

Phytochemical profiling and biofilm inhibitory potential of *Sphaerostephanos unitus* (L.) Holttum

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Research Article

Keywords: Antibiofilm, Antioxidant, Fatty acid fraction, GC–MS, *Pseudomonas aeruginosa*, *Sphaerostephanos unitus*

Posted Date: January 7th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8502915/v1>

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Additional Declarations: The authors declare no competing interests.

Abstract

Purpose

The aim of this study was to investigate the phytochemical composition and antioxidant, cytotoxic, antimicrobial, and antibiofilm activities of *Sphaerostephanos unitus* (L.) Holttum, a fern traditionally used in Northeast India.

Methods

Sequential solvent extraction was performed using methanol, ethanol, acetone, and ethyl acetate as solvents. Qualitative and quantitative phytochemical screening was followed by antioxidant assays, such as DPPH and nitric oxide scavenging, and cytotoxicity testing using brine shrimp lethality test. Antimicrobial and antibiofilm activities were studied against multidrug-resistant clinical isolates including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida* spp. The fatty acid-rich fraction was further analyzed using GC-MS.

Results

Ethanol extract had the highest phenolic and flavonoid content, exhibiting the most potent antioxidant activity with an IC_{50} of 35.74 μ g/mL. In addition, the fatty acid fraction exhibited the lowest minimum inhibitory concentration at 0.25 mg/mL and the strongest antibiofilm inhibition activity (94.8%). GC-MS analysis identified major fatty acids such as dodecanoic, tetradecanoic, and oleic acids, which are well known for their antimicrobial and anti-quorum sensing activities.

Conclusion

S. unitus demonstrates considerable antioxidant and antimicrobial potential, supporting its ethnomedicinal relevance and emphasizing its importance for future drug discovery.

Introduction

With the global increase in antimicrobial resistance, there has been an accelerated search for new alternatives. Based on animal behavior, traditional use, and folklore, the bioprospection of natural antimicrobial compounds from traditionally used plants and pteridophytes has plummeted. There are more than 12,000 species of pteridophytes distributed worldwide, which are underexplored, yet important repositories of phytochemicals with varied biological activities (Singh & Upadhyay, 2012). Despite their use in indigenous medical systems such as Ayurveda, Unani, and homeopathy, only a few ferns have been comprehensively studied for their pharmacological properties.

Sphaerostephanos unitus (L.) Holttum is a member of the Thelypteridaceae family with ethnomedicinal importance in India, especially in the Western Ghats and North-Eastern regions. Young fronds are traditionally used as topical washes for treating dermatological diseases such as scabies and wounds, in addition to being used as a centrifuge (Varaprasadham & Marimuthu, 2011). Among the tea-tribe communities of Assam, the plant is also used as an antiparasitic agent in poultry cages during brooding. It is also a medicinal herb employed by folk communities in starter cultures for producing traditional beverages. Earlier studies have revealed the presence of secondary metabolites, such as phenolics, flavonoids, triterpenoids, and saponins. This corroborates with its antioxidant, anti-inflammatory, antidiabetic, and antibacterial properties (Johnson et al., 2020). However, studies of its antimicrobial and antibiofilm efficacy against clinically relevant pathogens are limited. In addition, biofilm-associated bacteria exhibit an inherent tolerance to conventional antibiotics. To address this, in this study, we sought to explore the antimicrobial and antibiofilm properties of the solvent- and fatty acid-rich fractions of this plant, profile a few phytochemicals in the fatty acid-rich fractions, and corroborate their pharmacological potential, antioxidant, and cytotoxic activities.

Materials and methods

Chemicals

The chemicals and reagents used were of analytical grade. 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, quercetin, ascorbic acid, and the solvents methanol, ethyl acetate, ethanol, and acetone were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL) and HiMedia Pvt. Ltd.

Collection and authentication of plant material

Fresh fronds of *Sphaerostephanos unitus* (L.) Holttum were collected from Jorhat, Assam, India (26°46'33.012" N, 94°15'29.772" E). Herbarium specimens were prepared and deposited for taxonomic authentication in the Weed Herbarium of Assam Agricultural University (AAU), Jorhat. A voucher specimen, Collection No. 04, was deposited at the Weed Herbarium for reference. Plant samples were washed, shade-dried at $27 \pm 2^\circ\text{C}$, pulverized to a fine powder, and stored in airtight containers until extraction.

