Deciphering nature's secrets: the antibacterial power and phytochemical characterization of six medicinal fern species

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Abstract

Plants have been key in the development of novel antimicrobial agents and other drugs. However, studies on empirical evidence of the antimicrobial properties of plants have largely focused on higher plants while neglecting lower vascular plants, including ferns. This study investigated the antibacterial potency and characterized the phytochemical profile of six fern species originating from Bwindi Impenetrable National Park, Uganda. The antibacterial potency of the extracts was assessed against pathogenic bacteria using the microtiter plate and agar well diffusion assays. Key generic phytochemicals were quantified through Ultra-Violet (UV)-vis spectrophotometric techniques. Fingerprinting of the extraction components was attained using High Performance Liquid Chromatography (HPLC)-based methods. The fern species contain bioactive compounds such as phenolics and saponins, with species such as *Dicranopteris linearis* giving high yields of such compounds 158.01±0.95 Gallic Acid Equivalent (GAE) mg/g and (479.40±1.07 DE mg/g) of extract, respectively. Fern extracts demonstrated substantial antimicrobial activity against common pathogenic bacteria such as *K. pneumonia, E. coli, P. aeruginosa,* and *S. aureus* with Minimum Inhibitory Concentrations (MICs) as low as 0.39% w/v and Minimum Bactericidal Concentrations (MBCs) as low as 3.13 w/v for some fern species such as *Oleandra distenta*, *Asplenium friesiorum, Dicranopteris linearis,* and *Marattia fraxinea*. The ethanolic extract of *Marattia fraxinea* was potent against *P. aeruginosa* with the highest zones of inhibition of 32.67±0.58mm at 50% w/v concentration while *D. linearis* and *O. distenta* aqueous extracts were potent against the gram-positive bacteria with the highest zones of inhibition of 27.7±1.15 and 26.67±1.53 mm, respectively, at 50% w/v concentration. This study has elucidated that extracts of fern species can be effective against common pathogenic bacteria.

Introduction

Infections caused by bacteria rank among the top causes of death yearly, with poorer nations accounting for the most significant share of these deaths. Bacterial pathogens were estimated to be associated with over 7 million deaths annually in 2019.[1] Increasingly worrying trends in antimicrobial resistance will likely make the situation worse. Antimicrobial resistance occurs when microbes like viruses, bacteria, and fungi adapt and stop responding to antibiotics. It threatens conventional medicine and global public health response to infectious diseases.[2] Longer illnesses, prolonged hospitalization, greater mortality, and increased expen-

ditures are all direct outcomes of infection with resistant microbes.[2,3] On the other hand, the indirect impact of antimicrobial resistance goes beyond higher health risks and has a wide range of public health repercussions, including implications for development.[4] The global economy is negatively impacted by antimicrobial resistance, leading to increased treatment costs and decreased output due to illness in humans and animals.[5] The global action plan on antimicrobial resistance emphasizes the need to research and develop new antimicrobial drugs to address the antimicrobial resistance crisis.

Plants have been an essential component in discovering new antimicrobials and other pharmaceuticals. Medicinal plants contain secondary metabolites used worldwide to cure several diseases.[6] It has been estimated that the herbal medicine industry supports the health care of more than five billion people worldwide, with the biggest consumers stemming from developing nations.[7] For instance, many communities in Africa and Asia use medicinal plants to treat and manage chronic and acute conditions.[8,9] Herbal medicines are widely used by several communities because they are economically feasible, readily accessible, and believed to be less toxic than synthetic pharmaceuticals.[10]

Numerous investigations have examined the effectiveness of therapeutic plants in combating prevalent microbial infections. However, many of these investigations have mostly concentrated on angiosperms, with limited studies investigating the antibacterial properties of ferns. Ferns are non-flowering, lower-vascular plants that are classified under the Filicinophyta division.[11] The few antimicrobial studies that have been conducted on this group of plants have largely focused on fern species from Europe and Asia,[12-14] with hardly any studies exploring the antimicrobial activity of Uganda's fern species. Given their ecological diversity and rich phytochemical composition, ferns may harbor bioactive compounds with significant therapeutic potential.

Traditional knowledge depicts the medicinal importance of several fern species. For instance, the leaf decoction of *Cyathea manniana* Hook., has been used as medicine for sexually transmitted diseases, and as a remedy for gastrointestinal conditions and respiratory disorders.[15,16] In Asia and Africa, decoctions of several *Asplenium* species are traditionally used for treating respiratory infections, urinary disorders, fevers, filariasis, and skin infections.[17] Some members of the family Dennsteadtiaceae such as species from the genus *Pteridium* and *Blotiella* are traditionally used for pain management, treatment of gastrointestinal parasites, and treating bacterial and fungal infections.[15,18] *Dicranopteris linearis* (Burm.f.) Underw., commonly known as the Old World forked fern, has been traditionally used in various Asian cultures for its medicinal properties. The fronds are used in the management of respiratory disorders, and in treating external wounds, ulcers, and boils.[19] *Marattia fraxinea* Sm. has been traditionally used in treating infectious diseases, it has also been widely used as an anthelmintic in Tanzania and India.[20,21]

Despite their well-documented traditional medicinal uses, the antimicrobial properties of these ferns remain largely unexplored, particularly in Uganda. This study aimed to bridge this gap by achieving two key objectives: i) characterizing the selected fern species based on their phytochemical constituents and ii) evaluating their antibacterial potential against pathogenic bacterial strains. By scientifically assessing their phytochemical profiles and antimicrobial activities, this study sought to validate their ethnobotanical significance and explore their potential as sources of novel antibacterial compounds for pharmaceutical applications.

Materials and Methods

Study area

The study was conducted on ferns found in Bwindi Impenetrable National Park (Figure 1). The park was initially designated as a forest reserve in 1932 and elevated to a national park in 1991 to safeguard its gorilla population and diverse avian and plant life.[22] The Park is located in the southwestern region of Uganda (Figure 1). It is positioned on the eastern boundary of the Western Rift Valley and covers the highest area of the Rukiga Highlands.[23] The forest covers an area of roughly 331 square kilometers and is distinguished by its rugged hills and small valleys at altitudes ranging from 1400 m to 2600 m. The climate is tropical, with two distinct periods of increased rainfall occurring from March to May and September to November. The park has a unique natural range of forests at medium altitudes, including Afromontane forests, which have a high level of plant species diversity.[24]

Sample collection

Given that fern species are infrequently used and cited in Uganda's traditional practice, we selected six based on literature indicating relatedness to the species belonging to families that were reportedly used in traditional medicinal remedies in various parts of the globe. The six-fern species selected included *Oleandra distenta* Kunze., *Cyathea manniana* Hook., *Asplenium friesiorum* C.Chr., *Blotiella crenata* (Alston) Schelpe., *Dicranopteris linearis* (Burm.f.) Underw., and *Marattia fraxinea* Sm. Prior to sample collection, a botanist at the Institute of Tropical Forest Conservation (ITFC) identified each fern species, and reference samples were pressed and deposited in the ITFC herbarium.

