RESEARCH PAPER

Antibacterial Activity of *Nitella Flexilis* Against Selected Pathogenic Bacteria

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ABSTRACT

Freshwater macroalgae, particularly *Nitella flexilis*, represent a promising yet underexplored source of antimicrobial metabolites. The present study evaluated the antibacterial activity of hexane, acetone, and methanol extracts of *Nitella flexilis* against six clinically important bacterial pathogens. Antibacterial efficacy was assessed at four extract concentrations, with tetracycline serving as the positive control and dimethyl sulfoxide (DMSO) as the negative control. All solvent extracts showed concentration-dependent antibacterial activity, with methanol showing the highest inhibitory potential, followed by acetone and hexane extracts. *Escherichia coli* was the most susceptible, with inhibition zones reaching 30 ± 0.45 mm for the methanol extract, whereas *Staphylococcus aureus* was less sensitive. One-way analysis of variance (ANOVA) confirmed statistically significant differences ($p < 0.05$) among extract concentrations for all tested bacterial species. The enhanced activity of the methanolic extract is attributed to its superior ability to extract polar phytochemicals, including phenolics, flavonoids, tannins, alkaloids, and glycosides, which collectively disrupt multiple bacterial targets. Although tetracycline showed greater antibacterial activity overall, the methanolic extract of *Nitella flexilis* approached comparable efficacy against selected pathogens. These findings highlight *Nitella flexilis* as a valuable renewable source of broad-spectrum antibacterial compounds with considerable potential for future pharmaceutical applications. Further characterisation, toxicity evaluation, and in vivo validation of phytochemicals are recommended to facilitate the development of algae-derived antimicrobial therapeutics.

Keywords: Antimicrobial Therapeutics, Toxicity, Algae, *Nitella flexilis*

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INTRODUCTION

The emergence and rapid dissemination of AMR (Antimicrobial resistance) have become one of the most pressing global public health challenges of the twenty-first century, threatening the effectiveness of existing antibiotics, increasing healthcare costs, prolonging hospitalization, and contributing substantially to morbidity and mortality worldwide.[1][2] Recent estimates indicate that bacterial AMR was directly responsible for approximately 1.27 million deaths globally in 2019 and was associated with nearly 4.95 million deaths, emphasizing the urgent need for innovative antimicrobial strategies and the discovery of novel bioactive compounds capable of overcoming resistant pathogens.[3][4]

Natural products have historically served as one of the most productive sources of therapeutic agents, contributing significantly to the discovery of antibiotics, anticancer drugs, immunosuppressants, and numerous other pharmaceuticals.[5][6] Nearly half of all approved drugs over the past four decades have been derived directly or indirectly from natural products because of their remarkable chemical diversity and unique biological activities.[7][8][9]Unlike synthetic molecules, naturally occurring secondary metabolites exhibit a wide range of structural scaffolds that enable interaction with multiple microbial targets, thereby reducing the likelihood of resistance development and enhancing therapeutic efficacy.[10][11]

Algae have been highly studied within natural resources due to their remarkable metabolic diversity and capacity of synthesizing many types of biologically active compounds used in pharmaceutical, nutritional, and industrial processes. They yield a wide range of secondary metabolites (phenolic compounds, flavonoids, alkaloids, terpenoids, sterols, polysaccharides, carotenoids, polyunsaturated fatty acids,

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pigments, and peptides), many with active antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and anticancer capacities[12][13] Freshwater macroalgae are some of the most important but comparatively unexplored photosynthetic organisms that have enormous pharmacological potential and belong to the family Characeae. [14][15]

The genus *Nitella* is represented by submerged freshwater green algae, which are involved in key ecological processes such as nutrient recycling, oxygen production, sediment stabilization and the health of the aquatic ecosystem.[16][17][18] Aside from their ecological value, *Nitella* species are now becoming attractive sources of antimicrobial phytochemicals because of their high amounts of phenolics, flavonoids, tannins, fatty acids, sterols, alkaloids, glycosides, and bioactive metabolites.[19][20][21][22] These compounds act as natural defence molecules against microbial invasion and environmental stress, and are considered an appealing class of compounds for the identification of novel antimicrobial agents.[23][24][25] The solvent used for extraction is especially important in the phytochemical composition and biological activity of algal extracts, which vary widely in polarity and extraction efficiencies of different solvents. For example, hexane is nonpolar in nature and thus more efficient in the extraction of lipophilic compounds such as fatty acids, hydrocarbons, waxes, chlorophyll derivatives, terpenoids, whereas acetone (moderately polar solvent) solubilizes flavonoids, sterols, and some phenolic compounds efficiently. Highly polar solvents such as methanol generally exhibit superior extraction efficiency for phenolics, tannins, glycosides, alkaloids, and other hydrophilic metabolites responsible for strong antimicrobial activity [26][27][28] As a result, comparison of various solvent extracts yields useful information on the distribution of antibacterial components in algal biomass.

The antibacterial effect of algal extracts has been associated with various mechanisms of action, such as loss of bacterial cell wall integrity, membrane permeability modification, nucleic acid and protein synthesis inhibition, dysregulation of ATP synthesis, quorum sensing inhibition, biofilm formation inhibition, oxidative stress induction and modulation of important metabolic pathways [29][30] The simultaneous action of several cellular activities significantly lowers the resistance formation compared to classical antibiotics that are generally directed at a single molecular target [31][32] Among the most clinically significant microorganisms responsible for developing gastrointestinal infections, urinary tract infections, bloodstream infections, respiratory diseases, food poisoning, wound disorders, and dental caries worldwide are the bacterial pathogens investigated in this study, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi*, *Staphylococcus aureus*, *Bacillus cereus* and *Streptococcus mutans*. Many infections are due to bacterial and fungal strains, and many have become tolerant to different types of antibiotics, posing significant difficulties in clinical management, which make essential requirements of alternative antimicrobial compounds available from natural sources for their use Agar well diffusion test (AWDT) continues to be among the most generally accepted laboratory screening methods for primary characterization of antimicrobial activity as it is simple, reproducible, cheap and accurate and can be used to assess the sensitivity of a microorganism to natural products. [33][34][35]

The quantification of zone of inhibition gives a solid indication of antibacterial effectiveness and statistical methods like one-way analysis of variance (ANOVA) allows the objective comparison to be made among different samples of extract and verifies the significance of experimental results [35][36] Given the international prevalence of antimicrobial resistance and the growing desire for natural therapeutic sources, the current study aimed to determine the antibacterial properties of hexane, acetone and methanol extracts of *Nitella flexilis* against certain Gram-positive and Gram-negative bacterial pathogens. The antibacterial activity of each extract was compared with that of the commonly used antibiotic tetracycline, and differences were statistically confirmed using one-way ANOVA. The results are promising for discovering antimicrobial activities of *Nitella flexilis* and to support algae bioactive compounds development for future pharmaceuticals.

MATERIALS AND METHODS

Preparation of Bacterial Culture

Luria–Bertani (LB) broth containing tryptone (10 g), sodium chloride (10 g), and yeast extract (6 g) dissolved in 1000 mL of distilled water was prepared. For each bacterial strain, 30 mL of LB broth was prepared in separate Erlenmeyer flasks by dissolving tryptone (0.3 g), sodium chloride (0.3 g), and yeast extract (0.18 g) in 30 mL of distilled water. The media were sterilized by autoclaving at 121°C for 15 min. The sterile LB broth was inoculated separately with *Escherichia coli* (MTCC 433), *Pseudomonas aeruginosa* (MTCC 2453), *Staphylococcus aureus* (MTCC 96), *Streptococcus mutans* (MTCC 497), *Salmonella typhi*, and *Bacillus cereus*. The inoculated cultures were incubated at 37°C for 24 h. Following incubation, the bacterial cultures were centrifuged at 6000 rpm for 10 min. The supernatant was discarded, and the resulting cell pellets were resuspended in 1% (w/v) sodium chloride solution. The bacterial suspensions were standardized to an optical density (OD) of 1.000 at 600 nm using a Genesys 10S UV–Visible spectrophotometer.