Preparation of extracts

Five grams of plant powder, passed through a 60-mesh sieve, was extracted in a Soxhlet apparatus for 6 h using different solvents based on increasing polarity, that is, ethyl acetate, acetone, ethanol, and methanol. The solvent-to-material ratio was 1:20 w/v, and extraction was performed until the siphon solvent became colorless. The extracts were concentrated under reduced pressure at 37°C and were stored at 4°C. The yield of the extracts was calculated as follows:

$$\text{Yield (\%)} = \left(\frac{\text{Weight of solvent free extract (g)}}{\text{Dry plant weight}} \right) * 100$$

The dried extracts were re-dissolved in 10% DMSO (v/v) and diluted with sterile distilled water for antimicrobial and antibiofilm assays to ensure that the final DMSO concentration in the antimicrobial test wells did not exceed 1% (v/v).

Phytochemical screening

Qualitative phytochemical screening for alkaloids, phenols, flavonoids, tannins, saponins, terpenoids, cardiac glycosides, coumarins, phlobatannins, xanthoproteins, and carboxylic acids was performed on the extracts using standard color reaction assays (Subba et al., 2016). The details of the procedures and observations of these tests are presented in **Table S1**.

Determination of total phenolic compounds

The Folin-Ciocalteu test was used to determine the total phenolic content. A 100 µl of the sample and 100 µl of Folin-Ciocalteu reagent (MERCK-DJ4D640513) were mixed. Then, 1 ml of 7.5% sodium carbonate solution was added to the mixture after 5 min. Then, 3 mL of distilled water was added. After 2 h in the dark, the absorbance was measured at 765 nm using a SYSTRONIC PC-based double-beam UV-VIS spectrophotometer-2202. The total phenolic content was calculated by extrapolating the calibration curve produced by gallic acid (25, 50, 100, 150, and 250 µg/ml) solutions. All tests were performed in triplicate. The total phenolic content of the dried sample was estimated in milligrams of gallic acid equivalents (GAE) (Sulastri et al., 2018). Reagent blanks were prepared using an identical volume of methanol instead of the extract, in addition to the respective assay reagents, and their absorbance was subtracted from the sample readings.

Determination of total flavonoids

Aluminium chloride assay was used to determine the total flavonoid concentration. A 100 µL sample was mixed with 300 µL of 95% ethanol, followed by 100 µL of 10% aluminium trichloride and 100 µL of 1 M potassium. The volume was increased to 3 ml. The absorbance was measured at 415 nm using a SYSTRONIC PC-based double-beam UV-VIS spectrophotometer-2202 after 30 min of incubation. The total flavonoid content was calculated by extrapolating the calibration curve obtained by preparing solutions with quercetin (5, 10, 15, 25, and 50 µg/ml). The flavonoid content was determined thrice. The total flavonoid content of the dried sample was determined in mg of quercetin equivalents (QE) (Sulastri et al., 2018). Reagent blanks were prepared using an identical volume of ethanol instead of the extract, in addition to the respective assay reagents, and their absorbance was subtracted from the sample readings.

Determination of total tannins

Insoluble polyvinyl-polyrrolidone (PVPP), which binds to tannins, was used to assess tannin concentrations. A total phenolic sample (100 µL) was vortexed and centrifuged at 3000 rpm for 10 min after being maintained at 4°C for 15 min. Non-tannin phenolics were quantified in the transparent supernatant, similar to total phenolics. The difference between the total phenol and non-tannin phenolic content was used to estimate the total tannin content (Pulipati et al., 2014). Reagent blanks were prepared using an identical volume of ethanol instead of the extract, in addition to the respective assay reagents, and their absorbance was subtracted from the sample readings.

DPPH free radical scavenging activity

All extracts were evaluated for their ability to eliminate free radicals using 1,1-diphenyl-2-picryl-hydrazyl (DPPH). DPPH solution (100 µL DPPH solution in methanol (0.1 mM) was mixed with 100 µL methanol solution of each crude extract at different concentrations (31.25, 62.5, 125, and 250 µg/ml). The mixtures were thoroughly mixed and incubated at room temperature in the dark for 30 min. The absorbance was measured in the visible range at 517 nm using a Model Nova BioEra Life Science Microplate Reader. Ascorbic acid was used as a standard. In the reaction mixtures, lower absorbance values indicated significant free radical scavenging activity. The tests were performed in triplicate. The DPPH free radical scavenging activity was calculated using the following formula:

$$\text{Scavenging Activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) * 100$$

Here, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of all samples (Sravani & Paarakh, 2012).