Preparation of fern extracts

Samples of mature fronds of each fern species were collected, packed in sample bags, labeled, and then transported to ITFC. The samples were washed and dried under shade for three weeks. The dried samples were taken to the Pharmaceutical Analysis Laboratory at Mbarara University of Science and Technology for further processing. The dried fern material was ground into a powder using an electric grinder. The ethanol extract was obtained using the cold maceration method. This involved soaking 20 g of the powder in 180 mL of absolute ethanol in amber glass bottles; the mixture was occasionally agitated for 72 hours.[25] The aqueous extract was prepared using the infusion technique, where 20 g powder was immersed in 180 mL of boiling water and covered for 30 minutes.[25] First, a muslin cloth was used to filter each extract, followed by a Whatman no. 1 filter paper. The filtrate was then concentrated *in vacuo* using a rotary evaporator. Using a freeze-drier, the aqueous extracts were subsequently lyophilized to powdered form. On the other hand, the ethanol extracts were air-dried.

The extraction yield was determined using the formula:

$$Extraction\ Yield = \frac{Weight\ of\ extract\ after\ evaporation\ of\ solvent}{Dry\ weight\ of\ the\ plant\ powder\ before\ extraction} x100$$

Determination of total phenolic content of the fern extracts

The Folin-Ciocalteau technique was used to determine the Total Phenolic Content (TPC) in the fern extracts.[26] The procedure included combining 1 mL of plant extract (at a concentration of

1 mg/mL) with 2 mL of Folin-Ciocalteu reagent (10% v/v). Subsequently, 2 mL of 7.5% (w/v) sodium carbonate (Na_2CO_3) solution was introduced to the mixture. The sample was then incubated in the oven at 40°C for 30 minutes. A Jenway™ UV-vis spectrophotometer was used to determine TPC by measuring the absorbance of the extract solutions at a wavelength of 760 nm using gallic acid as the reference standard. The TPC was recorded in mg Gallic Acid Equivalent (GAE)/g of dry extract computed from the linear equation $y = 0.0223x - 0.0374$, with a coefficient of determination (R^2) value of 0.990.

Determination of total flavonoid content of the fern extracts

The extracts' Total Flavonoid Content (TFC) was evaluated using the aluminum chloride ($AlCl_3$) colorimetric assay.[26] First, 1 mL of a 1 mg/mL fern extract solution was combined with 4 mL of methanol. Next, 0.3 mL sodium nitrite solution of 5% (w/v) was added, and the solution was kept in the dark at room temperature of 25°C for 6 minutes. Following this, 0.3 mL of $AlCl_3$ and 2 mL of sodium hydroxide (NaOH) solution were added, and the solution was kept at 25°C for 6 minutes. A Jenway™ UV-VIS spectrophotometer was then used to measure the absorbance of the solution at a wavelength of 420 nm using Quercetin as the reference standard. The TFC was then reported in mg Quercetin equivalent (QE)/g of dry extract, calculated using the expression $y = 0.0024x - 0.0078$, with an R^2 value of 0.9974.

Determination of total saponin content of the fern extracts

To quantify the Total Saponin Content (TSC) in fern extracts, the vanillin-sulfuric acid method was employed. In this approach, 1 mL of the fern extract was combined with 0.5 mL of 8% vanillin and 5 mL of 72% sulfuric acid. The solution was heated in a shaking water bath at 60°C for 15 minutes. Upon completion of heating, the mixture was then rapidly cooled in ice-cold water for 5 minutes, and its absorbance was measured at a wavelength of 550 nm, using Diosgenin as the reference standard. The TSC was quantified as mg of Diosgenin equivalent (DE)/g calculated from the regression equation $y = 0.0024x - 0.0078$, $R^2 = 0.9951$.

Phytochemical fingerprinting using reverse phase-high-performance liquid chromatography

The 1 mg/mL extract solution was filtered using a membrane filter (Whatman cellulose nitrate, pore size of 0.45 µm). The chemical profile of the filtered solution was then characterized using High-Performance Liquid Chromatography (HPLC). For this analysis, a Shimadzu Prominence UFLC® system was utilized in a reverse-phase mode. A solvent system made of methanol and

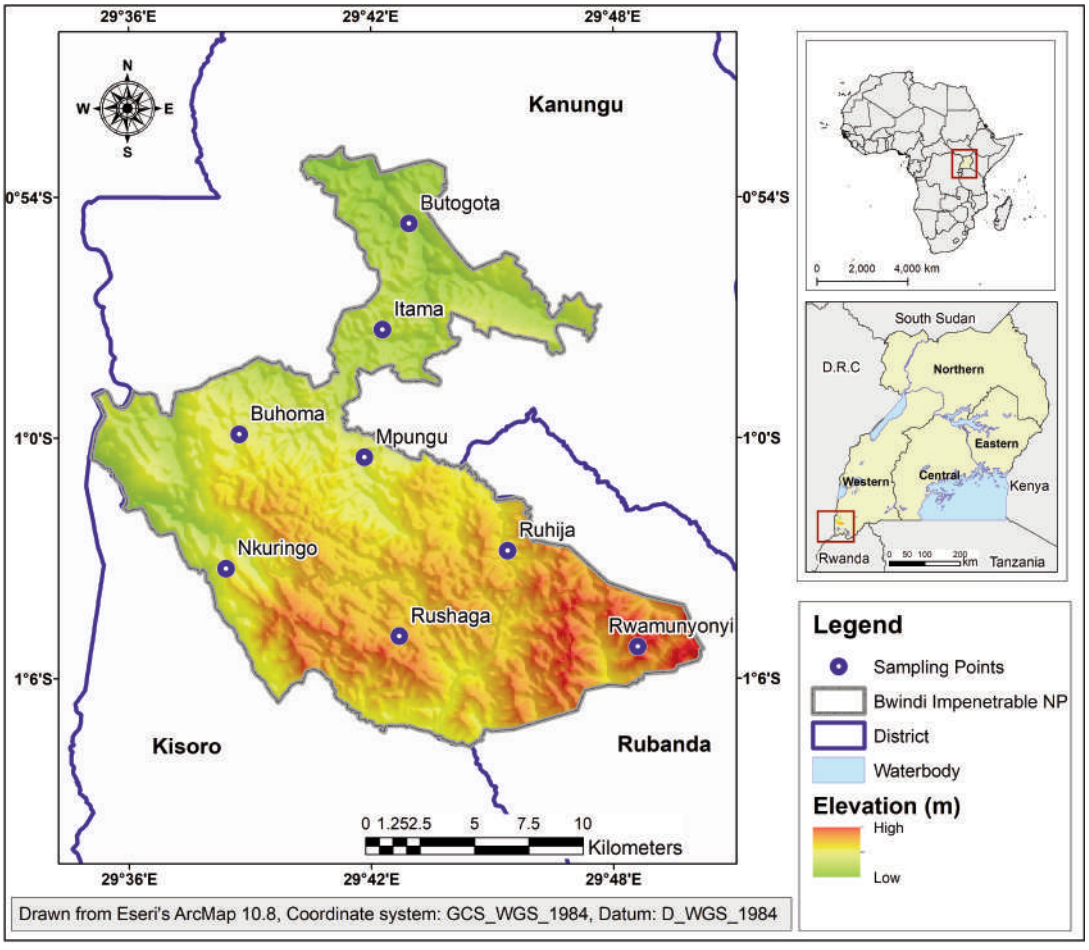

Figure 1. Map showing the fern sampling sites in Bwindi Impenetrable National Park in southwestern Uganda.