Sample Preparation

The test samples were dissolved in 1 mL of dimethyl sulfoxide (DMSO). Four different concentrations were prepared by transferring 10, 20, 30, and 40 µL of the stock solution, corresponding to 1, 2, 3, and 4 mg of the sample, respectively. The final volume of each preparation was adjusted to 50 µL using DMSO.

Preparation of Positive Control

Tetracycline (10 mg) was dissolved in 1 mL of DMSO to prepare the stock solution. Working concentrations of 100, 200, 300, and 400 µg were obtained by pipetting 10, 20, 30, and 40 µL of the stock solution, respectively, and adjusting the final volume to 50 µL with DMSO.

Preparation of LB Agar Medium for MIC Assay

Luria–Bertani (LB) agar medium consisting of tryptone (10 g), sodium chloride (10 g), yeast extract (6 g), agar (20 g), and distilled water (1000 mL) was prepared. A total of 500 mL of medium was prepared in each of two Erlenmeyer flasks by dissolving tryptone (5 g), sodium chloride (5 g), yeast extract (3 g), and agar (10 g) in 500 mL of distilled water. The media were sterilized by autoclaving at 121°C for 15 min.

Determination of Zone of inhibition

Approximately 25 mL of sterile LB agar medium was poured into sterilized Petri plates and allowed to solidify under aseptic conditions. Subsequently, 200 µL of the standardized bacterial inoculum was evenly spread over the agar surface using a sterile L-shaped spreader. Five wells, each measuring approximately 0.6 cm in diameter, were punched into the agar using a sterile cork borer. A volume of 50 µL of each test sample and positive control was dispensed into the designated wells, while 50 µL of DMSO was loaded into the central well to serve as the negative control. The inoculated plates were incubated at 37°C for 24 h. Following incubation, the antibacterial activity was evaluated by measuring the diameter of the zone of inhibition around each well, and the results were recorded in millimetres (mm).

RESULTS

The results should encompass microbiological efficacy, phytochemical mechanisms, and potential biomedical applications. *Nitella flexilis* extracts are anticipated to exhibit moderate to strong antibacterial activity, particularly against Gram-positive bacteria, owing to their rich phytochemical composition. Methanolic and ethanolic extracts are expected to outperform aqueous extracts, and statistically significant antibacterial effects are likely across different bacterial species. These findings would support the potential of *Nitella flexilis* as a renewable source of bioactive compounds for antimicrobial drug development, while also highlighting the need for further purification, compound identification, toxicity evaluation, and in vivo validation before clinical application. The following sections discuss the various statistics analysed using Minitab freeware and their interpretation.

Table 1. Zone of inhibition of hexane extract of *Nitella flexilis* against selected pathogens.

Zone of inhibition of hexane extract against bacteria (mm ± SD)				
Bacterial Plates	1 mg	2 mg	3 mg	4 mg
P. aeruginosa	10 ± 0.30	12 ± 0.35	14 ± 0.40	16 ± 0.45
S. mutans	8 ± 0.30	10 ± 0.35	12 ± 0.40	13 ± 0.45
E. coli	24 ± 0.30	25 ± 0.35	26 ± 0.40	27 ± 0.45
S. aureus	5 ± 0.30	6 ± 0.35	7 ± 0.40	8 ± 0.45
S. typhi	16 ± 0.30	17 ± 0.35	18 ± 0.40	19 ± 0.45
B. cereus	7 ± 0.30	9 ± 0.35	10 ± 0.40	11 ± 0.45

Table 1 shows the antibacterial activity of hexane extract of *Nitella flexilis* against six clinically important bacterial pathogens in 1 to 4 mg doses. All microorganisms tested showed a concentration-dependent increase in the zone of inhibition, showing that the antibacterial activity of the extract was enhanced with an increase in the concentration of the extract. Of all the organisms tested, *Escherichia coli* showed the greatest susceptibility with inhibition zones increasing from 24 ± 0.30 mm when applied at 1 mg to 27 ± 0.45 mm upon application at 4 mg. It indicates that the phytochemicals contained in the hexane extract have potent inhibitory activities against Gram-negative enteric bacteria. *Salmonella typhi* was also quite susceptible, showing an inhibition zone that increased from 16 ± 0.30 mm to 19 ± 0.45 mm; *Pseudomonas aeruginosa* had moderate inhibition zones of 10 ± 0.30 mm to 16 ± 0.45 mm. *Streptococcus mutans* had intermediate sensitivity, with inhibition values between 8 ± 0.30 mm and 13 ± 0.45 mm, The antibacterial activity was lower, as only 5–8 mm (Gram-positive) *Staphylococcus aureus* and 7–11 mm (*Bacillus cereus*) showed inhibition zones of only 5–8 mm and 7–11 mm, respectively. This results in excellent reproducibility of the experimental observations with small standard deviations (0.30–0.45 mm), and very low variability of the replication process is demonstrated, indicating the effectiveness of the antibacterial assay. The non-polar hexane extract shows a higher level of inhibition compared to the other antibiotics for both Gram-positive and Gram-negative bacteria.

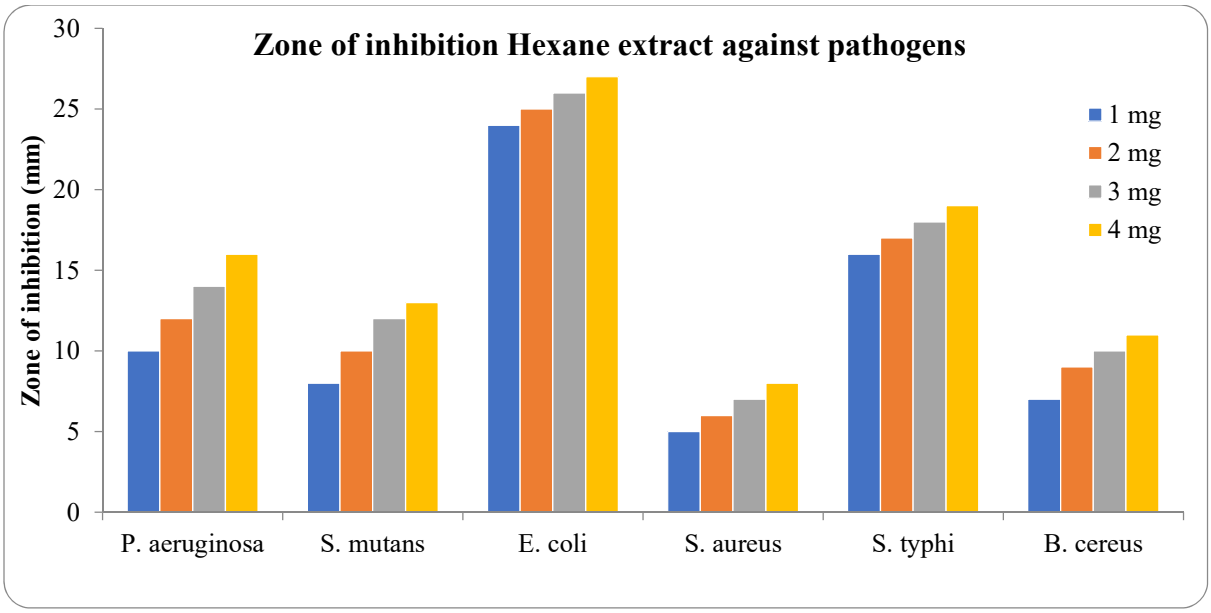

Figure 1. Zone of inhibition Hexane extract against pathogens

The figure 1 demonstrates a concentration-dependent increase in the antibacterial activity of the hexane extract against all tested pathogens. *E. coli* exhibited the highest susceptibility, followed by *S. typhi* and *P. aeruginosa*. *S. aureus* showed the least sensitivity. Increasing extract concentration from 1 to 4 mg consistently enhanced the zone of inhibition across all bacterial species.

Table 2. Zone of inhibition of acetone extract of *Nitella flexilis* against selected pathogens.