Nitric oxide scavenging activity

A 500 μ L extract solution at different concentrations (31.25, 62.5, 125, and 250 μ g/mL) was combined with 500 μ L of 10 mM sodium nitroprusside solution and incubated at 37°C for 2 h to determine whether it could scavenge NO. Finally, 500 μ L Griess reagent (1% sulfanilamide in 2.5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) was added. The absorbance was then measured in the visible range at 570 nm using a Model Nova BioEra Life Science Microplate Reader. Ascorbic acid was used as a standard. The tests were performed in triplicate. The NO-scavenging ability was calculated using the following formula:

$$\text{Scavenging Activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) * 100$$

Here, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of samples and a comparison of the extract (Shanmugapriya et al., 2012). The IC_{50} values of the antioxidant assays were calculated by linear regression of percentage inhibition values versus extract concentration using Microsoft Excel (2019). A straight-line trend line was applied, and a regression equation was employed to interpolate the IC_{50} values. The curve plots and regression outputs are provided in the Supplementary Data.

Brine Shrimp Lethality Assay

The brine shrimp lethality assay (BSLA) was conducted as a preliminary screen for the cytotoxic activity of crude solvent extracts of *Sphaerostephanos unitus* using a previously described method earlier (Krishnaraju et al., 2005) with slight modifications. Eggs of *Artemia salina* were hatched in artificial seawater (25 g/L sea salt in distilled water) under continuous aeration for 48 h at room temperature ($27 \pm 2^\circ\text{C}$). For the test, actively motile nauplii free of eggshells were collected from the illuminated side of the hatching vessel. Ten nauplii were delivered to each test tube filled with 4.5 mL of artificial seawater. Crude extracts dissolved in a minimal volume of DMSO ($\leq 1\%$ v/v) and added to obtain final concentrations of 10, 100, 300, 800, and 1000 μ g/mL. Podophyllotoxin, purchased from the local stores, was used as the positive control, and 1% DMSO in seawater was used as the negative control. All treatments were performed in triplicate. The treated nauplii were incubated at room temperature ($\sim 27^\circ\text{C}$) under ambient laboratory lighting for 24 h. Survival was assessed by counting live nauplii, and the percent lethality was calculated using the following formula:

$$\text{Lethality (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) * 100$$

where A_0 represents the initial number of nauplii, and A_1 represents the number of survivors after 24 h. LC_{50} values were calculated by linear regression of extract concentrations against percentage lethality using Microsoft Excel 2019. A straight-line trend line was applied, and the regression equation was used to interpolate the LC_{50} values. Data are presented as the mean $\pm$ SEM; $n = 3$.

Preparation of fatty acid-rich fraction

A fatty acid-rich fraction was extracted from the *S. unitus* methanol extract using the modified Twitchell precipitation method (Twitchell, 1921). In summary, 2 g of dried extract was treated with 10% lead acetate to remove polyphenols and the supernatant was acidified with 1% HCl before being refluxed for 2 h. The precipitate formed was extracted with ethanol and further purified by fractional crystallization at 4°C to yield a fatty acid-rich fraction (SU-F). This fraction was concentrated, reconstituted in 10% DMSO, and stored at 4°C until use. The antimicrobial activities of SU-F fraction and crude extracts were evaluated against clinical pathogens.

