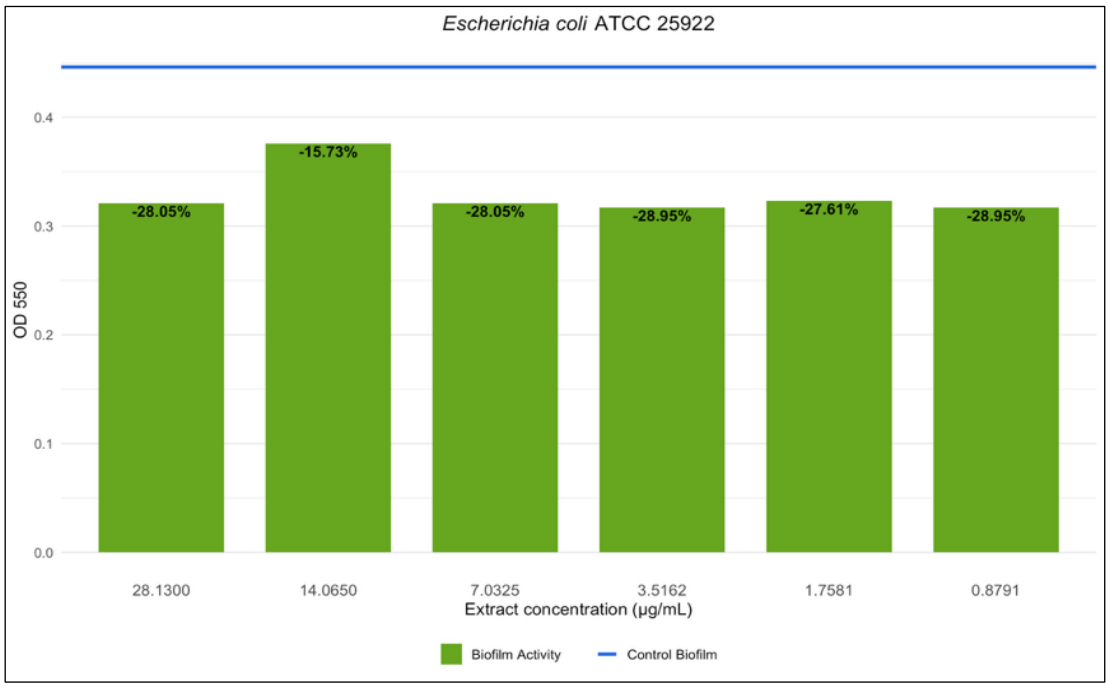


Figure 16: Graph illustrating the antibiofilm effect of the water extract of *Rhynchostegium riparioides* against *Escherichia coli* ATCC 25922

