



Identification of antigonorrhoeal phytochemical lead compounds using a metabolomics-guided approach

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Abstract

Neisseria gonorrhoeae remains a high-priority pathogen according to the World Health Organisation Bacterial Priority Pathogens List. In South Africa, increasing antimicrobial resistance to tetracycline, ciprofloxacin, and penicillin is limiting available treatment options for *N. gonorrhoeae* infections. This challenge necessitates the exploration of alternative therapeutics, with natural products representing a promising source of novel scaffolds. This study employed a metabolomics-guided approach to tentatively identify antigonorrhoeal compounds from South African medicinal plants. Sixteen crude extracts prepared by ultrasonic extraction, and 112 solid-phase extraction (SPE) fractions, were screened against *N. gonorrhoeae* ATCC 49981 using broth microdilution. Leaves from *Helichrysum odoratissimum* (L.) Sweet and leaves and twigs from *Terminalia phanerophlebia* Engl. & Diels yielded the most active samples, with minimum inhibitory concentrations (MICs) as low as 6.25 µg/mL. SPE fractions from both plants were analysed by Ultra-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UPLC-HRMS). Data were processed using the Waters UNIFI® platform and analysed by Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) in MetaboAnalyst 6.0. Putative compounds were annotated using *in silico* tool, including SIRIUS, complemented by MassLynx, and a literature search, enabling tentative annotation of 23 compounds. Of these, 12 were prioritised by the *Neisseria* Bayesian model (score ≥ 0.5), including kaempferol and carvacrol in *H. odoratissimum*, and vitexin and isoscapoletin in *T. phanerophlebia*. Tanimoto similarity analysis revealed low structural similarity to ciprofloxacin (< 0.30), indicating novelty relative to fluoroquinolone scaffolds. These findings provide candidate compounds for further validation and demonstrate that integrating metabolomics with computational prediction can accelerate antigonorrhoeal discovery.

Keywords Antibacterial · Gonorrhoea · *Helichrysum odoratissimum* · Metabolomics · *Terminalia phanerophlebia*

Introduction

Gonorrhoea remains among the most widespread Sexually Transmitted Infections (STIs) globally. In 2020, it was estimated that 82.4 million new infections occurred in individuals aged 15–49 years (Mylonas 2025; Zanotta et al. 2025).

The World Health Organisation (WHO) reported the African Region to have the highest incidence of cases of gonorrhoea in 2020, closely followed by the Western Pacific Region, with particularly high prevalence also documented in Southern Africa (Jubair et al. 2021; Raccagni et al. 2023). South Africa bears a substantial burden in the Southern Africa, with approximately 2 million cases reported annually, contributing about 2% of the worldwide burden (De Vos et al. 2023). The burden of this disease is quite disconcerting considering the clinical emergence and spread of drug-resistant *Neisseria gonorrhoeae* strains (Hooshier et al. 2024). Surveillance data indicate that fluoroquinolone resistance (e.g., ciprofloxacin) exceeds 70%, while resistance to tetracycline and penicillin is reported above 80% and 65%, respectively, rendering these agents ineffective for empirical treatment (Kularatne et al. 2018; Maduna et al. 2020; Yakobi et al. 2024).

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N. gonorrhoeae has a well-documented history of rapidly developing resistance, with the drug-refractory strains reported soon after the initial use of antibiotic therapy (Maduna et al. 2020). Since then, strains of *N. gonorrhoeae* have become resistant against first-line agents such as sulfonamides, penicillin, and tetracycline, as well as to second-line treatment drugs (fluoroquinolones) (Unemo et al. 2016; Kularatne et al. 2018). Of greater concern is the recent emergence and spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, including those resistant to cephalosporins (ceftriaxone and cefixime) and azithromycin (Raccagni et al. 2023; Fifer And Johnson 2025). This incessant increase in resistance poses a significant global health challenge since the pathogen has developed resistance to nearly all available antibiotics, including the last-line treatment regimen (Quillin and Seifert 2018).

Against this background, in 2024, the WHO updated its priority list of pathogens, originally published in 2017, with *N. gonorrhoeae* retaining its high-priority status (Tacconelli et al. 2018; Jesudason 2024). Beyond the significant morbidity associated with gonorrhoea, the growing challenge of controlling the disease also presents substantial global economic costs. Given the clinical and economic challenges posed by the spread of gonococcal infections, largely driven by the pathogen's ability to acquire resistance, priority needs to be placed on the discovery of novel antigonorrhoeal agents for its treatment. One viable option worth interrogating in search of alternative treatment options is medicinal plants (Quillin and Seifert 2018; Vică et al. 2021).

Plants have been extensively used in traditional medicine across the globe due to their ability to remedy a wide range of ailments, including bacterial infections (Abdallah et al. 2023). In South Africa, it is reported that up to 70% of the country's population relies on traditional medical practice, with medicinal plants serving as a primary source of healthcare (Scott et al. 2008; Adeosun et al. 2023). Given the extensively documented anecdotal efficacy of this traditional medicine, there is merit in the continued investigation in search of potential lead compounds for drug development for gonorrhoea treatment (Tshikalange et al. 2025). Several plants have demonstrated *in vitro* antigonorrhoeal activity against *N. gonorrhoeae*. For example, *Eucalyptus cinerea*, *Cannabis sativa*, *Allium sativum*, *Aloe vera* and *Aloe ferox*, exhibited antigonorrhoeal potential against this pathogen (Ruddock et al. 2005; Kambizi et al. 2008; Nazer et al. 2019). These plant-based remedies highlight the potential of natural products in addressing antimicrobial resistance (AMR) and supporting the development of alternative treatments (Vică et al. 2021).

The antigonorrhoeal activity of these plants is largely attributed to their rich repertoire of phytochemicals, including flavonoids, terpenoids, alkaloids, and phenolic

compounds. Flavonoids exert antibacterial effects through mechanisms including membrane disruption, inhibition of nucleic acid synthesis, and interference with essential enzymatic processes (Górniak et al. 2019; Álvarez-Martínez et al. 2024). Similarly, terpenoids have been shown to compromise membrane integrity and enhance permeability, contributing to bacterial cell death. These bioactive constituents position plant-derived phytochemicals as promising candidates for novel therapeutic development against *N. gonorrhoeae* (Patra 2012; Othman et al. 2019).

Despite their promise, the complex chemical nature of plants poses challenges in the identification and isolation of their bioactive compounds (Sasidharan et al. 2011). To address this challenge, over the last decade, bioprospecting projects have adopted metabolomics-guided workflows, used in conjunction with advanced computational tools, to accelerate the discovery of phytochemical lead compounds (Turi et al. 2015; Salem et al. 2020). Metabolomics, particularly when coupled with ultra-performance Liquid Chromatography-High-Resolution Mass Spectrometry (UPLC-HRMS), enables the comprehensive profiling of small-molecule metabolites in biological samples (Anand et al. 2019; Vaou et al. 2021). Chemometric techniques, such as Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), support metabolomics analyses by identifying discriminant features associated with biological activity, which can subsequently be linked to putative bioactive compounds (Okada et al. 2010; Tugizimana et al. 2013). Furthermore, machine learning models, such as the *Neisseria* Bayesian model, enable the prediction of the antigonorrhoeal potential of compounds based on their structural features and existing activity data. These cutting-edge analytical approaches hold great promise for expediting the discovery of compounds with potential pharmacological and clinical applications (Pereira et al. 2020).

The present study investigated the antigonorrhoeal activity of various plant extracts and their gradient solvent fractions, aiming to identify bioactive compounds that could contribute to the documentation and development of new antigonorrhoeal agents. Through metabolomics and computational tools, we sought to uncover new natural products with the potential to address the growing challenge of antimicrobial resistance in gonorrhoea.

Materials and methods

Plant selection and collection

Sixteen medicinal plants (Table 1) were selected for extraction, fractionation, and initial screening. These species were part of a larger collection gathered by the Council for

Scientific and Industrial Research (CSIR) and the South African Biodiversity Institute (SANBI) between 1998 and 2005 (Khorombi et al. 2006). This plant collection was transferred to the Biodiscovery Centre at the University of

Table 1 Medicinal plants selected for screening, including their plant part, voucher number, and GPS coordinates

Plant species ^a	Family ^b	Plant part	Voucher number	GPS coordinates
<i>Crinum sp</i>	Amaryllidaceae	Bulbs	QM00150	27°03'33"S, 27°29'57"E
<i>Drimis sp</i>	Asparagaceae	Whole plant	FP00938	34°07'39"S, 25°26'05"E
<i>Helichrysum mundtii</i> Harv.	Asteraceae	Roots	JM0126	25°39'02"S, 30°17'21"E
<i>Helichrysum odoratis-simum</i> (L.) Sweet.	Asteraceae	Leaves	FP01629	34°09'56"S, 24°29'20"E
<i>Helichrysum rutilans</i> (L.) D.Don	Asteraceae	Leaves	FP00646	33°37'41"S, 26°47'03"E
<i>Catha edulis</i> (Vahl.) Endl.	Celastraceae	Leaves	PRU121392	23°59'09"S, 29°29'19"E
<i>Terminalia phanerophlebia</i> Engl. & Diels	Combretaceae	Leaves and Twigs	JM00243	24°47'34"S, 30°52'16"E
<i>Terminalia prunoides</i> M.A.Lawson	Combretaceae	Stems	MM00160	24°55'24"S, 30°24'22"E
<i>Terminalia prunoides</i> M.A.Lawson	Combretaceae	Roots	MM00160	24°55'24"S, 30°24'22"E
<i>Terminalia sericeae</i> Burch. Ex DC.	Combretaceae	Leaves and Twigs	JM00313	33°55'37"S, 24°40'11"E
<i>Terminalia sericeae</i> Burch. Ex DC.	Combretaceae	Roots	JM00313	25°40'48"S, 29°25'42"E
<i>Crassula tetragona</i> L.	Crassulaceae	Leaves	FP00920	33°51'57"S, 24°44'26"E
<i>Cassia abbreviate beareana</i> Oliv.	Fabaceae	Leaves	FP00851	27°36'05"S, 32°54'57"E
<i>Geranium robustum</i> Kuntze	Geraniaceae	Whole plant	EV00201	34°07'39"S, 25°26'05"E
<i>Salvia africana-lutea</i> L.	Lamiaceae	Twigs	BP01349	34°01'22"S, 25°18'59"E
<i>Syzygium cordatum</i> Hochst. ex C.Krauss	Myrtaceae	Leaves	JM00187	27°36'03"S, 32°54'52"E