0.01% trifluoroacetic acid in water in a 30:70 ratio for pumps A and B, respectively, was used as the mobile phase. The chromatography was carried out on a C_{18} Phenomenex Luna® column (5 µm, 250x4.6 mm) set at a constant temperature of 40ºC. The flow rate was maintained at 0.6 mL/min with the injection volume of 20 µL, and the eluted compounds were detected at a wavelength of 254 nm, providing a detailed chemical fingerprint of the extract. The acquisition time for each injection was 40 minutes.

Determination of antimicrobial activity

Preparation of inoculum

Standard strains of pathogenic bacteria, including *Staphylococcus aureus* ATCC 25923, a gram-positive bacterium, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922, which are gram-negative bacteria were used in assessing the antibacterial activity of the fern extracts. The standard stock cultures were provided by the Microbiology Laboratory at Mbarara University of Science and Technology. The bacterial strains were initially grown on Mueller Hinton Agar (MHA) at 37°C for 24 hours. The test bacterial strains were collected as fresh colonies and then suspended in sterile saline. The concentrations of the bacterial strains were adjusted to a 0.5 McFarland standard, which is equivalent to $1.5x10^8$ CFU/mL.

Antibacterial assay

The antibacterial activity of the fern extracts was evaluated using the agar well diffusion method described by Parekh *et al*. [27] In this technique, 1 mL of freshly prepared bacterial suspension was evenly spread across sterile MHA plates. Afterward, a sterile borer was used to create 8 mm wells in the agar. One hundred (100) µL of fern extracts, prepared in concentrations of 0.78% to 50% (w/v), were carefully dispensed. Aqueous extracts were prepared with sterile distilled water, while ethanolic extracts were diluted using 1% DMSO to ensure proper solubility and dispersion. Ciprofloxacin was used as a positive control to confirm the assay's reliability and effectiveness. The experiment was conducted in triplicate, and all plates were incubated at 37°C for 24 hours. Following the incubation period, the extracts' antibacterial potency was assessed by measuring the diameter of the inhibition zones around each well.

The fern extracts' Minimum Inhibitory Concentrations (MICs) were evaluated using the procedures described by Mogana *et al*.[28] Briefly, 100 µL of Mueller Hinton Broth was carefully pipetted into each well of a sterile 96-well microplate to serve as a nutrient medium for bacterial growth. Next, 100 µL of fern extracts, prepared at various concentrations ranging from 0.391% (w/v) to 25% (w/v), were added to the respective wells to evaluate their inhibitory potential. Following this, 100 µL of a bacterial suspension, standardized to $1.5x10^6$ CFU/mL, was introduced into each well. The microplates were placed in an incubator set at 37°C for 24 hours to give ample time for any inhibitory effects to become apparent. To determine the MIC, 40 µL of INT (p-iodonitrotetrazolium chloride) solution was added to each well as an indicator. After an additional 2-hour incubation at 37°C, the wells were examined for color changes, indicating bacterial growth and inhibition potency of the fern extracts.

To further assess the bactericidal potential of the fern extracts, the Minimum Bactericidal Concentration (MBC) was determined. This involved transferring 50 µL from each well that displayed no visible bacterial growth during the MIC assay onto fresh MHA plates and incubating at 37°C for 24 hours to allow any remaining viable bacteria to grow. The MBC was determined as the concentration of the fern extract at which no bacterial colonies were observed on the agar plates.

Data analysis

The quantitative measurements of general phytochemical constituents and antimicrobial activity were presented as mean ± Standard Deviation (SD) where appropriate. Data were subjected to a two-way Analysis of Variance (ANOVA). All statistical analyses were executed at a 5% significance level in GraphPad Prism version 9.0 (GraphPad® Software Inc., USA). We used classical hierarchical cluster analysis to generate groups based on Euclidean distances in PAST® version 4.11 by Hammer *et al*.[29] These clusters were then used to categorize the fern extracts according to similarities in phytochemical parameters such as Total Phenolic Content (TPC), extraction efficiency, Total Saponin Content (TSC), and Total Flavonoid Content (TFC). We further performed a Principal Component Analysis (PCA) in Origin® version 9.8 (OriginLab Corporation, 2020) to identify patterns, relationships, and clusters associated with the measured phytochemical parameters of the fern extracts. Prior to conducting PCA, the dataset was standardized to ensure comparability among variables. The selection of Principal Components (PCs) for further analysis was based on Kaiser's Criterion, considering eigenvalues ≥1 and a threshold of at least 70% cumulative variance explained.[30] The contributions of individual variables to the retained PCs were examined through their loadings. Additionally, PCA biplots were constructed to illustrate clustering patterns among the samples.

Results

Phytochemical profiling of fern extracts

Extraction index

In this work, we investigated the effects of ethanol and water on the yield and concentration of saponins, phenolics, and flavonoids of fern extracts. The extraction yield recorded from fern extracts ranged from 4.60 to 19.82% (Figure 2a). The water extracts generally had a higher extraction efficiency, with the highest yield recorded in *Dicranopteris linearis* (16.66%), while the lowest yield was recorded in *Cyathea manniana* (8.50%) fern species extracts. Ethanol generally had a lower yield when compared to water, except for *Dicranopteris linearis* (19.82%). *Oleandra distenta* had the lowest yield of 4.60% compared to ethanol extracts of other fern species.

Total phenolic content

In this study, we examined the variation of phenolics in the extracts of different fern species. Results indicated that the TPC of the fern extracts varied significantly among the species and solvent of extraction ($p<0.0001$). The TPC recorded ranged from 8.35±0.19 to 175.52±1.50 GAE mg/g dry extract (Figure 2b). Considering the water extracts, the highest values of TPC were recorded in extracts of *Dicranopteris linearis* (158.01±0.95 GAE mg/g), while *Marattia fraxinea* had the lowest TPC (10.84± 0.27 GAE mg/g). In the ethanol extracts, *Dicranopteris linearis* had the highest (175.52±1.50 GAE mg/g) TPC value, while extracts from *Oleandra distenta* had the lowest TPC (8.35±0.19 GAE mg/g). The water extracts generally had a higher TPC than the ethanol extracts, with the exception of extracts from *Dicranopteris linearis* and *Marattia fraxinea*.

Total flavonoid content

We examined the variability in the TFC in extracts of fern species, and results indicated that the TFC of the fern extracts varied significantly among the species and extraction solvent ($p<0.0001$), as shown in Figure 2c. The TFC recorded in this study ranged from 0.85±0.08 to 48.95±0.13 QE mg/g of dry extract, as indicated in Figure 2c. Results also suggested that the ethanol extracts generally had higher concentrations of flavonoids compared to water extracts. In the ethanol extracts, *Asplenium friesiorum* (48.95±0.13 QE mg/g) had the highest TFC, while *Cyathea manniana* (15.53±0.07 QE mg/g) had the lowest TFC. Regarding the water extracts, *Dicranopteris linearis* (13.19±0.08 QE mg/g) had the highest TFC, while *Cyathea manniana* (0.85±0.08 QE mg/g) had the lowest TFC.