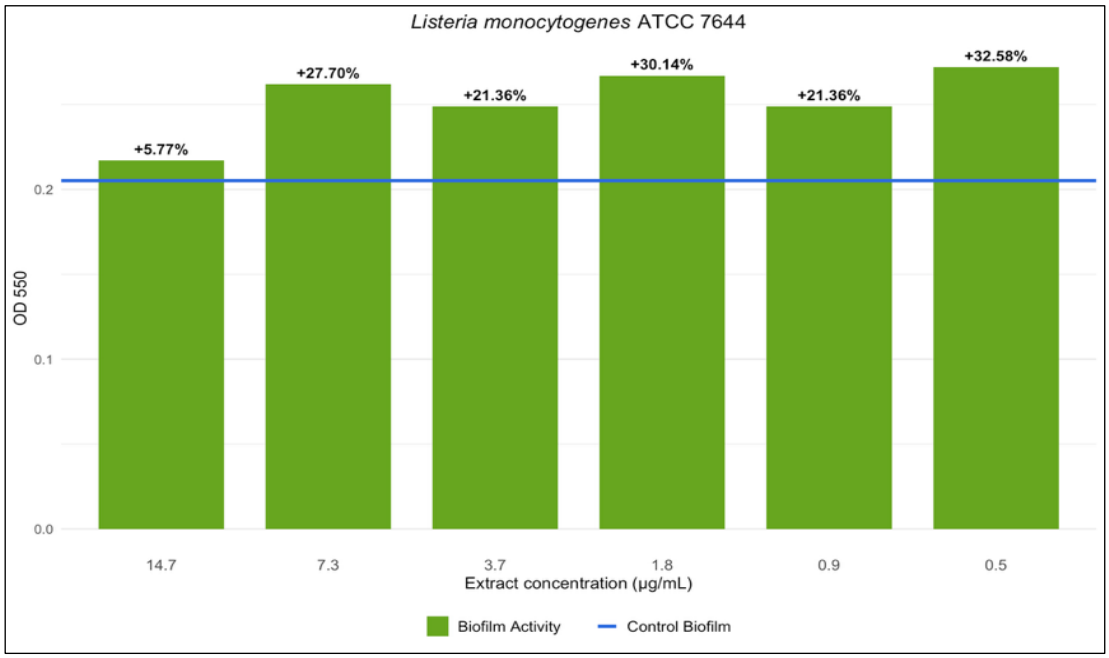


Figure 17: Graph illustrating the antibiofilm effect of the ethanol extract of *Rhynchostegium riparioides* against *Listeria monocytogenes* ATCC 7644

In the *E. coli* ATCC 25922 strain, treatments with the water extract of *R. riparioides* were found to reduce biofilm formation at all tested concentrations. The highest reduction was recorded at concentrations of 3.5162 µg/mL and 0.8791 µg/mL, with a biofilm inhibition rate of 28.95%. At the other concentrations, inhibition rates of 28.05% at 28.1300 µg/mL, 28.05% at 7.0325 µg/mL, 27.61% at 1.7581 µg/mL, and 15.73% at 14.0650 µg/mL were observed.

In the *L. monocytogenes* ATCC 7644 strain, treatments with the ethanol extract of *R. riparioides* were found to increase biofilm formation at all tested concentrations. The highest increase was observed at a concentration of 0.5 µg/mL, with a 32.58% rise in biofilm formation. At the other concentrations, increases of 30.14% at 1.8 µg/mL, 27.70% at 7.3 µg/mL, 21.36% at 0.9 µg/mL, 21.36% at 3.7 µg/mL, and 5.77% at 14.7 µg/mL were recorded.

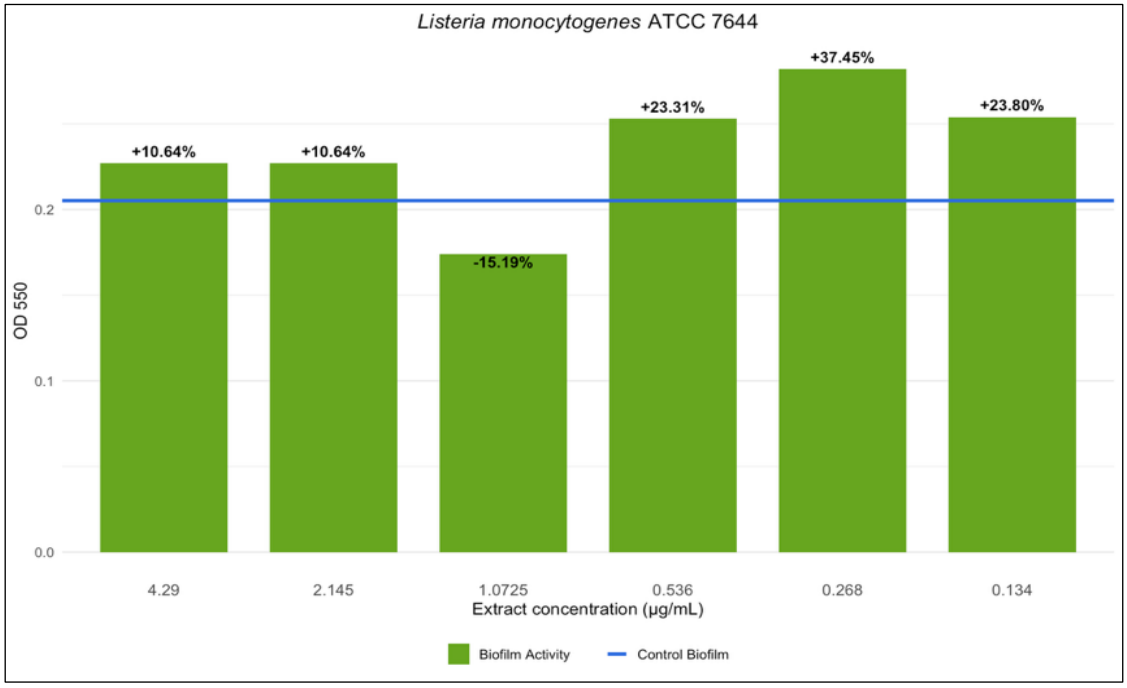


Figure 18: Graph illustrating the antibiofilm effect of the methanol extract of *Rhynchostegium riparioides* against *Listeria monocytogenes* ATCC 7644