^a, ^bPlant species names and families were identified on the World Flora Online (WFO) plant list <http://wfoplantlist.org>

Pretoria in the Department of Chemistry, where it remains available for research and bioprospecting purposes. Botanical names and families of the selected species were confirmed through the World Flora Online (WFO) database (<http://wfoplantlist.org>). Plant materials used for this research included bulbs, leaves, roots, stems, and twigs. Species identification was performed by botanists affiliated with the CSIR and the SANBI at the time of collection. Herbarium voucher specimens are preserved at the SANBI and the H.G.W.J. Schweickerdt Herbarium at the University of Pretoria (Supplementary Information Table 1).

Extraction and fractionation

Plant materials were extracted and fractionated using a slightly modified method from that used by Invernizzi et al. (2022). The choice of solvents was guided by polarity-based extraction principles to ensure broad recovery of phytochemical classes. The powdered samples (7.2 g) were placed in sintered glass extraction vessels and extracted in two rounds. In the first round, extraction was performed using 50 mL of dichloromethane (DCM): methanol (MeOH) (1:1, v/v) (UnivAR, Sigma Aldrich, USA). The glass vessels were immersed in an ultrasonication bath (Labotech, South Africa) and sonicated for 60 min, ensuring the temperature did not exceed 40 °C. The mixture was then drained and collected into a 100 mL round bottom flask. In the second round, the remaining plant residue was sonicated with 50 mL of MeOH for 30 min, followed by filtration. Solvents were evaporated from the combined extracts using a rotary evaporator (R-100, Büchi, South Africa). The resulting crude extracts were transferred to pre-weighed, labelled glass vials and left to dry further under a fume hood for 24 h. The extraction yield percentage was determined from a single extraction per sample, using the equation:

$$\% \text{ yield} = \frac{\text{mass of dry extract}}{\text{mass of initial dry plant material}} \times 100$$

Once the crude extract was completely dry, the fractionation process was carried out as previously reported by Invernizzi et al. (2022). Approximately 600 mg of each crude extract was reconstituted in 6 mL of MeOH and absorbed onto cotton wool prior to loading onto the cartridge. Fractionation was performed on a positive-pressure Solid-Phase Extraction (ppSPE) workstation (Thermo Scientific Hypersep C8, 2 g/6 mL). Elution was performed using a stepwise polarity gradient (8 mL per step) as follows: fraction 1, water (H₂O): MeOH (95:5); fraction 2, H₂O: MeOH (80:20); fraction 3, H₂O: MeOH (60:40); fraction 4, H₂O: MeOH (40:60); fraction 5, H₂O: MeOH

(20:80); fraction 6, MeOH (100%); and fraction 7, acetonitrile (MeCN): MeOH (50:50). This gradient elution enabled the progressive separation of compounds from highly polar to less polar, yielding seven fractions per extract and a total of 112 SPE fractions across all 16 crude extracts. Solvents were evaporated using a Genevac HT6 centrifugal evaporator (SP Scientific). The resulting dried fractions were weighed and dissolved in 100% dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA) to prepare stock solutions of 20 mg/mL, which were stored at 4 °C until further use in antgonorrhoeal screening.

Antigonorrhoeal screening of plant extracts and their fractions

The broth microdilution method was employed to determine the minimum inhibitory concentration (MIC) of the extracts and fractions, following the procedure used by Mashamba et al. (2022) with slight modifications.

Neisseria gonorrhoeae ATCC 49981 (ThermoFisher, Waltham, USA) was used for this assay. For purity, the strain was streaked on Luria Bertani (LB) agar plate (Lasec, South Africa) and incubated at 37 °C for 24 h to obtain single colonies. Thereafter, a single colony was transferred into the LB broth (Lasec, South Africa) and incubated overnight to allow bacterial growth. Ciprofloxacin (Merck, Darmstadt, Germany) was used as the positive control, a stock solution at 0.18 µg/mL was prepared to achieve a final concentration of 0.06 µg/mL upon mixture. DMSO (1% stock concentration) (Sigma-Aldrich, USA) served as the negative control, and the extracts and fractions (200 µg/mL), were prepared in sterile LB broth. In a 96-well microplate, 100 µL of LB broth was first dispensed into each well, followed by 100 µL of the test samples, which were serially diluted. An equal volume (100 µL) of bacterial suspension (OD₆₀₀ nm adjusted to 0.1–0.08) was added to each well, resulting in final concentrations ranging from 50 µg/mL to 0.39 µg/mL. After 24 h of incubation at 37 °C with shaking (120 rpm), MIC values were determined by adding 40 µL (200 µg/mL) of *p*-iodonitrotetrazolium (INT) (Sigma Aldrich, USA). The INT reflects general cellular dehydrogenase activity, resulting in the formation of a pink-coloured dye, which indicates bacterial growth (Kowalska-Krochmal and Dudek-Wicher 2021; Mulaudzi et al. 2024). The MIC was defined as the lowest concentration that prevented colourimetric change. The MIC value of ≤ 10 µg/mL was considered noteworthy and expressed in µg/mL as the mean value of triplicate experiments. After determining the MICs, plant species with the most active fractions were subjected to UPLC-HRMS based metabolomics.

UPLC-HRMS metabolomics analysis

Chromatographic analysis was performed following the method described by Moyo et al. (2024), with minor modifications. Fractions obtained from *H. odoratissimum* and *T. phanerophlebia* were used for this analysis.

Samples were diluted in HPLC grade MeOH to a final concentration of 1.5 mg/mL and centrifuged at 1500 rpm for 30 sec using a Mini Centrifuge (BOECO, Germany). The supernatant was then injected into a Waters ACQUITY UPLC system equipped with a BEH C18 column (2.1 mm × 100 mm, 1.7 µm) (Waters Inc., MA, USA). Chromatographic separation was achieved using a binary solvent system consisting of solvent A (H₂O with 0.1% CH₂O₂) and solvent B (MeOH with 0.1% CH₂O₂). The gradient elution was programmed as follows: 3% B at 0 min, increased to 100% B at 14 min, held at 100% B until 17 min, returned to 3% B at 17.5 min, and equilibrated at 3% B until 20 min. The flow rate was set at 0.3 mL/min, and the column temperature was maintained at 50 °C.

Mass spectrometric detection was performed using a Xevo G2-QTOF mass spectrometer (Waters Inc., MA, USA) operating in positive electrospray ionisation (ESI⁺) mode. Data were acquired using a data-independent acquisition (DIA) approach. The capillary voltage was set at 2.8 kV, with a source temperature of 120 °C, sampling cone voltage of 30 V, extraction cone voltage of 4.0 V, and nitrogen cone gas flow rate of 600 L/h. Ion fragmentation was achieved using high-energy Collision-Induced Dissociation (CID). Adduct detection was restricted to [M+H]⁺ and [M+Na]⁺ ions. Data acquisition was carried out using MassLynx software (v4.1, Waters Inc., MA, USA).

Data processing and chemometric analysis

Raw UPLC-HRMS data were processed using the multivariate analysis tool within the UNIFI[®] (Waters Inc., MA, USA) environment to generate a data matrix consisting of peak intensities, retention times, and corresponding *m/z* values. For feature alignment in UNIFI[®], the following parameters were applied: retention time (RT) tolerance of ± 0.1 min and target mass tolerance of ± 10.00 ppm along with additional parameters summarised in Table 2. The processed dataset was exported as a comma-separated values (.csv) file for downstream analysis.

Chemometric analysis was performed using MetaboAnalyst (v6.0), following a slightly modified protocol described by Wooding et al. (2025). Prior to analysis, data were subjected to log transformation (base 10) and Pareto scaling to reduce noise while preserving relevant biological variation. Principal Component Analysis (PCA) was used to enable

Table 2 UPLC-HRMS parameters for data processing

N ^o	Parameters	Value
01	Apply smoothing	0.00
02	Deisotope data	0.00
03	Function	1
04	Initial retention time	0.00
05	Final retention time	20.00
06	Low mass	50
07	High mass	1200
08	Marker intensity threshold	10.00
09	Mass window (Da)	0.05
10	Noise elimination level	0.00
11	Peak-to-peak baseline noise	0.00
12	Peak- width at 5% height (sec)	1.00
13	Type of analysis	Peak detection
14	Replicate % minimum	0.00

unbiased exploration of the dataset without prior class assumptions, while Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) was employed for supervised classification.

