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EVALUATIVE ANALYSIS OF ANTIBACTERIAL EFFICACY OF *CAMELLIA SINENSIS* AND *AZADIRACHTA INDICA* ON ORAL BIOFILM-FORMING BACTERIA

[1]Anitha Thomas, [2]Nagasri Ponnala
[1]Assistant Professor, [2]PG Student
Department of Microbiology
St. Francis College for Women, Begumpet, Hyderabad, India-500016

Abstract: This study explores the antimicrobial potential of *Camellia sinensis* and *Azadirachta indica* leaf extracts against bacteria that form oral biofilms. By examining their efficacy in combating oral biofilm formation, the research aims to provide valuable insights into alternative oral health management approaches. Both *Camellia sinensis* and *Azadirachta indica* have been traditionally known for their antimicrobial properties, and this study seeks to validate their effectiveness in addressing oral biofilm-related issues. The findings could pave the way for the development of natural antimicrobial strategies in oral care. To scientifically evaluate the efficacy of these plants against oral biofilm-forming bacteria, the study conducted phytochemical screening and assessed the antimicrobial activity of methanolic leaf extracts. Various bacterial species isolated from patients with dental caries and a standard strain *Streptococcus salivarius* (MTCC13009) were targeted for evaluation. Phytochemical screening revealed the presence of several bioactive compounds in both extracts, with catechin from *Camellia sinensis* and nimbidin from *Azadirachta indica* identified. Biofilm production was assessed through qualitative and quantitative methods, and the antimicrobial activity of the extracts was tested using agar-well diffusion and disc diffusion methods, comparing them with a standard antibiotic Chloramphenicol. *Camellia sinensis* demonstrated greater antimicrobial activity compared to *Azadirachta indica.* Additionally, a mouthwash was formulated using *Camellia sinensis* extract and compared with commercial Chlorhexidine mouthwash at different concentrations. Statistical analysis using ANOVA was performed, indicating significant antimicrobial activity for the *Camellia sinensis* mouthwash.

Keywords: Oral biofilm formation, antimicrobial potential, *Camellia sinensis, Azadirachta indica,* phytochemical screening.

I. INTRODUCTION:

Biofilms represent a unique survival strategy for microbes, enabling them to form dense, matrix-like structures on inert surfaces. These structures, composed of polymers secreted by microbes, offer protection against environmental stressors and antimicrobial agents, posing a serious public health risk (Berger et al. 2018). A diverse range of organisms, including *Pseudomonas, Vibrio, Escherichia, Salmonella, Listeria, Streptococcus,* and *Mycobacterium,* have been identified as culprits in biofilm-induced infections.(Berger et al. 2018). The oral cavity harbors diverse biofilms, including dental plaque, which is implicated in oral infections such as dental caries and periodontal disease. Additionally, biofilms on dental prostheses, such as dentures and implants pose significant challenges in oral health management. (Berger et al. 2018; Donlan and Costerton 2002). The adaptive nature of biofilms renders them resistant to traditional antimicrobial therapies, leading to chronic infections (Neppelenbroek 2015).

Plants as Natural Microbial Agents:

Natural products derived from plants offer potential solutions for combating biofilm-associated infections. These compounds target various stages of biofilm development, presenting promising alternatives to conventional antimicrobial agents. (Ananthi and Giri n.d.)

Camellia sinensis, belonging to the Theaceae family, is native to China and Southeast Asia. It is an evergreen shrub with yellow-white flowers and leaves harvested for tea production. (Mahmood, Naveed, and Khan 2010). It is said to possess antioxidant, anticancer, antidiabetic, antibacterial, antiviral, and neuroprotective properties. Green tea has anti-mutagenic, anti-thyroid, diuretic, and bone health benefits. (Zhao et al. 2022). Green tea's EGCG inhibits bacteria associated with gum diseases, promoting periodontal healing.

Azadirachta indica, or Neem, from the Meliaceae family, is a fast-growing tree found in tropical regions like India. (Alzohairy 2016). It possesses various pharmacological properties like antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory effects (Rahmani 2018). Neem extracts inhibit the growth of bacteria causing dental caries, including *Streptococcus mutans.* Neem leaf extract shows promise against microorganisms found in root canals. Neem-based mouth rinses are effective in treating chronic gingivitis and periodontal diseases (Naveed et al. 2014).

II. RESEARCH METHODOLOGY:

The study was conducted at St. Francis College for Women, Department of Microbiology, Louis Pasteur Laboratory, Hyderabad. This study was based on the comparative analysis of the antimicrobial activity of the oral biofilm-forming bacteria collected from patients suffering from dental caries with that of the standard strain *Streptococcus salivarius* (MTCC13009) procured from IMTECH, Chandigarh.

Sample Collection:

Dental Plaque-causing organisms (D1-D14) were isolated from patients with dental caries at Smile Dental Hospital and Implant Care, Hyderabad, along with the standard strain *Streptococcus salivarius* (MTCC13009) from IMTECH, Chandigarh. Samples were collected using sterile swabs by rubbing across teeth surfaces and oral cavity regions.