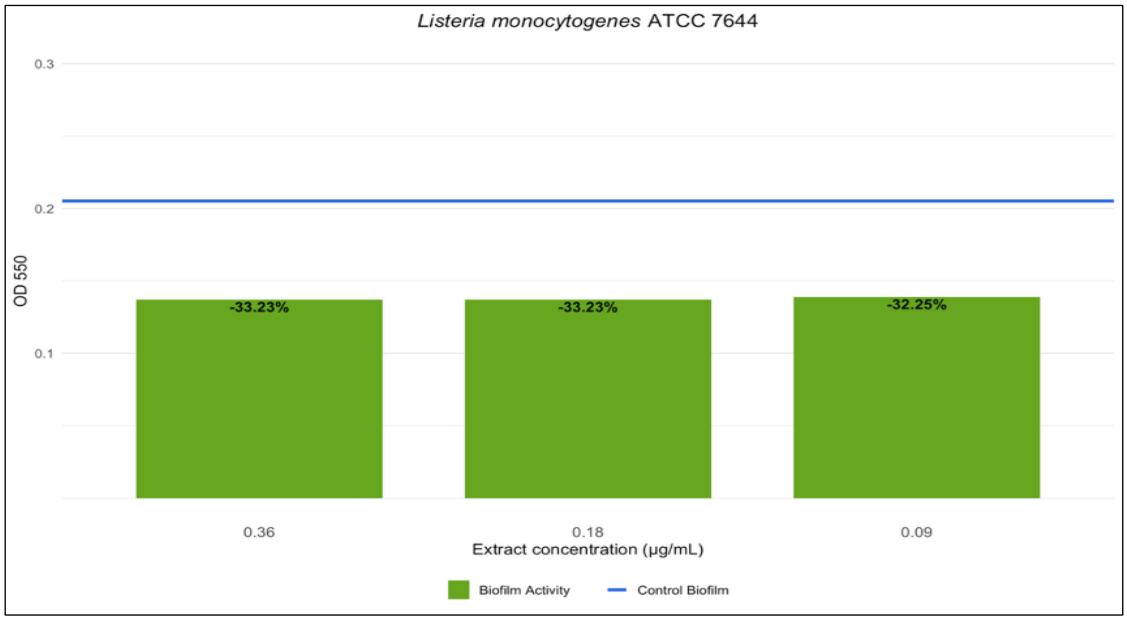


Figure 19: Graph illustrating the antibiofilm effect of the *n*-hexane extract of *Rhynchostegium riparioides* against *Listeria monocytogenes* ATCC 7644

The highest biofilm inhibition was observed at a concentration of 1.0725 µg/mL, with an inhibition rate of 15.19%. In contrast, biofilm formation increased at all other concentrations. The highest increases were recorded as 23.31% at 0.536 µg/mL, 23.80% at 0.134 µg/mL, 10.64% at 4.29 µg/mL, and 10.64% at 2.145 µg/mL.

In the *L. monocytogenes* ATCC 7644 strain, treatments with the n-hexane extract of *R. riparioides* were found to reduce biofilm formation at all tested concentrations. The highest inhibition rate was recorded at concentrations of 0.36 µg/mL and 0.18 µg/mL, both showing a 33.23% reduction in biofilm formation. At the lowest concentration of 0.09 µg/mL, a 32.25% decrease in biofilm formation was observed.