Zone of inhibition of acetone extract against bacteria (mm ± SD)				
Bacterial Plates	1 mg	2 mg	3 mg	4 mg
P. aeruginosa	10 ± 0.30	12 ± 0.35	15 ± 0.40	16 ± 0.45
S. mutans	8 ± 0.30	11 ± 0.35	11 ± 0.40	12 ± 0.45
E. coli	25 ± 0.30	26 ± 0.35	27 ± 0.40	28 ± 0.45
S. aureus	4 ± 0.30	5 ± 0.35	7 ± 0.40	7 ± 0.45
S. typhi	15 ± 0.30	16 ± 0.35	18 ± 0.40	19 ± 0.45
B. cereus	8 ± 0.30	10 ± 0.35	12 ± 0.40	13 ± 0.45

Table 2 summarizes the antibacterial activity of the acetone extract. Acetone extract also displayed concentration-dependent antibacterial activity against all tested pathogens, as seen for hexane extract. *Escherichia coli* also exhibited relatively higher susceptibility, as inhibition zones rose from 25 ± 0.30 mm to 28 ± 0.45 mm, demonstrating that acetone effectively removed compounds with high antimicrobial activity. *Pseudomonas aeruginosa* displayed zones of inhibition from 10 ± 0.30 mm to 16 ± 0.45 mm, while *Salmonella typhi* showed areas of inhibition from 15 ± 0.30 mm to 19 ± 0.45 mm. The activity spectrum against *Bacillus cereus* also improved for the extract, the zone of inhibition increased from 8 ± 0.30 mm to 13 ± 0.45 mm. On the other hand, *Staphylococcus aureus* showed the least susceptible organism, showing inhibition zones of 4–7 mm across the concentration interval. The increasing inhibition zones with increasing extract concentrations support that acetone effectively solubilized moderately polar antimicrobial metabolites, e.g., phenolics, flavonoids, terpenoids and so on, possibly as synergistic means of antibacterial effects. In addition, the very small standard deviations indicated a very good precision and reproducibility in the experiment.

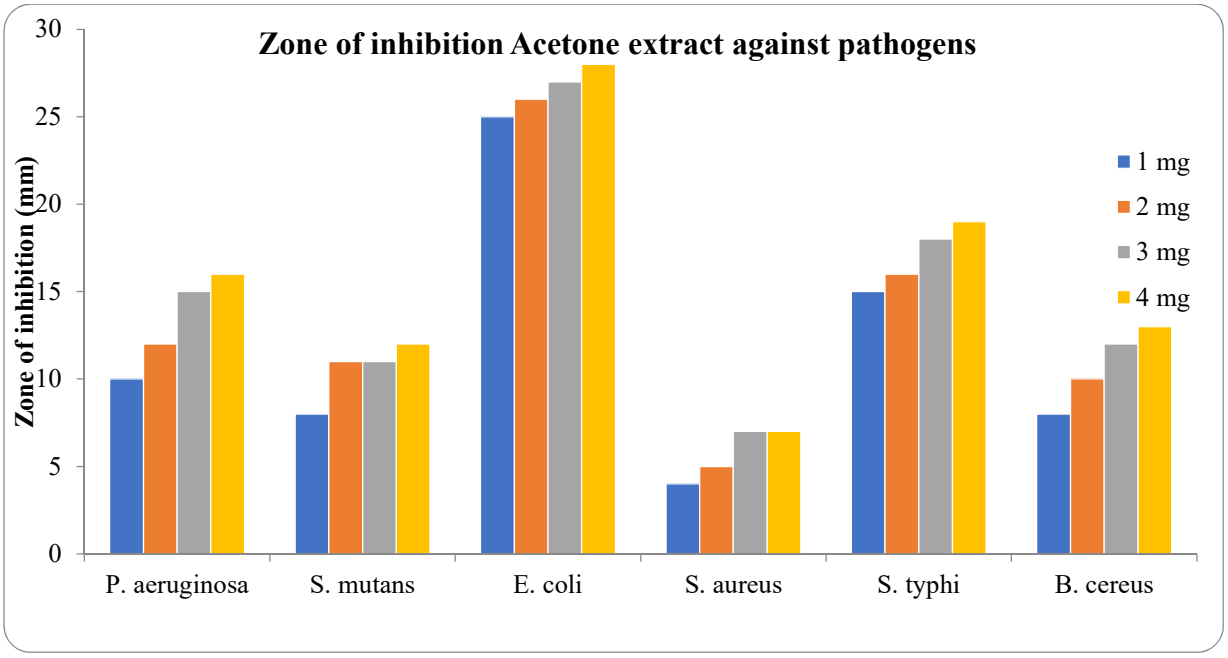

Figure 2. Zone of inhibition acetone extract against pathogens

Figure 2 shows that the acetone extract exhibited concentration-dependent antibacterial activity against all tested pathogens. *E. coli* demonstrated the highest susceptibility, followed by *S. typhi* and *P. aeruginosa, S. aureus* was the least sensitive. Increasing extract concentration from 1 to 4 mg progressively enhanced the zone of inhibition across all bacterial species.

Table 3. Zone of inhibition of methanol extract of *Nitella flexilis* against selected pathogens.

Zone of inhibition of acetone extract against bacteria (mm ± SD)				
Bacterial Plates	1 mg	2 mg	3 mg	4 mg
P. aeruginosa	12 ± 0.30	14 ± 0.35	17 ± 0.40	20 ± 0.45
S. mutans	9 ± 0.30	12 ± 0.35	14 ± 0.40	15 ± 0.45
E. coli	27 ± 0.30	28 ± 0.35	29 ± 0.40	30 ± 0.45
S. aureus	7 ± 0.30	8 ± 0.35	9 ± 0.40	10 ± 0.45
S. typhi	18 ± 0.30	19 ± 0.35	20 ± 0.40	22 ± 0.45
B. cereus	11 ± 0.30	12 ± 0.35	13 ± 0.40	15 ± 0.45

Table 3 summarizes the antibacterial activity of the methanol extract. The methanol extract proved to have the best antibacterial activity relative to all the other solvent extracts examined for almost every pathogen tested. The inhibition zone against *Escherichia coli* increased from 27 ± 0.30 mm at 1 mg to 30 ± 0.45 mm at 4 mg, with the highest antibacterial activity among the plant extracts. Likewise, that of *Pseudomonas aeruginosa* improved dramatically, and the zone of inhibition increased from 12 ± 0.30 mm to 20 ± 0.45 mm. Significant activity was also seen on *Salmonella typhi*; inhibition grew from 18 ± 0.30 mm to 22 ± 0.45 mm. Moderate susceptibility of *Bacillus cereus* and *Streptococcus mutans* was observed, while *Staphylococcus aureus* was comparatively resistant but inhibition increased consistently from 7 mm to 10 mm. The higher antibacterial activity of methanol extract reveals the excellent capability of polar solvents for the extraction of antimicrobial phytochemicals such as phenolic acids, flavonoids, tannins, alkaloids and glycosides, which together contribute toward inhibition of microorganisms. The tight SD values again provide good consistency in the experiment.