Isolation and characterization of oral biofilm-forming bacteria:

Samples were inoculated in nutrient broth and streaked on nutrient agar plates after incubation. Isolated colonies were observed for morphology, and pure cultures were obtained by sub-culturing. Gram staining and biochemical tests confirmed the isolates' identities, following Bergey's Manual of Systematic Bacteriology.

Biofilm production:

Biofilm production by Congo red agar:

For the Congo red agar method, Brain Heart Infusion agar and Congo red stain were autoclaved separately. After cooling to room temperature, they were mixed and poured into petri plates to solidify. Dental plaque-causing isolates and standard strain *Streptococcus salivarius* were streaked onto the agar and incubated at 37ºC for 24hrs to 48hrs. Biofilm-producing colonies appeared black with a dry crystalline consistency.(Basak et al. 2009; Jadhav and Tale 2015)

Biofilm production by Tube detection assay:

10ml of Tryptic soya broth with 1% glucose was inoculated with bacterial test organisms and incubated at 37ºC for 24hrs. After incubation, cultures were washed with phosphate buffer saline (pH 7.3), stained with 0.1% crystal violet, and then washed with deionized water. Tubes were dried in an inverted position after staining (Basak et al. 2009; Jadhav and Tale 2015).

Biofilm production by Microtiter plate method:

10ml of Tryptic soya broth with 1% glucose was inoculated with bacteria from an overnight nutrient agar culture. After incubation at 37ºC for 24hrs, cultures were diluted 1:100 with fresh medium. Diluted cultures

were added to 96-well flat bottom tissue culture plates, and sterile broth was maintained as a blank. Plates were incubated at 37ºC for 24hrs, then gently tapped. Wells were washed with phosphate buffer saline to remove free-floating bacteria. Adherent biofilms were fixed with 2% sodium acetate, stained with 0.1% crystal violet, and the excess stain was washed. Optical densities of stained biofilms were measured at 630nm wavelength using a micro-ELISA auto reader, with OD values of sterile medium subtracted from test organisms (Basak et al. 2009).

A comparative analytical study:

In biofilm production, evaluating different detection methods like Congo red agar, tube detection assay, and microtiter plate method is crucial. Calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) helps assess their accuracy and reliability. These metrics allow comparison of method performance, identification of false results, and ensuring quality assurance in biofilm detection (Basak et al. 2009).

Preparation of methanolic extracts of *Camellia sinensis* (Green tea) and *Azadirachta indica* (Neem) leaves:

Collection and processing of leaves:

Dried *Camellia sinensis* leaves were finely powdered after collection, while fresh *Azadirachta indica* leaves were washed and dried under sunlight for 3days before powdering. Both were stored in a refrigerator.

Methanolic extraction using Soxhlet method:

50g of *Camellia sinensis* and 26g of *Azadirachta indica* powder were separately extracted with 300 ml of methanol using a Soxhlet apparatus at 64.7ºC (Hashim et al. 2021) Extraction continued until the extract turned colourless. Methanolic extracts were then evaporated using a rotary vacuum evaporator, and the final extracts were stored in screw cap tubes in the refrigerator.

Phytochemical screening of methanolic extracts of *Camellia sinensis* and *Azadirachta indica:*

The methanolic extracts of green tea and neem leaves underwent quantitative analysis for various phytochemicals, including alkaloids, tannins, proteins, phytosterols, phenols, saponins, glycosides, acids, quinones, terpenoids, reducing sugar, flavonoids, and carbohydrates (Benisheikh et al. 2019; Geoffrey et al. n.d.; Latteef n.d.; Sayuri Nagano and Batalini 2021; Seriana et al. 2021). **(Table 2.1)**

Table 2.1 Phytochemical screening of methanolic extracts of *Camellia sinensis* and *Azadirachta indica*

Test	Observation
Alkaloids	Wagner's reagent was added to methanolic extracts, and the appearance of a brown to flocculent precipitate indicated the presence of alkaloids.
Tannins/Phenols	FeCl3 solution was added to the extracts, and the formation of a greenish-black colour confirmed the presence of tannins or phenols.
Proteins	Methanolic extracts were mixed with concentrated HNO3, and the observation of a white or yellow precipitate indicated the presence of proteins.
Phytosterols	Salkowski reaction was performed, and the appearance of red or brown colouration indicated the presence of phytosterols
Saponins	The formation of froth upon mixing extracts with sodium bicarbonate and distilled water indicated the presence of saponins
Glycosides	Treatment with glacial acetic acid and FeCl3 solution led the emergence of a reddish-brown precipitate, confirming the presence of glycosides.
Acids	Effervescence upon the addition of sodium bicarbonate solution confirmed the presence of acids.