Microorganisms, culture preparation and biofilm formation

The clinical isolates used in this study were obtained from the Ayursundra Superspecialty Hospital, Guwahati, Assam, India. The isolates included *Pseudomonas aeruginosa* (bronchial infection), *Salmonella typhi* (pus), *Klebsiella pneumoniae* (tracheal infection), *Acinetobacter baumannii* (tracheal infection), *Proteus mirabilis* (urine infection), *Candida albicans* (urine infection), and *Candida tropicalis* (tracheal infection). *Staphylococcus epidermidis* ATCC 35984 and *K. pneumoniae* ATCC BAA-1705 were used as the control strains. The antimicrobial resistance profiles of the clinical isolates were confirmed in the diagnostic laboratory of the hospital using CLSI-compliant protocols. Therefore, no antibiotic standards were included in the inhibitory studies. The *P. aeruginosa* clinical isolate is a β -lactamase-producing strain resistant to several antibiotics, including ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, and ciprofloxacin. Prior to antibiofilm assays, the intrinsic biofilm-forming ability of this *P. aeruginosa* clinical isolate was evaluated along with the ATCC strains *S. epidermidis* 35984 and *K. pneumoniae* BAA-1705, using the crystal violet tube adherence method (Das et al., 2017). Based on the OD_{492} readings, the *P. aeruginosa* isolate was marked as a strong biofilm former ($OD > 4 \times OD_{cut}$) (Hassan et al., 2011; Kirmusaoğlu, 2019) hence, only this isolate was selected for further antibiofilm activity testing.

All bacterial isolates were maintained on Luria-Bertani (LB) agar at 4°C, while yeast isolates were maintained on Potato Dextrose Agar (PDA). All bacterial isolates were sub-cultured in Mueller-Hinton broth prior to use and adjusted to a turbidity equivalent to 0.5 McFarland standard, which is approximately 1.5×10^8 CFU/mL. The inoculum was prepared similarly for antifungal assays using Sabouraud Dextrose Broth (SDB).

Determination of minimum inhibitory and bactericidal concentration

The MICs of the crude extracts and SU-F were determined using the resazurin-based broth microdilution method (Khanal et al., 2020). Two-fold serial dilutions (1000 – 7.81 µg/mL) were prepared in cation-adjusted Mueller-Hinton broth, with a final inoculum of 5×10^8 CFU/mL. Following incubation at 37°C for 24 h, the MIC was determined as the lowest concentration that prevented the color change from blue to pink due to resazurin. To determine the MBC, wells with no visible growth were subcultured on MHA plates. All experiments were performed in triplicate. For *Candida* spp., the MICs were determined in Sabouraud Dextrose Broth under identical conditions.

Antibiofilm activity assay

A 96-well microtiter plate assay was used to determine the inhibitory effect of *S. unitus* extract and SU-F on biofilm formation by *Pseudomonas aeruginosa* (Das et al., 2017; Kirmusaoğlu, 2019). Bacterial suspensions containing 1.5×10^8 CFU/mL were treated with the MIC concentration of each extract, incubated at 37°C for 48 h, and stained with 0.5% crystal violet. After washing with distilled water twice, the bound dye was solubilized in 30% acetic acid and the absorbance was measured at 492 nm. Biofilm inhibition (%) was calculated relative to that of untreated controls.

Estimation of biofilm matrix protein and EPS

The total protein content of the plant extract- and SU-F-treated *P. aeruginosa* biofilms was measured using the Lowry method. Briefly, the attached biofilm in culture tubes was washed with PBS and boiled in 0.5 N NaOH for 30 min to extract proteins. Supernatants were collected after centrifugation at 10,000 rpm for 5 min. Protein content was estimated at 650 nm using BSA as the standard. The total extracellular polysaccharide content in the treated biofilms was measured using the phenol-sulfuric acid assay method (Das et al., 2017). Biofilm fractions were treated with 5% phenol and concentrated sulfuric acid, incubated at 30°C, and the absorbance was measured at 490 nm. Glucose was used as a standard. The assays were performed in triplicate, and the absorbance was measured using a SYSTRONIC PC-based double-beam UV-VIS spectrophotometer-2202. The results were expressed relative to the untreated controls, where sterile distilled water was used instead of the extracts.

Statistical analysis

All experiments were performed in triplicate ($n = 3$), and the data are presented as the mean $\pm$ SEM. The IC_{50} and LC_{50} values for antioxidant and cytotoxicity assays were estimated by linear regression analysis of percent inhibition or lethality versus concentration using Microsoft Excel 2019. One-way analysis of variance (ANOVA) was used to determine significant differences among extracts, followed by Duncan's multiple range test at a significance level of $p < 0.05$, using WASP 2.0 software (ICAR Research Complex, Goa, India). For MIC/MBC determination, biological replicates were independently performed on three different days.

Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis

GC-MS analysis of the SU-F fraction was performed at Guwahati Biotech Park, Assam, India. The samples were dissolved in spectroscopy-grade ethanol, filtered through 0.2 µm filters, and analyzed using a PerkinElmer Clarus 680 GC system connected to a Clarus 600C single quadrupole MS fitted with an Elite-5MS capillary column (30 m $\times$ 0.25 mm, 0.25 µm film thickness). The following oven temperature program was applied: initial 60°C (1 min), ramped at 7°C/min to 200°C (3 min hold), and then ramped at 10°C/min to 300°C (5 min hold). Helium was used as the carrier gas in the split mode (10:1) with an injection volume of 1 µL at 280°C. The MS was operated in the EI mode (70 eV), scanning from m/z 50–600, with a source temperature of 150°C. Only chromatographic peaks with clear mass spectral patterns and a match $\geq 80\%$ similarity based on the NIST library were considered for compound identification. Low abundance or ambiguous peaks were not analyzed further.

Results and discussion

Phytochemical composition of *Sphaerostephanos unitus* (L.) Holtum

Phytochemical screening of crude solvent extracts of *Sphaerostephanos unitus* (L.) Holtum revealed the consistent presence of alkaloids, phenols, flavonoids, saponins, terpenoids, tannins, and cardiac glycosides (**Supplementary Table 1**). These phytochemical extracts in ethyl acetate, acetone, ethanol, and methanol are commonly associated with antioxidant, antimicrobial, and anti-inflammatory activities, as previously reported (Chopra & Doiphode, 2002; Johnson et al., 2020). Among the tested extracts, both ethanol and methanol extracts contained a wide array of phytochemicals. Polar solvents effectively recovered the phenolic and flavonoid compounds. This profile is consistent with earlier studies on *S. unitus* from South India, confirming its richness in secondary metabolites, which could be of pharmacological significance (Chopra & Doiphode, 2002).

Quantitative phytochemical analysis

The quantitative estimations of total phenolic, flavonoid, and tannin content in *S. unitus* extracts showed a wide range of variation among different solvents (**Table 1; Supplementary files**). The ethanol extract contained the highest concentrations of total phenolics (1166.67 ± 0.002 mg GAE/g), flavonoids (657.22 ± 0.002 mg QE/g), and tannins (763.47 ± 0.007 mg GAE/g), followed by the acetone and methanol extracts. Relatively low levels of these metabolites were identified in the ethyl acetate extract. The high polarity of ethanol may be the reason for the increased solubilization of hydroxylated compounds. Similar solvent-related differences have been reported for other pteridophytes (Subba et al., 2016) and *S. unitus* (Johnson et al., 2020). Such variations in metabolite yield could also be due to ecological and geographical factors that affect secondary metabolite biosynthesis (Yao et al., 2010). These compounds, particularly phenolics and flavonoids, are well-known contributors to the antioxidant and antimicrobial efficacy observed in further bioassays.

Antioxidant activity

The antioxidant activity of the crude solvent extracts was assessed using DPPH and nitric oxide (NO) radical scavenging assays to confirm the biological significance of the identified secondary metabolites. The ethanol extract exhibited the highest radical scavenging activity, with an IC_{50} of 35.74 ± 0.82 $\mu\text{g/mL}$ for DPPH and 73.29 ± 0.73 $\mu\text{g/mL}$ for NO among the tested extracts (**Table 2; Supplementary files**). Moderate activity was also observed for the acetone and methanol extracts, but the ethyl acetate extract was the least active. This agrees with the previously established strong positive correlation between total phenolic and flavonoid content and antioxidant efficiency (Manivannan & Johnson, 2020). The increased scavenging ability observed for the ethanol extract was consistent with the higher content of phenolic and flavonoid compounds, which are hydrogen-donating antioxidants that terminate free radical chain reactions. Similar trends have been described for other fern species rich in these metabolites. Therefore, *S. unitus* follows the same phytochemical activity relationship pattern reported for medicinal pteridophytes (Johnson et al., 2020; Sulaiman et al., 2011).