Total saponin content

Results indicated that the saponin content of the fern extracts varied significantly among the species and extraction solvent ($p<0.0001$), as shown in Figure 2d. The TSC recorded in this study ranged from 3.35±0.16 to 501.77±6.73 DE mg/g of extract. The ethanolic extracts of two fern species *i.e. Oleandra distenta* (501.77±6.73 DE mg/g of extract) and *Dicranopteris linearis* (479.40±1.07 DE mg/g of extract), had the highest TSC. On the other hand, *Marattia fraxinea* had the lowest TSC in both the water (3.35±0.16 DE mg/g) and ethanol (95.63±1.39 DE mg/g) extracts.

Classification of the fern species' phytochemical parameters

This study employed cluster analysis, a tool for multivariate analysis, to categorize the fern extracts according to the similarity in their phytochemical parameters, such as TPC, extraction efficiency, TSC, and TFC. As depicted in Figure 3, the hierarchical clustering results revealed four primary groups: A, B, C, and D. Group A contains fern extracts that generally possess moderate levels of the measured phytochemical parameters, Group B contains fern extracts with moderate extraction yield, and total phenolic content while possessing low levels of saponins and flavonoids. Group C includes a fern extract with moderately high levels of saponins and flavonoids, moderate extraction efficiency, and low total phenolic content. Group D contains extracts with generally high values of the phytochemical parameters. Further visualization of the contribution of the parameters to the observed variation was done through principal component analysis (Figure 4). Results indicated that total phenolic content and extraction yield are highly correlated and have a positive influence on PC1, and these two factors account for the variance in the aqueous extracts. Furthermore,

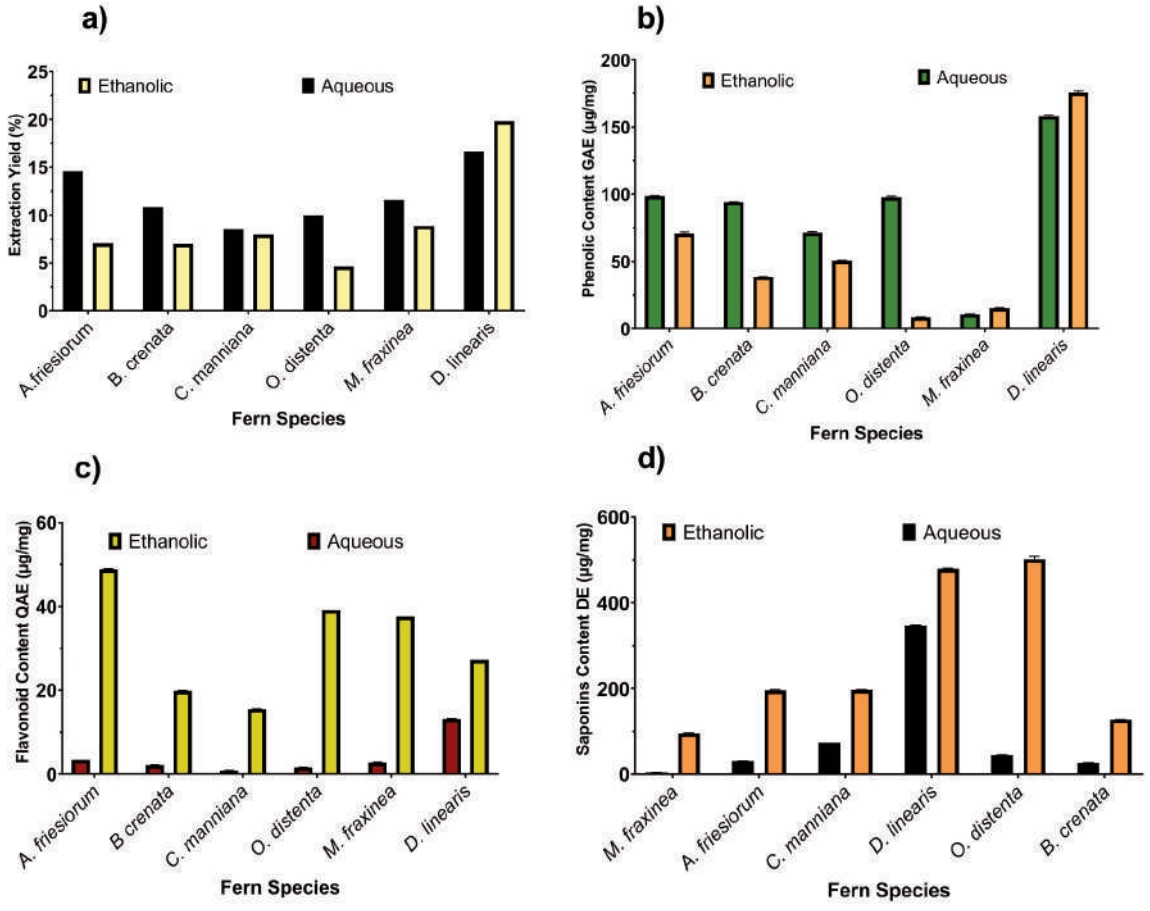

Figure 2. Phytochemical composition and extraction efficiency of selected fern species of Bwindi Impenetrable National Park, values are presented as means with Standard Deviation (SD) error bars for triplicate runs: **a**) Extraction efficiency expressed as a percentage (%) **b**) TPC expressed in Gallic Acid Equivalent (GAE), two-way ANOVA results $p<0.0001$, **c**) TFC expressed in Quercetin equivalent (QE) two-factor ANOVA results $p<0.0001$, **d**) TSC expressed in Diosgenin equivalent (DE) two-factor ANOVA results $p<0.0001$.

results indicated that total saponin content and total flavonoids are highly correlated and have a positive influence on PC2. These two factors account for the variance in the ethanol extracts. Results also indicated that cumulatively, PC1 and PC2 account for 89.06% of the variance.

Pattern recognition of high-performance liquid chromatography fingerprints of fern extracts

The visual examination of the 40-minute fingerprints revealed a certain degree of variation in the fern extracts for both the water and ethanol extracts (shown in Figure 5). The number of peaks in individual extract fingerprinting varied substantially. In the water extracts, the highest number of peaks were recorded in *Oleandra distenta* (70) and *Asplenium friesiorum* (62) while *Marattia fraxinea* had the fewest number of peaks (37). As for the ethanol extracts, the highest number of peaks were recorded in *Oleandra distenta* (46) and *Asplenium friesiorum* (41) while *Dicranopteris linearis* (26) had the fewest number of characteristic peaks.