Quinones	The appearance of a red colouration upon addition of concentrated H2SO4 indicated the presence of quinones
Terpenoids	The manifestation of reddish-brown colouration upon treatment with chloroform and concentrated H2SO4 confirmed the presence of terpenoids.
Reducing Sugar	Heating extracts with Benedict's reagent resulted in a brown-to-red colouration, indicating the presence of reducing sugar.
Flavonoids	The addition of concentrated H2SO4 led to a yellowish-orange colouration, confirming the presence of flavonoids.
Carbohydrates	Upon heating with Fehling's reagent, the appearance of a reddish-orange precipitate confirmed the presence of carbohydrates

GC-MS Analysis of methanolic extract of *Camellia sinensis:*

GC-MS analysis was conducted using GCMSQP2010, SHIMADZU, with fatty acids as standard. The analysis spanned 30 min, and the relative percentage of each component was calculated by comparing average peak areas to the total area in the chromatogram. This method assesses the proportional contribution of each compound, with higher peak areas indicating greater presence in the sample. The accuracy of these percentages relies on calibration and analysis conditions (Ananthi and Giri n.d.).

Purification of active components from methanolic extracts of *Camellia sinensis* and *Azadirachta indica* using Thin-layer chromatography:

Purification of Catechin from methanolic extract of green tea using thin-layer chromatography:

Catechin detection from green tea methanolic extract was performed using thin-layer chromatography. Green tea extract and methanol control were loaded onto a silica sheet and separated using acetic acid and chloroform (1:9), and chromatography continued until the solvent reached 3/4th of the sheet. The sheet was dried, and then examined under UV light at 280nm. The presence of catechin was indicated by the emergence of a dark orangish-pink spot ((Alasadiy 2013).

Purification of Nimbidin from methanolic extract of neem using thin-layer chromatography:

Nimbidin separation from neem extract was performed using thin-layer chromatography. Neem extract and methanol control were loaded onto a silica sheet, then developed in a tank saturated with ethanol and water (8:2). Chromatography continued until the solvent reached 3/4th of the sheet. The sheet was dried, and Nimbidin visualization was conducted under UV light at 560nm. The presence of Nimbidin was indicated by yellow and orange fluorescent spots (K.Gilbert Ross Rex, 2021).

Antimicrobial activity:

Muller Hinton Agar assessed the antimicrobial activity of green tea and neem methanolic extracts. Plaque-forming bacteria from dental caries patients and *Streptococcus salivarius* were cultured and spread on agar. Extracts, methanol solvent, and chloramphenicol were applied on agar discs, and incubated, and zone diameters were measured for analysis. Green tea showed stronger activity. The minimum inhibitory concentration (MIC) of green tea was determined at various concentrations against oral biofilm-forming bacteria using agar well-diffusion. Extracts were added into wells of agar, incubated, and zones measured for statistical analysis.

Synthesis of mouthwash using methanolic extract of *Camellia sinensis:*

Sucrose, sodium lauryl sulfate (a foaming agent), and potassium metabisulfite (a preservative) were weighed separately and then combined with 10ml of green tea extract (Nirumbama. K, 2022).

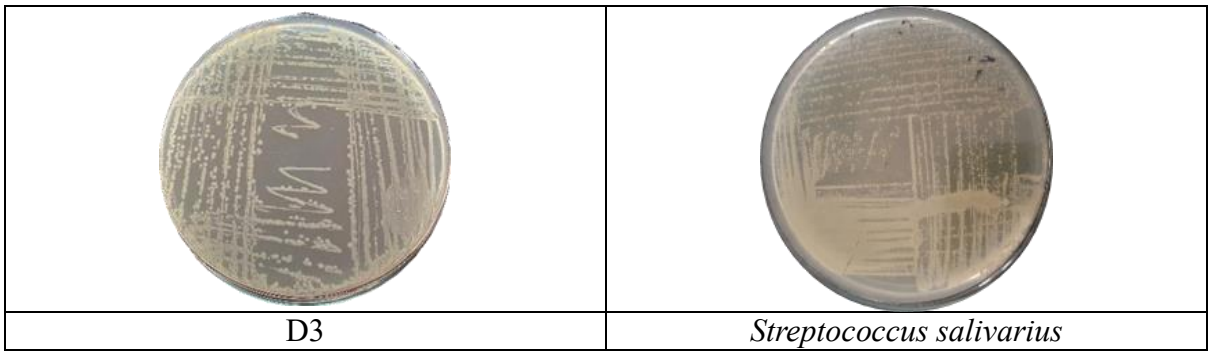
Antimicrobial activity of synthesized green tea mouthwash and commercial mouthwash (chlorhexidine) at different concentrations:

The antimicrobial activity of synthesized green tea mouthwash and commercial mouthwash (chlorhexidine) was compared using the agar well-diffusion assay. The culture broth was spread on Muller Hinton Agar, and wells were bored. Three wells each were filled with different concentrations of synthesized green tea mouthwash and chlorhexidine. The plates were then incubated at 37ºC for 16-18hrs. After incubation, the diameter of the inhibition zones was measured and analyzed statistically (Nirumbama. K, 2022).