Cytotoxic potential by brine shrimp lethality assay

Cytotoxicity tests of the crude solvent extracts were performed using the *Artemia salina* brine shrimp lethality test; the results are presented in **Table 3 (Supplementary files)**. Acetone exhibited the highest cytotoxicity, with an LC_{50} value of 468.53 $\mu\text{g/mL}$, followed by the ethanol, methanol, and ethyl acetate extracts. All extracts showed LC_{50} values less than 1000 $\mu\text{g/mL}$, which is considered to indicate significant cytotoxic activity (Anderson et al., 1991). The observed cytotoxicity is likely linked to the presence of alkaloids, flavonoids, and tannins, as similar plant extracts have been previously associated with apoptosis-inducing and enzyme-inhibitory effects (Patle et al., 2020). Mortality was concentration-dependent, confirming dose-responsive toxicity. These findings are supported by previous studies on ferns, which reported similar LC_{50} values for antitumor or antiproliferative activities. Accordingly, the results suggest that *S. unitus* contains bioactive compounds that show promise as cytotoxic and therapeutic agents, warranting further validation *in vitro*.

Antimicrobial and antibiofilm activities

The antimicrobial efficacy of various crude solvent extracts and SU-F against seven clinical and two ATCC reference isolates is shown in **Table 4**. All extracts exhibited measurable antimicrobial activity, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranging between 0.29 and 2.67 mg/mL and between 0.58 and 5.34 mg/mL , respectively. Among the crude extracts, the ethyl acetate extract was the most effective, with a MIC of 0.29 mg/mL and an MBC of 0.58 mg/mL against *Pseudomonas aeruginosa*, *Salmonella typhi*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Candida albicans* and *C. tropicalis*. Notably, SU-FA exhibited the strongest activity overall, with MIC and MBC values of 0.25 mg/mL and 0.5 mg/mL , respectively, against most bacterial and fungal isolates tested. This included the control strains *Staphylococcus epidermidis* ATCC 35984 and *K. pneumoniae* ATCC BAA-1705. These values were lower than those of the corresponding crude extracts, highlighting that the bioactive compounds responsible for the antimicrobial activity were concentrated in the fatty acid fraction. The presence of medium- and long-chain fatty acids such as dodecanoic acid, tetradecanoic acid, and oleic acid, as detected by GC-MS analysis, explains the high potential of *S. unitus* for antimicrobial action. These fatty acids disrupt microbial membranes and interfere with quorum-sensing pathways (Marimuthu et al., 2022), which could explain the pronounced inhibition of both the bacterial and fungal pathogens observed in this study. Overall, the low MIC/MBC ratios recorded for both the ethyl acetate extract and SU-F confirmed *S. unitus* is a promising source of lipid-derived antimicrobial agents.

Screening of the test organisms for their biofilm-forming ability showed that the clinical *Pseudomonas aeruginosa* isolate was a strong biofilm former, with $OD_{492} = 0.42 \pm 0.11$, while *Staphylococcus epidermidis* ATCC 35984 showed weak and the clinical *Klebsiella pneumoniae* isolates showed negligible biofilm formation. Therefore, *P. aeruginosa* was selected for further antibiofilm assay. Treatment with solvent extracts and SU-F significantly hindered biofilm development in microtiter plates (**Table 5; Supplementary files**). The methanol and ethyl acetate crude extracts showed moderate inhibition at approximately 79 %, while the highest inhibition of 94.80 ± 0.58 % was achieved for the fatty acid fraction, proving to be more efficient against biofilms. This result is in agreement with the finding that the lipidic components detected by GC-MS, including dodecanoic and oleic acids, play a role in disturbing surface adhesion and biofilm maturation. Consistent with the inhibition assay, SU-F caused a significant decrease in total extractable protein (0.131 ± 0.02 mg/mL) and exopolysaccharide (EPS) content (0.363 ± 0.01 mg/mL) compared with the untreated biofilms (0.87 ± 0.02 mg/mL protein; 1.03 ± 0.03 mg/mL EPS) (**Table 5; Supplementary files**). Reductions in both parameters indicate compromised biofilm matrix stability and diminished bacterial biomass. The correlation between reduced protein and EPS levels and increased inhibition percentage suggests that the fatty acid fraction impairs both initial adhesion and subsequent matrix production, thereby weakening the biofilm architecture. The strong antibiofilm activity of *S. unitus*, particularly its fatty acid-rich fraction, highlights its potential as a natural source of anti-quorum and antibiofilm agents that are effective against multidrug-resistant *P. aeruginosa*.