Fractions with MIC values ≤ 10 $\mu\text{g/mL}$ were classified as “active,” whereas those with MIC values > 10 $\mu\text{g/mL}$ were considered “inactive,” based on established antgonorrhoeal activity thresholds. Model robustness was evaluated using permutation testing ($n = 100$, $p < 0.01$), along with assessment of R^2 and Q^2 values, with $Q^2 > 0.5$ considered indicative of good predictive performance. Outliers were identified and excluded based on Hotelling’s T^2 test (95% confidence interval) (Eriksson et al. 2006). Model interpretation was conducted using score plots, loading plots, and contribution plots. Variables contributing significantly to group separation were further evaluated using Variable Importance in Projection (VIP) scores.

Compound annotation

For compound annotation, processed data were further analysed using UNIFI[®] software (v1.9.3.071, Waters Inc., MA, USA). Mass screening was performed using MS^E data-independent acquisition with automated peak detection and integration across the full mass and retention time range, along with automatic thresholding and peak width detection. Data smoothing (mean algorithm) was applied, and lock mass correction was performed using a reference mass of m/z 556.2766.

The processed data were exported in Mascot Generic Format (MGF) and imported into SIRIUS software (v6.0.0) for molecular formula prediction and structural elucidation, following the workflow described by Dührkop et al. (2019). The instrument type was set to Q-TOF, with a mass accuracy threshold of 10 ppm. Elemental composition was restricted to C, H, and O, with a maximum of 10 candidate formulas considered per feature. Structural annotation was performed using CSI: FingerID with searches conducted

against multiple biological and natural product databases integrated within SIRIUS, including BioCyc, Blood Exposure, ChEBI, and COCONUT, with PubChem used as a fallback. The following adducts were considered $[M+H]^+$ and $[M+Na]^+$. Molecular formula confidence was further supported by ZODIAC scoring as implemented within the SIRIUS workflow. Compound class prediction was supported using CANOPUS. In addition, known standards (caffeic acid and naringenin) were included to improve spectral alignment and annotation confidence.

Manual annotation of selected features was performed in MassLynx using a mass error tolerance of ± 5 ppm. Annotated compounds were further classified using the NPClassifier tool (<https://npclassifier.ucsd.edu/>) to assign structural classes (Kim et al. 2021). Finally, annotated compounds were subjected to activity prediction using a *Neisseria*-specific Bayesian model (Pereira et al. 2020).

Antigonorrhoeal activity prediction

Putative active compounds were analysed using the *Neisseria* Bayesian model available through the Ersilia Open-Source Initiative (<https://www.ersilia.io/>). This predictive model estimates the likelihood of small molecules acting as *N. gonorrhoeae* growth inhibitors. Model inference was initiated by submitting a request to the Ersilia GitHub repository (<https://github.com/ersilia-os/ersilia/issues>). The request included relevant details about the selected model, specifying the EOS model ID eos5cl7, developed by Pereira et al. (2020).

A csv file containing the SMILES representations of the identified compounds, along with the reference compound ciprofloxacin, was uploaded to the model interface. After processing, prediction results were downloaded and analysed.

Compounds with a probability score ≥ 0.5 were classified as likely active, while those below this threshold were classified as inactive. Model performance was evaluated using Receiver Operating Characteristic (ROC) curve analysis in GraphPad Prism 8. The area under the curve (AUC), standard error, 95% confidence interval (CI), and p -value were determined to assess the model’s ability to discriminate between active and inactive compounds. To evaluate structural similarity and scaffold novelty, Tanimoto similarity coefficients were computed between the predicted hit compounds and ciprofloxacin. Molecular fingerprints were generated using Molecular Access System (MACCS) keys in ChemMine Tools (<https://chemminetools.ucr.edu>). Pairwise distance matrices were converted to Tanimoto similarity values. Lower similarity values (< 0.3) were interpreted as evidence of structural novelty relative to ciprofloxacin. Representative values were integrated with the *Neisseria* Bayesian scores to guide compound prioritisation for subsequent experimental validation.

Results

Plant selection, extraction and fractionation

Plant selection was guided by ethnopharmacological knowledge, focusing on species traditionally used to treat infections, particularly those associated with Gram-negative bacteria. This approach resulted in the selection of 16 South African medicinal plants (Table 3) representing 10 plant families. The Combretaceae and Asteraceae were the most represented families, with five and three species, respectively, while the other families (Amaryllidaceae, Asparagaceae, Celastraceae, Crassulaceae, Fabaceae, Geraniaceae, Lamiaceae, and Myrtaceae) were each represented by a single species.

Plant materials comprising leaves, roots, stems, twigs, and whole plants were used in this research. The ground plant materials were sourced from the repository of the Biodiscovery Centre, Department of Chemistry, University of Pretoria. Relevant authorities in South Africa provided permission to collect the plant material in the repository, and the voucher specimens were deposited at the SANBI.

A summary of the plant species, their traditional preparation methods, traditional uses, and extraction yields is presented in Table 3. Extraction yields (w/w) ranged from 4.30% (*C. edulis*) to 8.84% (*H. rutilans*). Further details such as vernacular names and herbarium voucher specimens are provided in Supplementary Information Table 1.

A portion of each crude extract (300–600 mg) was fractionated using SPE, generating seven fractions per extract. The fraction masses are shown in Fig. 1, illustrating distribution patterns across solvent polarities.

For most species, the highest yields were obtained from moderately polar fractions, particularly fraction 3 (60:40 H₂O: MeOH) (*T. sericeae*, 275.62 mg). The less polar fractions, F5 (20:80 H₂O: MeOH), F6 (100% MeOH), and F7 (50:50 MeCN: MeOH) generally produced the lowest masses. An exception was observed in *H. odoratissimum*, where F6 yielded the most material (100.7 mg) compared to its other fractions. These differences may reflect metabolite variability and solvent affinity. In total, 16 crude extracts and 112 fractions were obtained and stored at 4 °C until *in vitro* testing against *N. gonorrhoeae* ATCC 49981.

Antigonorrhoeal activity of medicinal plants

The crude extracts and their fractions were evaluated for antigonorrhoeal activity against *Neisseria gonorrhoeae* ATCC 49981 using a microdilution assay. MIC values ranged from 6.25 to 50 µg/mL (Table 4). Both crude extracts (CE) and SPE-generated fractions (F1–F7) exhibited varying levels of activity against the tested strain.

Table 3 List of medicinal plants selected for screening, including their plant family, traditional uses, and extraction yields

Plant species ^a	Traditional preparation methods*	Traditional uses*	Extraction yield %
<i>Crinum sp</i>	Bulb roasting	Swellings and urinary tract infections (UTI)	8.25
<i>Drimia sp</i>	Whole plant grounding	Respiratory conditions and skin infections	4.50
<i>Helichrysum mundtii</i> Harv.	Root decoctions	Skin rashes and wound healing	8.33
<i>Helichrysum odoratissimum</i> (L.) Sweet.	Leaf infusions or smudging	Cough, diarrhoea, UTI and wound healing	7.72
<i>Helichrysum rutilans</i> (L.) D.Don	Leaf infusions	Wound healing	8.84
<i>Catha edulis</i> (Vahl.) Endl.	Leaf chewing	Diarrhoea, gonorrhoea, and pneumonia	4.30
<i>Terminalia phanerophlebia</i> Engl. & Diels	Leaf and twig decoctions	Cough, diarrhoea, UTI and tuberculosis	7.60
<i>Terminalia prunoides</i> M.A.Lawson	Stem decoctions	Pain and gastrointestinal disorders (GID)	7.23
<i>Terminalia prunoides</i> M.A.Lawson	Root decoctions	GID and skin rashes	8.43
<i>Terminalia sericeae</i> Burch. Ex DC.	Leaf and twig decoctions	Cough, fever, and GID	8.75
<i>Terminalia sericeae</i> Burch. Ex DC.	Root chewing	Diarrhoea and GID	8.56
<i>Crassula tetragona</i> L.	Leaf decoctions in milk	Diarrhoea, UTI and skin rashes	7.67
<i>Cassia abbreviate beareana</i> Oliv.	Leaf decoctions	Constipation, fever, and malaria	8.23
<i>Geranium robustum</i> Kuntze	Leaf infusions	Dysentery, fever, malaria, and wound healing	4.50
<i>Salvia africana-lutea</i> L.	Twig decoctions	Cough, bronchitis, and gynaecological issues	8.36
<i>Syzygium cordatum</i> Hochst. ex C.Krauss	Leaf infusions or decoctions	GID, tuberculosis, cold fever, and malaria	5.67

*Traditional uses and preparation provided are not exhaustive: <http://redlist.sanbi.org/species.php?species=576-6.>, <https://www.inaturalist.org/>, and <https://invasives.org.za/fact-sheet/brazilian-pepper-tree/> (Retrieved on the 22nd of July 2025); <https://prota.prota4u.org/> (Retrieved on the 08th of April 2026)

MIC classification criteria described by Kuete (2010) were adapted as follows: ≤10 µg/mL (noteworthy activity), 10–25 µg/mL (moderate activity), and ≥25 µg/mL (weak activity). The hit rate was calculated as the proportion of samples exhibiting noteworthy activity. Ciprofloxacin served as the positive control and inhibited *N. gonorrhoeae*.