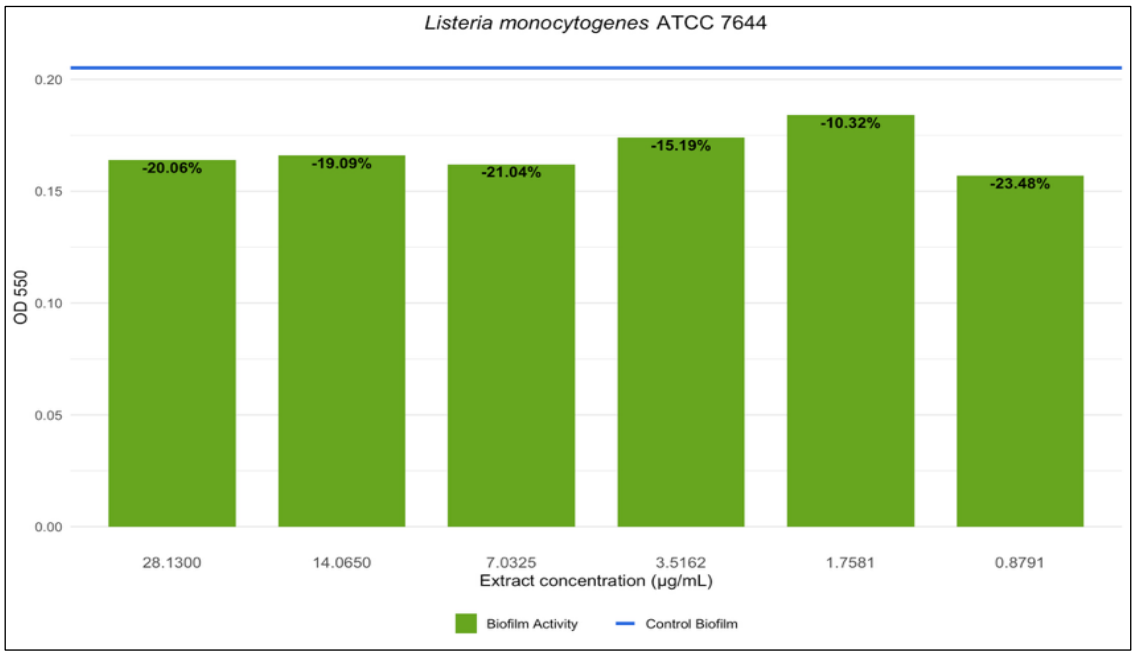


Figure 20. Graph illustrating the antibiofilm effect of the water extract of *Rhynchostegium riparioides* against *Listeria monocytogenes* ATCC 7644