III. RESULTS AND DISCUSSION:

Morphological and cultural characteristics:

From the study, various colonies of dental plaque-causing organisms were observed, characterized by different sizes, pigmentation, forms, elevations, margins, and translucency. Gram staining revealed the predominant presence of Gram-negative rods and Gram-negative cocci among the isolates, while the standard strain *Streptococcus salivarius* (MTCC13009) exhibited Gram-positive cocci Biochemical tests, following Bergey's Manual of Systematic Bacteriology, were conducted on dental isolates D1 to D14 for organism identification. **(Table 3.1) (Table 3.2) (Fig 3.1)**



D3 | *Streptococcus salivarius*

Figure 3.1 Morphological and cultural characteristics of Bacterial isolates and Standard strain *Streptococcus salivarius* (MTCC13009)

Table 3.1 Biochemical tests for gram negative rods (D1, D2, D3, D6, D7, D8, D9, D11, D12, D13)

Isolates	Oxidase test	Carbohydrate Fermentation		Fluorescent diffusible yellow pigment	Non- Fluorescent diffusible blue pigment	Alkaline peptone water required for growth	Motility	H2S production	Indole test	MR test	VP test	Urease test	Ornithine decarboxylase test	Lecithinase test	Organism
		Glucose	Lactose												
D1	+	-	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	*Pseudomonas sp.*
D2	+	-	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	*Pseudomonas sp.*
D3	+	+	NR	NR	NR	-	+	-	NR	NR	+	NR	NR	NR	*Aeromonas veronii*
D6	+	+	NR	NR	NR	-	NR	NR	NR	NR	-	NR	NR	NR	*Aeromonas sp.*
D7	+	+	NR	+	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	*Pseudomonas aeruginosa*
D8	-	NR	-	NR	NR	NR	-	NR	-	NR	NR	-	-	NR	*Yersinia sp.*
D9	-	NR	-	NR	NR	NR	-	NR	-	NR	NR	-	-	NR	*Yersinia sp.*
D11	-	NR	+	NR	NR	NR	+	-	-	+	-	NR	NR	NR	*Serratia fonticola*
D12	+	+	NR	+	-	NR	NR	NR	NR	NR	NR	NR	NR	+	*Pseudomonas cichorii*
D13	+	-	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	*Pseudomonas sp.*

Table 2.2 Biochemical tests for gram-negative cocci (D4, D5, D10, D14)

Isolates	Oxidase test	Catalase test	Indole test	Gelatin Hydrolysis test	Nitrate Reduction test	Maltose fermentation test	Organism
D4	-	-	-	+	-	+	*Veillonella sp.*
D5	-	-	-	+	-	+	*Veillonella sp.*
D10	+	+	-	-	-	-	*Moraxella sp.*
D14	+	+	-	-	-	-	*Moraxella sp.*

Biofilm production:

Biofilm production by Congo red agar method:

Bacterial isolates from D1-D14 and *Streptococcus salivarius* (MTCC13009) were inoculated on Congo red agar and incubated aerobically at 37ºC for 24hrs. Biofilm production was indicated by black colonies with a dry crystalline consistency. Isolates D3, D5, D6, D9, D10, D11, and *Streptococcus salivarius* (MTCC13009) showed strong biofilm production and hence they were categorized as strong biofilm producers. Isolates D1, D2, D13, and D14 showed moderate biofilm production whereas isolates D4, D7, D8, and D12 showed no biofilm production. Distribution of biofilm production showed 46.67% strong biofilm production, 26.67% showed moderate biofilm production and 26.67% showed no biofilm production. **(Table 3.3) (Fig 3.2)**

D4	D2	D1	*Streptococcus salivarius*
Not produced	Slightly produced	Moderately produced	Strongly produced

Figure 3.2 Biofilm production using Congo red agar method

Biofilm production by Tube detection assay:

Biofilm production was assessed using the tube detection assay by observing visible stained film lining on the wall and bottom of the tube. Isolates D2, D3, D6, D10, D11 and *Streptococcus salivarius* showed strong biofilm production, hence, are categorized as strong biofilm producers. Isolates D1, D5, D7, D8, D9, D13, and D14 showed moderate biofilm production whereas isolates D4 and D12 showed no biofilm production. Distribution of biofilm production showed 40% strong biofilm production, 46.67% moderate biofilm production, and 13.3% no biofilm production. **(Table 3.3) (Fig 3.3)**

