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Rodrigo de Souza Miranda

UFRJ: Universidade Federal do Rio de Janeiro

Marcos Vinicius Toledo e Silva

UFRJ FF: Universidade Federal do Rio de Janeiro Faculdade de Farmacia

Stephany Mendes Liporaci

UFRJ FF: Universidade Federal do Rio de Janeiro Faculdade de Farmacia

Ivana Correa Ramos Leal

UFRJ FF: Universidade Federal do Rio de Janeiro Faculdade de Farmacia

Roberto Carlos Martins

roberto@ippn.ufrj.br

UFRJ: Universidade Federal do Rio de Janeiro <https://orcid.org/0000-0002-2325-7912>

Rodrigo de Souza de Souza Miranda

UFRJ: Universidade Federal do Rio de Janeiro

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Bioassay-guided metabolomic profiling reveals flavanone-rich fractions as antibacterial agents in *Metrodorea nigra* (Rutaceae)

Authors

Rodrigo de Souza Miranda^{1,2} (0009-0002-0839-4836), Marcos Vinicius Toledo e Silva² (0000-0001-9105-1935), Stephany Mendes Liporaci¹ (0009-0000-8941-1858), Ivana Correa Ramos Leal² (0000-0002-6917-9323), Roberto Carlos Campos Martins¹ (0000-0002-2325-7912).

Affiliations

¹ Instituto de Pesquisa de Produtos Naturais Walter Mors, Universidade Federal do Rio de Janeiro, , Rio de Janeiro, RJ, Brazil.

² Departamento de Produtos Naturais e Alimentos, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

Contact: Roberto Carlos Campos Martins (roberto@ippn.ufrj.br)

Abstract

The increasing prevalence of multidrug-resistant bacteria highlights the urgent need for novel antimicrobial agents from natural sources. This study investigated the antibacterial, antibiofilm, and antioxidant activities of *Metrodorea nigra* leaves through a bioassay-guided approach combined with UHPLC-MS/MS and feature-based molecular networking (FBMN). The dichloromethane fraction and its subfraction Sp1D exhibited the highest antibacterial activity, with minimum inhibitory concentrations as low as 16–32 µg/ml against Gram-positive strains, including *Staphylococcus aureus*. Sp1D also demonstrated bactericidal effects and strong inhibition of biofilm formation. Metabolomic analysis revealed a predominance of flavonoids and identified pinostrobin, supported by NMR and MS/MS data, as a major constituent, along with the annotation of rhodomyrtone. FBMN analysis suggested a positive correlation between antibacterial activity and aglycone flavanones, whereas glycosylated flavonoids were mainly associated with inactive samples, indicating the influence of structural features on bioactivity. Antioxidant assays confirmed significant radical scavenging and reducing capacities, particularly for the crude extract and dichloromethane fraction. These findings expand the phytochemical knowledge of *M. nigra*, including the first report of flavanones in the genus, and highlight its potential as a source of bioactive compounds for the development of new antimicrobial agents.

Keywords: Metabolite annotation; Molecular networking; Bacterial resistance; Molecular networking

Introduction

Antibiotic resistance represents one of the most critical challenges in modern medicine, leading to persistent infections and increased mortality rates. Adaptive and selective mutations enable bacteria to develop diverse defense mechanisms, including target site modification, active efflux of antibiotics, and enzymatic inactivation of bioactive compounds. Consequently, many antimicrobial agents discovered decades ago have become increasingly ineffective due to their widespread use and the emergence of resistant strains. Therefore, the discovery of new bioactive compounds capable of overcoming multidrug-resistant bacteria remains a major priority in contemporary medicinal research (Nathan et al. 2014; Mundy et al. 2016).

In addition to mutation-driven resistance, the ability of certain microorganisms to form biofilms represents a significant obstacle in antimicrobial therapy. Biofilms are structured microbial communities embedded in a self-produced extracellular polymeric matrix composed of polysaccharides, proteins, and extracellular DNA. Compared to planktonic cells, biofilm-associated microorganisms exhibit enhanced survival and resistance due to improved surface adhesion, increased nutrient availability, cooperative stress responses, and reduced susceptibility to antimicrobial agents (Akinduti et al. 2022; Kang et al. 2022; Tao et al. 2022). Thus, biofilm formation constitutes an important survival strategy that further complicates infection control and treatment.

In response to this global health threat, the World Health Organization (WHO 2017) has established a priority list of multidrug-resistant pathogens to guide research and development of new antibiotics, categorizing them into critical, high, and medium priority groups. This list includes both Gram-positive and Gram-negative bacteria, such as carbapenem-resistant *Pseudomonas aeruginosa* and methicillin- and vancomycin-resistant *Staphylococcus aureus*. In this context, the search for novel antimicrobial agents is of paramount importance, and Brazil's vast biodiversity represents a valuable and largely untapped source of bioactive compounds.

Brazil harbors one of the richest floras worldwide, with numerous species still unexplored in terms of their chemical composition and pharmacological potential. *Metrodorea nigra* A. St.-Hil., a tropical species belonging to the Rutaceae family, is endemic to Brazil and widely distributed, particularly in the Atlantic Forest biome. This species is commonly known by several vernacular names, including "carrapateiro", "caputuna", "laranja-selvagem", and "chupa-ferro" (Pirani 2015).

Despite its broad distribution, the phytochemical and pharmacological properties of *M. nigra* remain poorly investigated. To date, only a limited number of studies have addressed the chemistry of the genus *Metrodorea*, reporting coumarins, terpenes, and alkaloids as major constituents, along with minor occurrences of saponins and phenolic acids (Baetas et al. 1999; Ferreira et al. 2006; Spletozer et al. 2015; Madeiro et al. 2017). The only phytochemical study specifically focused on *M. nigra* described the isolation of dihydrochalcones, coumarins, and a furoquinoline alkaloid from fruits, leaves, and peels; however, no biological activity assays were performed (Muller et al. 1995).

Within this framework, the present study aimed to evaluate the antimicrobial activity of the ethanolic extract of *M. nigra* leaves and its subfractions against clinically relevant multidrug-resistant bacteria, as well as to characterize their secondary metabolites using UHPLC-MS/MS. The exploration of Brazilian biodiversity, combined with bioassay-guided approaches, may provide valuable insights for the

discovery of novel antimicrobial agents and contribute to the development of alternative therapeutic strategies.

Material and methods

General experimental procedures

Plant material

Approximately 600 g of leaves and bark of *Metrodorea nigra* were collected in March 2018 at the Rio de Janeiro Botanical Garden (Rio de Janeiro, Brazil; coordinates: -22.923645, -43.454365) and taxonomically identified by Prof. Marcelo Trovó. A voucher specimen (Marquete O. 606) was deposited in the Herbarium of the Rio de Janeiro Botanical Garden (RB). This study was conducted in accordance with Brazilian regulations on genetic heritage and was authorized by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN; registration number A2EA1F4).

Preparation of the extracts and fractions

The leaves of *M. nigra* were dried in a ventilated oven at 40.0 °C for 48 h and subsequently ground using a knife mill to obtain 64.0 g of dried plant material. The crude extract was prepared by exhaustive maceration of the powdered leaves with absolute ethanol (250 ml), with solvent renewal every 24 h until complete extraction was achieved. The combined extracts were concentrated under reduced pressure using a rotary evaporator, yielding 7.9 g of crude ethanolic extract. This extract was subjected to liquid-liquid partitioning, affording four fractions according to increasing polarity: hexane (HEX-F), dichloromethane (DCM-F), ethyl acetate (EA-F), and aqueous (AQ-F). The crude extract and all fractions were evaluated for antibacterial activity through determination of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and inhibition of biofilm formation (IBF). The most active fraction (DCM-F) was further purified by column chromatography on Sephadex®, using methanol as the mobile phase, resulting in seven subfractions. Among these, subfractions Sp1B, Sp1C, Sp1D, and Sp1F were obtained in sufficient amounts for subsequent biological evaluation and chemical characterization, focusing on the identification of the compounds responsible for the observed activity.

