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Evaluation of the Phytochemical and Antimicrobial Activity of *Achyranthes aspera* Stem Extract against Dental Infection

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Abstract: The present study aims to evaluate the phytochemical and antimicrobial properties of *Achyranthes aspera* stem extract against *S. mutans* and *S. aureus*. Dental infections are a major problem in children and adults. Dental infections caused by *Streptococcus mutans* and *Staphylococcus aureus* are the main contributors to oral health problems, including periodontal disease and dental caries. The plant extracts were prepared using ethyl acetate, methanol, ethanol, and distilled water, and their efficiency was evaluated using the agar well diffusion method. The ethyl acetate extract exhibited the highest zone of inhibition against both bacterial strains, followed by ethanolic, methanolic, and aqueous extracts. Minimum Inhibitory Concentration (MIC) assays further confirmed the potency of the extracts, with the ethyl acetate extract exhibiting MIC values of 3.75 (µg/ml) against *S. mutans* and 7.5 (µg/ml) against *S. aureus*. These findings support the potential application of *Achyranthes aspera* as a natural antimicrobial agent for dealing with dental pathogens and highlight the importance of plant-based compounds in oral healthcare.

Keywords: *Achyranthes aspera*, Dental infection, *Streptococcus mutans*, and *Staphylococcus aureus*.

INTRODUCTION

Dental caries is one of the most prevalent, chronic, and irreversible localized infections affecting the oral cavity. It remains a major cause of tooth loss in both children and adults. The growth of dental caries is mainly linked to oral microbial pathogens, particularly species from the genus *Streptococcus* Seminario, *et al*., (2005) and *Staphylococcus (*Tsang, *et al*., 2002). The oral microbiota present in the mouth plays a main role in forming polymicrobial communities on the surface of the teeth, commonly known as dental biofilm (Bowen, *et al*., 2018; Chen, *et al*., 2020; Marsh and Zaura, 2017). The oral biofilm produced by these cariogenic microorganisms is kind of complex microbial community that develops in the oral cavity (Wang, *et al*., 2020). There is a large amount of evidence indicating that dental caries is more of a biofilm-induced disease, not an infectious disease (Sim, *et al*., 2016). The development of dental caries starts within the biofilm, which covers and forms on the surface of the teeth (Bowen and Koo, 2011; Chen, *et al*., 2020).

The primary virulence factors that required by the cariogenic bacteria to survive and sustain in the oral cavity include acidogenicity (the ability to produce acid), aciduricity (the ability to tolerate acidic environments), and the ability to synthesize substantial amounts of extracellular polysaccharides (EPS), mostly glucans, from using dietary carbohydrates as the source. These glucans play a vital role in the structural formation and stabilization of cariogenic dental plaque (Koo, *et al*., 2003). The synthesized glucan is responsible for the adhesion of cariogenic bacteria, particularly *Streptococcus mutans*, to the tooth enamel and each other. This interbacterial and surface attachment improves their resistance to mechanical removal by host clearance and increases tolerance to different antimicrobial agents (Ito, *et al*., 2020; Koo, *et al*., 2017). The progression of dental caries is primarily associated with the carbohydrate content in the diet and the frequency at which these carbohydrates are consumed (Ray, 2022). Furthermore, the use of chlorhexidine gluconate in mouthwashes is linked to many side effects, including increased mineral consumption, tooth staining, and irritation of the oral mucosa (Sakaue, *et al*., 2018).

Achyranthes aspera Linn. is a member of the Amaranthaceae family and commonly known as Apamarga or Chirchita. It is a well-known medicinal plant with significant importance in traditional medicine across the tropical regions of Africa and Asia. Widely distributed throughout the tropical world, it is commonly found as a weed across India (Yadav, *et al*., 2016).

Hence, the present study investigates *S. mutans* and *S. aureus* are bacterial strains selected for the target organism using ethyl acetate, ethanol, methanol, and distilled water extracts of the stem of *Achyranthes aspera*.

MATERIALS AND METHODS

Plant Collection and Identification

The stems of the *Achyranthes aspera* were collected from Shadevpur, Bhadrabad, Haridwar, Uttarakhand (29.8685°N, 78.0281°E). The plant was identified from BSI, Dehradun.