Fig. 1 Mass distribution of SPE fractions obtained from each plant extract. Crude extracts were fractionated using C8 SPE cartridges with stepwise elution using solvent systems of increasing polarity (H₂O: MeOH, MeOH, and MeCN: MeOH). Seven fractions (F1-F7) were collected, dried, and weighed for each extract. The figure shows the variation in fraction yields across solvent polarities and plant species, highlighting the enrichment of metabolites in specific fractions, particularly mid-polar fractions

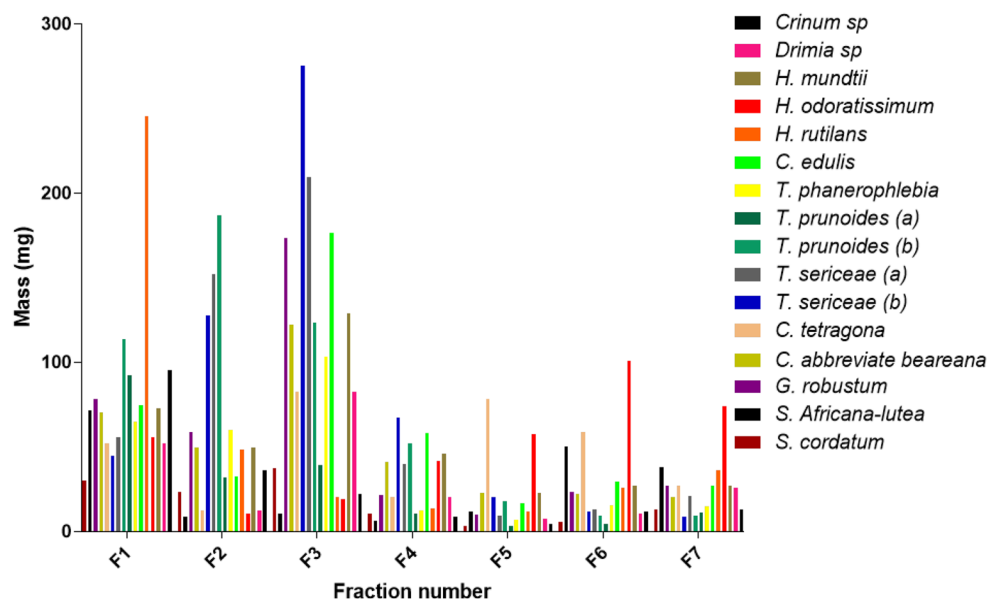


Table 4 Minimum inhibitory concentration of crude extracts and their SPE fractions (F1-F7) against *N. gonorrhoeae* ATCC 49981

Plant species	Plant part	MIC (μg/mL) ^a							
		F1	F2	F3	F4	F5	F6	F7	CE
<i>Crinum sp</i>	Bulbs	6.25	25	6.25	25	25	50	12.5	25
<i>Drimia sp</i>	Whole plant	6.25	6.25	6.25	12.5	50	25	50	12.5
<i>H. mundtii</i>	Roots	6.25	12.5	12.5	12.5	25	50	25	25
<i>H. odoratissimum</i>	Leaves	6.25	12.5	6.25	6.25	25	25	12.5	6.25
<i>H. rutilans</i>	Leaves	12.5	25	12.5	12.5	50	25	50	25
<i>C. edulis</i>	Leaves	25	50	25	12.5	6.25	12.5	6.25	12.5
<i>T. phanerophlebia</i>	Leaves & Twigs	12.5	6.25	6.25	6.25	25	50	25	6.25
<i>T. prunoides</i>	Stems	25	12.5	12.5	6.25	12.5	50	50	25
<i>T. prunoides</i>	Roots	12.5	12.5	6.25	6.25	50	12.5	25	6.25
<i>T. sericeae</i>	Leaves & Twigs	25	25	6.25	6.25	12.5	25	12.5	12.5
<i>T. sericeae</i>	Roots	12.5	6.25	6.25	12.5	50	50	25	25
<i>C. tetragona</i>	Leaves	25	12.5	12.5	6.25	6.25	12.5	25	12.5
<i>C. abbreviate beareana</i>	Leaves	50	25	25	50	25	12.5	25	50
<i>G. robustum</i>	Whole plant	50	12.5	25	12.5	50	50	12.5	25
<i>S. Africana-lutea</i>	Twigs	12.5	25	50	25	25	50	12.5	12.5
<i>S. cordatum</i>	Leaves	25	6.25	12.5	25	50	25	12.5	6.25

at 0.06 μg/mL, while 1% DMSO (final concentration 0.33%) was used as the negative control and showed no detectable inhibitory effect.

Based on these criteria, four crude extracts demonstrated noteworthy activity (MIC ≤ 10 μg/mL), namely *H. odoratissimum* (leaves), *T. phanerophlebia* (leaves and twigs), *T. prunoides* (roots), and *S. cordatum* (leaves), corresponding to a 25% extract hit rate (4/16). In addition, twenty-four fractions derived from multiple species, including *Crinum sp.* (bulbs), *Drimia sp.* (whole plant), *H. mundtii* (roots), *H. odoratissimum* (leaves), *C. edulis* (leaves), *T. phanerophlebia* (leaves and twigs), *T. prunoides* (roots and stems), *T. sericeae* (leaves, twigs, and roots), *G. robustum* (leaves), and *S. cordatum* (leaves), exhibited noteworthy activity, resulting in a fraction hit rate of 21.4% (24/112). Notably, active

fractions were distributed across multiple species; however, fractions F3 and F4 contributed the highest proportion of active hits, accounting for 43.75% (7/16) and 37.5% (6/16), respectively. *H. odoratissimum* and *T. phanerophlebia* yielded the highest number of bioactive fractions, highlighting their potential as candidates for further investigation to tentatively identify active constituents.

Chemometric analysis of the UPLC-HRMS data

Having assessed the potency of the fractions against *N. gonorrhoeae*, multivariate data analysis (MVDA) was employed to identify putative bioactive constituents in the fractions obtained from *H. odoratissimum* (leaves) and *T. phanerophlebia* (leaves and twigs). The UPLC-HRMS data

were processed using the Waters UNIFI[®] platform and subsequently exported as Excel spreadsheets for downstream chemometric analysis. Unsupervised principal component analysis (PCA) was initially conducted, followed by supervised orthogonal partial least squares discriminant analysis (OPLS-DA) from which an S-plot was constructed using MetaboAnalyst 6.0.

For *H. odoratissimum* (Fig. 2a), the PCA score plot of *H. odoratissimum* fractions (F1-F7) shows that the fractions do not overlap completely, suggesting they may have differences in the chemical composition. Fractions obtained with similar solvent polarity (F1 with F2; F3 with F4) tend to appear closer to each other, indicating some influence of polarity on metabolite distribution. However, the separation is not strict; some fractions (F5 to F7) with comparable polarity ranges are still positioned apart along the principal component. This indicates that, beyond polarity, differences in specific metabolites may contribute to the observed separation. Importantly, the PCA model explained 61.9% of the total variance, with all data points located within Hotelling's T^2 confidence ellipse, confirming the absence of outliers at the 95% confidence level.

The OPLS-DA score plot (Fig. 2b), generated by classifying fractions according to their activity, revealed a clear separation between active and inactive groups. Active fractions (green) clustered on the left quadrant, while inactive fractions (red) appeared on the right. Model performance parameters ($R^2X=0.770$, $R^2Y=0.996$, $Q^2=0.978$) indicated a good model fit and predictive capacity. The corresponding S-plot (Fig. 2c) highlighted 15 discriminatory metabolites positioned far from the origin, which contributed most significantly to the observed *in vitro* activity. A 100-permutation test produced $Q^2=0.981$ ($p<0.01$) and $R^2Y=0.997$, indicating that the model was not overfitted and had strong discriminatory power (Supplementary Information Fig. 3). Features with variable importance projection (VIP) ≥ 1.0 were identified as the most influential variables and are presented in Supplementary Information Table 2 (supplementary Figs. 5 and 6).

For *T. phanerophlebia* (Fig. 3a), the PCA score plot shows clear separation among the seven fractions. While several fractions appear in proximity (F1 to F5), their distribution along the plot may indicate differences in metabolite composition across fractions especially for F7. The PCA model accounted for 48.5% of the total variance. The OPLS-DA score plot (b) differentiated active (green, right side) and inactive (red, left side) fractions. Model quality metrics ($R^2X=0.667$, $R^2Y=0.996$, $Q^2=0.976$) confirmed both strong model fit and good predictive ability, with all samples positioned within the Hotelling's T^2 ellipse at the 95% confidence threshold. A 100-permutation test yielded $Q^2=0.974$ ($p<0.01$) and $R^2=0.997$, indicating that the

observed discrimination is less likely to be a random model (Supplementary Information Fig. 4). The associated S-plot (c) identified 8 discriminatory metabolites, located at the outer edges of the plot, that contributed most significantly to the *in vitro* anticonorrheal activity of *T. phanerophlebia* fractions. VIP analysis identified features with $VIP \geq 1.0$ as the most influential variables (Supplementary Information Table 2; Supplementary Figs. 7 and 8).

Compound annotation

Putative metabolites from *H. odoratissimum* and *T. phanerophlebia* were annotated using two complementary approaches: *in silico* fragmentation with SIRIUS (v6.0.0) and manual annotation with MassLynx combined with literature searches. The putative metabolites were identified according to the Metabolomics Standards Initiative (MSI) confidence level described by Blaženović et al. (2018). To assess the reliability of SIRIUS, two reference standards, caffeic acid and naringenin, were incorporated into the annotation workflow. Their mass spectral data were acquired under identical experimental conditions to those of the plant fractions. Raw data files were processed into *mgf* format using UNIFI before SIRIUS analysis. Both standards were successfully annotated by SIRIUS, thereby validating the accuracy of the mass spectral acquisition and processing protocols employed in this study.