GNPS MS/MS dereplication and Molecular Networking

Data was obtained using ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS). Analyses were performed in a Thermo Dionex Ultimate 3000 UHPLC coupled to a Thermo LCQFleet Ion Trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). An Acquity BEH C18 column (2.1 mm x 50 mm x 1.7µm, Waters, Millford, MA) was used in a flow rate of 0.3 ml/min. The column temperature was 40.0 °C and the mobile phase was water containing 0.1% formic acid (A) and acetonitrile (B). The gradient elution program is as follows: 0 to 5 min, 5% of B, 5 to 35 min, 5 to 95% of B, 35 to 40 min, 95% of B, 40 to 40.1 min, 95 to 5% and 40.1 to 45 min, 5% of B. The MS was equipped with an electrospray source (ESI) and was operated in both positive and negative polarities. High purity nitrogen (N₂) was used as sheath (35 arb) and auxiliary gas (10 arb), and high purity Helium

was used as collision gas. The MS parameters were as follows: source voltage 5kV, capillary voltage 4/-19 V, tube lens voltage 35/-66 V, and capillary temperature 400.0 °C. Mass spectra were acquired in full range acquisition, covering 100 to 1000 m/z, and fragments were obtained by Data Dependent Scan with a normalized collision-induced dissociation (CID) energy of 30%.

UHPLC-MS/MS data were processed using MZmine version 2.53. The resulting datasets were uploaded to the GNPS platform for dereplication and construction of feature-based molecular networks (FBMN). A metadata file was generated to classify samples according to their biological activity and incorporated into the molecular networking workflow. Following data processing, the annotated results were retrieved and critically assessed through comparison with literature data to validate compound assignments. The FBMN outputs were subsequently visualized and analyzed using Cytoscape software to support the interpretation of chemical relationships and their correlation with biological activity.

Antibacterial assays

The minimum inhibitory concentration (MIC) of the plant-derived samples was determined using the broth microdilution method in 96-well microplates, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016). The minimum bactericidal concentration (MBC) was assessed according to the methodology described by Isenberg (1988), using four concentration levels corresponding to sub-MIC, MIC, 2×MIC, and 4×MIC. Results for both assays were expressed in µg/ml. The inhibition of biofilm formation (IBF) was evaluated for the crude extract of *M. nigra* leaves, as well as for its hexane (HEX-F), dichloromethane (DCM-F), ethyl acetate (EA-F), aqueous (AQ-F) fractions, and subfraction Sp1D, at a fixed concentration of 128 µg/ml. The assay was performed against *Staphylococcus aureus* (ATCC 33591 and ATCC 29213) and *Staphylococcus epidermidis* (ATCC 35984), using an adapted protocol based on Stepanović et al. (2007). Bacterial suspensions were standardized to a final concentration of 10⁷ CFU/ml. Biofilm formation was conducted in tryptic soy broth (TSB) for *S. epidermidis* and in TSB supplemented with 2% glucose for *S. aureus*. Each well of a 96-well microplate was prepared with 160 µl of culture medium, 20 µl of bacterial inoculum, and 20 µl of the test sample. Plates were incubated at 37.0 °C for 24 h to allow biofilm development and surface adhesion. Following incubation, non-adherent cells were removed by washing with phosphate-buffered saline (PBS, pH 7.4), and the remaining attached biomass was fixed by heating at 60.0 °C for 1 h. The biofilms were then stained with 2% crystal violet solution for 15 min at room temperature, followed by rinsing with distilled water to remove excess dye. After drying at 60.0 °C for 15 min, the bound dye was solubilized with 150 µl of 95% ethanol for 30 min at room temperature. Absorbance was measured at 570 nm using a microplate reader (Stepanović et al. 2007; Valente et al. 2021).

Bacterial Growth curve

The bacterial growth curve assay was performed following an adapted protocol based on Hall et al. (2014), aiming to evaluate the effect of the samples on bacterial proliferation over time. *Staphylococcus aureus* strains (ATCC 33591 and ATCC 29213) were initially cultured on Mueller–Hinton agar plates and incubated at 37.0 °C for 24 h. Subsequently, isolated colonies were transferred to 3 ml of brain heart infusion (BHI) broth and incubated at 37.0 °C under agitation (100 rpm) for 16 h to obtain an overnight

culture. The bacterial suspension was then adjusted by measuring the optical density at 600 nm (OD₆₀₀) and diluting in BHI to a standardized OD of 0.05. The assay was conducted in 96-well microplates using three concentrations of the samples (0.5×MIC, MIC, and 2×MIC). Each well received 20 µl of the test sample and 180 µl of bacterial suspension in BHI, with all experiments performed in triplicate. Appropriate positive (growth control) and negative (sterility control) controls were included. Microplates were incubated at 37.0 °C under orbital shaking (100 rpm), and bacterial growth was monitored by measuring absorbance at 630 nm using a microplate reader. Readings were taken every 30 min during the first 3 h and subsequently at hourly intervals up to 8 h. Growth kinetics were expressed as absorbance over time, allowing evaluation of the impact of the samples on different phases of bacterial growth.

Antioxidant assays

Antioxidant activity was evaluated using DPPH, ABTS, and FRAP assays. The DPPH radical scavenging assay was performed according to Mensor et al. (2001), while the ABTS assay followed a modified protocol based on Re et al. (1999). Results for both methods were expressed as percentage of inhibition and EC₅₀ values. The ferric reducing antioxidant power (FRAP) assay was conducted as described by Benzie and Strain (1996), with results expressed as Fe²⁺ equivalents. All experiments were performed in triplicate.

Results and discussion

Bacterial strains, including ATCC and clinical isolates selected according to the WHO priority list (Supplementary Tables 1 and 2), were used to evaluate the antimicrobial activity of the ethanolic extract of *Metrodorea nigra* leaves and its fractions. A bioassay-guided approach was employed to identify active constituents, combining antibacterial assays with metabolomic profiling by UHPLC-MS/MS, NMR, and chemometric analyses.

Antimicrobial results.

Both the crude extract of *M. nigra* leaves and its dichloromethane fraction (DCM-F), obtained by liquid–liquid partitioning, exhibited significant antibacterial activity in MIC assays against *Staphylococcus aureus* (ATCC 33591 and ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus saprophyticus* (ATCC 15305). In contrast, *S. aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 35984), and *Klebsiella pneumoniae* (ATCC 700603) showed moderate to high resistance to the tested samples. The DCM-F was further subjected to Sephadex LH-20® column chromatography using isocratic methanol as the mobile phase, yielding subfractions Sp1B, Sp1C, Sp1D, and Sp1F. Among these, Sp1D exhibited the highest antibacterial activity, with a minimum inhibitory concentration of 32 µg/ml.

Table 1: MIC/MBC results of *M. nigra* crude extract and its fractions against standard ATCC strains

Bacterial strain	ATCC 33591 <i>S.</i> <i>aureus</i>	ATCC 29213 <i>S.</i> <i>aureus</i>	ATCC 25923 <i>S.</i> <i>aureus</i>	ATCC 27853 <i>P.</i> <i>aeruginosa</i>	ATCC 15305 <i>S.</i> <i>saprophyticus</i>	ATCC 12228 <i>S.</i> <i>epidermidis</i>	ATCC 35984 <i>S.</i> <i>epidermidis</i>	ATCC 700603 <i>K.</i> <i>pneumoniae</i>
Gram	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Negative
<i>M. nigra</i> fractions	MIC (µg/ml)							
Crude extract (MnBF1)	64	64	128	16	32	> 512	256	512
Hexane fraction (HEX-F)	> 512	> 512	> 512	256	512	> 512	512	> 512
Dichloromethane fraction (DCM-F)	32/64*	64/64*	256	16/32*	32	32/64*	≥ 128	512
Ethyl Acetate (EA-F)	512	> 512	> 512	128	128	> 512	>512	> 512
Aqueous (AQ-F)	> 512	> 512	-	> 512	> 512	> 512	-	-
Subfractions obtained from dichloromethane fraction purification								
Sp1 B	> 512	> 512	-	512	512	> 512	-	-
Sp1 C	64	128	-	32	64	64	-	-
Sp1 D	32/64*	32/64*	32	32/32*	32/64*	16/64*	256/256*	512/>512*
Sp1 F	256	≥ 256	-	256	≥ 256	≥ 256	-	-

*Fractions also subjected to Minimal Bactericidal Concentration (MBC) assay – Results in µg/ml.