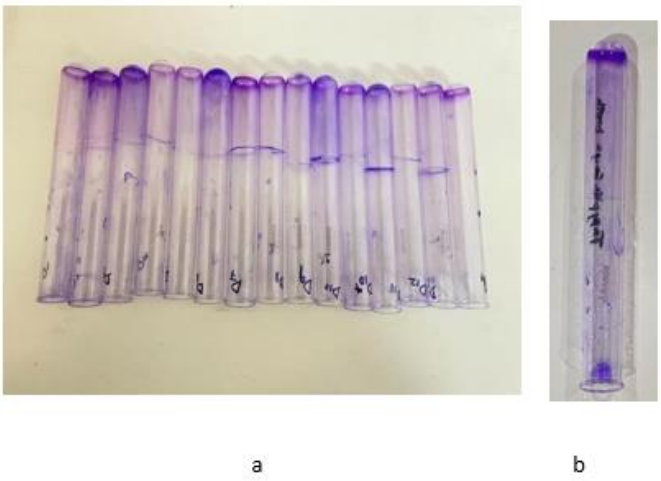


Figure 3.3 Biofilm production using Tube detection assay a) D1-D14 isolates b) *Streptococcus salivarius* (MTCC13009)

Biofilm production by Micro titer plate method:

In the assessment of biofilm production using the microtiter plate method, the optical density (OD) values of stained adherent biofilm were measured using a micro-ELISA auto reader at a wavelength of 630nm. The OD values of the sterile medium were established at 0.057, serving as the control reference against which all test values of bacterial isolates were compared. According to (Basak et al. 2009), the interpretation of OD values is as follows: OD values less than 0.120 suggest weak biofilm production, with values ranging from 0.120 to 0.240 indicating moderate biofilm production, and values exceeding 0.240 indicate strong biofilm production. These criteria provide a standardized framework for categorizing biofilm production levels based on OD measurements. Isolates D1, D2, D3, D4, D5, D6, D7, D11, D12, D13, D14, and *Streptococcus salivarius* showed weak biofilm production. Isolates D8, D9, and D10 showed moderate biofilm production and no isolates showed strong biofilm production using the microtiter plate method. **(Table 3.3) (Fig 3.4)**

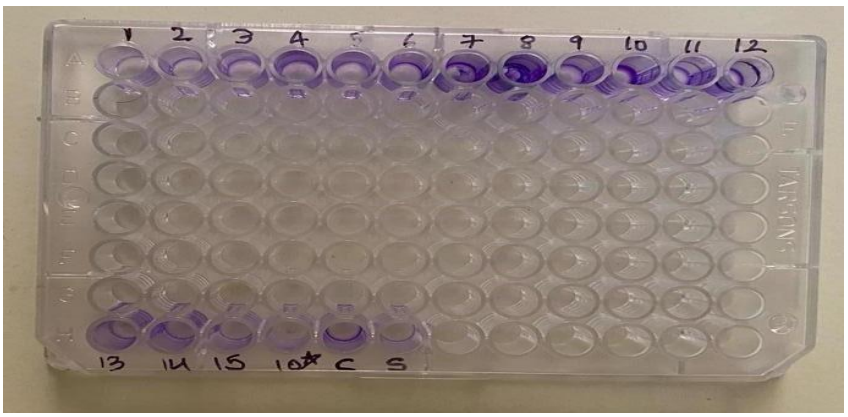


Figure 3.4 Biofilm production using Microtiter plate method

Table 3.3 Comparison of Biofilm production by qualitative and quantitative methods

ISOLATES	BIOFILM PRODUCTION		
	QUALITATIVE DETECTION		QUANTITATIVE DETECTION
	Congo red agar	Tube detection assay	Microtiter plate method (OD Values)
D1	+	+	0.060-0.057=0.003
D2	+	++	0.066-0.057=0.009
D3	++	++	0.108-0.057=0.051

D4	-	-	0.121-0.057=0.064
D5	++	+	0.091-0.057=0.034
D6	++	++	0.082-0.057=0.025
D7	-	+	0.100-0.057=0.043
D8	-	+	0.290-0.057=0.233
D9	++	+	0.181-0.057=0.124
D10	++	++	0.214-0.057=0.157
D11	++	++	0.110-0.057=0.053
D12	-	-	0.096-0.057=0.039
D13	+	+	0.097-0.057=0.04
D14	+	+	0.66-0.057=0.009
***Streptococcus salivarius* (MTCC13009)**	++	++	0.068-0.057=0.011

(+)- Biofilm producers, (++)- Good Biofilm producers, (-)- Poor Biofilm producers

A comparative statistical analysis:

Sensitivity measures the proportion of true positives; specificity measures the proportion of true negatives that are correctly identified by the test. Positive predictive value measures the proportion of positive results that are truly positive. Negative predictive value measures the proportion of negative test results that are truly negative. **(Table 3.4)**

The equations to calculate sensitivity, specificity, positive predictive value, and negative predictive value are as follows

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive+False negative}} \quad (3.1)$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative+False positive}} \quad (3.2)$$

$$\text{Positive predictive value} = \frac{\text{True positive}}{\text{True positive+False positive}} \quad (3.3)$$

$$\text{Negative predictive value} = \frac{\text{True negative}}{\text{True negative+False negative}} \quad (3.4)$$

Table 3.4 A comparative statistical analysis of biofilm production

Method	Sensitivity	Specificity	PPV	NPV
Congo red agar method	67%	60%	75%	50%
Tube detection assay	70%	50%	78%	40%
Microtiter plate method	80%	100%	100%	60%

Sensitivity: For Congo red agar, it correctly identifies 67% of biofilm producers. Similarly, for tube detection assay, it correctly identifies 70%, and for the microtiter plate method, it correctly identifies 80%.