Antibacterial activity

Six fern species were investigated to examine their antibacterial potential against common pathogenic bacteria using the agar well diffusion method. Results were expressed as inhibition zones, as illustrated in Figures 7 and 8. The results demonstrated that all fern extracts were efficacious in inhibiting bacterial growth, particularly at high concentrations. The antibacterial potential of the fern extracts was reduced with a decrease in concentrations. Furthermore, the antibacterial potency of extracts varied across species of ferns, extracts based on extraction solvent, and target bacterial strain. Both the ethanolic and aqueous extracts of

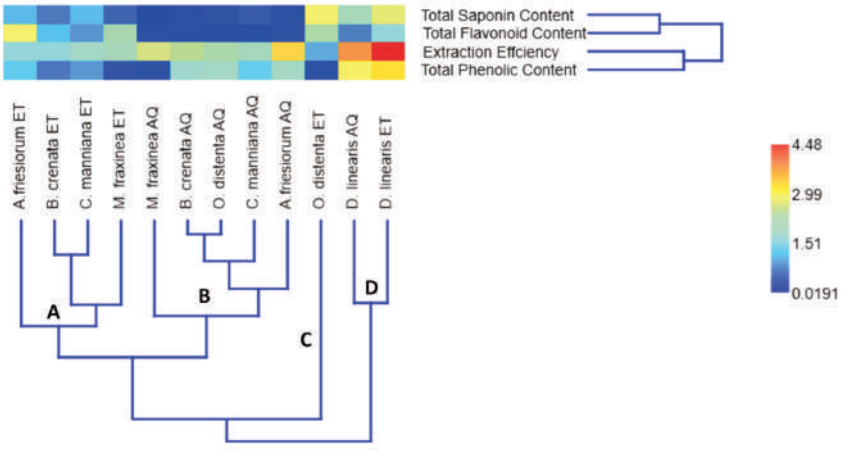

Figure 3. Classical hierarchical cluster analysis of fern extracts created using the unweighted pair group method with arithmetic mean and using Euclidian similarity index. Values used to generate the dendrogram are standardized values of the respective parameters (total phenolic content, extraction efficiency, total saponin content, and total flavonoid content). ET, ethanol extract; AQ, aqueous/water extract.

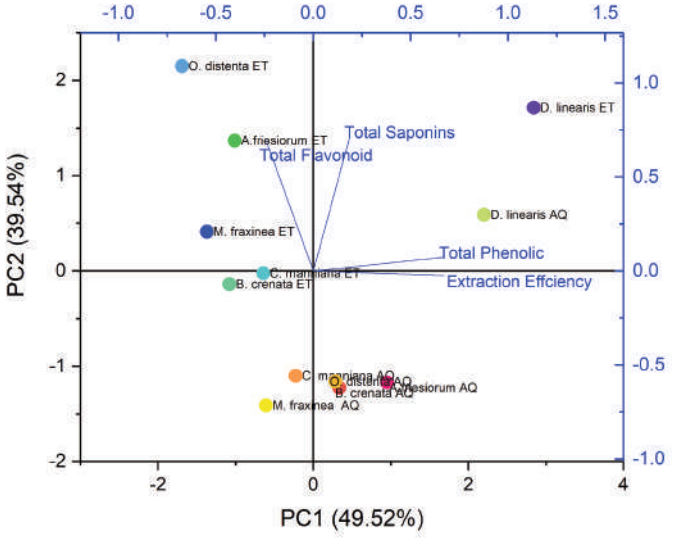

Figure 4. Principal Component Analysis (PCA) biplot indicating the variation of fern extracts based on phytochemical parameters of total phenolic content, extraction efficiency, total saponin content, and total flavonoid content. ET, ethanol extract; AQ, aqueous/water extract.

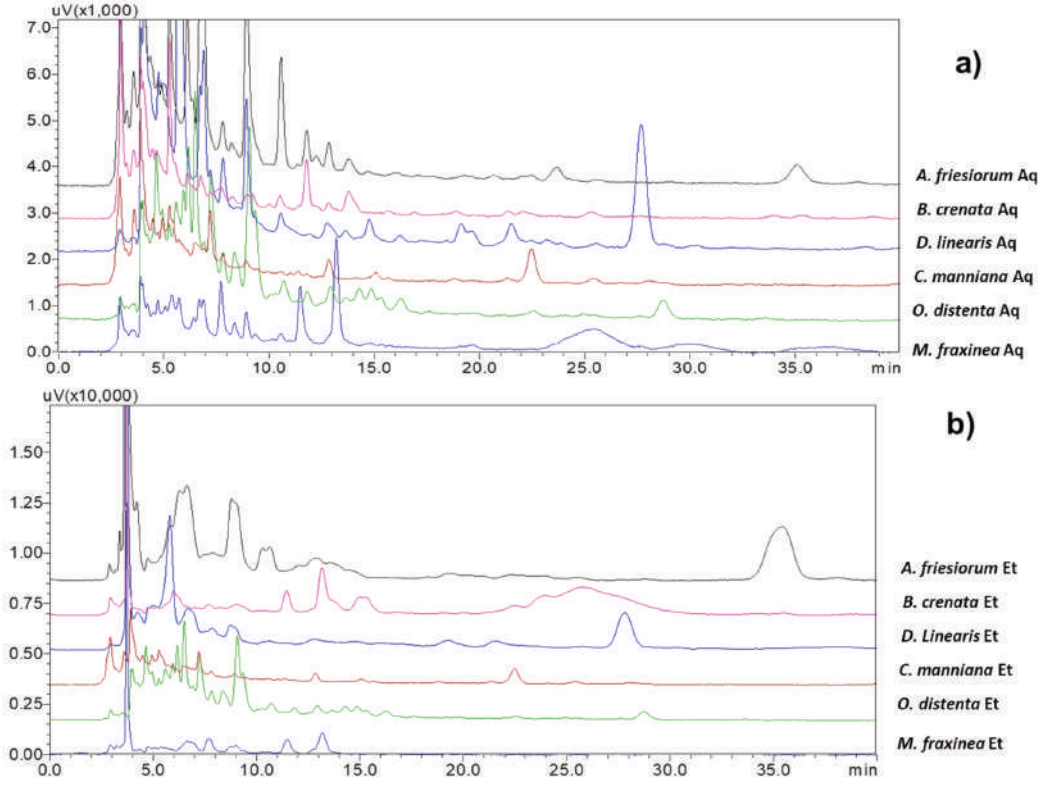

Figure 5. High-Performance Liquid Chromatography (HPLC) fingerprint overlay showing the variation in the extracts of different fern species: **a**) fingerprint of aqueous extracts, **b**) fingerprint of ethanol extracts.