SIRIUS generated molecular formulas for the 23 metabolites across the two plant species consistent with MassLynx. Among these, 10 compounds were fully annotated, including coumaric acid, quercetin, 5-O-methylnaringenin, undecylenic acid, isorhamnetin, isoscapoletin, vitexin, tricetin, hesperetin, and cucurbitacin F. The remaining compounds were annotated manually through elemental composition analysis in MassLynx, supported by literature searches. Importantly, many of the annotated metabolites have been previously reported to possess antimicrobial properties, suggesting a possible correlation between the presence of these compounds and the activity observed *in vitro*. The putative bioactive compounds identified from *H. odoratissimum* and *T. phanerophlebia* are presented in Table 5 and Fig. 4. Their Zodiac scores, CSI: FingerID scores, VIP scores and SMILES representations are presented in Supplementary Information Table 2.

Anticonorrheal prediction activity

The 23 tentatively identified compounds from *H. odoratissimum* and *T. phanerophlebia* were subjected to *in silico* evaluation using the *Neisseria* Bayesian model to predict their likelihood of acting as growth inhibitors. The model identified 12 compounds, including umbelliferone,

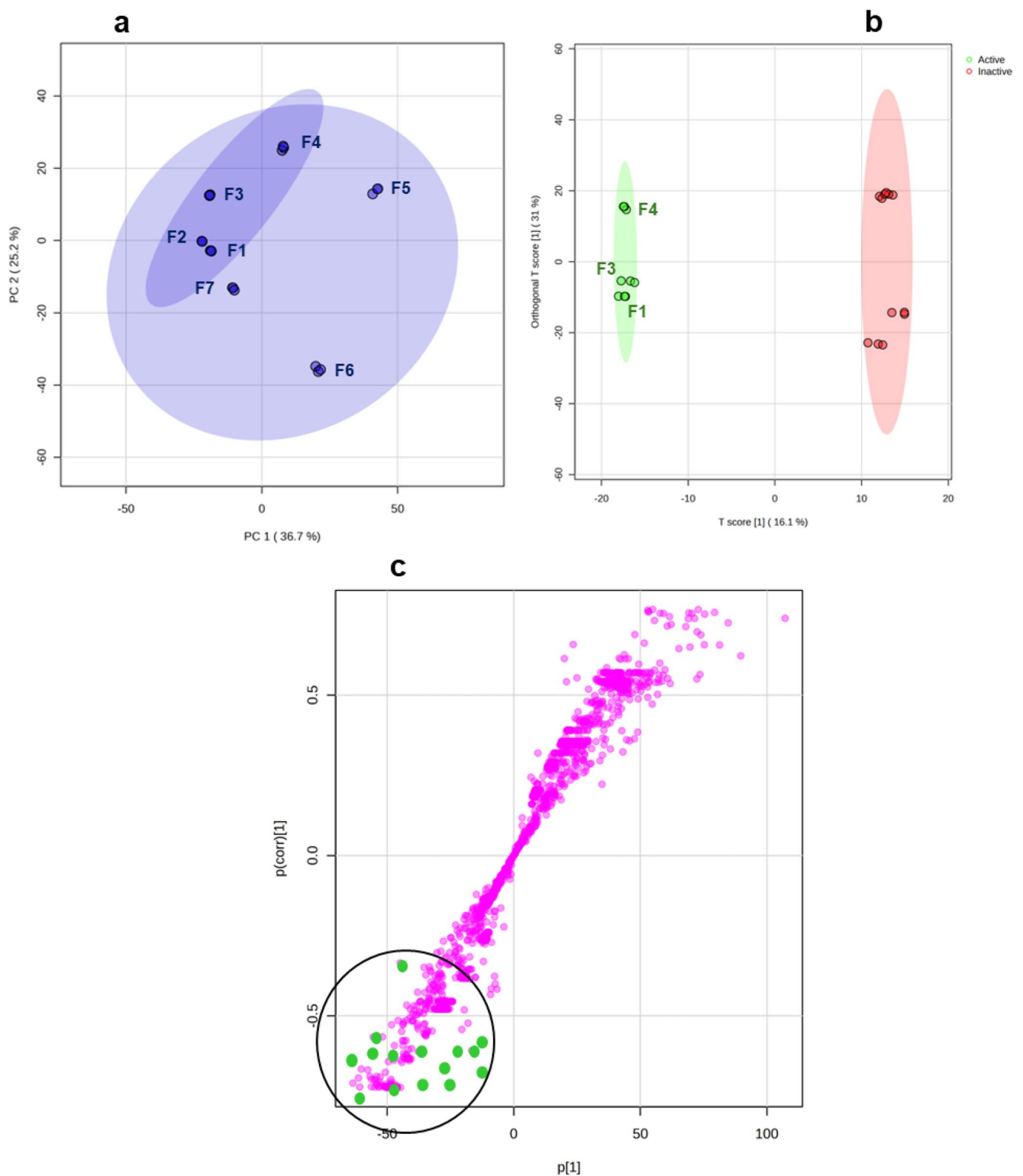


Fig. 2 Multivariate analysis of UPLC-HRMS data of *H. odoratissimum*, **(a)** PCA score plot showing the distribution of fractions, with variation reflecting potential differences in chemical composition. **(b)** OPLS-DA score plot demonstrating separation between active and

inactive fractions. **(c)** OPLS-DA loading S-plot highlighting variables contributing to class separation, where green features in the circled region are hypothesised to be associated with active fractions and may represent metabolites responsible for antibacterial activity

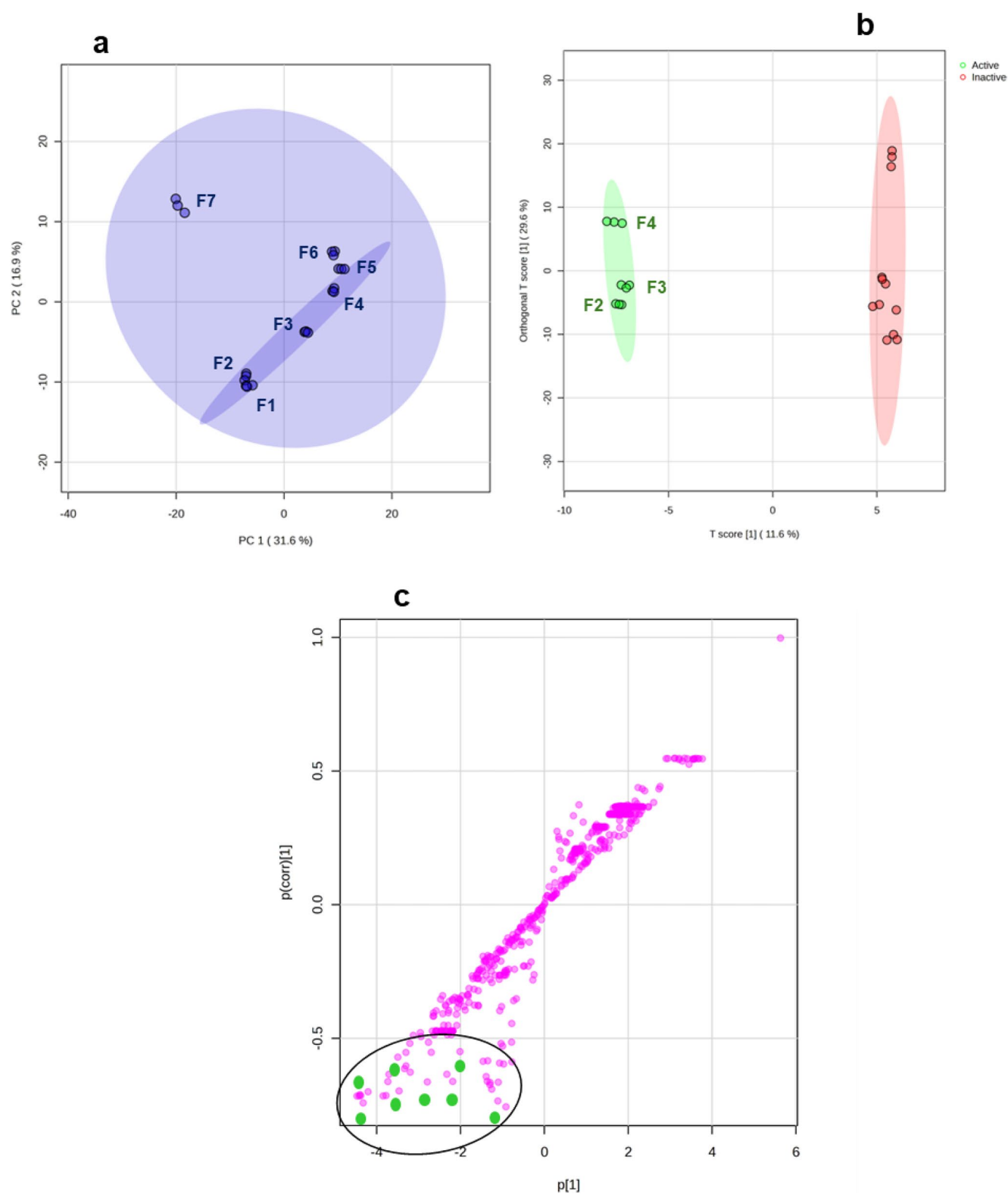


Fig. 3 Multivariate analysis of UPLC-HRMS data of *T. phanerophlebia*. **(a)** PCA score plot showing the overall distribution of fractions, with distinct separation of certain fractions reflecting differences in chemical composition. **(b)** OPLS-DA score plot demonstrating clear discrimination between active and inactive fractions. **(c)** OPLS-DA

loading S-plot highlighting variables contributing to class separation, where features in the circled region are potentially associated with active fractions and may represent metabolites responsible for anti-bacterial activity