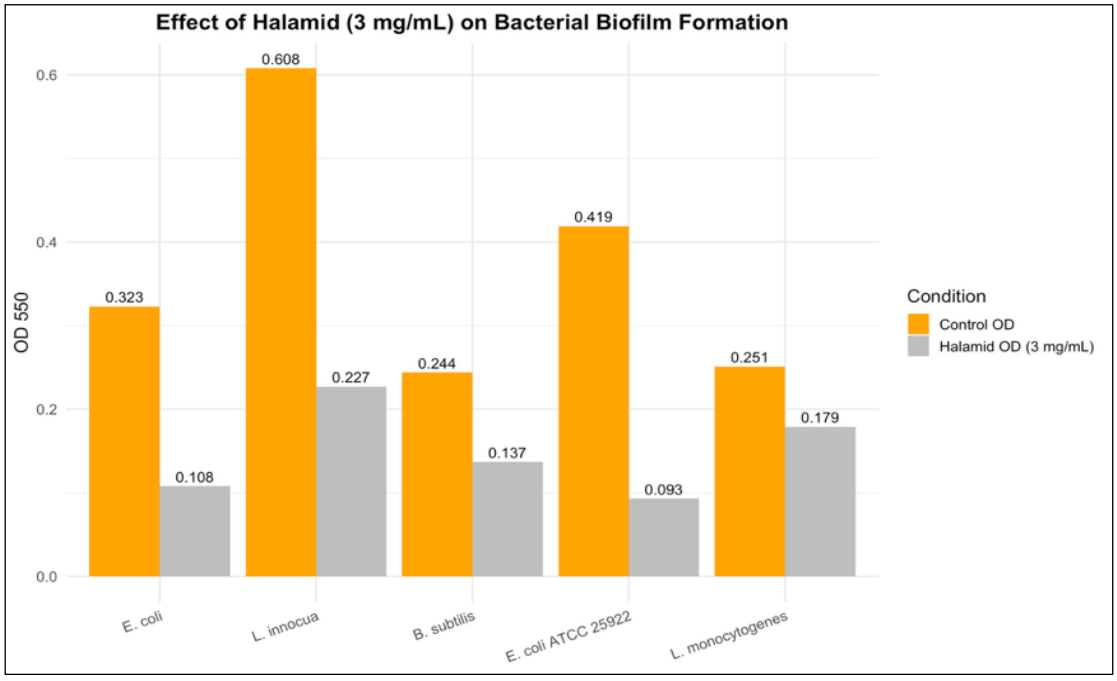


Figure 21. Biofilm graph showing the antibiofilm effect of Halamid (control group) at a concentration of 3 mg/ml

In the *L. monocytogenes* ATCC 7644 strain, treatments with the water extract of *R. riparioides* were observed to reduce biofilm formation at all tested concentrations. The highest inhibition rate was recorded at a concentration of 0.8791 µg/mL, with 23.48% inhibition. At the other concentrations, inhibition rates of 20.06% at 28.1300 µg/mL, 19.09% at 14.0650 µg/mL, 21.04% at 7.0325 µg/mL, 15.19% at 3.5162 µg/mL, and 10.32% at 1.7581 µg/mL were observed.

At a concentration of 3 mg/mL, treatment with Halamid resulted in a decrease in the optical density (OD_{550}) value of the *E. coli* strain from 0.323 in the control group to 0.108 in the treatment group. For *Listeria innocua*, these values were 0.608 and 0.227, respectively; for *Bacillus subtilis* DSMZ 1971, 0.244 and 0.137; for *E. coli* ATCC 25922, 0.419 and 0.093; and for *Listeria monocytogenes* ATCC 7644, 0.251 and 0.179, respectively.

4. Discussions and Conclusions

4.1. Biochemical compounds

In this study, the phytochemical contents of *R. riparioides* extracts obtained using different solvents (ethanol, methanol, and n-hexane) were evaluated based on percentage area data from GC-MS analysis. The findings indicated that solvent polarity significantly influenced both the diversity of extracted compounds and their relative abundance within the extracts. Across all three extracts, palmitic acid and stearic acid were identified as common major compounds. Stearic acid was found in the highest concentration (6.07%) in the ethanol extract, whereas palmitic acid was most abundant (18.65%) in the methanol extract.

The ethanol and methanol extracts displayed similar compositional profiles, sharing numerous common compounds. Notably, tetradecanoic acid, neophytadiene, phytol, squalene, methyleicosapentaenoate, and diploptene were detected in both extracts. These components are known to be widespread in moss species and are thought to play important roles in the chemical defense mechanisms of these plants (Benek, 2024)[2]. The methanol extract presented a more balanced profile in terms of compound diversity and concentration, with significant amounts of neophytadiene (21.55%) and linolenic acid (10.45%). Meanwhile, the ethanol extract was characterized by a particularly high proportion of neophytadiene (41.16%), although other compounds were present at lower percentages. This suggests that the methanol extract may possess a broader spectrum of biological activity, while the ethanol extract may exhibit its effects based on a few major compounds.

In the n-hexane extract, unlike the other extracts, a large majority of the total peak area (79.29%) was dominated by a single compound, tris(2,4-di-tert-butylphenyl) phosphate. Tris(2,4-di-tert-butylphenyl) phosphate (Irgafos 168) is an organophosphorus antioxidant and stabilizer widely used in the plastics industry due to its ability to reduce oxidative degradation and enhance polymer stability (Chen et al., 2020). This finding suggests that the biological activity of the n-hexane extract may largely depend on the presence of this specific compound. Additionally, the effective biofilm inhibition observed at the lowest tested concentration of 0.4 µg/ml for the n-hexane extract is thought to be related to the presence of its major compound, tris(2,4-di-tert-butylphenyl) phosphate. The data indicate that the methanol extract exhibits a more favorable profile in terms of phytochemical diversity and balanced percentage distribution compared to other solvent extracts. Furthermore, GC-MS analyses revealed greater compound diversity and richness in the methanol extract compared to the n-hexane extract. This rich chemical profile explains why the methanol extract showed the second highest biofilm inhibition rate after the n-hexane extract. Therefore, the synergistic effect of multiple active compounds in the methanol extract appears to play a significant role in inhibiting biofilm formation. While the ethanol extract is characterized by dominance of a few main compounds with high biological activity potential, the n-hexane extract displays limited diversity and an imbalanced compound distribution.