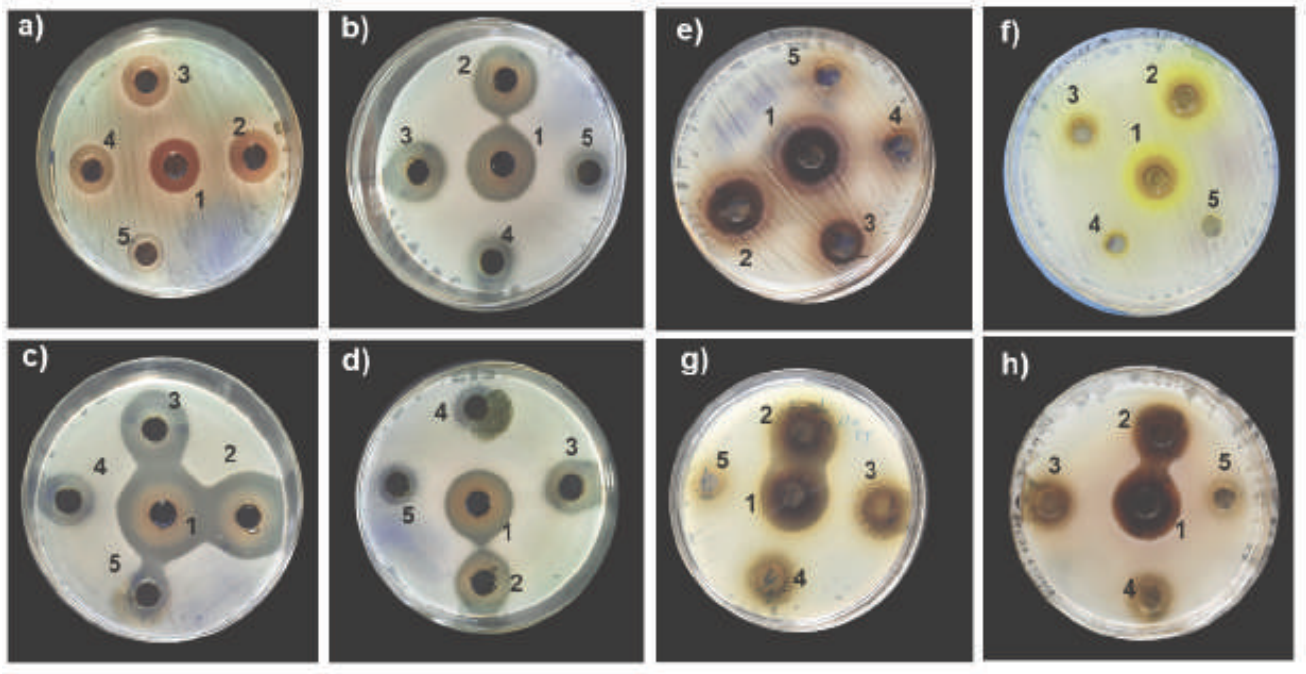

Figure 6. Photographic illustration of antimicrobial potential of fern extracts. **a**) Zones of inhibition of *S. aureus* induced by *Dicranopteris linearis* ethanol extract; **b**) zones of inhibition of *K. pnemoniae* induced by *Marattia fraxinea* ethanol extract; **c**) zones of inhibition of *E. coli* induced by *Marattia fraxinea* ethanol extract; **d**) zones of inhibition of *P. aeruginosa* induced by *Marattia fraxinea* ethanol extract; **e**) Zones of inhibition of *E. coli* induced by *Blotiella crenata* water extract; **f**) zones of inhibition of *S. aureus* induced by *Blotiella crenata* water extract; **g**) zones of inhibition of *E. coli* induced by *Marattia fraxinea* water extract; **h**) zones of inhibition of *K. pneumoniae* induced by *Marattia fraxinea* water extract.

Marattia fraxinea were the most potent on all the bacterial strains at a concentration of 50% w/v. The extracts of *Marattia fraxinea* were more potent against gram-negative strains of *P. aeruginosa, K. pnemoniae, and E. coli,* with the highest zones of inhibition recorded by its ethanolic extract against the *P. aeruginosa* (32.67±0.58mm) at 50% w/v concentration. The aqueous extracts of *Dicranopteris linearis* and *Oleandra distenta* extracts were the most potent against the gram-positive bacteria, producing the highest zones of inhibition of 27.7±1.15 and 26.67±1.53 mm, respectively, at 50% w/v concentration. The extracts of *Cyathea manniana* were generally less potent against all the pathogenic bacterial strains examined in this study. The aqueous extracts produced the lowest zone of inhibition, with the lowest value recorded against *P. aeruginosa* (13.3±0.58 mm). Results indicated that the gram-positive bacterial strain, *S. aureus*, was the most susceptible to fern extracts, followed by *E. coli*, while *P. aeruginosa and K. pnemoniae* were the least sensitive to the fern extracts.

In this study, the Minimum Inhibitory Concentrations (MICs) of the fern extracts were evaluated using the microplate technique, and the findings are detailed in Table 1. The MIC values ranged from 0.39 to 25% (w/v), indicating a spectrum of antimicrobial efficacy among the different fern extracts. Notably, *Oleandra distenta* extracts demonstrated the highest antibacterial potency, showing the lowest MIC values against both *S. aureus* and *E. coli* strains. In contrast, the aqueous extract of *Cyathea manniana* exhibited the least inhibitory effect, with a significantly higher MIC of 25% (w/v) against *E. coli*, suggesting reduced antimicrobial activity. Meanwhile, the aqueous extracts of *Marattia frax-*

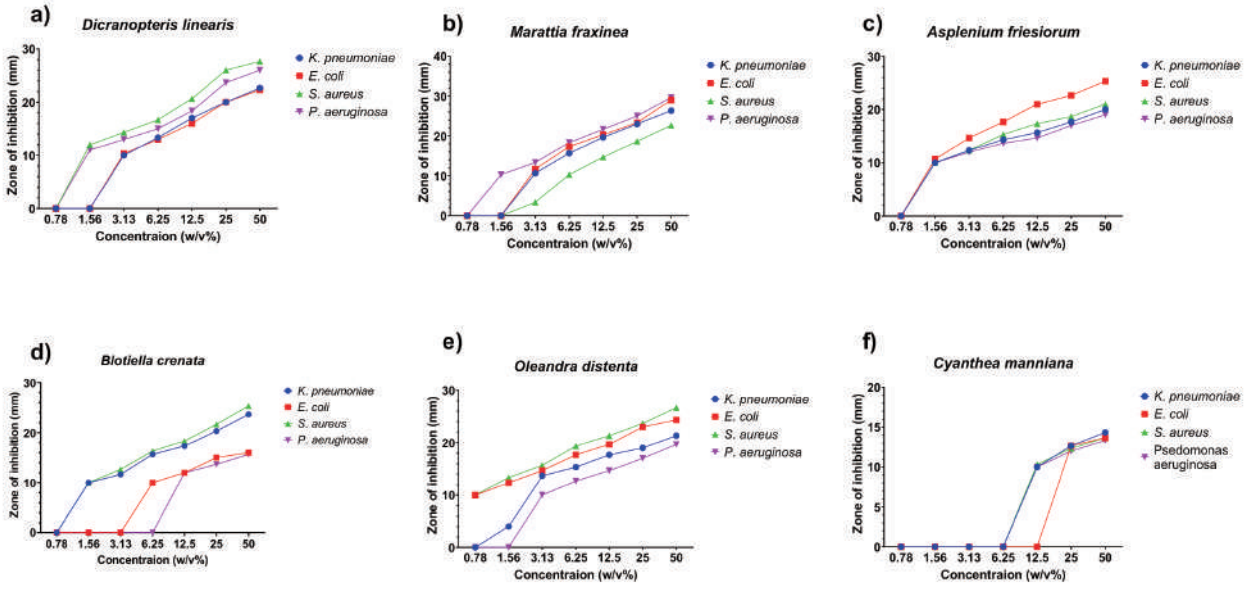

Figure 7. Antimicrobial activity of aqueous extracts of selected fern species from Bwindi Impenetrable National Park, values are presented as means of triplicate runs: **a**) variation in Zone of Inhibition (ZOI) with concentration of the aqueous extract of *Dicranopteris linearis*; **b**) variation in ZOI with concentration of the aqueous extract of *Marattia fraxinea*; **c**) variation in ZOI with concentration of the aqueous extract of *Asplenium friesiorum*; **d**) variation in ZOI with concentration of the aqueous extract of *Blotiella crenata*; **e**) variation in ZOI with concentration of the aqueous extract of *Oleandra distenta*; **f**) variation in ZOI with concentration of the aqueous extract of *Cyathea manniana.*

Table 1. Minimum Inhibitory Concentration (MIC) of selected fern extracts tested against common pathogenic bacterial strains.