GC-MS characterization of fatty acid-rich fraction

GC-MS analysis of SU-F revealed six major peaks corresponding to the major bioactive compounds (**Fig S9; Table S2**). Peak identification was based on NIST library matches with ≥ 80 % similarity and retention time concordance with known standards. Only well-resolved dominant peaks were considered for the compound assignment. The main constituents summarized in **Table S2** include medium- to long-chain fatty acids and their related esters: dodecanoic acid (base peak, 30.77 min), tetradecanoic acid (26.83 min), oleic acid (32.42 min), and N-decanoic acid (23.58 min), as well as 13-methyltetradec-9-enoic acid methyl ester and 3-N-hexylthiolane, S,S-dioxide. These fatty acids are known for their antibacterial, antibiofilm, and anti-inflammatory activities (Juárez-Rodríguez et al., 2021; Ralte et al., 2022). Saturated fatty acids, such as dodecanoic and tetradecanoic acids, disrupt

microbial membranes, whereas their unsaturated analogs, such as oleic acid, inhibit quorum-sensing pathways and subsequent biofilm development in *Pseudomonas aeruginosa*. The sulfur-containing compound 3-N-hexylthiolane (S,S-dioxide) has been reported to exhibit antitumor and antimicrobial activities. Therefore, the GC–MS profile underpins the dominance of lipidic metabolites in *S. unitus* and provides a mechanistic basis for its strong antimicrobial and antibiofilm activities against some MDR bacteria in the current study.

Conclusion

This study highlights the fern species *Sphaerostephanos unitus* (L.) Holttum as a potential candidate with profound bioactivity. The ethanol and ethyl acetate fractions showed high phenolic, flavonoid, and tannin content, which correlated with strong antioxidant activity. Among all solvents, the fatty acid-rich fraction (SU-F) exhibited pronounced antimicrobial and antibiofilm activities, effectively diminishing the formation of *Pseudomonas aeruginosa* biofilms and their matrix components. GC–MS analysis revealed the presence of bioactive lipidic metabolites, such as dodecanoic, tetradecanoic, and oleic acids, which may explain these effects. Considering resource limitations, only the fatty acid fraction was characterized using GC-MS. Nevertheless, the data obtained provided a sound justification for further chromatographic purification and molecular docking studies that could outline specific mechanism-metabolite interactions. Overall, the present study justifies the traditional use of *S. unitus* in folk medicine and highlights this species as a candidate for the development of safe plant-derived antioxidant and antimicrobial agents.

Declarations

Acknowledgement

The authors thank Assam Agricultural University for providing plant identification support. Gratitude was extended to Dr. Rajiv Goswami, Guwahati Biotech Park, Guwahati, and Assam for GC-MS analysis.

Ethics statement

The plant materials were collected with the knowledge, verbal permission, and assistance of members of the Boloma Mishing village community. The scholar received approval and support directly from local stakeholders and no endangered or protected species were involved.

Funding

The authors declare that no funds, grants, or other support was received during the preparation of this manuscript.

Data Availability

Detailed data are available in the manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the conception and design of the study. The material preparation, data collection, and *in vitro* analyses were performed by the first author. The first draft of the manuscript was written by both authors who commented on the previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this study, we used ChatGPT (OpenAI) to improve the clarity and readability of the manuscript. After using this tool, the author(s) reviewed and edited the content as required and took (s) full responsibility for the content of the published article.

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Tables

Table 1. Quantitative determination of total phenolic, flavonoid, and tannin content in solvent extracts of *Sphaerostephanos unitus* (L.) Holttum.

Solvent	Total Phenolic Content (TPC) (mg GAE/g)	Total Flavonoid Content (TFC) (mg QE/g)	Total Tannin Content (TTC) (mg GAE/g)
Methanol	424.56 ^d ± 0.003	237.98 ^d ± 0.002	6.365 ^d ± 0.005
Ethyl Acetate	506.67 ^c ± 0.005	550 ^b ± 0.006	272.423 ^c ± 0.141
Ethanol	1166.67 ^a ± 0.002	657.223 ^a ± 0.002	763.473 ^a ± 0.007
Acetone	635 ^b ± 0.031	532.78 ^c ± 0.002	408.033 ^b ± 0.002

Note: Data are expressed as mean ± SEM (n = 3). Values followed by different superscript letters (a–d) within a column differ significantly at $p < 0.05$ according to Duncan's multiple range test.