These results underscore the direct impact of solvent choice on the resulting phytochemical profile and biological activity, highlighting the need to optimize extraction strategies according to the targeted biological activity.

4.2. Antibiofilm effects

This study evaluated the antibiofilm activities of *R. riparioides* extracts prepared with ethanol, methanol, n-hexane, and water solvents against standard bacteria (*Bacillus subtilis* DSMZ 1971, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, clinical isolate *Escherichia coli*, and food isolate *Listeria innocua*. The results demonstrated that the biofilm inhibition potential of the extracts varied depending on the solvent type and the target bacterial species.

Other than the n-hexane extract, all other solvent extracts induced biofilm formation in at least one strain, whereas the n-hexane extract exhibited varying degrees of biofilm inhibition across all strains. GC-MS analysis revealed that its compound profile was predominantly composed (79.29%) of tris(2,4-di-tert-butylphenyl) phosphate, an organophosphorus antioxidant widely used in the plastics industry for its oxidative degradation prevention and polymer stabilization properties (Chen et al., 2020). This compound likely explains the observed high inhibition rates. The use of a nonpolar solvent like hexane facilitated the extraction of lipophilic secondary metabolites, which are thought to contribute to the biofilm inhibition effect observed here, potentially including terpenoids, sterols, or lipophilic phenolic compounds. Future studies are recommended to isolate the active components, elucidate biofilm inhibition mechanisms, and perform extensive testing on different pathogenic species.

The methanol extract was characterized by the presence of active compounds such as palmitic

acid, phytol, linoleic acid, and oleic acid according to GC-MS analysis. Given the reported antimicrobial properties of these compounds, it is expected that the methanol extract provides strong biofilm inhibition at low concentrations against various bacteria. For instance, fatty acids like linoleic and oleic acid are known to disrupt cell membrane permeability in gram-positive bacteria, exhibiting antimicrobial effects, while phytol has demonstrated significant antibacterial and antibiofilm activity particularly against *P. aeruginosa* (Desbois & Smith, 2010; Saini et al., 2016). These findings align well with the suppressive effects on biofilm formation observed for the methanol extract. For L. monocytogenes, a distinct behavior was observed (Figure 18). Although biofilm formation was induced, a 15% inhibition was recorded at a concentration level of 1.0725 µg/mL. This finding further emphasizes the critical importance of the dose–response relationship.

Significant biofilm inhibition rates of the ethanol extract were demonstrated. Compounds detected by GC-MS, such as stearic acid, may have indirect effects on biofilm inhibition. Polar solvents like ethanol are effective in extracting phenolic compounds, flavonoids, and certain alkaloids. Thanks to its biofilm inhibitory properties, the ethanol extract of *R. riparioides* holds potential as a natural agent for biotechnological, industrial hygiene, and possible medical applications. Future work should focus on isolating active constituents, investigating molecular mechanisms of biofilm inhibition, and assessing activity under varied environmental conditions.

When evaluating the antibiofilm effects of the water extract of *R. riparioides* against different bacterial species, marked strain-specific differences were observed. The extract exhibited strong biofilm suppression across all tested concentrations in *E. coli*, with inhibition rates ranging from 69.07% to 74.76%, indicating that the extract can effectively inhibit biofilm formation even at lower doses and possesses high antibiofilm potential (Figure 4). In *L. innocua*, biofilm suppression of 88.69% and 94.35% was observed at higher concentrations (28.13 and 14.06 µg/mL, respectively), whereas the effect decreased at lower concentrations, with a 24.39% increase in biofilm formation recorded at 0.8791 µg/mL. These results suggest that the effect is concentration-dependent and that biofilm formation may be stimulated at low doses (Figure 8). Against *B. subtilis* DSMZ 1971, the extract promoted biofilm formation across all concentrations, with increases ranging from 50.40% to 67.39%, indicating a stimulatory effect in this gram-positive strain (Figure 12). In *E. coli* ATCC 25922, inhibition rates ranged from 15.73% to 28.95%, reflecting a more limited effect (Figure 16), while *L. monocytogenes* ATCC 7644 exhibited weaker biofilm suppression, ranging from 10.32% to 23.48% (Figure 20). Overall, the aqueous extract of *R. riparioides* demonstrated significant antibiofilm potential, particularly against *E. coli* and *L. innocua,* while promoting biofilm formation in *B. subtilis*. These findings indicate that the extract's effect depends on the bacterial species and the structure of the biofilm, showing selective activity across strains.