Both DCM-F and Sp1D were evaluated for minimum bactericidal concentration (MBC) to determine their mode of antimicrobial action. The results indicated that Sp1D exhibited predominantly bactericidal effects against most tested strains, with MBC values equal to or up to one dilution above the corresponding MIC values. In contrast, *Klebsiella pneumoniae* (ATCC 700603) was resistant to all samples (MIC = 512 µg/ml) and was the only strain for which Sp1D showed a bacteriostatic effect (Table 1). Based on the activity observed against ATCC strains, selected samples (crude extract, DCM-F, Sp1C, and Sp1D) were further evaluated against clinical isolates. Three *Staphylococcus aureus* strains (517, 664, and 1025), obtained from different clinical sources, and one *Pseudomonas aeruginosa* strain (i059) were tested. The results (Table 2) confirmed the susceptibility of *S. aureus* clinical isolates, with Sp1D showing the highest activity. Conversely, the *P. aeruginosa* clinical strain remained resistant to all samples (MIC = 512 µg/ml).

Table 2: MIC results of *M. nigra* crude extract and its fractions against clinical bacterial strains (µg/ml)

Metrodorea nigra Fractions	Clinical bacterial strain	<i>Staphylococcus aureus</i>			<i>Pseudomona aeruginosa</i>
		517	664	1025	i059
	Gram	Positive	Positive	Positive	Negative
Crude ethanolic extract		64	128	64	512
Dichloromethane fraction		64	64/128*	32	512

Subfractions obtained from dichloromethane fraction purification				
SP1 C	64	128	32	512
SP1 D	16	32/128*	32	512

*Fractions also subjected to Minimal Bactericidal Concentration (MBC) assay – Results in µg/ml.

The ability of *M. nigra* leaf extracts and fractions to inhibit biofilm formation was evaluated using the crude ethanolic extract, dichloromethane (DCM-F), and ethyl acetate (EA-F) fractions, as well as subfractions Sp1C, Sp1D, and Sp1F, against *Staphylococcus aureus* (ATCC 33591 and ATCC 29213) and *Staphylococcus epidermidis* (ATCC 35984).

The crude extract showed marked inhibition of *S. aureus* biofilm formation, with activity primarily associated with the DCM-F. In contrast, EA-F exhibited lower activity. Subfractions derived from DCM-F (Sp1C, Sp1D, and Sp1F) inhibited *S. aureus* biofilm formation by more than 80%. Notably, Sp1D completely inhibited biofilm formation in both *S. aureus* strains and was the only sample to significantly inhibit *S. epidermidis* (~80%). These findings, together with MIC and MBC results, indicate that Sp1D is the most bioactive fraction.

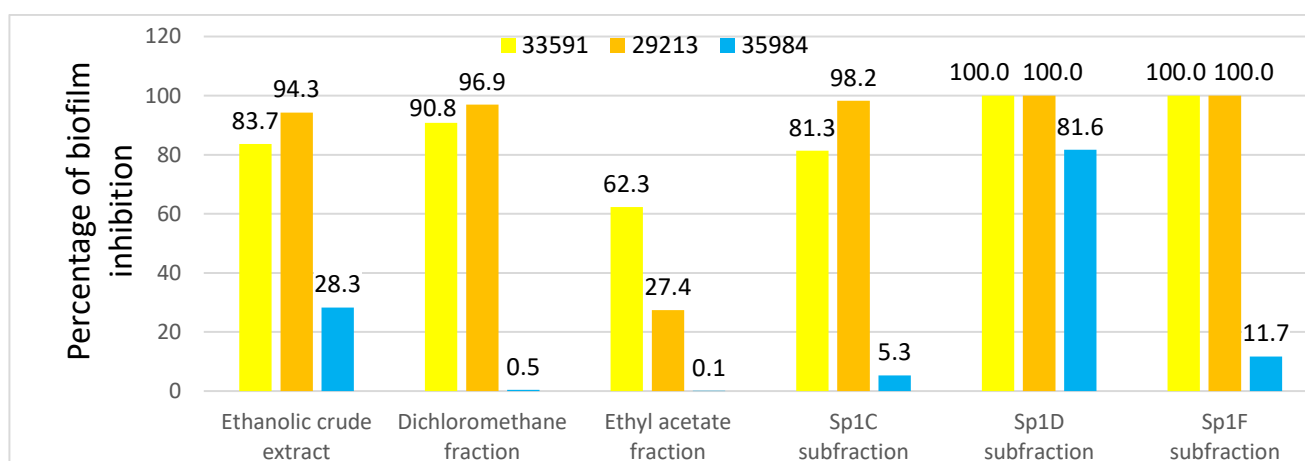


Fig 1. Percentage of inhibition of *M. nigra* fractions (in triplicates) against bacterial biofilm formation on strains ATCC 33591, 29213 and 35984 at 570 nm.

* Inhibition (%) of bacterial biofilm formation for *M. nigra* fractions. Absorbance of samples (128 µg/ml) obtained in triplicate (At) at 570 nm. Positive control of bacteria + medium (C+), negative controls of pure medium (C-) and medium + sample (C-#), obtained in triplicate. The percentage of inhibition was determined in excel using the formula $(C+ - (At - (C-# - C-)))/C+$. Standard deviation calculated on the values of each single and expressed in the graph.

The effects of the ethanolic crude extract (MnBF1), dichloromethane fraction (DCM-F), and subfraction Sp1D on bacterial growth kinetics were evaluated against *Staphylococcus aureus* strains ATCC

29213 and ATCC 33591, as shown in the corresponding figures.

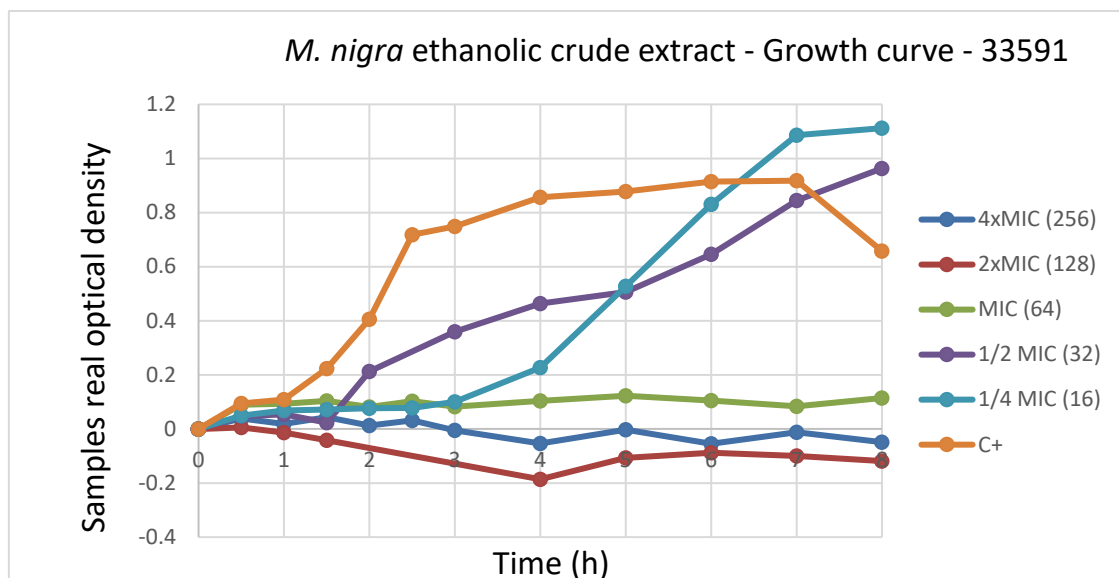


Fig 2. *M. nigra* ethanolic crude extract influence over bacterial growth curve inhibition for ATCC 33591