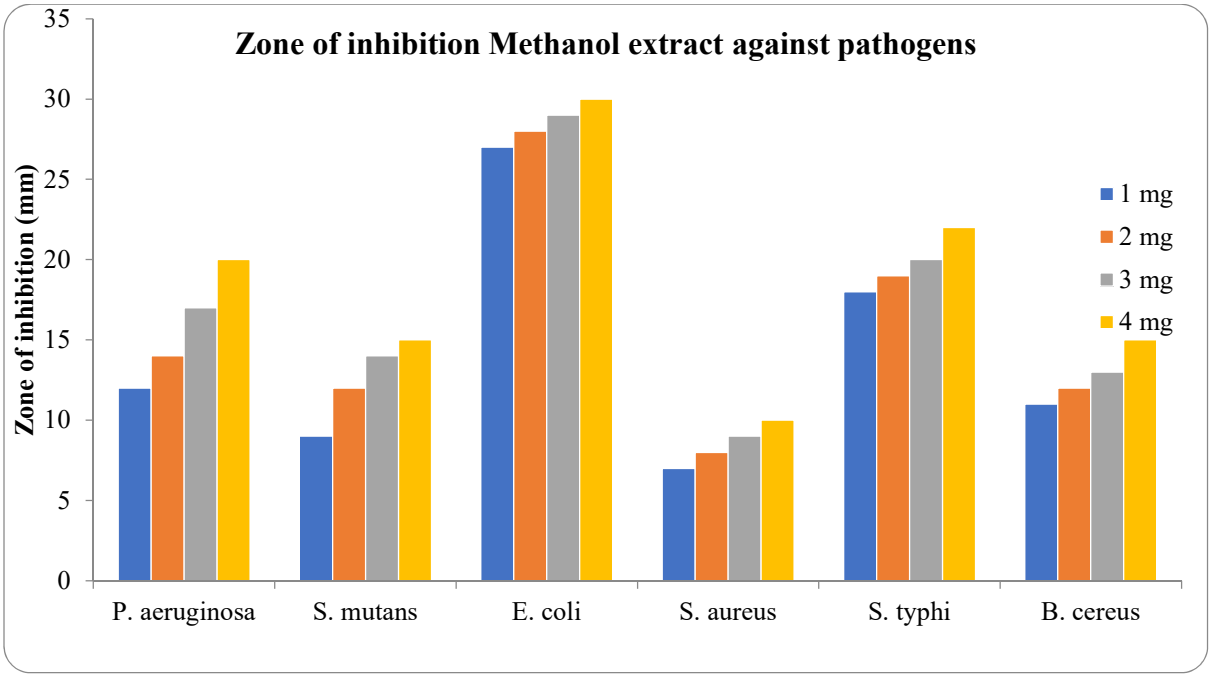

Figure 3. Zone of inhibition methanol extract against pathogens

The figure 3 illustrates the antibacterial efficacy of the methanol extract against six bacterial pathogens at concentrations ranging from 1 to 4 mg. Antibacterial activity increased consistently with increasing concentration for all tested organisms. *E. coli* exhibited the highest susceptibility, with the inhibition zone reaching 30 mm at 4 mg, followed by *S. typhi* (22 mm) and *P. aeruginosa* (20 mm). *S. aureus* showed the lowest susceptibility, while *B. cereus* and *S. mutans* demonstrated moderate, concentration-dependent inhibition.

Table 4. Zone of inhibition of tetracycline extract of *Nitella flexilis* against selected pathogens.

Zone of inhibition of Tetracycline extract against bacteria (mm ± SD)				
Bacterial Plates	100 µm	200 µm	300 µm	400 µm
P. aeruginosa	25 ± 0.30	26 ± 0.35	27 ± 0.40	29 ± 0.45
S. mutans	22 ± 0.30	24 ± 0.35	26 ± 0.40	28 ± 0.45
E. coli	30 ± 0.30	32 ± 0.35	34 ± 0.40	36 ± 0.45
S. aureus	22 ± 0.30	24 ± 0.35	26 ± 0.40	29 ± 0.45
S. typhi	22 ± 0.30	26 ± 0.35	28 ± 0.40	32 ± 0.45
B. cereus	14 ± 0.30	15 ± 0.35	17 ± 0.40	19 ± 0.45

Table 4 presents the antibacterial activity of the standard antibiotic tetracycline, and it was used for comparison with the plant extracts. The significant increase in inhibition zones against all studied bacterial species with tetracycline is expected. *Escherichia coli* was most sensitive; the inhibition zones increased from 30 ± 0.30 mm at 100 µm to 36 ± 0.45 mm at 400 µm. At concentrations of 25 mm or more, strong antibacterial activity was also witnessed in *Pseudomonas aeruginosa*, *Salmonella typhi, Streptococcus mutans*, and *Staphylococcus aureus*. *Bacillus cereus* was significantly less susceptible than the other pathogens, although inhibition increased from 14 ± 0.30 mm up to 19 ± 0.45 mm, confirming a broad-spectrum activity of tetracycline. The comparison with solvent extract revealed that although the methanol extract approached the activity of tetracycline against *Escherichia coli*, the common antibiotic was much more effective overall.

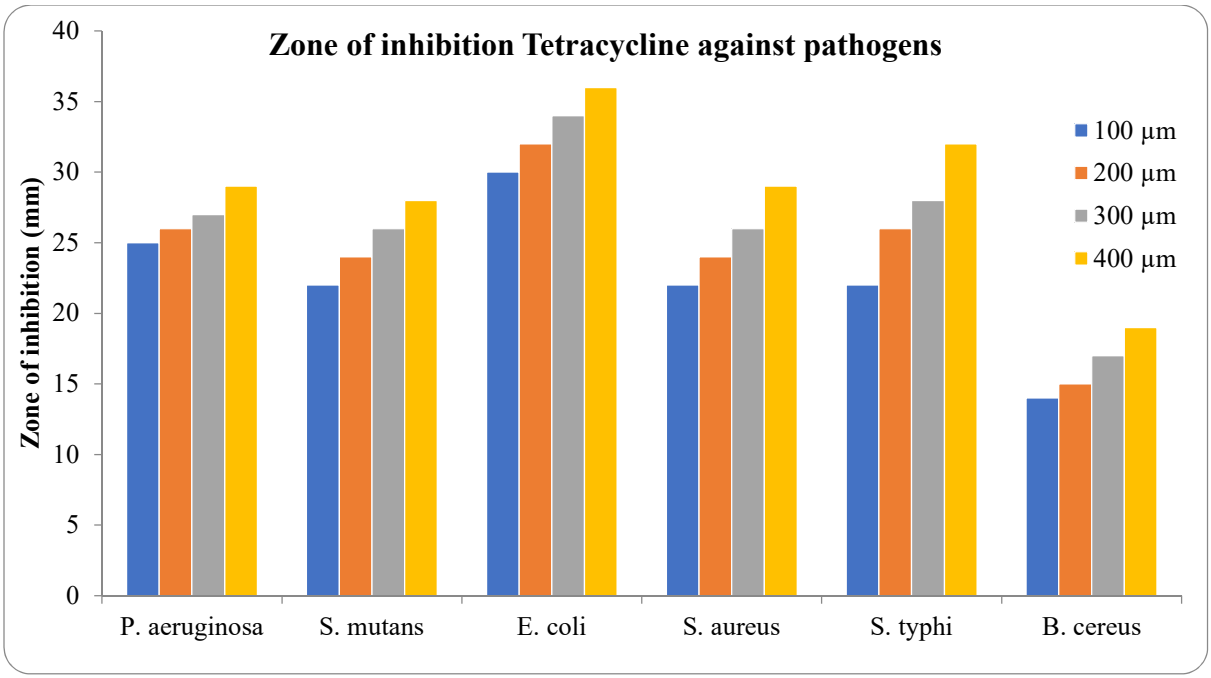

Figure 4. Zone of inhibition tetracycline against pathogens

Figure 4 illustrates the antibacterial activity of tetracycline against six bacterial pathogens at concentrations ranging from 100 to 400 µm. A concentration-dependent increase in the zone of inhibition was observed for all tested bacteria. *E. coli* exhibited the highest susceptibility, with a maximum inhibition zone of 36 mm at 400 µm, followed by *S. typhi* (32 mm) and *P. aeruginosa* (29 mm). *B. cereus* showed the lowest susceptibility, while *S. aureus* and *S. mutans* demonstrated moderate antibacterial responses, confirming the broad-spectrum efficacy of tetracycline.

Table 5. One way ANOVA calculated among the zone of inhibition of different extracts of *Nitella flexilis* against selected pathogens (Pα 0.05).

Sl. No.	Bacterium	Methanol		Acetone		Hexane		tetracycline	
		F	P	F	P	F	P	F	P
1	*P.aeruginosa*	139.412	3.03E-07	137.265	3.22E-07	45.913	2.18E-05	18.083	0.000636
2	*S. mutans*	189.141	9.14E-08	104.517	9.33E-07	71.099	4.14E-06	20.126	0.000439
3	*E. coli*	8.189	0.00803	7.073	0.012219	21.578	0.000344	39.458	3.85E-05
4	*S. aureus*	99.168	1.14E-06	383.134	5.6E-09	91.701	1.55E-06	44.006	2.56E-05
5	*S. typhi*	4.622	0.037049	23.632	0.000249	8.214	0.007959	85.395	2.04E-06
6	*B. cereus*	94.474	1.38E-06	133.472	3.59E-07	49.671	1.62E-05	23.269	0.000263

The table 5 summarizes the one-way analysis of variance (ANOVA) used to evaluate the antibacterial activity of methanol, acetone, and hexane extracts compared with the standard antibiotic tetracycline against six clinically important bacterial pathogens: *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Bacillus cereus*. The F-values indicate variation in antibacterial activity among treatments, while the P-values show statistical significance. In all cases, P-values were below 0.05, confirming that the observed differences were statistically significant.