Specificity: For Congo red agar, it correctly identified 60% of non-biofilm producers. For the tube detection assay, it correctly identifies 50%, and for the microtiter plate method, it correctly identifies 100%. A higher specificity means fewer false positives.

Positive predictive value: For Congo red agar, out of all positive results, 75% are truly positive. For tube detection assay, it is 78%, and for microtiter plate method, it is 100%

Negative predictive value: For Congo red agar, out of all negative results, 50% are truly negative. For the tube detection assay, it is 40%, and for the microtiter plate method, it is 60%. A higher negative predictive value indicates a lower chance of false negatives.

Preparation and Phytochemical screening of methanolic extracts of *Camellia sinensis* and *Azadirachta indica:*

The Soxhlet extraction method was employed to prepare methanolic extracts of *Camellia sinensis* (Green tea) and *Azadirachta indica* (Neem). Following extraction, the methanol was evaporated from the extracts using a rotatory vacuum evaporator. Qualitative phytochemical screening was conducted to identify secondary metabolites within the methanolic extracts of both plants. Green tea extracts displayed positive results for alkaloids, tannins, phytosterols, phenols, saponins, glycosides, quinones, terpenoids, reducing sugars, and flavonoids while proteins, acids, and carbohydrates were absent. Neem extracts exhibited positive results for alkaloids, phytosterols, phenols, saponins, acids, tannins, quinones, and terpenoids, with negative results for proteins, glycosides, reducing sugars, and carbohydrates. **(Table 3.5)**

Green tea extracts contain caffeine, tannins, phytosterols, and polyphenols like catechins, contributing to their antimicrobial and antioxidant properties. These compounds may aid in oral health maintenance by promoting saliva production, controlling bacterial growth, and reducing plaque formation. Similarly, Neem extracts, rich in alkaloids, phenols, and terpenoids, exhibit antibacterial and anti-inflammatory effects, which could combat oral pathogens, and inflammation, thus preventing oral diseases. The synergistic action of these phytochemicals may offer various health benefits beyond oral health, such as antioxidant, anti-inflammatory, and potential cancer-preventive effects.

Table 3.5 Phytochemical screening of secondary metabolites in methanolic extracts of green tea and neem

TESTS	PHYTOCHEMICALS	GREEN TEA EXTRACT	NEEM EXTRACT
Wagner's test	**ALKALOIDS**	Positive	Positive
Xanthoprotein test	**PROTEINS**	Negative	Negative
Salkowski reaction	**PHYTOSTEROLS**	Positive	Positive
FeCl3 test	**PHENOLS**	Positive	Positive
Foam test	**SAPONINS**	Positive	Positive
Acetic acid test	**GLYCOSIDES**	Positive	Positive
Sodium bicarbonate test	**ACIDS**	Negative	Negative
FeCl3 test	**TANNINS**	Positive	Positive
H2SO4 test	**QUINONES**	Positive	Positive
Chloroform test	**TERPENOIDS**	Positive	Positive
Benedict's reagent test	**REDUCING SUGAR**	Positive	Negative
Fehling-s test	**CARBOHYDRATE**	Positive	Negative
H2SO4 test	**FLAVONOIDS**	Positive	Positive

Interpretation of GC-MS analysis:

The Gas chromatography-mass spectroscopy (GC-MS) analysis revealed a diverse array of chemical compounds present in the samples, each characterized by specific retention times, peak areas, and molecular properties. Compounds such as 3-Decyn-2-ol, Ethanoe-1-chloro-1-fluoro, and Diisopropyl ether were identified, along with cyclohexane derivatives, aromatic compounds like Benzene 1,3-dichloro, and complex structures like Cyclonosiloxane. These compounds exhibit pharmacological significance, with alkaloids, flavonoids, phenolic compounds, plant-derived steroids, and tannins known for their antibacterial, anti-diabetic, and analgesic properties. **(Fig 3.5)**