Table 2. DPPH and nitric-oxide free-radical scavenging activity (IC₅₀ µg/mL) of *Sphaerostephanos unitus* extracts and standard ascorbic acid

Sample / Solvent Extract	IC ₅₀ (µg/ml)	
	DPPH free radical scavenging activity	Nitric Oxide free radical scavenging activity
Ascorbic acid (Standard)	44.330 ^{def} ± 0.30	68.047 ^{cde} ± 0.1
Methanol	127.19 ^a ± 0.56	79.99 ^b ± 0.47
Ethyl Acetate	97.77 ^a ± 0.76	74.54 ^c ± 0.38
Ethanol	35.74 ^b ± 0.82	73.29 ^c ± 0.73
Acetone	112.91 ^a ± 1.2	84.92 ^a ± 0.75

Note: Values represent mean ± SEM (n = 3). Different superscript letters within a column indicate significant differences ($p \leq 0.05$, Duncan's multiple range test).

Table 3. Brine shrimp (*Artemia salina*) lethality of *Sphaerostephanos unitus* extracts and standard podophyllotoxin

Sample / Solvent Extract	LC ₅₀ (µg/ml±SEM), 24hours
Podophyllotoxin (Standard)	0.03
Methanol	600.32
Ethyl acetate	510.55
Ethanol	573.72
Acetone	468.53

Note: Each LC₅₀ value represents the mean of three independent experiments.

Table 4. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations (mg/mL) of *Sphaerostephanos unitus* extracts and fatty acid-rich fraction (Su-F) against bacterial, fungal, and reference strains

Sample / Solvent Extract	<i>P. aeruginosa</i>	<i>S. typhi</i>	<i>A. baumannii</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>S. epidermidis</i> ATCC 35984	<i>K. pneumoniae</i> ATCC BAA-1705
	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)	MIC / MBC (mg/mL)
Methanol	0.67 / 1.33	0.67 / 2.67	0.67 / 1.33	0.67 / 1.33	1.33 / 2.67	0.67 / 1.33	0.67 / 1.33	1.33 / 2.67	0.67 / 1.33
Ethyl acetate	0.29 / 0.58	0.29 / 1.17	0.29 / 0.58	0.29 / 0.58	0.58 / 1.17	0.29 / 0.58	0.29 / 0.58	0.58 / 1.17	0.58 / 1.17
Ethanol	1.33 / 2.67	1.33 / 5.34	1.33 / 2.67	1.33 / 2.67	2.67 / 5.34	1.33 / 2.67	1.33 / 2.67	2.67 / 5.34	2.67 / 5.34
Acetone	0.52 / 1.04	0.52 / 2.10	0.52 / 1.04	0.52 / 1.04	1.04 / 2.10	0.52 / 1.04	0.52 / 1.04	1.04 / 2.10	1.04 / 2.10
Fatty acid (Su-F)	0.25 / 1.0	0.25 / 1.0	0.25 / 0.5	0.25 / 0.5	0.5 / 1.0	0.25 / 0.5	0.125 / 0.25	0.25 / 0.5	0.25 / 0.5

Note: MIC – Minimum inhibitory concentration; MBC – Minimum bactericidal concentration. Values represent the mean of three independent observations. Bold values indicate the lowest MIC and MBC recorded for each organism.

Table 5. Effect of *Sphaerostephanos unitus* extracts and fatty acid fraction on biofilm formation and biofilm matrix components in *Pseudomonas aeruginosa*

Sample / solvent extract	Biofilm inhibition %	Protein content (mg/ml)	Exopolysaccharide content (mg/ml)
Untreated sample	-	0.87 ± 0.02	1.03 ± 0.03
Methanol	79.054 ± 0.61	0.265 ± 0.05	0.749 ± 0.03
Ethyl acetate	79.122 ± 0.6	0.396 ± 0.02	0.925 ± 0.03
Ethanol	57..094 ± 0.3	0.145 ± 0.02	0.972 ± 0.05
Acetone	70.743 ± 0.3	0.467 ± 0.03	0.630 ± 0.01
Fatty acid	94.797 ± 0.58	0.131 ± 0.02	0.363 ± 0.01

Note: Values are mean ± SEM (n = 3). Biofilm inhibition % was calculated relative to the untreated control. Protein and exopolysaccharide content were quantified from attached biofilms after treatment at MIC levels of each extract/fraction.

Supplementary Files

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