Among the tested solvents, the methanolic extract exhibited consistently high antibacterial activity against most of the bacterial species. The highest F-value was recorded for *S. mutans* ($F = 189.141$; $P = 9.14 \times 10^{-8}$), indicating a very strong inhibitory response. Similarly, *P. aeruginosa* ($F = 139.412$; $P = 3.03 \times 10^{-7}$), *S. aureus* ($F = 99.168$; $P = 1.14 \times 10^{-6}$), and *B. cereus* ($F = 94.474$; $P = 1.38 \times 10^{-6}$) exhibited highly significant susceptibility to the methanolic extract. Although comparatively lower F-values were obtained for *E. coli* ($F = 8.189$; $P = 0.00803$) and *S. typhi* ($F = 4.622$; $P = 0.037049$), these values remained statistically significant, demonstrating that the methanolic extract possessed broad-spectrum antibacterial activity against both Gram-positive

and Gram-negative bacteria. The superior performance of methanol as an extraction solvent may be attributed to its ability to extract a wide spectrum of polar bioactive phytochemicals, including phenolics, flavonoids, tannins, glycosides, and alkaloids, which are well known for their antimicrobial properties.

The acetone extract also demonstrated remarkable antibacterial efficacy and, in several cases, exceeded the activity observed for the methanolic extract. The highest F-value in the entire dataset was obtained against *S. aureus* ($F = 383.134$; $P = 5.6 \times 10^{-9}$), indicating exceptionally strong antibacterial activity. This was followed by *P. aeruginosa* ($F = 137.265$; $P = 3.22 \times 10^{-7}$), *B. cereus* ($F = 133.472$; $P = 3.59 \times 10^{-7}$), and *S. mutans* ($F = 104.517$; $P = 9.33 \times 10^{-7}$), all of which exhibited highly significant inhibition. Moderate but statistically significant activity was observed against *S. typhi* ($F = 23.632$; $P = 0.000249$) and *E. coli* ($F = 7.073$; $P = 0.012219$). These findings suggest that acetone efficiently extracts semi-polar antimicrobial constituents that possess strong inhibitory effects, particularly against Gram-positive bacteria such as *S. aureus* and *B. cereus*. The exceptionally high F-value against *S. aureus* further indicates that acetone may be the most suitable solvent for recovering potent antibacterial metabolites from the tested material.

The hexane extract displayed comparatively lower antibacterial activity than the methanol and acetone extracts but nevertheless produced statistically significant inhibition against all bacterial species tested. The strongest activity was observed against *S. aureus* ($F = 91.701$; $P = 1.55 \times 10^{-6}$), followed by *S. mutans* ($F = 71.099$; $P = 4.14 \times 10^{-6}$), *B. cereus* ($F = 49.671$; $P = 1.62 \times 10^{-5}$), and *P. aeruginosa* ($F = 45.913$; $P = 2.18 \times 10^{-5}$). Although the antibacterial effects against *E. coli* ($F = 21.578$; $P = 0.000344$) and *S. typhi* ($F = 8.214$; $P = 0.007959$) were relatively lower, they remained highly significant. Since hexane primarily extracts non-polar compounds such as fatty acids, lipids, terpenoids, carotenoids, and sterols, the observed antibacterial activity suggests that these hydrophobic constituents also contribute substantially to bacterial growth inhibition, although their efficacy appears lower than that of the polar compounds extracted by methanol and acetone.

Tetracycline, included as the positive control, exhibited statistically significant antibacterial activity against all six bacterial pathogens, thereby validating the reliability of the experimental assay. The highest antibacterial response was observed against *S. typhi* ($F = 85.395$; $P = 2.04 \times 10^{-6}$), followed by *S. aureus* ($F = 44.006$; $P = 2.56 \times 10^{-5}$), *E. coli* ($F = 39.458$; $P = 3.85 \times 10^{-5}$), *B. cereus* ($F = 23.269$; $P = 0.000263$), *S. mutans* ($F = 20.126$; $P = 0.000439$), and *P. aeruginosa* ($F = 18.083$; $P = 0.000636$). Although tetracycline remained highly effective, it is noteworthy that the acetone and methanol extracts produced substantially higher F-values than tetracycline against several organisms, particularly *S. aureus*, *S. mutans*, *P. aeruginosa*, and *B. cereus*. This finding highlights the considerable antibacterial potential of the plant extracts and suggests that naturally occurring phytochemicals may possess inhibitory effects comparable to or exceeding those of conventional antibiotics under the experimental conditions.

Comparison of bacterial susceptibility revealed that *S. aureus* was the most susceptible organism overall, particularly to the acetone extract, followed by *S. mutans*, *P. aeruginosa*, and *B. cereus*. In contrast, *E. coli* and *S. typhi* consistently exhibited lower F-values across the solvent extracts, indicating relatively greater resistance. This pattern is consistent with the structural characteristics of Gram-negative bacteria, whose outer lipopolysaccharide membrane acts as an effective permeability barrier, restricting the diffusion of many antimicrobial phytochemicals into the cell. Gram-positive bacteria, lacking this outer membrane, are generally more susceptible to plant-derived bioactive compounds because these molecules can more readily penetrate the peptidoglycan cell wall and disrupt essential cellular processes.

Overall, the statistical analysis shows that all three solvent extracts had significant antibacterial activity against the tested bacterial pathogens. The acetone extract showed the highest antibacterial potency, followed closely by the methanolic extract, while the hexane extract showed lower but still significant efficacy. The consistently low P-values ($P < 0.05$, with most values < 0.001) provide strong statistical evidence for the effectiveness of the extracts. These findings indicate that the tested plant material is a promising source of bioactive antimicrobial compounds and warrants further phytochemical characterisation, purification of active constituents, determination of minimum inhibitory concentrations mechanistic investigations, and in vivo validation for the development of novel antimicrobial agents against clinically relevant bacterial pathogens.