Halamid (3 mg/ml), used as a positive control, showed notable biofilm inhibition particularly against *E. coli* strains but unexpectedly low activity against *B. subtilis*. These results suggest that Halamid may exhibit selective activity depending on differences in bacterial cell wall structures.

Another notable observation is that the results obtained for L. innocua (Figures 5–8) indicated that biofilm structure was almost completely eradicated across all solvents. Although L. monocytogenes is phenotypically and genotypically highly similar to L. innocua, differences such as weaker virulence factors, generally non-pathogenic nature, and higher sensitivity to stress distinguish the two species (Milillo et al., 2012). Considering these differences, the divergence observed for L. monocytogenes (Figures 17–21) becomes meaningful. These findings suggest that the commonly used model strain of L. monocytogenes may not always serve as an appropriate model under all conditions. Indeed, several studies have highlighted that using L. innocua as a model organism can yield misleading results (Mohan et al., 2019; Bruschi et al., 2017; Friedly et al., 2008).

Although L. innocua is considered a non-infectious bacterium, its high genomic similarity with other Listeria species facilitates the potential transfer of resistance and virulence genes (Li et al., 2021). Considering the implications of resistance gene dissemination today and in the future, the findings of this study gain further significance.

4.3.Antimicrobial effects

Disk diffusion tests revealed that the inhibition zones all had a baseline diameter of 7 mm. This suggests that increasing the amount of tested substance could enhance the antimicrobial effect and that strains showing no inhibition at lower doses might respond if tested with higher concentrations.

Among the compared extracts, the n-hexane extract, with the lowest substance amount of 0,37

mg, exhibited antimicrobial activity against four different strains: *C. glabrata*, *S. lugdunensis*, *K. pneumoniae*, and *S. epidermidis*. The ethanol extract, containing 2,71 mg of substance, showed activity against two strains: *L. innocua* and *E. faecalis*. The methanol extract, with the highest substance amount of 4,09 mg, demonstrated antimicrobial effects against *E. coli* and *E. faecalis*.

Tris(2,4-di-tert-butylphenyl) phosphate (TDTBPP), which comprises 79.29% of the n-hexane extract and is the only major compound detected, is considered the primary contributor to the observed antimicrobial activity. Although no studies have directly investigated the antimicrobial effects of TDTBPP, some toxicological research highlights its adverse effects on biological systems. For example, Kang et al. (2025) reported disrupted lipid metabolism in mouse liver cells exposed to TDTBPP. Similarly, Zhang et al. (2024) demonstrated cardiac morphology and function impairments in zebrafish following TDTBPP exposure.

Given these known toxic effects, it is plausible that TDTBPP is responsible for the antimicrobial activity of the extract. The correlation between inhibition zones in disk diffusion tests and MIC values supports this relationship. MIC tests showed that the extract exhibited bactericidal activity at low concentrations such as 0.35 mg/mL. This antimicrobial effect, observed across gram-positive and gram-negative bacteria as well as yeast cells, indicates that TDTBPP's toxicity is not limited to animal cells but extends to microorganisms.
The ethanol extract showed antimicrobial activity exclusively against gram-positive bacteria. MIC and MBC tests revealed bacteriostatic effects at 7,34 mg/mL and bactericidal effects at 14,68 mg/mL concentrations against *E. faecalis* and *L. innocua* strains.

A study by Ceyhan-Güvensen & Keskin (2016) on *Mentha pulegium* (commonly known as pennyroyal) leaves, whose extract consists of 69.95% neophytadiene, reported antimicrobial effects against 11 bacterial strains including *E. faecalis*. This finding is supported by other literature (Stojanović et al., 2000; Alagić et al., 2002; Herrero et al., 2006), indicating that plant extracts containing neophytadiene possess antimicrobial potential.