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Extraction of Leaves of *Achyranthes aspera*

Fresh stems of *Achyranthes aspera* were collected carefully, systematically washed with water, air-dried, and then ground in the mixture into a coarse powder. Approximately 150 grams of the powdered plant material was placed for the extraction using a Soxhlet apparatus with 1500 ml each of solvents ethyl acetate, ethanol, methanol, and distilled water separately. The solvents were heated to allow for vaporization, followed by condensation, permitting the extract to be collected in the flask. The collected extract was then concentrated by evaporating at 70 °C in the oven and dried (Chaudhary, *et al*., 2023).

The percentage yield of the plant extract was calculated using the formula:

$$Percentage\ yeild = \frac{Weight\ of\ the\ extract}{Weight\ of\ the\ sample} \times 100$$

Test Microorganism

The culture of clinical isolate of *Staphylococcus aureus* MTCC-744 was obtained from the stock samples preserved at the Department of Botany and Microbiology, Gurukula Kangri (Deemed to be University), Haridwar, and *Streptococcus mutans* MTCC-497 was obtained from the MTCC, Chandigarh.

Qualitative Phytochemical Analysis of *Achyranthes aspera*

Tannins: 2 ml of the *Achyranthes aspera* plant extract was put in the test tube, and a few drops of 1% ferric chloride solution were added. The formation of blue-green precipitate indicates the existence of tannins (Ujah, *et al*., 2021).

For Alkaloids

Dragendroff's Reagent Test: 0.5 g of the crude extract of *Achyranthes aspera* was dissolved in 1% hydrochloric acid and filtered with filter paper. To 2 ml of the resulting filtrate was treated with Dragendorff's reagent. The formation of a red colour precipitate confirmed the presence of alkaloids (Ujah, *et al*., 2021).

For Phenol

Lead Acetate Test: 10 mg of the plant extract was treated with a few drops of lead acetate, resulting in a yellow precipitate that confirmed the existence of phenols (Santhi and Sengottuvel, 2016).

For Saponins: 0.5 mg of crude plant extract was shaken with 5 ml of distilled water. Formation of frothing or appearance of creamy small bubbles detected the presence of saponins (Santhi and Sengottuvel, 2016).

For Glycosides: 5 ml of extract, 0.3 ml of Fehling's A and B solutions were added to the solution until it turns alkaline, indicating the presence of glycoside (Ujah, *et al*., 2021).

For Amino Acid: According to Al-Hashemi, *et al*., (2016), 1 ml of the plant extract was taken in a test tube and treated with some drops of Ninhydrin reagent. The purple colour indicated the presence of amino acids.

For Steroid: 2 ml of the plant extract of *Achyranthes aspera* was dissolved in 2 ml of chloroform, followed by the addition of 2 ml of concentrated sulfuric acid. The appearance of a red colour showed the presence of steroids (Raphael, 2012).

For Flavonoids

Pew's Test: 0.1 g of metallic zinc and 8 ml of concentrated sulfuric acid were mixed with 5 ml of plant extract. The appearance of the red colour indicates the presence of flavonoids (Santhi and Sengottuvel, 2016).

For Coumarin: 3 ml of 10% NaOH was added to the plant extract, and a yellow colour was observed. This confirms the positive results for coumarins (Le Thi, *et al*., 2021).

Quantitative Analysis of the Plant Extract of *Achyranthes aspera*

Determination of Total Tannin Content

Tannin content was estimated by using the Folin-Ciocalteu reagent method. 0.1 ml of the plant extract was mixed with distilled water, Folin-Ciocalteu reagent, and sodium carbonate solution, and the volume was adjusted to 10 ml. The mixture was properly shaken and allowed to stand at room temperature for 30 minutes. A standard calibration curve was prepared using tannic acid solutions ranging from 20 to 100 μg/ml, following the same procedure. Absorbance was recorded at 725 nm using a UV-Visible spectrophotometer. The tannin content was determined as tannic acid equivalents (TAE) (Islam, *et al*., 2015).

Determination of Total Phenol Content

1 ml of plant extract (100 μg/ml) and standard gallic acid solutions (20–100 μg/ml) were transferred into labeled test tubes. 5 ml of Folin–Ciocalteu reagent (10-fold diluted) to each test tube, and 4 ml of 7.5% sodium carbonate were added. The test tubes with mixtures were incubated at room temperature for 30 minutes for

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standards and 60 minutes for the extract. Absorbance was measured at 550 nm using a UV-Vis spectrophotometer against a blank. Total phenolic content was determined as gallic acid equivalents (GAE) (Islam, *et al*., 2015).