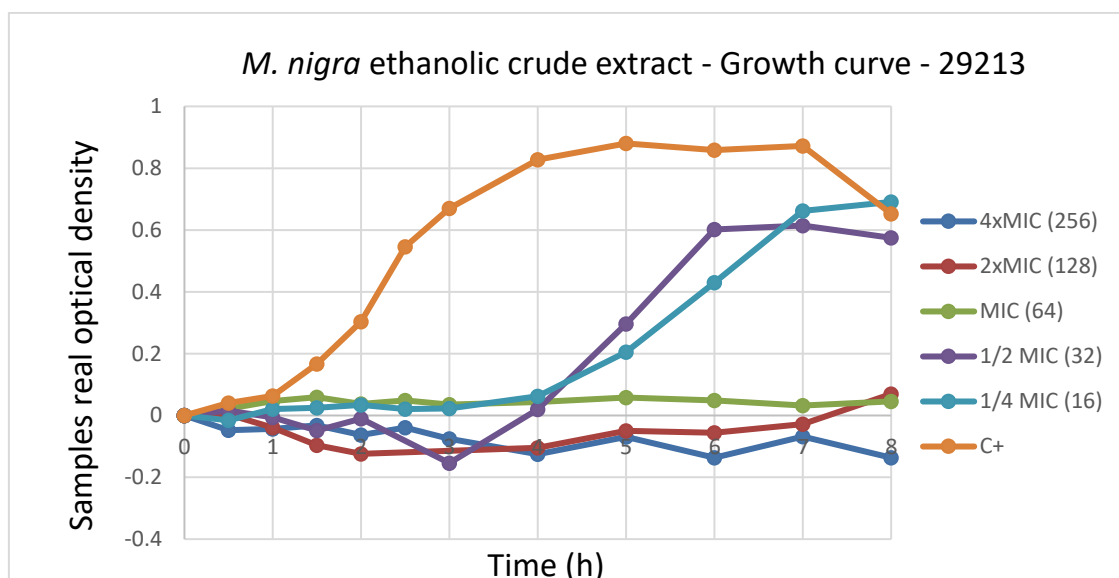


Fig 3. *M. nigra* ethanolic crude extract influence over bacterial growth curve inhibition for ATCC 29213

Growth curve analysis demonstrated that the ethanolic crude extract markedly suppressed the proliferation of both *S. aureus* strains (Fig. 3), particularly at MIC and supra-MIC levels, where bacterial growth was almost completely arrested. The delay observed at sub-MIC concentrations suggests interference with early cellular processes, likely associated with membrane destabilization and metabolic disruption. Such effects are consistent with the amphiphilic nature of phenolic compounds, which can

partition into lipid bilayers, increasing membrane permeability and impairing essential functions.

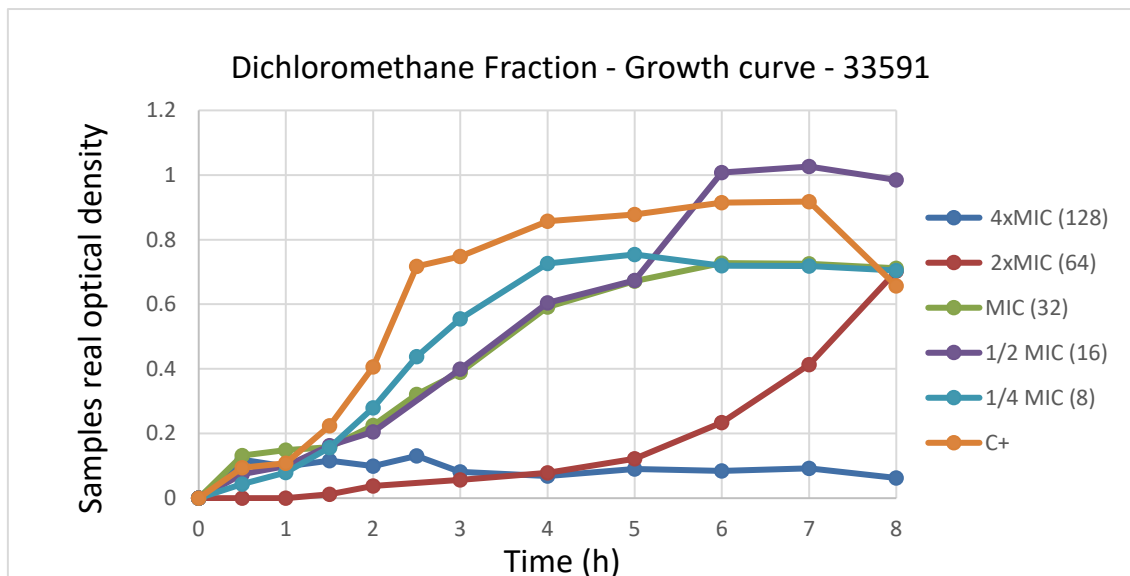


Fig 4. *M. nigra* dichloromethane partition influence over bacterial growth curve inhibition for ATCC 33591

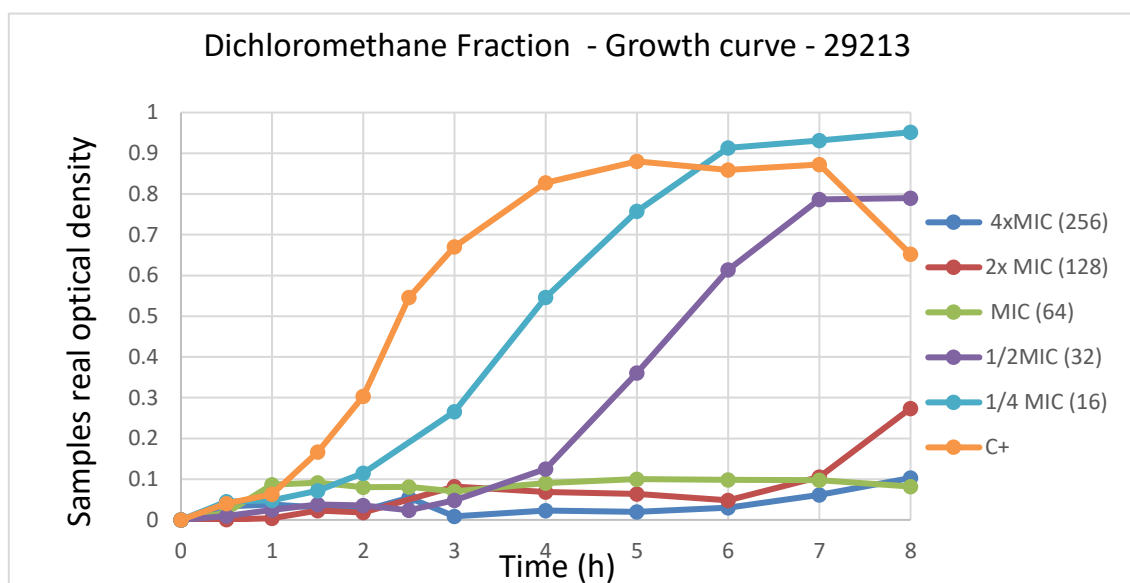


Fig 5. *M. nigra* dichloromethane partition influence over bacterial growth curve inhibition for ATCC 29213

The dichloromethane fraction (DCM-F) (Figs. 4–5) exhibited a similar but more selective profile, with sustained inhibition of ATCC 29213 and partial recovery in ATCC 33591 at lower concentrations. This behavior supports the enrichment of moderately lipophilic metabolites in DCM-F, particularly aglycone flavonoids, whose increased hydrophobicity enhances interaction with phospholipid membranes. Previous studies indicate that such compounds may disrupt membrane integrity, collapse proton gradients,

and interfere with membrane-associated enzymes, ultimately leading to growth inhibition.

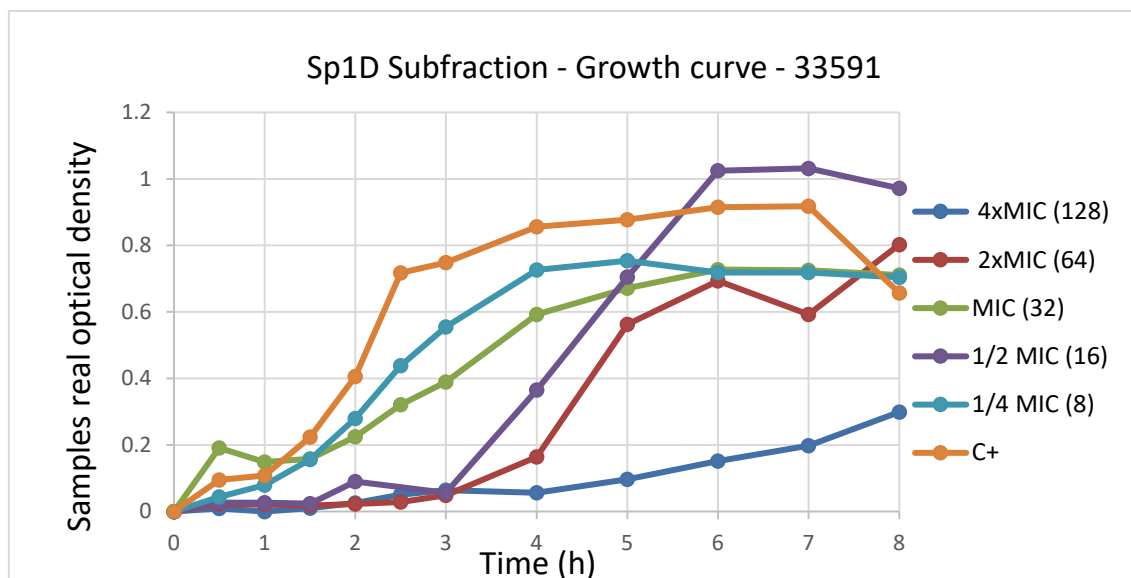


Fig 6. *M. nigra* Sp1D subfraction influence over bacterial growth curve inhibition for ATCC 33591