Table 5 Putative active compounds from *H. odoratissimum* and *T. phaneroplebia* from the OPLS-DA loading S-plot

RT (min)	Observed mass (m/z)	Calculated mass (m/z)	Adduct	Molecular formula	Fragment ions	Mass error (ppm)	Tentative compound	Class
Standard								
3.28	181.0496	181.0500	[M + H] ⁺	C ₉ H ₈ O ₄	163.0386 164.0428 179.0337	−2.2	Caffeic acid	Phenolic compounds
4.08	273.0757	273.0762	[M + H] ⁺	C ₁₅ H ₁₂ O ₅	147.0441 153.0180 273.0759	−1.8	Naringenin	Flavonoids
<i>H. odoratissimum</i>								
6.23	449.1435	449.1447	[M + H] ⁺	C ₂₂ H ₂₄ O ₁₀	147.0438 167.0330 287.0904	−2.6	Sakuranin	Flavonoids
6.37	163.0387	163.0394	[M + H] ⁺	C ₉ H ₆ O ₃	145.0280 147.0802 152.0093	−4.5	Umbelliferone	Coumarins
6.71	165.0542	165.0551	[M + H] ⁺	C ₉ H ₈ O ₃	71.0506 129.0689 163.0741	−4.2	m-Coumaric acid	Phenolic compounds
6.93	303.4328	303.4343	[M + H] ⁺	C ₁₅ H ₁₀ O ₇	165.0331 175.0740 257.0842	−4.9	Quercetin	Flavonoids
7.55	287.0833	287.0842	[M + H] ⁺	C ₁₆ H ₁₄ O ₅	124.0161 152.0097 167.0332	−3.1	5-O-methylnaringenin	Flavonoids
7.56	309.0371	309.0371	[M + H] ⁺	C ₁₅ H ₁₀ O ₆	137.0225 152.0092 287.0891	−0.9	Kaempferol	Flavonoids
8.40	241.0839	241.0841	[M + Na] ⁺	C ₁₃ H ₁₄ O ₃	121.1013 147.0818 217.1193	−0.8	12-hydroxytremetone	Terpenoids
8.76	181.0492	181.0500	[M + H] ⁺	C ₉ H ₈ O ₄	145.1016 163.0223 179.0286	−4.4	Caffeic acid	Phenolic compounds
8.80	207.1361	207.1360	[M + Na] ⁺	C ₁₁ H ₂₀ O ₂	109.0652 135.0435 177.0534	0.46	Undecylenic acid	Fatty acids
9.49	317.0646	317.0661	[M + H] ⁺	C ₁₆ H ₁₂ O ₇	159.1162 229.1209 275.1257	−4.7	Isorhamnetin	Flavonoids
9.90	289.0704	289.0711	[M + H] ⁺	C ₁₅ H ₁₂ O ₆	151.0754 173.0583 271.0927	−2.4	Eriodictyol	Flavonoids
10.52	195.0653	195.0657	[M + H] ⁺	C ₁₀ H ₁₀ O ₄	143.0844 157.1002 175.1105	−2.0	Ferulic acid	Phenolic compounds
10.89	151.1119	151.1122	[M + H] ⁺	C ₁₀ H ₁₄ O	119.0861 131.0854 135.1164	−1.9	Carvacrol	Terpenoids
12.10	225.0754	225.0762	[M + H] ⁺	C ₁₁ H ₁₂ O ₅	103.0070 138.9708 182.9610	−3.5	4-hydroxy-3,5-dimethoxycinnamic acid	Phenolic compounds
13.25	439.3560	439.3575	[M + H] ⁺	C ₃₀ H ₄₆ O ₂	163.0746 205.0485 389.1542	−3.4	Deoxyflindissone	Terpenoids
<i>T. phaneroplebia</i>								
6.16	193.0497	193.0500	[M + H] ⁺	C ₁₀ H ₈ O ₄	133.0286 153.0175 193.0474	−1.5	Isoscopoletin	Coumarins

Table 5 (continued)

RT (min)	Observed mass (m/z)	Calculated mass (m/z)	Adduct	Molecular formula	Fragment ions	Mass error (ppm)	Tentative compound	Class
6.55	433.1118	433.1134	[M + H] ⁺	C ₂₁ H ₂₀ O ₁₀	283.0589 303.0115 313.0693	−3.7	Vitexin	Flavonoids
6.56	435.0549	435.0563	[M + H] ⁺	C ₁₉ H ₁₄ O ₁₂	283.0589 303.0133	−3.2	Ellagic acid pentoside	Tannins
7.13	303.0860	303.0868	[M + H] ⁺	C ₁₆ H ₁₄ O ₆	153.1163 177.0854 260.0819	−2.4	Hesperetin	Flavonoids
7.14	601.1183	601.1193	[M + H] ⁺	C ₂₈ H ₂₄ O ₁₅	153.0171 287.1266 309.1536	−1.6	Isoorientin 2-O-gallate	Flavonoids
7.52	303.0480	303.0504	[M + H] ⁺	C ₁₅ H ₁₀ O ₇	153.0172 241.0262 277.0320	−7.9*	Tricetin	Flavonoids
8.54	519.3305	519.3312	[M + H] ⁺	C ₃₀ H ₄₆ O ₇	207.1368 287.0533 303.0489	−1.3	Cucurbitacin F	Terpenoids
14.78	381.1299	381.1313	[M + Na] ⁺	C ₂₀ H ₂₂ O ₆	357.1343 381.1415	−3.6	Pinoresinol	Furofuranoid lignans

*Compound annotation outside the <5 ppm mass error

coumaric acid, quercetin, 5-O-methylnaringenin, caffeic acid, ferulic acid, isorhamnetin, isoscapoletin, vitexin, tricetin, hesperetin, and kaempferol, as putative bioactive hits exceeding the prediction threshold. Ciprofloxacin, included as a reference compound, achieved a predicted probability score of 0.51, which falls within the established activity threshold of the model.

Receiver Operating Characteristic (ROC) curve analysis yielded an area under the curve (AUC) of 0.6714 (standard error=0.1080, 95% CI=0.3598–0.7831, $p=0.4281$) (Supplementary Information Fig. 9), indicating moderate discriminatory ability between active and inactive compounds. The wide confidence interval reflects the limited sample size ($n=24$, including the reference compound), but the model provides a useful prioritisation framework for subsequent experimental validation. To further assess structural novelty, Tanimoto similarity analysis based on Molecular Access System (MACCS) fingerprints was performed between the 12 predicted hits and ciprofloxacin. All plant-derived compounds exhibited low Tanimoto similarity values (0.068–0.191) relative to ciprofloxacin, indicating distinct chemical scaffolds (Supplementary Information Table 3).

Discussion

South African medicinal plants continue to be a valuable source of biologically active compounds for treating various infectious diseases (Baloyi et al. 2021). In this study, sixteen medicinal plants (Table 1) traditionally used against

infections associated with Gram-negative bacteria were evaluated for their anticonorrhoeal activity. The predominance of traditional applications targeting conditions such as urinary tract, gastrointestinal, and wound-associated infections suggests that these plants may possess broad-spectrum antimicrobial properties relevant to Gram-negative pathogens such as *N. gonorrhoeae*. Furthermore, the use of decoctions and infusions in traditional preparation reflects a reliance on extraction methods that favour polar constituents, which may limit the recovery of the full phytochemical diversity present within these plants (Hlatshwayo et al. 2025). In this regard, the preparation of plant material plays a critical role in preserving and enriching bioactive compounds (Baloyi et al. 2022; Adeosun et al. 2023). Ultrasonic-assisted extraction was therefore employed to enhance the recovery of phytochemicals. Subsequently, SPE fractionation enabled the separation of metabolites based on polarity, facilitating the enrichment of potentially active constituents. Such polarity-guided approaches are recognised as effective strategies for improving the detection and localisation of bioactive compounds within complex plant matrices (Abubakar and Haque 2020; Badawy et al. 2022; Brendler et al. 2023).

Extraction yields varied across plant species (Table 3); however, it is imperative to note that a higher yield does not necessarily correlate with significant biological activity, as phytochemical composition is a more critical determinant (Badawy et al. 2022). This observation aligns with previous studies by Eloff et al. (2018) and Baloyi et al. (2022), which highlight that biologically active compounds are not

necessarily present in the most abundant extracts. Fractionation revealed a polarity-driven distribution of metabolites (Fig. 1), with higher yields generally associated with more polar fractions and lower yields observed with less polar fractions. This trend suggests that a substantial proportion of extractable plant metabolites are polar to moderately polar in nature. Notably, the comparatively higher yields in less polar fractions for certain species indicates variability in metabolite composition, reflecting differences in chemical profiles between plant species. Importantly, these differences are likely to influence the observed biological activity as solvent polarity governs the selective extraction and biological activity of phytochemicals (Azzahra et al. 2025). In this context, the antigonorrhoeal activity of the extracts and fractions was further evaluated.

The observed MIC values represent the concentration at which plant extracts and fractions inhibit the growth of the target bacterium, indicating its antigonorrhoeal potential (Andrews 2001; Kowalska-Krochmal and Dudek-Wicher 2021). Among the tested samples, *H. odoratissimum* and *T. phanerophlebia* emerged as the most promising candidates, exhibiting notable antigonorrhoeal activity. Both species demonstrated consistent activity across crude extracts and their derived fractions, suggesting a progressive enrichment of bioactive constituents during fractionation. This supports the effectiveness of polarity-guided separation in concentrating compounds potentially responsible for the observed antigonorrhoeal effects. The relevance of these findings is reinforced by ethnopharmacological evidence, as both species have a history of use in the traditional treatment of infections affecting the urinary and reproductive systems (Matrose et al. 2021; Anokwuru et al. 2022). Given that *N. gonorrhoeae* primarily targets the urogenital tract, the observed activity strengthens the link between traditional use and biological efficacy.