Determination of Total Flavonoid Content

Total flavonoid content was estimated by using the aluminium chloride method (Rakesh, *et al*., 2021). 1 ml of plant extract (100 µg/ml), 4 ml of methanol, 1 ml of 10% aluminium chloride, and 1 ml of 1 M sodium acetate were added successively and vortexed. The mixture was incubated in the dark at room temperature for 45 minutes. Absorbance was measured at 415 nm using a UV-Vis spectrophotometer (Shimadzu UV-2450). A blank was prepared without the extract. Quercetin (20–100 µg/ml) served as the standard, and results were expressed as quercetin equivalents (QE).

GC-MS of *Achyranthes aspera* Extract

Ethyl acetate extract exhibited the most potent antimicrobial activity against *Staphylococcus aureus* and *Streptococcus mutans*. Therefore, it was selected for further analysis using Gas Chromatography-Mass Spectrometry (GC-MS). The compound identification of the plant extract through GC-MS followed a standardized and accurate protocol. An autosampler (AOC-20i+s) was used to ensure accurate and efficient sample handling, performing multiple rinses and rapid injections with high precision.

Antimicrobial Activity of *Achyranthes aspera* Extract

The determination of the antibacterial activity of *Achyranthes aspera* extracts in ethyl acetate, ethanol, methanol, and distilled water against two microbial pathogens, *S. aureus* and *S. mutans*, by using the well diffusion method. Bacterial cultures were grown in nutrient broth and spread on Muller-Hinton agar plates (CLSI, 2018). Well (6mm diameter) were filled with 50 µl of the herbal extract at concentrations of 125mg/ml, 250mg/ml, and 500mg/ml. Dimethyl sulfoxide (DMSO) was used as the negative control, and streptomycin was used as the positive control. The plates were incubated at 37°C for 24 hours, and zones of inhibition were measured in millimeters.

MIC of Plant Extract

Minimum inhibitory concentration was determined by the microdilution assay. The concentration of the extract is 60, 30, 15, 7.5, 3.75, and 1.87 (µg/ml) was prepared in DMSO. The lowest concentration of plant extract that prevents bacterial growth. To confirm the inhibition of bacterial growth, the inoculum was streaked onto freshly prepared agar plates (Kumar, *et al*., 2024).

RESULTS AND DISCUSSION

Percentage Yield of Plant Extracts

Table 1 represents the extract percentage yield for *Achyranthes aspera* using different solvents, ethyl acetate, ethanol, methanol, and distilled water. Distilled water obtained the highest yield at 4.2%, producing 6.4 grams of extract. Methanol achieved a yield of 2.2%, extracting 3.4 grams of plant material. Ethanol resulted in a 1.7% yield, yielding 2.6 grams of extract, while ethyl acetate produced the lowest yield at 1.6%, with 2.4 grams of extract. A study done by Rahmawati, *et al*., (2020) reported the percentage yield of *Achyranthes aspera* in ethyl acetate (17.58%) and methanol (29.77%). The percentage yield of the crude ethanol extract was found to be 6.47% for the stem of *Achyranthes aspera* (Sunthamala, *et al*., 2021). *Achyranthes aspera* demonstrates promising antibacterial properties against *Streptococcus mutans*, supporting its potential use in developing herbal oral care products to prevent dental caries (Yadav, *et al*., 2016).

Table 1: Extract percentage yield of *Achyranthes aspera*

S. No.	Solvent	Weight of plant extract in grams	% yield
1.	Ethyl acetate	2.4	1.6
2.	Ethanol	2.6	1.7
3.	Methanol	3.4	2.2
4.	Water	6.4	4.2

Qualitative Phytochemical Analysis of *Achyranthes aspera*

Table 2 presents the results of the qualitative phytochemical analysis of *Achyranthes aspera* using various solvents. None of the solvents tested showed the presence of tannins, phenols, and saponins. alkaloids present in ethyl acetate and methanol, lycosides present in ethyl acetate, ethanol, and methanol. Steroids were detected only in ethanol. Amino acids were found in both ethanol and methanol. Coumarin and flavonoids were present in all four solvents tested, including ethyl acetate, ethanol, methanol, and distilled water. *A. aspera* exposed the presence of several

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bioactive components, including alkaloids, saponins, tannins, cardiac glycosides, and steroids, which were identified in the solvents (Sharma, *et al*., 2013). The phytochemical analysis of *Achyranthes aspera* extracts shows that alkaloids, flavonoids, saponins, and tannins/phenolic compounds are present in both methanol and aqueous extracts. Amino acids are present only in the aqueous extract and are absent in the methanol extract (Sureshkumar, 2021).