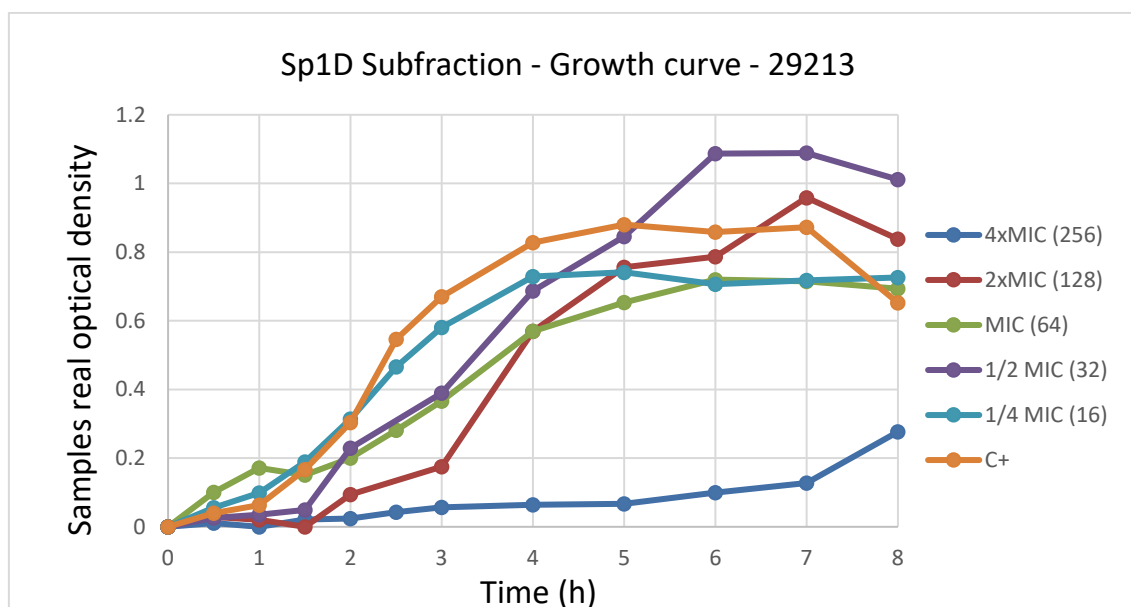


Fig 7. *M. nigra* Sp1D subfraction influence over bacterial growth curve inhibition for ATCC 29213

In contrast, the Sp1D subfraction (Fig. 6), although enriched in flavanones, showed reduced and less sustained inhibition, especially for ATCC 33591. This apparent decrease in efficacy may reflect the loss of synergistic interactions present in the parent fraction. Flavonoid SAR studies suggest that antibacterial activity is strongly influenced by structural features such as hydroxylation patterns, methoxylation, and glycosylation. In particular, aglycone flavanones (e.g., pinostrobin-like structures) exhibit enhanced membrane affinity and biological activity compared to their glycosylated counterparts, which are generally less permeable and therefore less active.

Taken together, these findings indicate that the antibacterial effect of *M. nigra* is not solely attributable to individual compounds, but rather to a combination of structurally complementary metabolites acting through membrane-targeted mechanisms and possibly additional intracellular targets.

Chemical composition results

The chemical composition of the bioactive fractions from *M. nigra* leaves was investigated by HPLC-DAD, UHPLC-MS/MS, and NMR analyses. The HPLC-DAD profile of the most active fraction (Sp1D) exhibited prominent absorption bands at 230–235 nm and 284–288 nm, characteristic of flavanone-type chromophores, indicating a predominance of flavonoid constituents (Sisa et al. 2010; Lin et al. 2012). Flavonoids are well-recognized antimicrobial agents, acting primarily through membrane disruption and interference with essential cellular processes. Their amphiphilic nature facilitates interaction with phospholipid bilayers, while their capacity to bind proteins enables modulation of enzymes, transporters, and regulatory pathways, ultimately compromising microbial viability (Shamsudin et al. 2022; Tao et al. 2022). The enrichment of flavonoids in Sp1D is consistent with its pronounced antibacterial and antibiofilm activities, as well as its bactericidal behavior. Flavonoid enrichment in Sp1D aligns with its strong antibacterial, antibiofilm, and bactericidal activities. Likewise, the activity observed for DCM-F likely reflects the accumulation of these moderately lipophilic phenolics.

Further purification of Sp1D by preparative HPLC yielded four subfractions, corresponding to peaks with retention times of 8.37, 9.01, 10.00, and 10.53 min (Fig. 8).

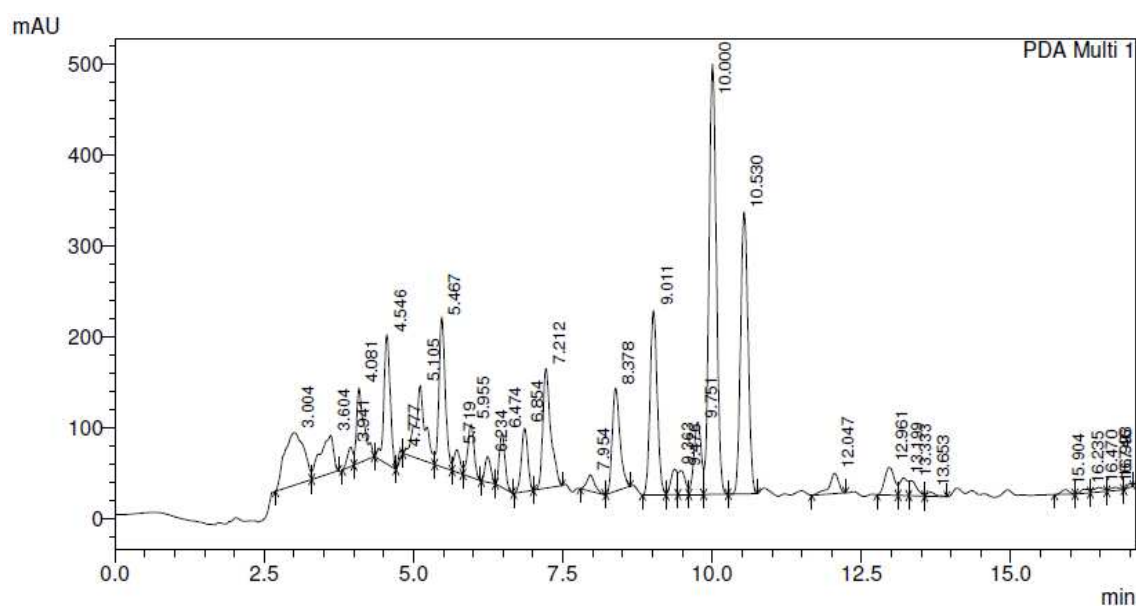


Fig. 8. HPLC-DAD chromatographic profile of the bioactive Sp1D subfraction from *M. nigra*

Reference flavonoid standards were analyzed by HPLC-DAD under identical conditions to those used for Sp1D, allowing comparison of retention times. Several peaks matched those of known compounds, suggesting the presence of 6-hydroxynaringenin/naringenin (1/2), sakuranetin (3), pinocembrin (4), and pinostrobin (5).

The compound eluting at 8.3 min was isolated by preparative HPLC in high purity and structurally elucidated by 1D and 2D NMR experiments, confirming its identity as the flavanone pinostrobin (5) (table 3).

Table 3: Comparison of δ values, obtained experimentally, with those observed in the literature for pinostrobin (**5**), in the 1D and 2D NMR spectra.