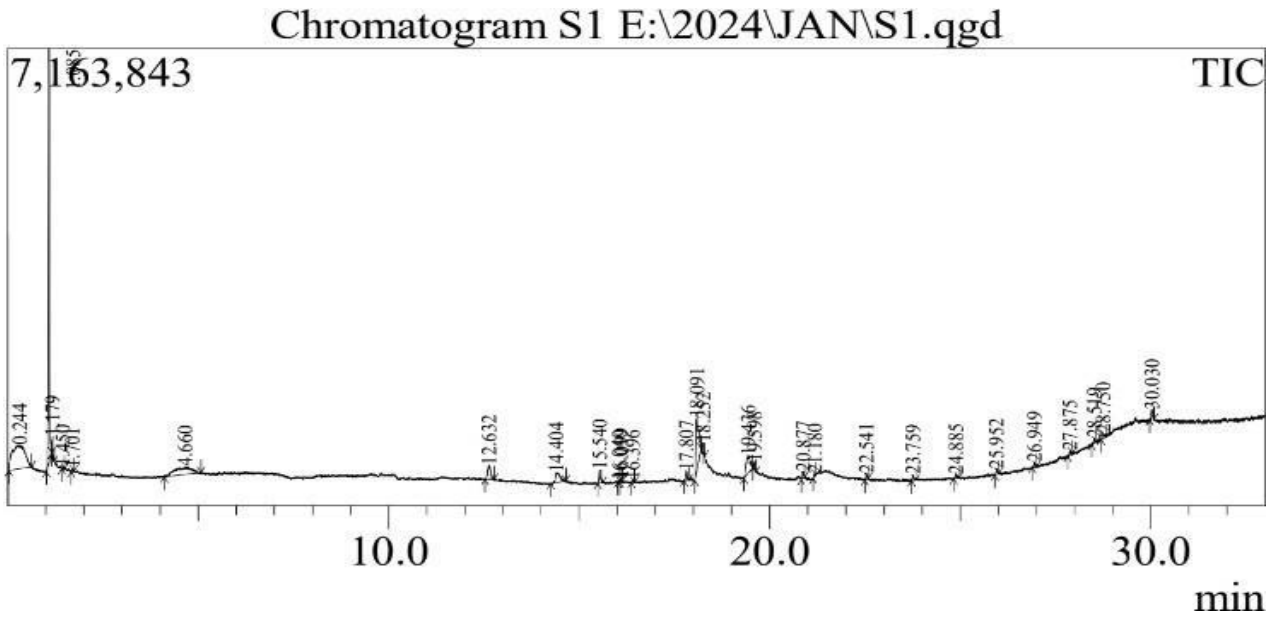


Figure 3.5 GC MS Chromatogram

Purification of active components using thin-layer chromatography:

Thin-layer chromatography was employed for the purification of active components from the methanolic extracts of *Camellia sinensis* (Green tea) and *Azadirachta indica* (Neem). Catechin, a compound isolated from green tea, was visualized as an orangish-pink fluorescent spot under UV light at 280nm. Similarly, Nimbidin, extracted from neem, was visualized as an orange fluorescent spot under UV light at 560nm. **(Fig 3.6) (Fig 3.7)**

Figure 3.6 Purification of Catechin from green tea using TLC

Figure 3.7 Purification of Nimbidin from neem using TLC

Antimicrobial activity of green tea extract and neem extract using agar well-diffusion:

The antimicrobial activity of methanolic extracts from *Camellia sinensis* (green tea) and *Azadirachta indica* (neem) was assessed using the agar well-diffusion method. The extracts were tested against various bacterial isolates, and the zone diameter of inhibition was measured. Green tea extract demonstrated significant antimicrobial activity against several isolates, while neem extract showed limited efficacy. Methanol, used as a solvent, did not exhibit antimicrobial properties. **(Table 3.6) (Fig 3.8)**

Table 3.6 Interpretation of the Diameter of the Zone of Inhibitions using agar well-diffusion method

Isolates	**Zone of inhibition**		
	Green tea extract	**Neem extract**	**Methanol solvent**
D1	2.8cm	0cm	0cm
D2	1.7cm	0cm	0cm
D3	1.0cm	0cm	0cm
D4	2.1cm	0cm	0cm
D5	2.7cm	1.7cm	0cm
D6	2.0cm	0cm	0cm
D7	2.1cm	0cm	0cm
D8	3.2cm	0cm	0cm
D9	3.1cm	0cm	0cm
D10	1.4cm	0cm	0cm
D11	0.7cm	0cm	0cm
D12	2.3cm	0cm	0cm
D13	2.6cm	0cm	0cm
D14	5.6cm	0cm	0cm
***Streptococcus salivarius* (MTCC13009)**	3.7cm	1.3cm	0cm