Table 2: Qualitative phytochemical analysis of *Achyranthes aspera*

S. No.	Phytochemicals	Solvents			
		Ethyl acetate	Ethanol	Methanol	Dist. Water
1.	Tannins	-	-	-	-
2.	Alkaloids	+	-	+	-
3.	Phenol	-	-	-	-
4.	Glycosides	+	+	+	-
5.	Saponins	-	-	-	-
6.	Steroid	-	+	-	-
7.	Amino acid	-	+	+	-
8.	Coumarin	+	+	+	+
9.	Flavonoids	+	+	+	+

Quantitative Analysis of Phytochemical

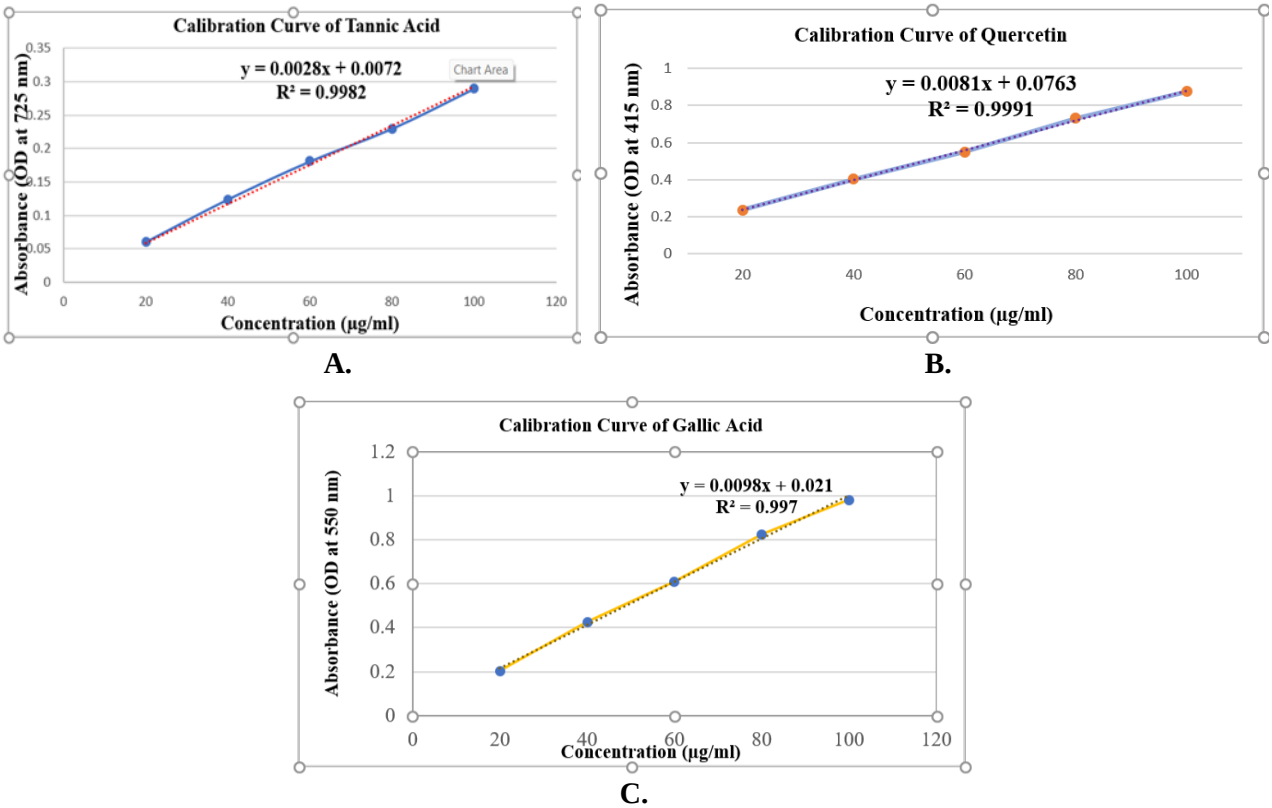

Figure 1- Calibration curve of A. Tannic acid B. Gallic acid C. Quercetin

Table number 3 *Achyranthes aspera* demonstrated flavonoid levels of 44.42 mg/g QE in ethyl acetate, 47.44 mg/g QE in ethanol, 32.66 mg/g QE in methanol, and 56.24 mg/g QE in distilled water; tannin and phenol were absent in all solvents.