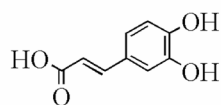
Beyond this, the distribution of activity across both crude extracts and selected fractions suggests that bioactive compounds are not confined to a single polarity range, however are instead present across multiple fractions, with enrichment in moderately polar extracts. This pattern may indicate that polarity-guided fractionation not only concentrates active metabolites but also reveals the chemical diversity contributing to antigonorrhoeal effects (Segwatibe et al. 2023). The observed activity is likely attributable to the complex phytochemical composition of these plants, where multiple compounds may act synergistically to enhance antigonorrhoeal efficacy. Such synergistic interactions are commonly reported in plant-derived extracts and may contribute to overcoming the structural and functional barriers associated with Gram-negative bacteria (Stefanovic 2017; Ayaz et al. 2019).

The variability in activity across fractions may also be linked to the structural complexity of Gram-negative

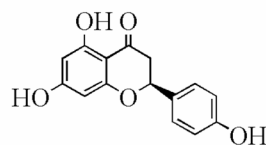
bacteria. *N. gonorrhoeae* possesses an outer membrane rich in lipooligosaccharides, which acts as a permeability barrier and limits the penetration of antigonorrhoeal agents (Saxena et al. 2023). In addition, restricted porin-mediated uptake and the presence of multidrug efflux systems further reduce the intracellular accumulation of bioactive compounds (Webber and Coldham 2010). Despite these barriers, the observed activity suggests that certain plant-derived metabolites may overcome these limitations, potentially through mechanisms such as membrane disruption, enzyme inhibition, or modulation of efflux systems. These multifaceted interactions may have contributed to the enhanced antigonorrhoeal effects observed in specific fractions.

To the best of our knowledge, no previous studies have specifically evaluated *H. odoratissimum* and *T. phanerophlebia* for antigonorrhoeal activity. However, several reports describe their antigonorrhoeal effects against other Gram-negative bacteria, generally with moderate activity. For example, Serabele et al. (2021) reported that the methanolic extract of *H. odoratissimum* was active against urinary, gastrointestinal, and respiratory pathogens, including *Escherichia coli*, *Proteus mirabilis*, and *Moraxella catarrhalis* with MIC values ranging from 130 to 2000 µg/mL. Extracts derived from a mixture of chloroform and methanol in the same study exhibited lower MIC values (63–1000 µg/mL), respectively, against the same bacterial strains (Serabele et al. 2021). In contrast these findings, *H. odoratissimum* extract and fractions in our study inhibited *N. gonorrhoeae* at much lower MIC values. Additionally, Madikizela et al. (2014), reported that the methanolic extract of *T. phanerophlebia* has antibacterial activity against *Klebsiella pneumoniae*. Another study by Florenca et al. (2024) showed that the *T. phanerophlebia* aqueous extract had activity against *K. pneumoniae* (453 µg/mL). The present study's results demonstrated stronger activity of *T. phanerophlebia* extract and fractions against *N. gonorrhoeae*, reinforcing the potential of this plant in treating STIs.

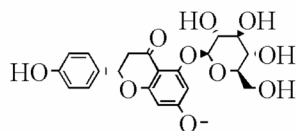
It is worth noting that some studies have focused on plants within the same *Helichrysum* and *Terminalia* genera. For example, when Seleteng-Kose et al. (2019) investigated medicinal plants for the treatment of STIs, their study demonstrated that the organic extract from the whole plant of *Helichrysum caespitium* exhibited high activity against *N. gonorrhoeae*, with an MIC value of 60 µg/mL, while Muganza et al. (2017) found an MIC in the range of 37–300 µg/mL against four WHO *N. gonorrhoeae* strains. In another study by da Silva et al. (2010), the activity of hydro-ethanolic extracts from the leaves and roots of *Terminalia boivinii*, *Terminalia sambesiaca*, and *Terminalia spinosa* was tested against nine penicillin and tetracycline-resistant and sensitive *N. gonorrhoeae* strains; the MIC

a) Standard compounds

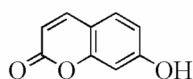
Caffeic acid



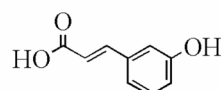
Naringenin

b) Compounds from *H. odoratissimum*

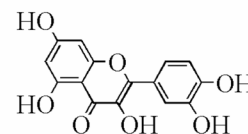
Sakuranin



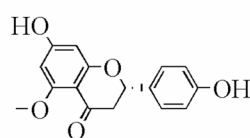
Umbelliferone



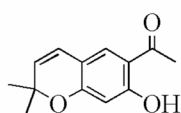
Coumaric acid



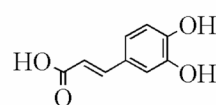
Quercetin



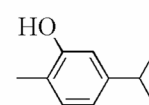
5-O-methylnaringenin



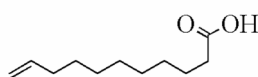
12-hydroxytremetone



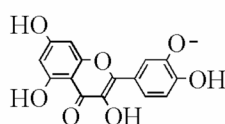
Caffeic acid



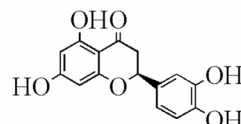
Carvacrol



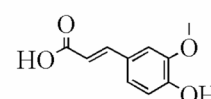
Undecylenic acid



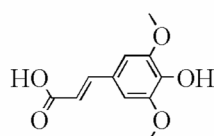
Isorhamnetin



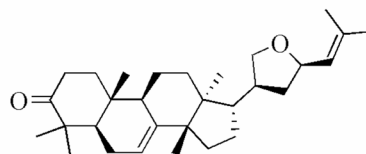
Eriodictyol



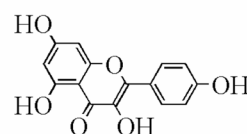
Ferulic acid



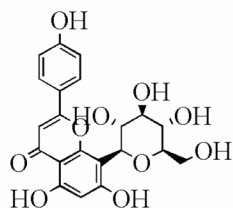
4-hydroxy-3,5-dimethoxycinnamic acid



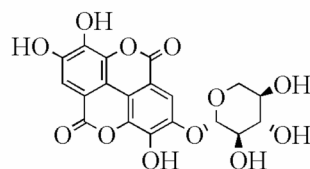
Deoxyflindissone



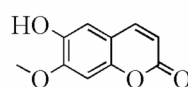
Kaempferol

c) Compounds from *T. phanerophlebia*

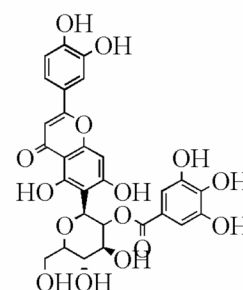
Vitexin



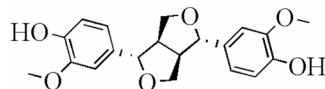
Ellagic acid pentoside



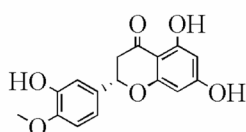
Isoscooletin



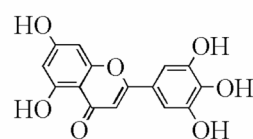
Isoorientin-2-O-gallate



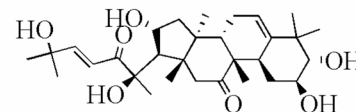
Pinoresinol



Hesperetin



Tricetin



Cucurbitacin F

Fig. 4 Chemical structures of (a) standard compounds, (b) compounds from *H. odoratissimum* and (c) *T. phanerophlebia* tentatively identified using SIRIUS and manual annotation using MassLynx with literature search

values for these extracts ranged between 100 and 400 µg/mL.

Although the findings from the abovementioned studies differ from this study's results, the variation may be attributed to factors such as extraction and fractionation methods, solvent ratios, and bacterial strain type. Nonetheless, this study's findings provide valuable evidence for the broad spectrum of antimicrobial potential of *H. odoratissimum* and *T. phanerophlebia* as sources of antigonorrhoeal agents and underscore the importance of optimised extraction methodologies in enhancing biological activity. These literature comparisons highlight the novelty of these findings. In search of chemical scaffolds playing a pivotal role in the observed *in vitro* activity, the integration of UPLC-HRMS enabled the rapid association of metabolites with antigonorrhoeal effects, providing a time-efficient alternative to traditional bioassay-guided fractionation (Okada et al. 2010; Tugizimana et al. 2013; Moyo et al. 2024). The application of SIRIUS-based *in silico* fragmentation, supported by manual annotation and literature comparison, facilitated the tentative identification of metabolites associated with bioactive fractions. Subsequent prioritisation using a *Neisseria*-specific Bayesian predictive model identified several candidate compounds belonging predominantly to phenolic, flavonoid, and coumarin classes (Fig. 5), which are well-documented for their antimicrobial properties, particularly against Gram-negative bacteria. The low structural similarity of these candidates to ciprofloxacin further suggests potential novelty in their mechanisms of action, which may enable them to circumvent existing resistance pathways. These findings highlight the value of combining metabolomics, chemometric analysis, and *in silico* prediction as a robust strategy for identifying candidate bioactive compounds and advancing the discovery of novel antigonorrhoeal scaffolds.