C	¹ H NMR		¹³ C NMR	
	<i>Experimental</i> CD ₃ OD – 500MHz	<i>Literature</i> CDCl ₃ – 400MHz	<i>Experimental</i> CD ₃ OD – 500MHz	<i>Literature</i> CDCl ₃ – 400MHz
2	5.49 ppm (<i>dd</i> , $J = 12.7$ Hz e $J = 3.1$ Hz)	5.43 ppm (<i>t</i> , $J = 6.7$)	79.08 ppm	79.25 ppm
3-H₁	3.12 ppm (<i>dd</i> , $J = 17.2$ Hz e $J = 12.7$ Hz)	3.10 ppm (<i>trans</i>)	42.71 ppm	43.36 ppm
3-H₂	2.81 ppm (<i>dd</i> , $J = 17.2$ Hz e $J = 3.1$ Hz)	2.81 ppm (<i>cis</i>)	42.71 ppm	43.36 ppm
4	-	-	193.33 ppm	195.79 ppm
5	-	-	163.7 ppm	163.16 ppm
6	6.05 ppm (<i>d</i> , $J = 2.3$ Hz)	6.04 ppm	94.36 ppm	95.49 ppm (C-6, C-8)
7	-	-	168.15 ppm	164.36 ppm
8	6.09 ppm (<i>d</i> , $J = 2.3$ Hz)	6.03 ppm	93.05 ppm	95.49 ppm (C-6, C-8)
9	-	-	163.03 ppm	164.63 ppm
10	-	-	102.63 ppm	103.20 ppm
1'	-	-	138.8 ppm	138.30 ppm
2'	7.5 a 7.3 ppm (<i>m</i>)	7.28-7.49 ppm (H-2'-H6')	125.93 ppm	126.17ppm (C-2', C-6')
3'	7.5 a 7.3 ppm (<i>m</i>)	7.28-7.49 ppm (H-2'-H6')	128.29 ppm	128.92 ppm (C-3', C-4', C-5')
4'	7.5 a 7.3 ppm (<i>m</i>)	7.28-7.49 ppm (H-2'-H6')	128.24 ppm	128.92 ppm (C-3', C-4', C-5')
5'	7.5 a 7.3 ppm (<i>m</i>)	7.28-7.49 ppm (H-2'-H6')	128.29 ppm	128.92 ppm (C-3', C-4', C-5')
6'	7.5 a 7.3 ppm (<i>m</i>)	7.28-7.49 ppm (H-2'-H6')	125.93 ppm	126.17 ppm (C-2', C-6')
5-HO	4.57 ppm (<i>s</i>)	12.07 ppm	-	-
7-OMe	3.81 ppm (<i>s</i>)	3.86 ppm	54.8 ppm	55.60 ppm

*Singlet (*s*); Duplet (*d*); Double duplet (*dd*); Triplet (*t*); Multiplet (*m*).

Literature reference NMR values for pinostrobin can be found at Nyokat et al. 2017.

UHPLC-MS/MS data from the crude extract, fractions, and subfractions were analyzed using the GNPS platform for dereplication. Library matching enabled the annotation of two compounds in Sp1D, namely the flavanone pinostrobin (**5**), previously confirmed by NMR, and the xanthone rhodomyrton (**6**).

Several additional features within this fraction remained unannotated, suggesting either the absence of corresponding entries in current databases or the presence of structurally novel flavonoid derivatives.

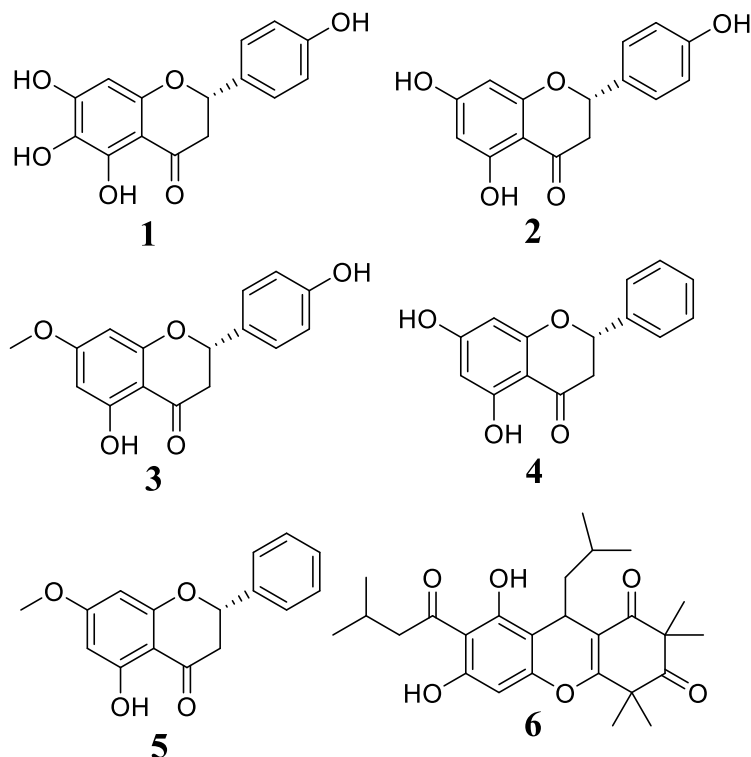


Fig.9. Dereplicated metabolites identified in the bioactive fractions of *Metrodorea nigra* leaves

Pinostrobin (5), first reported from *Pinus strobus* L., is a widely distributed flavanone found in natural products such as honey and propolis. It has also been isolated from plant sources including *Cajanus cajan* and *Boesenbergia pandurata*, which are currently considered among the richest sources of this compound. Pinostrobin exhibits a broad spectrum of pharmacological activities, including anti-inflammatory, antioxidant, anticancer, gastroprotective, antimicrobial, and antiviral effects (Patel et al. 2015).

Notably, it displays significant antibacterial activity against pathogenic strains such as *Staphylococcus aureus* and has been reported to enhance biofilm inhibition when combined with conventional antibiotics, such as ciprofloxacin, suggesting synergistic effects against *Escherichia coli* and *Pseudomonas aeruginosa* (Christena et al. 2015; Tasco et al. 2018). In addition, pinostrobin has demonstrated cytoprotective effects in ulcer models and a favorable safety profile, with no significant toxicity or mutagenicity observed in experimental studies (Charoensin et al. 2010; Rosdianto et al. 2020).

Rhodomyrton (6), a bioactive acylphloroglucinol originally isolated from *Rhodomyrton tomentosa*, has emerged as a potent antibacterial agent, particularly against multidrug-resistant Gram-positive pathogens such as MRSA and *Streptococcus pneumoniae* (Limsuwam et al. 2009; Mitsuwan et al. 2017; Nwabor et al. 2021). From a structure–activity perspective, its lipophilic scaffold, combined with

multiple hydroxyl functionalities, favors strong interactions with bacterial membranes and membrane-associated proteins.

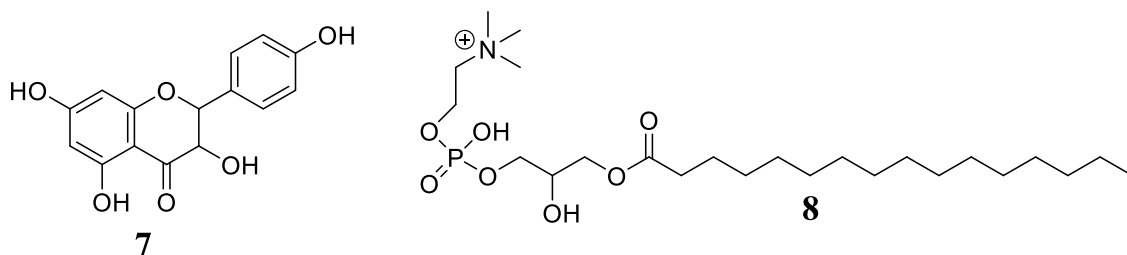
Mechanistically, rhodomyrtone disrupts membrane organization and induces alterations in lipid domains, impairing membrane fluidity and permeability. This effect is accompanied by broad metabolic interference, including inhibition of pathways related to DNA, RNA, protein, and lipid biosynthesis, ultimately compromising cell envelope integrity and cellular homeostasis (Bach et al. 2019; Nwabor et al. 2021). In addition, sub-inhibitory concentrations have been shown to attenuate virulence by reducing the production of key pathogenic factors, indicating a dual antibacterial and anti-infective mode of action (Limsuwan et al. 2011).

These properties highlight the importance of amphiphilicity and functional group distribution in governing the potent and multifaceted biological activity of rhodomyrtone.