Table 3: Total phytochemical content

Phytochemicals	Ethyl acetate	Ethanol	Methanol	Distilled water
Tannin	-	-	-	-
Phenol	-	-	-	-
Flavonoids	44.42	47.44	32.66	56.24

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GC-MS of *Achyranthes aspera* Extract

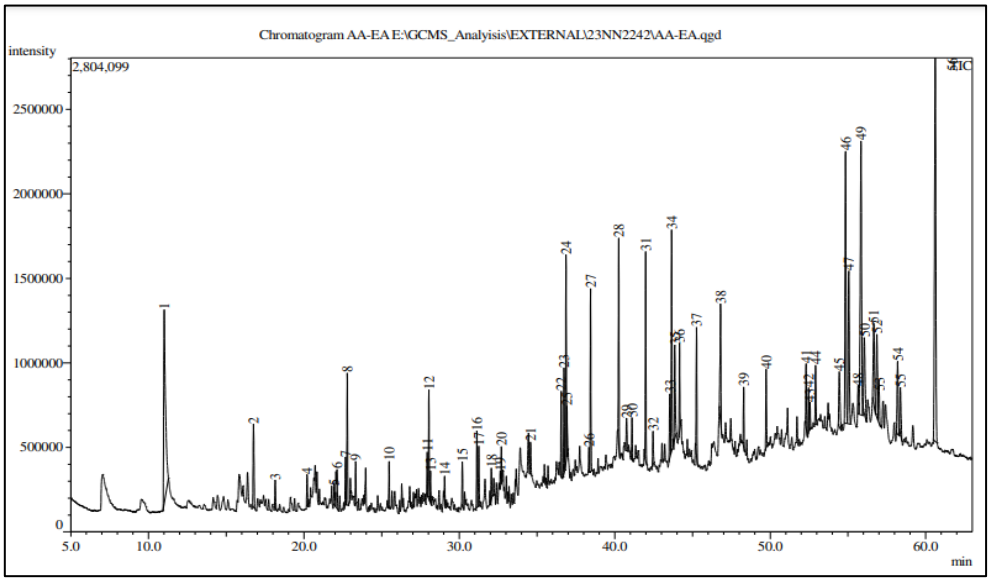

Figure 1: GC-MS chromatogram for stem in ethyl acetate extracts of *Achyranthes aspera*

Table 4 demonstrates that the GC-MS analysis of *Achyranthes aspera* provides a detailed insight into its chemical composition, showcasing a diverse range of compounds. Mostly, the profile includes various alcohols, ketones, fatty acids, and sterols. Noteworthy components identified include phytol, a precursor to vitamin E, which contributes to antioxidant properties. Sterols like stigmasterol, γ-sitosterol, and stigmastanol are indicative of potential pharmacological activities, such as anti-inflammatory and cholesterol-lowering effects. **Phytol, stigmasterol, γ-sitosterol,** and **stigmastanol** are compounds that are known for numerous bioactivities, but **phytol** stands out as the most likely candidate responsible for **antimicrobial activity**. Additionally, the presence of unique compounds, such as tris(2,4-di-tert-butylphenyl) phosphate, suggests environmental adaptations or contamination. This comprehensive chemical fingerprint highlights *Achyranthes aspera* as a valuable resource for both medicinal and industrial applications, due to its diverse array of bioactive compounds with potential health benefits.