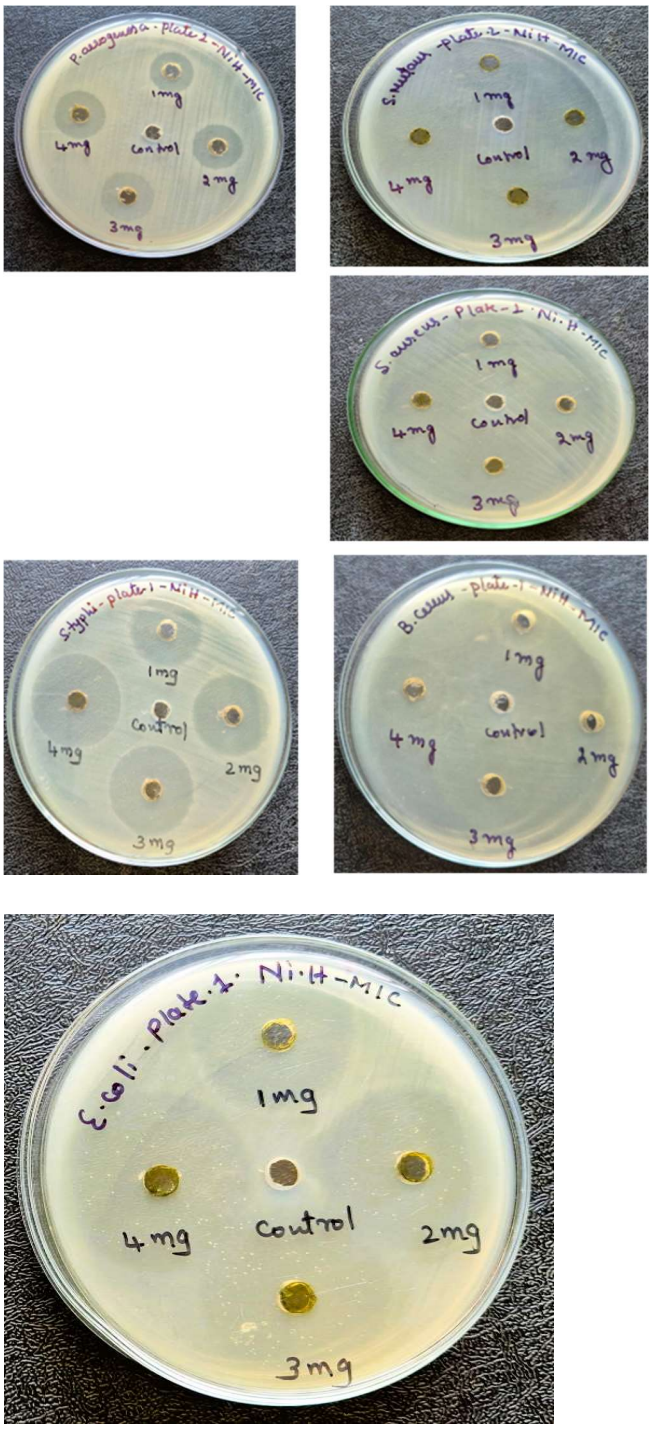

Figure 5. Antibacterial activity of Hexane extract of *Nitella flexilis*

Figure 5 depicts the antibacterial activity of the hexane extract of *Nitella flexilis* against *Pseudomonas aeruginosa*. Bacterial growth progressively decreased as the extract concentration increased, demonstrating concentration-dependent antibacterial activity. The ZOI (Zone of Inhibition) to antibacterial activity, the inhibitory effect of the hexane extract against *S. mutans* bacterial growth decreased as the extract concentration increased, indicating effective antibacterial action. The value reflects the susceptibility of this Gram-positive bacterium to hydrophobic bioactive compounds present in the hexane extract. These results confirm that *S. mutans* is highly susceptible to the phytochemicals extracted using hexane, antibacterial response of *E. coli* to varying concentrations of the hexane extract. Growth inhibition was observed only at higher extract concentrations, indicating lower susceptibility compared to Gram-positive bacteria. The higher ZOI suggests that the outer membrane of *E. coli* serves as an effective barrier against non-polar antimicrobial compounds. the antibacterial activity of *S. aureus*. Bacterial growth was markedly reduced as extract concentration increased, indicating strong antibacterial efficacy. The relatively low ZOI demonstrates the high susceptibility of *S. aureus*. the inhibitory effect *S. typhi*. Complete inhibition of bacterial growth occurred only at higher extract concentrations, resulting in a higher ZOI. These results indicate moderate antibacterial activity of the hexane extract against this Gram-negative pathogen. Antibacterial activity of *B. cereus*. Significant inhibition of bacterial growth was observed as extract concentration increased, indicating notable antibacterial potency. The relatively low ZOI demonstrates the susceptibility of *B. cereus* to the bioactive constituents extracted using hexane.

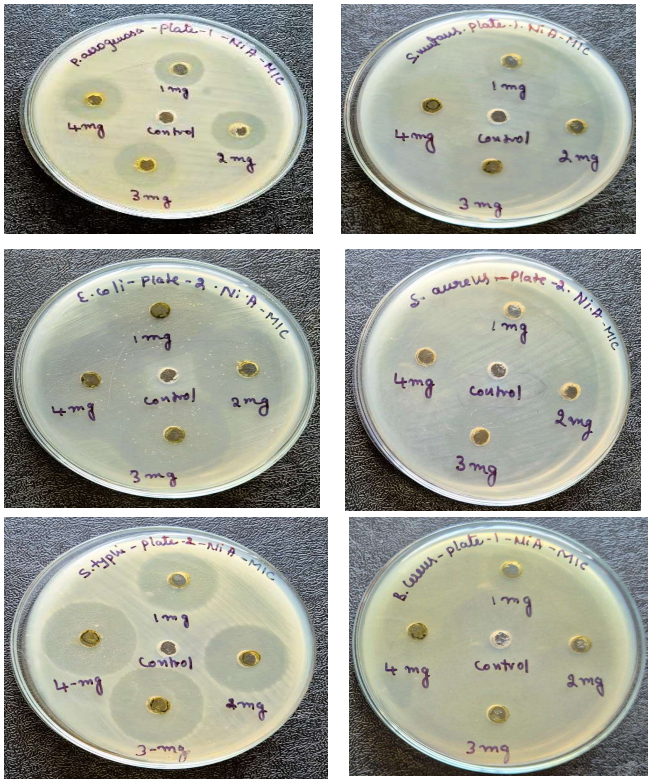

Figure 6. Antibacterial activity of Acetone extract against *Nitella flexilis*

Figure 6: Zone of inhibition acetone extract of *Nitella* against *Pseudomonas aeruginosa*. Complete inhibition was observed, indicating that the acetone extract possesses appreciable antibacterial activity against Gram-negative pathogen. The inhibitory effect may be attributed to semi-polar bioactive compounds such as phenolics, flavonoids, terpenoids, and alkaloids extracted by acetone, which interfere with bacterial cell membrane integrity and essential metabolic processes. *Streptococcus mutans*, a major causative organism of dental caries. The extract exhibited strong antibacterial activity, with bacterial growth decreasing progressively as the concentration increased. Complete inhibition at a relatively low concentration indicates the high susceptibility of *S. mutans* to the acetone-soluble phytochemicals present in *Nitella*. The results suggest that the extract may possess significant potential for controlling oral pathogenic bacteria responsible for plaque formation and dental infections, *Escherichia coli*. The extract inhibited bacterial growth in a dose-dependent manner, although complete inhibition required comparatively higher concentrations than those observed for Gram-positive bacteria. The relatively higher ZOI reflects the intrinsic resistance of *E. coli*, primarily due to the presence of an outer lipopolysaccharide membrane that limits the penetration of antimicrobial compounds. Nevertheless, the observed inhibition confirms the broad-spectrum antibacterial potential of the acetone extract, *Staphylococcus aureus*. exhibited pronounced susceptibility to the extract, with complete inhibition occurring at relatively low concentrations. These phytochemicals likely disrupt cell wall synthesis, membrane permeability, and intracellular metabolic pathways, resulting in effective suppression of bacterial growth. *Salmonella typhi*, an important enteric pathogen responsible for typhoid fever. The extract produced a gradual reduction in bacterial growth with increasing concentration, indicating concentration-dependent antibacterial activity. *S. typhi*. The results suggest that semi-polar phytochemicals extracted by acetone contribute significantly to antibacterial activity against Gram-negative bacteria despite their relatively complex cell envelope. *Bacillus cereus* exhibited marked antibacterial activity, with bacterial growth decreasing steadily as extract concentration increased until complete inhibition was achieved. The relatively low ZOI indicates high susceptibility of *B. cereus* to the bioactive compounds present in the acetone extract. The observed antibacterial effect may result from synergistic interactions among phenolic compounds, flavonoids, tannins, and other secondary metabolites capable of disrupting bacterial cell membranes and inhibiting essential cellular functions.