As can be seen, both pinostrobin and rhodomyrtone express potent antimicrobial activity, which justifies, the inhibition observed in MIC, MBC and biofilm assays together with other compounds of the mixture, since compounds **2**, **3** and **4** also have antimicrobial activity related in literature (Salunkhe et al. 2023; Rasul et al. 2013; Park et al. 2014).

In addition to pinostrobin (**5**) and rhodomyrtone (**6**), GNPS-based dereplication revealed a flavonoid-rich chemical profile for *M. nigra*, with most annotated compounds belonging to flavone and flavanone subclasses, present as both aglycones and glycosides (e.g., kaempferol (**7**), rutin (**11**), 5,6,2-trimethoxyflavone (**14**), and gancaonin E (**17**)).

From a structure–activity standpoint, this chemical diversity is particularly relevant, as subtle structural variations—such as hydroxylation patterns, methoxylation, and glycosylation—are known to modulate lipophilicity, membrane permeability, and target interactions, thereby influencing antibacterial efficacy. In general, aglycone flavonoids tend to exhibit higher bioactivity due to enhanced membrane affinity, whereas glycosylated derivatives often display reduced activity but may contribute to antioxidant effects. A comprehensive list of annotated metabolites and their spectral data is provided in the Supplementary Material.



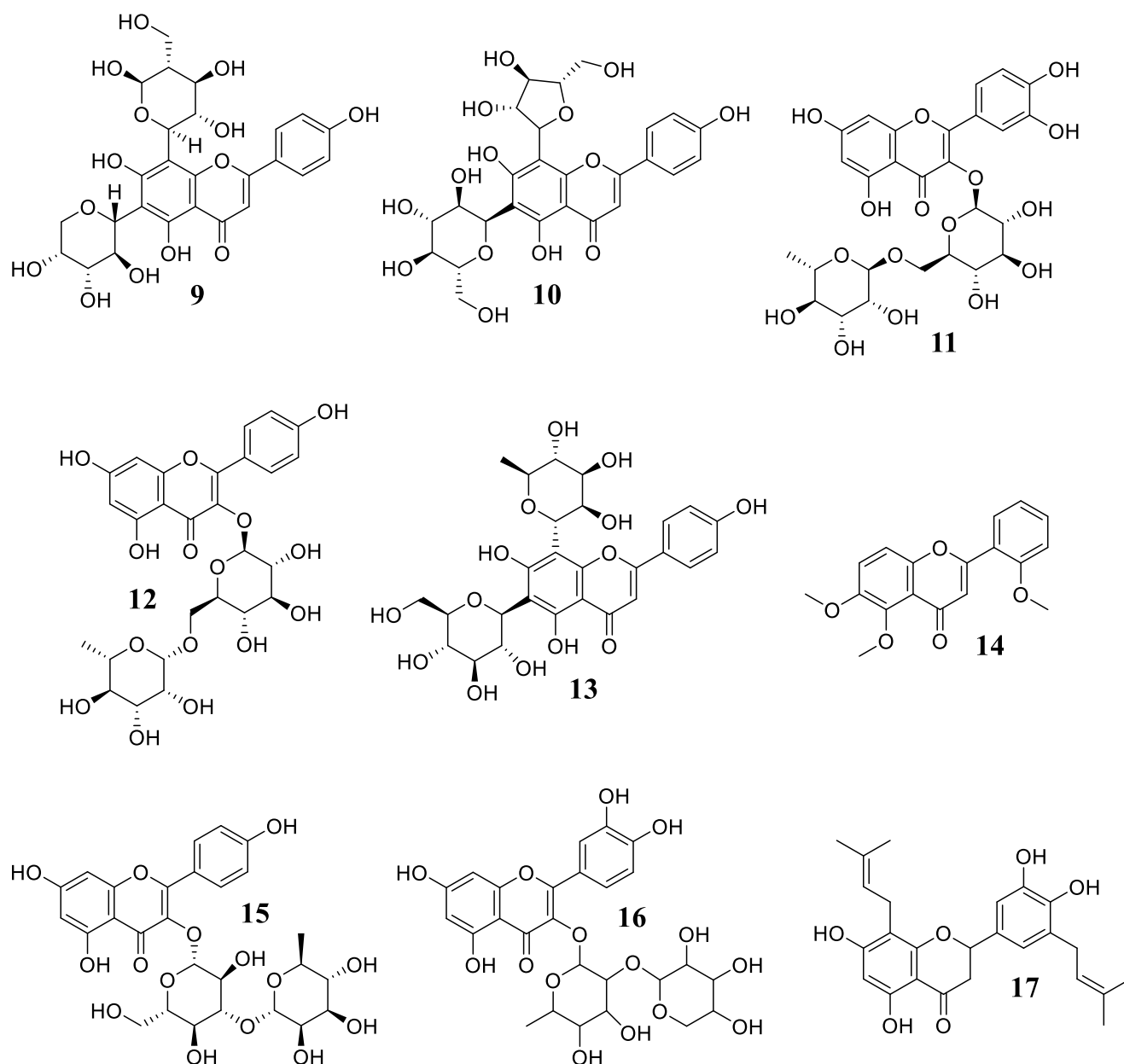


Fig. 10. Chemical structures of flavonoids and related metabolites (7–17) annotated by UHPLC-MS/MS dereplication in *M. nigra* bioactive fractions

Antioxidant results.

Antioxidant activity, assessed by DPPH, ABTS, and FRAP assays, was significant across most *M. nigra* samples. The crude extract (MnBF1) and dichloromethane fraction (DCM-F) showed the highest DPPH scavenging activity ($EC_{50} = 6.08 \pm 0.90$ and 6.79 ± 1.80 $\mu\text{g/ml}$), surpassing rutin. In the ABTS assay, DCM-F and subfraction Sp1D were the most active ($EC_{50} = 2.76 \pm 0.03$ and 2.42 ± 0.07 $\mu\text{g/ml}$), indicating strong radical cation neutralization. FRAP results revealed MnBF1 as the most potent reducing agent ($5,432.8 \pm 305.7$ $\mu\text{mol Fe}^{2+}/\text{g}$), consistent with its broader metabolite diversity.

The activity profile correlates with flavonoid composition: DCM-F and Sp1D are enriched in aglycone flavanones (e.g., naringenin, pinocembrin, pinostrobin), while EA-F contains mainly glycosylated

flavonoids, which retain redox activity but generally exhibit lower antimicrobial potency. In contrast, HEX-F showed negligible activity, likely due to its nonpolar composition and limited compatibility with the assay systems. Detailed results are provided in the Supplementary Material.

Dereplication and Feature-based molecular networking (FBMN)

Due to the limited phytochemical information available for *M. nigra*, LC-MS/MS-based dereplication was performed to annotate metabolites potentially associated with the observed antimicrobial activity. A total of 12 compounds were tentatively identified (Table 4), predominantly flavonoids, detected in both positive and negative ionization modes.

This chemical profile is consistent with the biological results, as flavonoids are widely recognized for their antibacterial properties. Structure–activity studies indicate that lipophilicity is a key determinant of activity against Gram-positive bacteria, facilitating interaction with and disruption of phospholipid membranes, which represent the primary site of action (Yuan et al. 2022). In contrast, Gram-negative bacteria generally exhibit higher resistance due to the presence of an outer membrane barrier; however, flavonoids may still exert activity through additional mechanisms, including enzyme inhibition (e.g., DNA gyrase) and interference with intracellular targets (Yan et al. 2024).

Table 4: Annotated molecules by Feature-based molecular networking in modes positive and negative

Item	[M+H] ⁺	[M-H] ⁻	MS ² (+)	MS ² (-)	Activity in MN	Compound
1	271.2	-	(167), 131	-	Active	Pinostrobin chalcone
2	271.2	-	(167), 131, 103	-	Active	(+)-Pinostrobin
3	433.0	-	(415), 397, 367, 337, 313	-	Inactive	Isovitexin
4	271.9	-	(167), 131	-	Non tested	Strobopinin
5*	565.0	563.1	(547), 529, 511, 499, 481, 445	545, 503, 473, (443), 383, 353	Inactive	Isoschaftoside
6	-	609.2	-	(301), 271, 255	Inactive	Rutine
7	-	593.1	-	(285), 257, 229	Inactive	Kaempferol 3- <i>O</i> -rhamnopyranosil
8*	-	593.1	-	327, (285), 255	Inactive	Kaempferol 3- <i>O</i> -rutinoside
9	-	545.0	-	371, (353), 341, 338	Inactive	3-[[3-[2,3-Dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-5-benzofuranyl]-1-oxo-2-propen-1-yl]oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid (ACI)
10	-	577.2	-	487, (457), 413, 383, 353	Inactive	Isoviolanthin
11*	-	563.1	-	545, 503, 473, 443, (383), 353	Inactive	Schaftoside
12	-	577.2	-	559, 503, 473, 457, 383, (353)	Inactive	Violanthin

*Compounds with no absolute chemistry described in the GNPS database annotation.