Phenolic compounds were identified among the candidate metabolites and are likely to contribute to the observed antigonorrhoeal activity. This class, including compounds such as caffeic, coumaric, and ferulic acids, have long received interest in antimicrobial drug discovery due to their diverse and potent antibacterial, antifungal, and antiviral properties. These compounds are characterised by at least one hydroxyl (-OH) group attached to an aromatic ring and are known for their broad biological activities, including antioxidant, anti-inflammatory, and antibacterial properties (Ecevit et al. 2022). They exert antimicrobial effects by disrupting bacterial membranes, chelating essential metal ions, and inhibiting enzymatic processes,

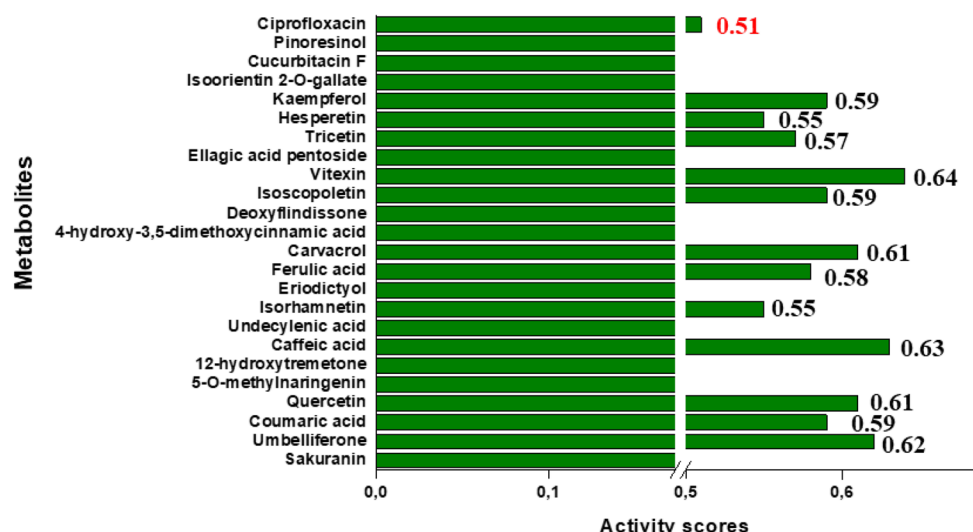
making them promising therapeutic agents (Oliver et al. 2016). Caffeic acid has been extensively studied for its bactericidal activity, as noted in Xu et al. (2022) against *E. coli* and *K. pneumoniae* strains. Lou et al. (2012) showed coumaric acid exhibited antibacterial activity against *E. coli*, *Shigella dysenteriae*, and *Salmonella typhimurium*, respectively, while ferulic acid and coumaric acid demonstrated activity against *Acinetobacter baumannii* strains (Korkut et al. 2025). This activity is likely influenced by their weak organic acid properties and varying lipophilicity, which affect their integration into microbial membranes (Sánchez-Maldonado et al. 2011).

The putative annotation of these compounds in bioactive fractions suggests an imperative role in the antigonorrhoeal effects observed in this study. Although previous reports have demonstrated moderate activity of these compounds against Gram-negative bacteria, their presence within complex plant extracts may enhance their efficacy through synergistic interactions. This highlights the importance of considering both individual compound activity and collective phytochemical effects when interpreting antigonorrhoeal potential.

Flavonoids also emerged as prominent contributors, with compounds such as quercetin, 5-O-methylnaringenin, vitexin, tricetin, isorhamnetin, hesperetin, and kaempferol. They are commonly found not only in plants but also in certain microalgae and fungi. They are known to exert antibacterial effects by disrupting the bacterial cytoplasmic membrane, altering its permeability, and causing leakage of intracellular contents (Orsat And Routray 2017). Furthermore, antibacterial activity of flavonoids is influenced by their chemical structure, particularly the type, number, and position of methoxyl (-OCH₃) and hydroxyl (-OH) groups (Liang et al. 2022). As example, quercetin has been shown to inhibit *N. gonorrhoeae* in Mulaudzi et al. (2024), while Adeosun et al. (2023) also reported activity against *K. pneumoniae*. Vitexin exhibited an inhibitory effect against *P. aeruginosa* (Das et al. 2016). A comparative study by Choi et al. (2022) demonstrated the antibacterial activity of hesperetin against *E. coli* and *P. aeruginosa*. Although hesperetin's activity against *N. gonorrhoeae* has not yet been reported, its structural similarity to other antibacterial flavonoids suggests it may have a similar mode of action, warranting further investigation.

Several studies have explored kaempferol for its antibacterial potential (Periferakis et al. 2023) and identified it as a potent flavonoid in promoting bacterial protein leakage into the extracellular environment of *E. coli* (He et al. 2014). Additionally, kaempferol has been reported to act as an efflux pump inhibitor (EPI), enhancing the intracellular retention of antibiotics. Since *N. gonorrhoeae* relies heavily on efflux pump-mediated drug resistance, kaempferol may serve as a

Fig. 5 Growth inhibitor scores predicted by the Bayesian model. Compounds with scores above the threshold of 0.5 are considered to have a high likelihood of inhibitory activity, while those below 0.5 are considered less likely to exhibit inhibition



promising EPI, potentially increasing the effectiveness of antibiotics against this pathogen (Ashraf et al. 2023).

Overall, the presence of these compounds in bioactive fractions suggests that they may play a significant role in the antgonorrhoeal effects observed in this study. This raises the possibility that flavonoid-rich fractions may contribute to overcoming intrinsic resistance mechanisms in this pathogen and highlights them as promising candidates for further investigation.

In addition to phenolics and flavonoids, other compound classes such as terpenoids with carvacrol. The antimicrobial activity of terpenoids is closely linked to their structural features, particularly their lipophilic hydrocarbon backbone combined with polar functional groups such as hydroxyls. This amphipathic nature allows compounds like carvacrol to insert into bacterial lipid bilayers. The hydrophobic ring structure interacts with membrane lipids, while the hydroxyl group forms hydrogen bonds, destabilising the membrane architecture. This dual interaction increases membrane permeability, leading to leakage of ions and intracellular constituents, ultimately causing cell death. Consistent with this mechanism, a study conducted by Mulaudzi et al. (2024) reported that carvacrol inhibited *N. gonorrhoeae*.

Coumarin derivatives, including umbelliferone and isoscapoletin, were also identified as potential contributors. These compounds are characterised by a 1,2-benzopyrone structure, structurally similar to quinolones, and are a well-known class of antibacterial agents (Singh et al. 2017). Given their structural resemblance to quinolones, these compounds may potentially act by inhibiting bacterial topoisomerases, a mechanism exploited by fluoroquinolones in *N. gonorrhoeae* treatment. Umbelliferone demonstrated complete inhibition against *B. cereus* and *K. pneumoniae* (Mandal et al. 2025).

The combined presence of phenolics, flavonoids, terpenoids, and coumarins within bioactive fractions provides

a plausible explanation for the antgonorrhoeal effects observed in this study. These compound classes are known to act through diverse and complementary mechanisms, which may enhance overall efficacy through synergistic interactions. These findings highlight the potential of plant-derived metabolites as promising candidates for the development of novel antgonorrhoeal agents.

Conclusion, limitations, and future prospects

This study demonstrates the potential of polarity-guided fractionation in uncovering bioactive metabolites with antgonorrhoeal activity from South African medicinal plants. *Helichrysum odoratissimum* and *Terminalia phanerophlebia* emerged as promising sources of antgonorrhoeal compounds, supporting their ethnopharmacological relevance. The identification of phenolic, flavonoid, terpenoid, and coumarin compounds within bioactive fractions provides an explanation for the observed activity, highlighting the contribution of diverse phytochemical classes that may act synergistically to enhance antibacterial efficacy. These findings demonstrate the potential of plant-derived metabolites to overcome resistance mechanisms in *Neisseria gonorrhoeae* and support their relevance in the search for novel therapeutic agents.

Nevertheless, several limitations must therefore be acknowledged. Compound identification was putative, based on UPLC-HRMS spectral matching rather than isolation and structural confirmation, and the observed bioactivity reflects the effects of complex fractions rather than individual constituents, preventing definitive attribution of activity or confirmation of synergistic interactions. In addition, no direct mechanistic experiments were performed to

validate effects on resistance pathways, and all assays were conducted on reference strains, leaving activity against clinical and resistant isolates unresolved.

Further research should focus on the isolation, *in silico* and experimental validation of these metabolites, as well as the elucidation of their mechanisms of action and evaluation against clinical isolates to assess their translational potential.

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Author contributions **Perfey Lumu Lumu: ** investigation, methodology, formal analysis and data curation, writing of the original draft, review and editing; **Phanankosi Moyo: ** dataset collection, formal analysis, review and editing; **Alphonse Onana: ** methodology, review and editing; **Patrick Matthew Mc Millan: ** dataset acquisition, review and editing; **Madelien Wooding: ** Raw data processing using UNIFI, metabolomics, review and editing; **Samkelo Malgas: ** supervision, reviewing and editing; **Vinesh J. Maharaj: ** equipment and funds acquisition, review and editing; **Sekelwa Cosa: ** conceptualisation, project administration, supervision, funds acquisition, review and editing.

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Data availability All data generated or analysed during this study are presented within the article and its supplementary files.

Declarations

Institutional review board statement Ethical clearance for this research was obtained from the University of Pretoria, Faculty of Natural and Agricultural Sciences Ethics Committee (Reference: NAS182/2023; date of final approval: 20 May 2025).

Competing interests The authors declare no competing interests.

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