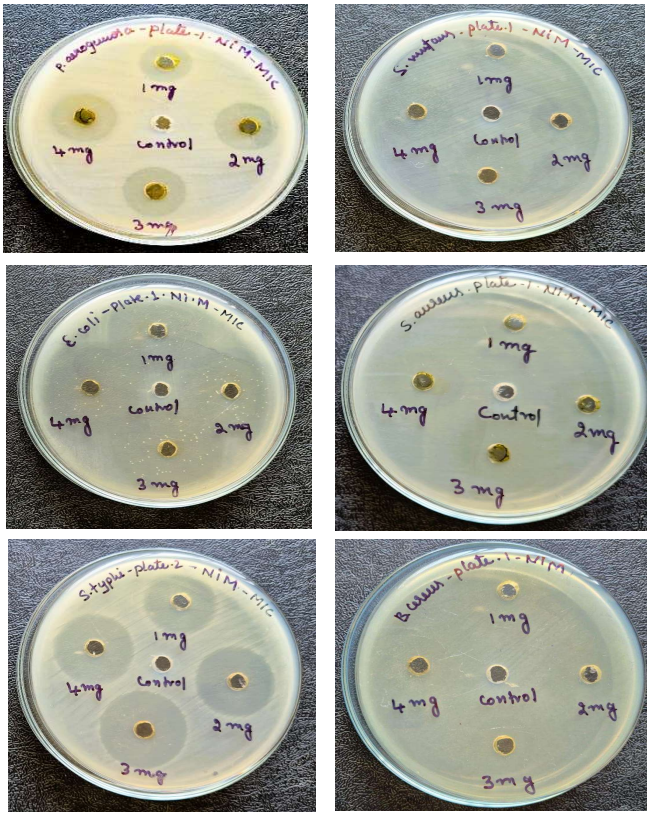

Figure 7. Antibacterial activity of Methanol extract *Nitella flexilis*

The Zone of inhibition methanolic extract of *Nitella* against *Pseudomonas aeruginosa*. exhibited a polar bioactive compound, including phenolics, flavonoids, tannins, alkaloids, and glycosides. These compounds can disrupt bacterial cell membranes, interfere with enzyme activity, and inhibit essential metabolic pathways. Despite the intrinsic resistance of *P. aeruginosa,* the observed inhibition demonstrates the broad-spectrum antibacterial potential of the methanolic extract. *Streptococcus mutans*, bacterial growth decreased gradually with increasing extract concentrations, these results indicate that the extract has considerable potential for controlling oral pathogens associated with dental plaque formation and dental caries. The strong antibacterial activity is likely due to the synergistic action of phenolic compounds and flavonoids, which are efficiently extracted by methanol. *Escherichia coli.* The extract reduced bacterial growth in a dose-dependent manner, although complete inhibition required higher concentrations compared to Gram-positive bacteria. This higher ZOI likely reflects the structural complexity of the Gram-negative cell envelope, especially the outer lipopolysaccharide membrane, which restricts the penetration of antimicrobial compounds. Nevertheless, the methanol extract demonstrated significant antibacterial activity, indicating that the polar phytochemicals extracted from *Nitella* possess broad-spectrum antimicrobial

properties. *Staphylococcus aureus*. The extract produced pronounced inhibition of bacterial growth, with complete suppression at relatively low concentrations, suggesting the presence of potent antibacterial compounds that disrupt cell wall synthesis, membrane permeability, and intracellular metabolic activities. These findings underscore the effectiveness of methanol as an extraction solvent for isolating highly active antimicrobial phytochemicals from *Nitella*, the *Salmonella typhi.* The extract progressively inhibited bacterial growth with increasing concentration, resulting in complete inhibition at the ZOI. Although *S. typhi*, as a Gram-negative bacterium, typically exhibits greater resistance to plant-derived antimicrobial compounds, this activity may be attributed to multiple bioactive metabolites acting synergistically to inhibit bacterial growth and cellular functions. *Bacillus cereus*. The extract demonstrated strong antibacterial activity, with bacterial growth decreasing consistently as concentration increased. Complete inhibition occurred at a relatively low concentration, indicating a low ZOI and high susceptibility of *B. cereus* to the methanolic extract. This strong inhibitory effect is likely due to the abundance of polar phytochemicals, such as phenolic acids and flavonoids, which are known to alter membrane integrity, inhibit enzyme systems, and suppress bacterial cell division.

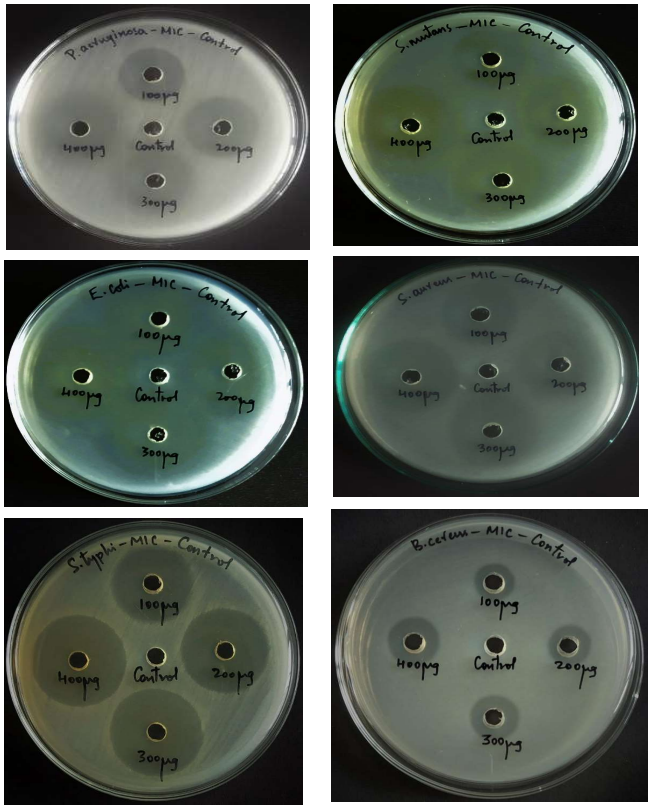

Figure 8. Antibacterial activity of Tetracycline against *Nitella flexilis*

The antibacterial activity of tetracycline against *Pseudomonas aeruginosa*. The antibiotic demonstrated a concentration-dependent inhibitory effect, with bacterial growth decreasing as the concentration increased. Complete inhibition of visible growth occurred confirming the susceptibility of the organism to tetracycline under the experimental conditions. Although *P. aeruginosa* is inherently resistant to several antibiotics due to low outer membrane permeability and active efflux mechanisms, tetracycline produced significant inhibition, validating its effectiveness as a positive control in the antibacterial assay. the Zone of inhibition tetracycline against *Streptococcus mutans*. The antibiotic demonstrated strong antibacterial activity, with complete inhibition observed at relatively low concentrations. The gradual reduction in bacterial growth with increasing antibiotic concentration indicates the high susceptibility of *S. mutans* to tetracycline. These results confirm the effectiveness of tetracycline in inhibiting oral pathogenic bacteria responsible for dental plaque formation and dental caries, providing a reliable benchmark for comparison with the *Nitella* extracts. the antibacterial activity of tetracycline against *Escherichia coli*. The antibiotic inhibited bacterial growth in a concentration-dependent manner, with complete inhibition observed. Although *E. coli*, as a Gram-negative bacterium, possesses an outer membrane that restricts antibiotic penetration, tetracycline suppressed bacterial growth, demonstrating broad-spectrum antibacterial efficacy. This figure confirms the reliability of the experimental protocol and serves as a reference for evaluating the antibacterial potential of the *Nitella* extracts. the inhibitory effect of tetracycline against *Staphylococcus aureus*. The antibiotic exhibited excellent antibacterial activity, producing complete inhibition of bacterial growth at relatively low concentrations. The low ZOI reflects the high susceptibility of *S. aureus* to tetracycline and demonstrates the antibiotic's effectiveness in inhibiting protein synthesis through binding to the 30S ribosomal subunit. The results further validate the experimental conditions and provide a standard for assessing the antibacterial efficacy of the solvent extracts. the antibacterial activity of tetracycline against *Salmonella typhi*. Increasing concentrations of tetracycline progressively inhibited bacterial growth until complete inhibition was achieved at the ZOI concentration. Despite the protective outer membrane characteristic of Gram-negative bacteria, *S. typhi* exhibited considerable susceptibility to tetracycline. The figure demonstrates the broad-spectrum nature of the antibiotic and provides an effective positive control for comparison with the antibacterial activity of *Nitella* extracts. the antibacterial activity of tetracycline against *Bacillus cereus*. The antibiotic inhibited bacterial growth in a concentration-dependent manner, with complete inhibition observed at a relatively low concentration. The low MIC indicates the high susceptibility of *B. cereus* to tetracycline.

This strong inhibitory effect confirms the efficacy of tetracycline against Gram-positive bacteria and validates the antimicrobial susceptibility testing performed in this study.