Given the bioassay-guided nature of this study, antimicrobial data were integrated with MS/MS profiles from the crude extract and derived fractions to construct molecular networks (MN), enabling the correlation of chemical features with biological activity. Feature-based molecular networking (FBMN), a quantitative approach, allowed the relative distribution of metabolites across active and inactive samples to be assessed, thereby facilitating the identification of compounds potentially responsible for the observed activity (Nothias et al. 2020).

Two FBMNs were generated, corresponding to positive and negative ionization modes, encompassing all samples. This strategy enabled the prioritization of candidate bioactive metabolites and supported the annotation of previously unassigned features based on spectral similarity within molecular clusters. By integrating node abundance with bioactivity metadata, it was possible to associate specific chemical entities with active, inactive, or untested fractions, and to infer the potential contribution of structurally related compounds.

In the positive ion mode network (Fig. 11), two major flavonoid families were identified: C-glycosylated flavones (cluster 11) and aglycone flavanones (cluster 15). The former were predominantly associated with inactive fractions, indicating a limited contribution to antibacterial activity. In contrast, aglycone flavanones were enriched in active or untested samples, suggesting a positive correlation with bioactivity. This trend is consistent with reported structure–activity relationships, in which aglycones exhibit enhanced membrane affinity and antimicrobial potency compared to their glycosylated counterparts (Shamsudin et al. 2022). Notably, the isolation of pinostrobin and its significant antibacterial activity further supports the central role of flavanone aglycones in the observed biological effects.

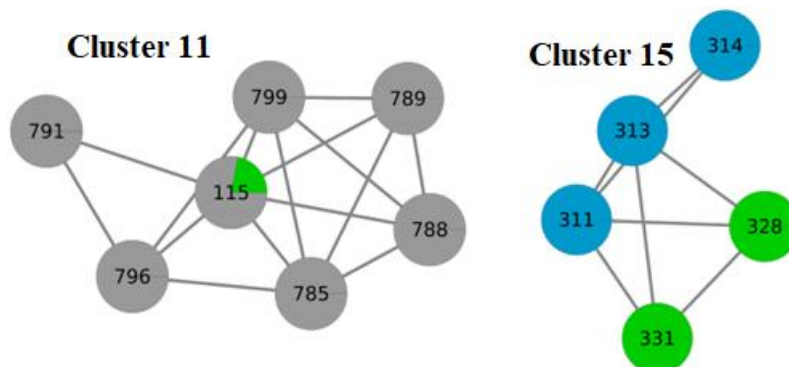


Fig. 11. Clusters 11 and 15 obtained in FBMN under positive mode.

*Antimicrobial activity: Green = active; Gray = inactive; Blue = non tested; ** Node identification: 115 – Isoshaftoside; 311 – Strobopinin; 331 – Pinostrobin; 228 – Pinostrobin chalcone; 785 – Isovitetxin.

In the negative ion mode FBMN (Fig. 12), two major flavonoid families were identified: O-glycosylated flavonols (cluster 5) and C-glycosylated flavones (cluster 4). In both clusters, nodes were predominantly associated with inactive samples, indicating a limited contribution of these metabolites to the antibacterial activity.

Although flavonoids are widely recognized as bioactive compounds with antimicrobial potential, structure–activity relationship studies demonstrate that glycosylation generally reduces their efficacy. This

reduction is primarily attributed to decreased lipophilicity, which limits membrane permeability, as well as altered interactions with intracellular targets (Shamsudin et al. 2022). These factors likely explain the low activity observed for glycosylated flavonoids in the present study.

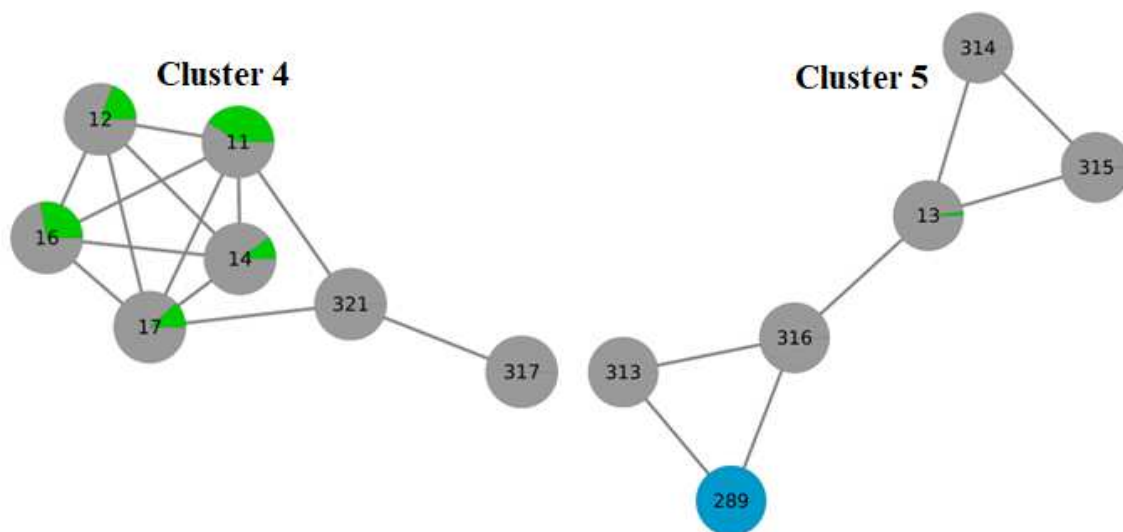


Fig. 12. Clusters 4 and 5 obtained in FBMN under negative mode.

*Antimicrobial activity: Green = active; Gray = inactive; Blue = non tested; ** Node identification: 11 – Isoshaftoside; 13 - Kaempferol 3-*O*-rhamnopyranosil; 14 – violanthin; 17 – Isoviolanthin; 314 - Kaempferol 3-*O*-rutinoside; 315 – Ruthin.

Both molecular networks revealed clusters of features that could not be annotated through GNPS dereplication, indicating the presence of potentially unknown metabolites. Although this limits structural interpretation at this stage, several of these unassigned nodes are associated with bioactive fractions in the FBMN, suggesting a possible contribution to the observed antibacterial effects. These findings highlight *M. nigra* as a promising source of chemically unexplored metabolites and support further isolation and structural characterization efforts aimed at identifying novel antibacterial agents.

Conclusions:

Metrodorea nigra exhibited significant antibacterial and antibiofilm activities, particularly against Gram-positive bacteria. Bioassay-guided fractionation identified the dichloromethane fraction and subfraction Sp1D as the most active samples, with bactericidal effects. Metabolomic analysis indicated that the activity is mainly associated with flavonoid-rich fractions, especially aglycone flavanones such as pinostrobin, while glycosylated flavonoids were predominantly linked to inactive samples. These findings suggest that structural features, particularly glycosylation, influence antibacterial activity. This study also reports, for the first time, the occurrence of flavanones in the genus *Metrodorea*. Overall, the results highlight *M. nigra* as a promising source of antimicrobial compounds and support further investigation of its bioactive constituents.

Declarations:

Ethics approval:

Not applicable

Competing interests:

The authors declare that they have no competing interests

Authors contributions:

Miranda, R.S.: Project idealization, crude extract preparation and purification, development of antimicrobial and antioxidant bioassays, results compilation and analysis, bibliography, original draft and manuscript correction.

Silva, M.V.T.: Mass spectrometry data mining and molecular networking development

Liporaci, S.M.: Chemical purification, bioactivity analysis support, and bibliography.

Leal, I.C.R.: Project idealization, antimicrobial assays conceptualization and supervision, results evaluation, funding acquisition, manuscript correction.

Martins, R.C.C.: Project idealization, purification and chemical investigation, conceptualization and supervision, results evaluation, manuscript correction and editing.

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Availability of data and material:

All data generated or analyzed during this study are included in this published article and supplementary information files.

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