Fern species	Extract	Minimum Inhibitory Concentration (% w/v)			
		K. pnemoniae	*E. coli*	*S. aureus*	*P. aeruginosa*
Oleandra distenta	Aqueous	1.56	0.39	0.39	3.13
	Ethanolic	3.13	3.13	1.56	1.56
Cyathea manniana	Aqueous	12.50	25.00	12.50	12.50
	Ethanolic	6.25	6.25	3.13	3.13
Asplenium friesiorum	Aqueous	1.56	1.56	1.56	1.56
	Ethanolic	6.25	12.5	3.13	1.56
Blotiella crenata	Aqueous	1.56	6.25	1.56	12.50
	Ethanolic	1.56	3.13	6.35	6.35
Dicranopteris linearis	Aqueous	3.13	3.13	1.56	1.56
	Ethanolic	6.25	3.13	3.13	6.25
Marattia fraxinea	Aqueous	3.13	3.13	3.13	1.56
	Ethanolic	3.13	3.13	6.25	1.56

inea, *Asplenium friesiorum*, *Oleandra distenta*, and *Dicranopteris linearis* displayed broad-spectrum antibacterial activity against all tested pathogenic strains, with MIC values ranging from 0.39 to 3.13% (w/v), highlighting their potential as effective antimicrobial agents.

In this study, the Minimum Inhibitory Concentrations (MICs) of the fern extracts were evaluated using the microplate technique, and the findings are detailed in Table 1. The MIC values ranged from 0.39 to 25% (w/v), indicating a spectrum of antimicrobial efficacy among the different fern extracts. Notably, *Oleandra distenta* extracts demonstrated the highest antibacterial potency, showing the lowest MIC values against both *S. aureus* and *E. coli* strains. In contrast, the aqueous extract of *Cyathea manniana* exhibited the least inhibitory effect, with a significantly higher MIC of 25% (w/v) against *E. coli*, suggesting reduced antimicrobial activity. Meanwhile, the aqueous extracts of *Marattia fraxinea*, *Asplenium friesiorum*, *Oleandra distenta*, and *Dicranopteris linearis* displayed broad-spectrum antibacterial activity against all tested pathogenic strains, with MIC values ranging from 0.39% to 3.13% (w/v), highlighting their potential as effective antimicrobial agents. Table 2 presents the findings of assessing the bactericidal properties of the fern extracts by determining the Minimum Bactericidal Concentration (MBC). Variability in the bactericidal potency of the extracts of test fern species was observed. The minimum bactericidal concentrations ranged from 3.13 to 25% % w/v of the fern extracts. Aqueous extracts of *Oleandra distenta*, *Asplenium friesiorum,* and *Marattia fraxinea* had the highest bactericidal activity across the pathogenic test microorganisms, with

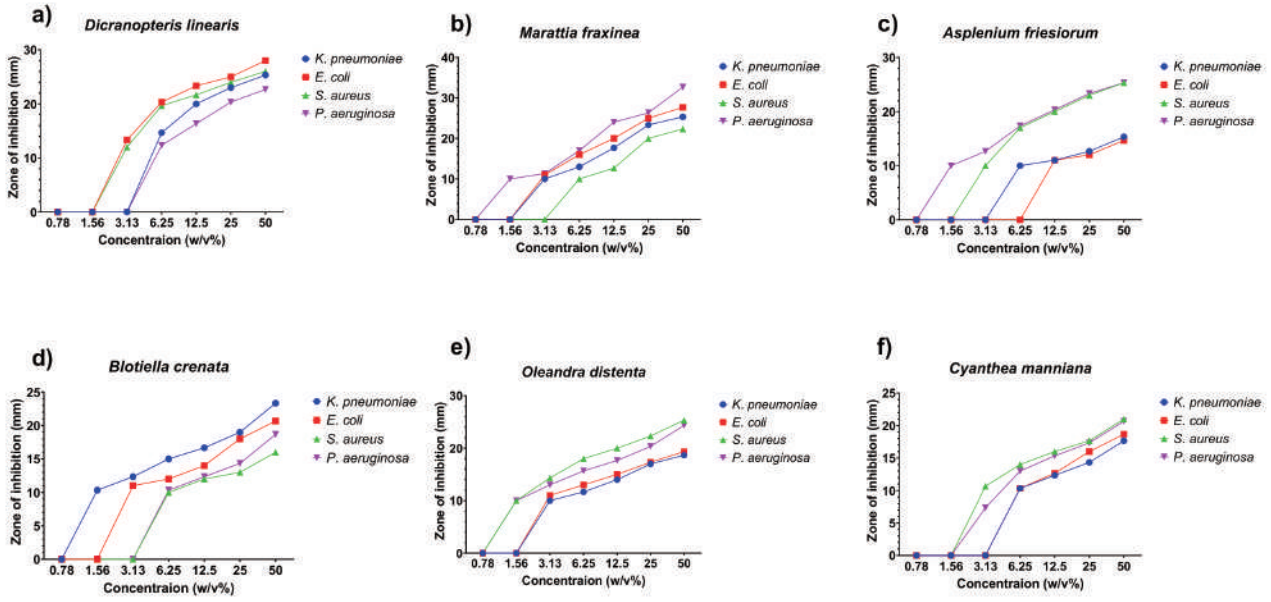

Figure 8. Antimicrobial activity of ethanol extracts of selected fern species from Bwindi Impenetrable National Park, values are presented as means of triplicate runs: **a**) variation in ZOI with concentration of the ethanol extract of *Dicranopteris linearis*; **b)** variation in ZOI with a concentration of the ethanol extract of *Marattia fraxinea*; **c**) variation in ZOI with a concentration of the ethanol extract of *Asplenium friesiorum*; **d**) variation in ZOI with concentration of the ethanol extract of *Blotiella crenata*; **e**) variation in ZOI with concentration of the ethanol extract of *Oleandra distenta*; **f**) variation in ZOI with concentration of the ethanol extract of *Cyathea manniana.*

Table 2. Minimum Bactericidal Concentration (MBC) of selected fern extracts tested against common pathogenic bacterial strains.

Fern species	Extract	Minimum Inhibitory Concentration (% w/v)			
		K. pnemoniae	*E. coli*	*S. aureus*	*P. aeruginosa*
Oleandra distenta	Aqueous	12.5	6.25	6.25	12.5
	Ethanolic	25.0	6.25	12.5	12.5
Cyathea manniana	Aqueous	N	N	N	N
	Ethanolic	N	N	25	N
Asplenium friesiorum	Aqueous	3.13	3.13	3.13	6.25
	Ethanolic	12.5	25.0	12.5	6.25
Blotiella crenata	Aqueous	12.5	25.0	12.5	25.0
	Ethanolic	12.5	12.5	25.0	25
Dicranopteris linearis	Aqueous	12.5	12.5	6.25	6.25
	Ethanolic	25.0	12.5	12.5	12.5
Marattia fraxinea	Aqueous	6.25	6.25	6.25	6.25
	Ethanolic	6.25	6.25	12.5	12.5

N, no minimum bactericidal concentration observed.