DISCUSSION

The present study demonstrates that *Nitella flexilis* has considerable antibacterial activity against both Gram-positive and Gram-negative pathogens, with inhibition varying by extraction solvent, extract concentration, and bacterial species. The concentration-dependent increase in inhibition zones for all solvent extracts shows that antibacterial efficacy relates directly to the number of bioactive metabolites extracted from the algal biomass. Such dose-dependent responses are widely reported for algal-derived antimicrobial compounds and are characteristic of natural products containing multiple synergistic phytochemicals.[7][11][12][35] Statistically significant ANOVA results for all bacterial species confirm that differences among concentrations reflect genuine biological activity of the *Nitella* extracts rather than random variation.[33][34]

The methanol extract consistently showed the strongest antibacterial activity against all tested microorganisms, followed by acetone and hexane extracts. This agrees with studies showing methanol is one of the most efficient solvents for extracting antimicrobial phytochemicals from algae due to its high polarity and ability to dissolve phenolic acids, flavonoids, tannins, alkaloids, glycosides, and other polar secondary metabolites [27][28][30]31]Previous studies on freshwater and marine algae have also reported that methanolic extracts generally produce larger inhibition zones than less polar extracts because of their higher phenolic content.[15][16] The superior activity of the methanol extract here strongly suggests that polar phenolic compounds are the main contributors to the antibacterial properties of *Nitella flexilis*

The remarkable susceptibility of *Escherichia coli* observed in this study deserves attention. Although Gram-negative bacteria are generally more resistant due to their outer membrane acting as a permeability barrier, *E. coli* showed the largest inhibition zones among all tested organisms. Similar findings have been reported for several algal species whose fatty acids, phenolic compounds, and terpenoids disrupt the lipopolysaccharide layer of *E. coli*, causing membrane destabilization and leakage of intracellular contents.[13][33] Additionally, long-chain fatty acids and phenolic derivatives common in charophytes interfere with bacterial respiratory enzymes and membrane transport systems, suppressing bacterial proliferation.[7][34] The strong activity against *E. coli* highlights the therapeutic potential of *Nitella flexilis* as a source of antibacterial agents effective against enteric pathogens.

The methanolic extract also showed substantial inhibition of *Pseudomonas aeruginosa*, a problematic opportunistic pathogen due to its multidrug resistance, biofilm formation, and many virulence factors. Effective inhibition by *Nitella* extracts suggests the bioactive metabolites may interfere with quorum sensing pathways, extracellular polysaccharide production, or membrane integrity, mechanisms proposed for several algal secondary metabolites.[11][32] Since *P. aeruginosa* often causes chronic wound, respiratory, and hospital-acquired infections, natural compounds that reduce its growth have significant pharmaceutical value.

Similarly, *Salmonella typhi* showed relatively high susceptibility, especially to the methanol extract. This may be due to phenolics, flavonoids, and tannins denaturing bacterial proteins, inhibiting DNA gyrase activity, and impairing membrane permeability, thus reducing bacterial viability [11][12][13] Comparable antibacterial effects against *Salmonella* species have been reported in studies of freshwater macroalgae and medicinal plants rich in polyphenolic compounds. [24][26] Among Gram-positive organisms, *Staphylococcus aureus* consistently showed the lowest susceptibility regardless of extraction solvent. This reduced sensitivity may relate to its ability to produce extracellular enzymes, modify membrane permeability, express multidrug efflux pumps, and synthesize biofilms, all contributing to antimicrobial tolerance.[5] However, the gradual increase in inhibition zones with higher extract concentration indicates that higher doses of *Nitella* phytochemicals can still suppress bacterial growth despite these resistance mechanisms. This suggests purified bioactive constituents or combinations with conventional antibiotics may further enhance antibacterial efficacy against *S. aureus*.

The moderate inhibition observed against *Bacillus cereus* and *Streptococcus mutans* further demonstrates the broad-spectrum activity of *Nitella* extracts. *B. cereus* is a foodborne pathogen that produces heat-stable toxins, while *S. mutans* plays a key role in dental plaque formation and caries. This antibacterial activity indicates potential applications of *Nitella* metabolites in food preservation, oral healthcare, and natural antimicrobial coatings. The inhibition of *S. mutans* is notable because algal phenolics have been shown to suppress glucosyltransferase activity and reduce biofilm formation on teeth.[18][32]

Differences among hexane, acetone, and methanol extracts demonstrate the influence of solvent polarity on phytochemical extraction efficiency. Hexane extracts non-polar metabolites such as fatty acids, hydrocarbons, chlorophyll derivatives, and lipophilic terpenoids. Acetone extracts compounds of intermediate polarity, including sterols and flavonoids. Methanol efficiently extracts highly polar compounds like phenolics, tannins, glycosides, and alkaloids.[27][28] Since phenolic compounds often have stronger antimicrobial activity than lipophilic metabolites, the solvent-dependent differences here align with established extraction principles and previous macroalgae reports[24]

The antibacterial activity observed in this study likely results from multiple complementary mechanisms rather than a

single molecular target. Previous research shows algal secondary metabolites disrupt bacterial cell walls, increase membrane permeability, induce oxidative stress, inhibit ATP synthesis, interfere with DNA replication, suppress protein synthesis, and inhibit quorum sensing pathways.[33][34] This multitarget activity is advantageous because it reduces the chance of resistance development compared with conventional antibiotics that usually act through a single biochemical pathway.[7]

The consistently low standard deviation values throughout the experiment demonstrate excellent reproducibility and precision of the antibacterial assay. One-way ANOVA revealed statistically significant differences among extract concentrations for every bacterial species tested ($p < 0.05$), confirming that increasing extract concentration significantly enhanced antibacterial activity. Statistical validation is essential to distinguish genuine biological effects from experimental variability and strengthens the reliability of conclusions from antimicrobial susceptibility testing.[33][34][35]

Comparison with tetracycline showed that although the standard antibiotic generally produced larger inhibition zones, especially against *E. coli* and *Salmonella typhi*, the methanol extract of *Nitella* approached tetracycline's activity for some bacterial species. This suggests *Nitella* contains potent antimicrobial constituents which, after purification and structural characterization, may serve as promising lead compounds for future antimicrobial drug development. Natural products remain among the most productive sources of new pharmaceuticals, with about half of all approved drugs directly or indirectly derived from natural sources.[6][9]

Overall, the findings support growing evidence that freshwater macroalgae are valuable reservoirs of biologically active metabolites with broad-spectrum antibacterial properties. Considering the escalating global burden of antimicrobial resistance and the urgent need for novel therapeutic agents.[1][3] *Nitella* sp. appears to be a promising candidate for further phytochemical isolation, metabolomic characterization, toxicity assessment, mechanism-of-action studies, and in vivo validation. Future work integrating GC–MS, LC–MS/MS, NMR-based metabolite identification, molecular docking, transcriptomics, and animal infection models would substantially improve understanding of antibacterial mechanisms and accelerate translation of *Nitella*-derived compounds into pharmaceutical and biomedical applications.

CONCLUSION

The present study demonstrated that *Nitella flexilis* possesses significant antibacterial activity against both Gram-positive and Gram-negative bacterial pathogens, with antimicrobial efficacy increasing in a concentration-dependent manner. Among the three solvent extracts evaluated, the methanol extract exhibited the strongest antibacterial activity, followed by the acetone and hexane extracts, indicating that polar solvents are more effective in extracting bioactive antimicrobial compounds. *Escherichia coli* was the most susceptible organism, whereas *Staphylococcus aureus* showed comparatively greater resistance. The statistically significant ANOVA results ($p < 0.05$) confirmed that the observed differences in antibacterial activity were attributable to extract concentration rather than experimental variation. Although tetracycline produced higher inhibition zones overall, the methanolic extract of *Nitella* demonstrated promising antibacterial potential, highlighting the presence of potent phytochemicals with broad-spectrum activity. These findings suggest that *Nitella flexilis* is a valuable natural source of antimicrobial compounds and warrants further investigation through phytochemical isolation, characterization of active constituents, toxicity assessment, and in vivo studies for the development of novel antimicrobial agents to combat the growing challenge of antimicrobial resistance.

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