Table 4: GC-MS of *Achyranthes aspera*

S. No.	Retention time	Area%	Name	Formula
1.	11.011	7.82	1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$
2.	23.327	0.63	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$
3.	31.127	1.23	2-Hexadecen-1-ol, 3,7,11,15 tetramethyl-,	$C_{20}H_{40}O$
4.	31.245	1.05	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$
5.	32.625	0.27	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$
6.	36.732	1.58	Phytol	$C_{20}H_{40}O$
7.	41.106	0.57	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$
8.	42.458	0.53	Heneicosanoic acid, methyl ester	$C_{22}H_{44}O_2$
9.	43.541	1.31	Trichloroacetic acid, pentadecyl ester	$C_{17}H_{31}Cl_3O_2$
10.	43.858	1.80	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl	$C_{19}H_{38}O_4$
11.	44.165	1.30	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$
12.	52.308	0.91	Nonacosan-10-one	$C_{29}H_{58}O$
13.	52.558	0.42	n-Tetracosanol-1	$C_{24}H_{50}O$
14.	52.914	1.02	Vitamin E	$C_{31}H_{52}O_3$
15.	54.444	1.49	Ergost-5-en-3-ol, (3. beta.)-	$C_{28}H_{48}O$
16.	54.847	6.22	Stigmasterol	$C_{29}H_{48}O$
17.	55.050	3.59	Tetrahydrosmilagenin	$C_{27}H_{48}O_3$

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18.	55.840	8.10	.gamma. -Sitosterol	$C_{29}H_{50}O$
19.	56.051	2.22	Stigmastanol	$C_{29}H_{52}O$
20.	56.666	2.40	.beta. -Amyrin	$C_{30}H_{50}O$
21.	57.017	1.12	4,22-Stigmastadiene-3-one	$C_{29}H_{46}O$
22.	58.375	1.04	9,19-Cyclolanostan-3-ol, 24-methylene-, (3. beta.)-	$C_{31}H_{52}O$
23.	60.615	10.85	Tris(2,4-di-tert-butylphenyl) phosphate	$C_{42}H_{63}O_4P$