In the methanol extract, neophytadiene was detected at 21.55%, along with palmitic acid (18.65%) and linolenic acid (10.45%) as major components. When comparing the antimicrobial effects of ethanol and methanol extracts, both showed similar activity against *E. faecalis*, suggesting neophytadiene's possible role in this effect. Other fatty acids present in the methanol extract have also been previously reported to possess antimicrobial properties (Huang et al., 2011). The higher concentrations of these compounds in the methanol extract compared to ethanol extract might have created a synergistic effect, explaining the enhanced efficacy at lower concentrations observed in MIC and MBC tests. For instance, the MIC and MBC values for the methanol extract against *E. faecalis* were 2,14 mg/mL and 4,28 mg/mL, respectively. Additionally, the methanol extract showed direct bactericidal activity against *E. coli* at 4,28 mg/mL concentration.

In the literature, the only study investigating the antimicrobial activity of *Rhynchostegium riparioides* was conducted by Basile et al. (1998), in which only an acetone extract was used and tested against a limited number of bacterial strains. In that study, minimum inhibitory concentrations (MIC) were reported as 4 µg/mL for *Escherichia coli* ATCC 11229 and *Klebsiella pneumoniae* ATCC 27736, 8µg/mL for *Enterobacter cloacae* ATCC 10699 and *Bacillus subtilis* ATCC 10774, 8µg/mL for *Proteus mirabilis* ATCC 7002, 16 µg/mL for *Pseudomonas aeruginosa* ATCC 27853, and 64 µg/mL for *Enterobacter aerogenes* ATCC 13048, while no antimicrobial activity was observed against *Streptococcus faecalis* ATCC 14428, *Staphylococcus aureus* ATCC 13709, *Salmonella typhi* ATCC 19430, and *Proteus vulgaris* ATCC 12454.

In the present study, however, not only standard strains but also food-derived and clinical isolates were included, and a much broader range of microorganisms was evaluated. Moreover, instead of relying solely on acetone, solvents with different polarities such as methanol, ethanol, and n-hexane were used in the extraction process. This approach demonstrates that both the type of solvent and the origin of the microorganism play a significant role in antimicrobial activity and suggests that *R. riparioides* may possess a broader and more diverse antimicrobial profile than previously reported. In both studies, *Enterobacter aerogenes* ATCC 13048 was tested; however, while Basile et al. (1998) reported activity of the acetone extract at 64 µg/mL, no antimicrobial effect was observed with the methanol, ethanol, and n-hexane extracts used in the present study.

Overall, this study demonstrated that extracts obtained from the moss *R. riparioides* exhibit significant antimicrobial and antibiofilm activities, which are highly influenced by solvent polarity

and the resulting phytochemical profiles. The n-hexane extract, characterized predominantly by tris(2,4-di-tert-butylphenyl) phosphate (79.29%), showed notable antimicrobial and antibiofilm effects at very low concentrations, suggesting that this compound is primarily responsible for the observed biological activity. The methanol extract displayed a broader and balanced phytochemical composition, including bioactive compounds such as neophytadiene, palmitic acid, and linolenic acid, which contributed to its effective broad-spectrum antimicrobial and antibiofilm performance. In contrast, the ethanol extract showed moderate activity predominantly against gram-positive bacteria, likely driven by its high neophytadiene content. These findings highlight the importance of selecting appropriate extraction solvents to target desired biological activities. The potent antimicrobial and antibiofilm properties of *R. riparioides* extracts, particularly from n-hexane and methanol solvents, position this moss as a promising natural source for developing novel antimicrobial and antibiofilm agents, paving the way for future pharmacological studies and practical applications.

Declarations

Authors' contributions

Idea/Concept: MEB, KC, CY. Conceptualization and design: CY, DT, AB. Auditing/ Consulting: KC, AB, DT, CY. Resources: ADU, MEB, KOD. Materials: ADU, MEB, ED. Data Collection & Processing: DT, ADU, GG. Analysis & Interpretation: GG, KOD, ED, GG. Literature Review: GG, KOD, ED. Writing: CY, GG, GG. Critical Review: KC.

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Conflict of interest

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

Ethics approval and consent to participate

The authors declare that ethical approval was not required for this study.

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