MBC values ranging from 3.13 to 12.5 % w/v. The aqueous extracts of *Cyathea manniana* did not show bactericidal activity against all test pathogenic bacterial strains. Nevertheless, the ethanol extract of this species proved to be bactericidal, particularly against gram-positive strains (*S. aureus*). This suggests that gram-negative bacteria are less susceptible to the extracts derived from this species.

Discussion

Plants contain bioactive compounds, including phenolics, alkaloids, coumarins, terpenoids, and flavonoids.[31] The bioactive compounds are obtained from the plant material through a process of extraction, which involves separating the soluble plant metabolites from the insoluble cellular components using appropriate solvents.[32] Any extraction process aims to achieve the maximum quantity of the desired bioactive compound, thereby optimizing its bioactivity.[33] Therefore, the yield and phytochemical composition of plant extracts are heavily influenced by the extraction methods and the type of solvent used. In this study, the water extracts generally had a higher extraction efficiency than ethanol. The differences in extraction efficiency may be due to the ability of the active compounds to dissolve in the solvent, the strength of the solute-matrix interaction, and the rate at which mass is transferred.[33] The results regarding extraction yield provide a starting point for assessing the uniformity of the raw materials utilized in the extraction procedure for medicinal fern species.

The molecular composition of phenolics consists of a cyclic aromatic ring combined with at least one hydroxyl functional group.[34] Phenolic compounds are known to have a broad spectrum of biochemical functions, such as inhibiting bacterial growth, preventing oxidation, inhibiting mutations, and combating cancer.[35] It is, therefore, essential to quantify phenols in plant extracts. The variation of TPC among species of ferns observed in this study may be attributable to the variations in the genetic makeup and environment in which species occur, these two factors are known to influence the phytochemical constituents of plants.[36] It is worth noting that the variation in the TPC among fern extracts could affect their biological activity. Nevertheless, this study has established a baseline regarding the presence of phenols in water and ethanol extracts of fern species.

Flavonoids belong to a larger category of phenolic compounds that come in different structural forms, including glycosides or aglycones.[37] This group of compounds is known to possess numerous biological activities, including anti-inflammatory, anti-hepatotoxic, anti-tumor, antibacterial, antiviral, and antioxidant properties.[38] For this reason, they are now seen as essential compounds with many nutraceutical and pharmacological applications. In this study, the high concentration of flavonoids registered in the ethanol extracts when compared with water extracts may indicate that the fern extracts contain higher amounts of free flavonoids, which are generally soluble in organic solvents like ethanol.[39] This trend in results is comparable to findings by Truong *et al*.[32] The variability in the TFC among extracts exhibited in this study could consequently have varying effects on the biological activity of fern extracts, specifically the antibacterial activity that was investigated in the present study.

Saponins are a group of structurally related compounds that are secondary metabolites, consisting of a triterpenoid, aglycone, or steroid linked to oligosaccharide groups.[40] Saponins exhibit numerous biological and pharmacological properties, including cytotoxicity, anticancer, and antibacterial activity. Saponins are thus acknowledged as the main active compounds in traditional healing practices, which have been employed for centuries to prevent and treat a wide range of ailments.[41] For this reason, investigating the concentration of saponin content in fern extracts was deemed necessary in this study to highlight the saponin concentrations in fern species in Uganda. Results indicated that ethanol was more efficient in extracting saponins than water as a solvent. This can be ascribed to the varying solubility of saponins in the two different solvents. Furthermore, prior studies have demonstrated that the choice of extraction solvents significantly affects the effectiveness of saponin extraction.[42]

The results of multivariate analysis based on four parameters of phenolic content, flavonoid content, saponin content, and extraction yield expressly revealed that *Dicranopteris linearis* extracts are generally distinct from other extracts due to the high extraction yield and high phenolic and saponin contents. Therefore, the classes generated from this analysis can guide herbal medicine practitioners and product developers in selecting the best fern species and extract for use as a raw material in herbal medicine formulations. For instance, if formulations require high concentrations of saponins and phenolics, then both aqueous and ethanol extracts of *Dicranopteris linearis* provide the best yield of these bioactive compounds. The findings also demonstrated marked phytochemical variability in the extracts of the various fern species. The fact that the two solvents (ethanol and water) have different extraction abilities explains the variation in the peak densities with species. However, the variation in the characteristic peaks between species could be attributed to the differences in the genetic makeup and microenvironment conditions such as soil and climate. These factors are known to have remarkable effects on the phytochemical composition of plants.[43] Nonetheless, the fingerprints provide substantial insight into the presence of the active phytochemicals of selected fern species. Common peaks, in this case, would mean the compounds are likely the same or closely related. Conversely, the uncommon peaks could account for the variations in the antibacterial efficacy of the extracts studied in this work. The results of this investigation have shown that variation in the antibacterial efficacy of extracts from ferns exists. This variation may be related to variations in the phytochemical contents of various species of ferns. Results of MIC and MBC indicated that extracts from most fern species investigated in this study had both inhibitory and bactericidal potential, thus suggesting that ferns contain active compounds that could be potential sources of antimicrobial agents. The presence of flavonoids, saponins, and phenolics (Figure 2) explains the antibacterial activity demonstrated by the fern extracts. Flavonoids and saponins are examples of phytochemicals that can either stop extracellular microbial enzymes from making substrates that microorganisms need to grow or can directly affect microorganisms' metabolism by suppressing oxidative phosphorylation.[44] Other studies have proposed that the bioactive substances found in the plant extracts interact with the proteins that make up the microbial cell membrane, ultimately resulting in the membrane rupturing and, eventually, cell lysis.[45] In addition to interfering with normal cell communication, plant secondary metabolites may also change the structure and function of membranes and the production and function of DNA and RNA.[46] So, the differences in antimicrobial activity observed among the test pathogenic bacteria looked at in this study may be due to the different cell membrane compositions and structures of gram-negative and gram-positive bacteria. Nevertheless, this study has proved that fern extracts are potentially effective against common pathogenic bacteria and thus could be potential sources of potent antibacterial agents. Though the *in vitro* study reported the

promising antibacterial activity of ferns, there is a need for an *in vivo* study of the extracts in the future to inform on the metabolism of the active molecules and their pharmacokinetics, which will contribute to drug development and assessment.

Conclusions

Bacterial infections remain a major global health challenge, exacerbated by the rising antibiotic resistance. This study highlights ferns as a promising source of antimicrobial agents, with species like *Dicranopteris linearis* containing significant amounts of bioactive compounds such as phenols, saponins, and flavonoids. Both water and ethanol extracts of the investigated fern species exhibited antibacterial activity, highlighting their potential applications in nutraceuticals, pharmacology, and herbal medicine. These findings contribute to drug development pipelines by identifying ferns as a valuable source of plant-based bioactive compounds that could be refined into novel antimicrobial agents.

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