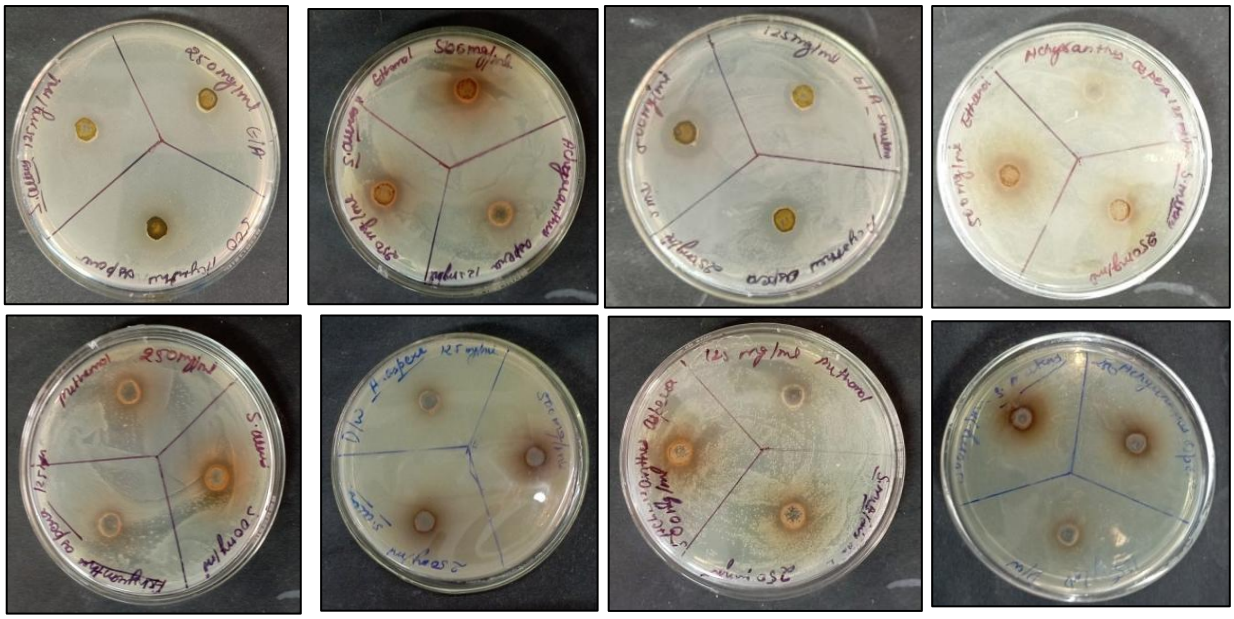
Antimicrobial Activity of Plant Extract

Table 5 provides data on the antibacterial activity of *Achyranthes aspera* plant extracts against *Staphylococcus aureus* and *Streptococcus mutans* using different solvent types (ethyl acetate, ethanol, methanol, and distilled water) at varying concentrations (150, 250, and 500 mg/ml). Specifically, for *S. aureus*, the maximum zone of inhibition detected was 14.80 ± 0.60 mm at 500 mg/ml using ethyl acetate, while for *S. mutans*, the maximum was 19.83 ± 0.32 mm at 500 mg/ml using ethyl acetate as well. According to Mishra, (2018), the plant extract of *Achyranthes aspera* showed the greatest antibacterial activity against *Bacillus subtilis*, with a zone of inhibition of 18 mm, while *Staphylococcus aureus* showed 16 mm. Among gram-negative bacteria, *Pseudomonas aeruginosa* showed a 12 mm zone of inhibition, while *Escherichia coli* exhibited the lowest inhibition at 9 mm. The plant extract exhibited a determinate antibacterial effect against *Staphylococcus aureus*, as shown by a zone of inhibition with an average diameter of 14.5 ± 1.5 mm (Sri Ramakrishna, *et al*., 2022).

Table 5: Zone of inhibition of *Achyranthes aspera* plant extract

Microorganisms	**Extract conc. (mg/ml)**	**Diameter of the Zone of Inhibition (mm)**				
		Ethyl acetate	**Ethanol**	**Methanol**	**Dist. water**	**Streptomycin**
S. aureus	**500**	14.80 ± 0.60	NA	NA	NA	17.10 ± 0.30
	250	11.10 ± 0.17	NA	NA	NA	15.20 ± 0.27
	150	10.00 ± 0.00	NA	NA	NA	12.20 ± 0.27
S. mutans	**500**	19.83 ± 0.32	NA	NA	NA	20.23 ± 0.35
	250	17.43 ± 0.35	NA	NA	NA	16.33 ± 0.62
	150	15.93 ± 0.41	NA	NA	NA	13.43 ± 0.24

NA- not appear



A. *Staphylococcus aureus* MTCC-7443 **B.** *Streptococcus mutans* MTCC-497

Figure 2: Showing antimicrobial activity of *Achyranthes aspera* (stem) extracts against A. *Staphylococcus aureus* and B. *Streptococcus mutans*

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MIC of Plant Extract

The Minimum Inhibitory Concentration (MIC) antibacterial potential against *Staphylococcus aureus* and *Streptococcus mutans of Achyranthes aspera extract*. Exhibited an MIC of 7.5 (µg/ml) against *S. aureus* and 3.75 (µg/ml) against *S. mutans*. The Minimum Inhibitory Concentration (MIC) data of *Achyranthes aspera* extracts disclose different antibacterial efficacy against diverse microorganisms. Ethyl acetate, ethanol, and aqueous extracts showed reasonable to low activity, generally with MICs of 50–100 µg/ml, although all extracts showed effectiveness (12.5 µg/ml) against *S. epidermis (*Sharma, *et al.*, 2013). The ethanol, methanol, and aqueous (distilled water) extracts of *Achyranthes aspera* s exhibit different concentrations of antimicrobial activity against different microorganisms. Methanol extract exhibited strong inhibitory effects, with the lowest MIC value of 62.5 µg/ml against *Klebsiella pneumoniae*, *Candida albicans*, and *Saccharomyces cerevisiae*, and moderate activity (125–250 µg/ml) against most other organisms. Ethanol extract also showed considerable antimicrobial potential, mainly against *Klebsiella pneumoniae* and *Saccharomyces cerevisiae* (62.5 µg/ml), whereas showing reasonable efficacy (125–250 µg/ml) against other tested strains. Aqueous extract was comparatively less effective but still active, maintaining MIC values of 125–250 µg/ml across most organisms (Owk, *et al*., 2014).

Table 6: Table MIC of plant extract

S. No.	Microorganism	MIC (µg/ml)	
		Formulation	Streptomycin
1.	***S. aureus***	7.5	3.75
2.	***S. mutans***	3.75	1.87

CONCLUSION

In this study, the *Achyranthes aspera* ethyl acetate extract exhibited high antimicrobial activity against both bacterial strains. The findings indicate that the stem contains bioactive compounds responsible for antimicrobial action against dental infectious bacteria.

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