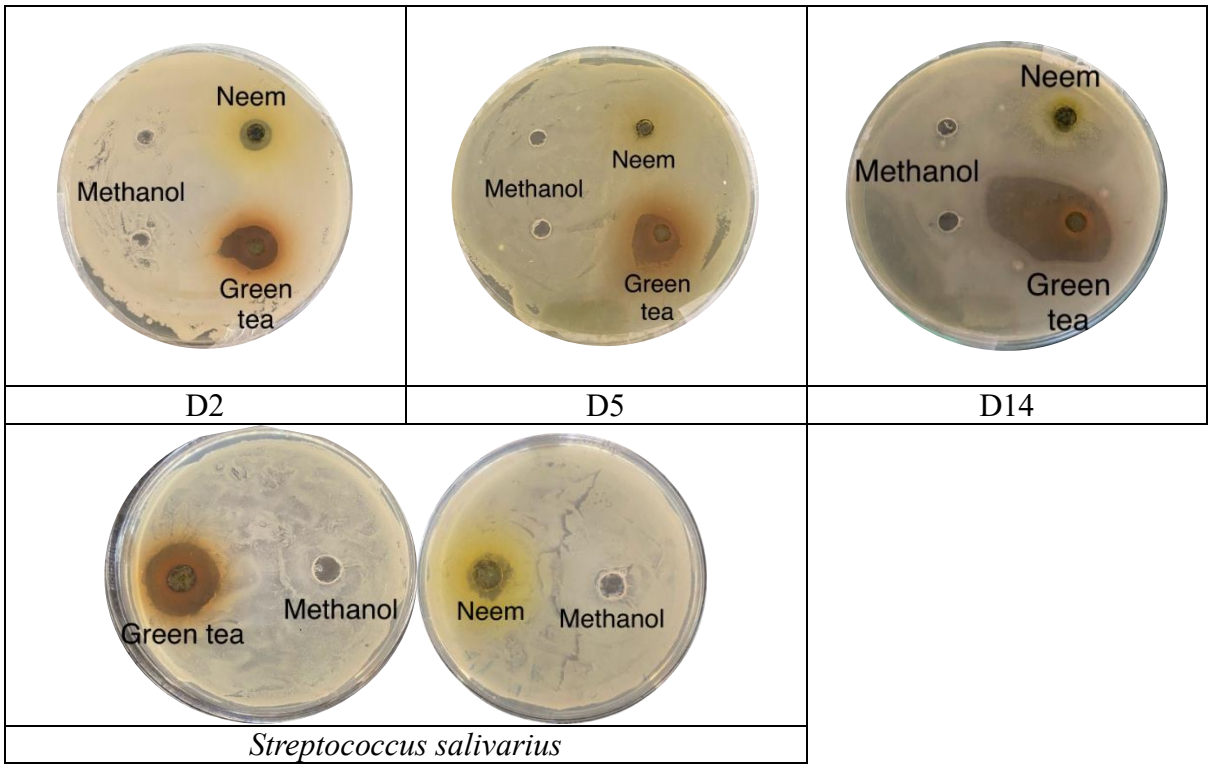


Figure 3.8 Antimicrobial activity of methanolic extracts of *Camellia sinensis* and *Azadirachta indica* using agar well-diffusion method

Descriptive statistical analysis:

Descriptive statistical analysis was conducted to evaluate the effectiveness and variability of the extracts. Green tea extract displayed the highest mean effectiveness, indicating its potency. Neem extract showed lower efficacy compared to green tea. Methanol exhibited no antimicrobial activity. Variability was highest for green tea extract, followed by neem extract, while methanol showed consistent behaviour across samples. **(Table 3.7)**

Table 3.7 Descriptive statistical analysis of antimicrobial activity using agar well-diffusion method

Descriptive statistical analysis	Green tea extract	Neem extract	Methanol (Solvent)
Mean	2.466667	0.2	0
Standard deviation	1.194432	0.533185	0
Standard error	0.308401	0.137668	0
Sample variance	1.426667	0.284286	0

Comparative analysis using the disc-diffusion method:

A comparative analysis was performed between green tea extract, neem extract, chloramphenicol (an antibiotic), and methanol solvent using the disc-diffusion method. Green tea extract demonstrated the highest mean effectiveness, while neem extract showed lower efficacy. Methanol exhibited no antimicrobial activity. **(Table 3.8) (Table 3.9) (Fig 3.9)**

Table 3.8 Interpretation of diameter of zone of inhibitions using disc-diffusion method

Isolates	Green tea extract	Neem extract	Chloramphenicol	Methanol (Solvent)
D1	2.1cm	1.9cm	1.5cm	0cm
D2	2.9cm	2.1cm	1.5cm	0cm
D3	1.9cm	1.5cm	2.9cm	0cm
D4	1.6cm	0cm	2.1cm	0cm
D5	3.1cm	2.1cm	2.6cm	0cm
D6	1.4cm	1.2cm	1.8cm	0cm
D7	1.5cm	1.3cm	1.0cm	0cm
D8	1.7cm	0cm	1.5cm	0cm
D9	2.1cm	1.2cm	1.6cm	0cm
D10	1.2cm	0cm	1.5cm	0cm
D11	1.9cm	0cm	1.4cm	0cm
D12	2.1cm	1.1cm	1.8cm	0cm
D13	1.9cm	0cm	1.7cm	0cm
D14	3.8cm	2.0cm	1.7cm	0cm
***Streptococcus salivarius* (MTCC13009)**	2.3cm	2.2cm	2.8cm	0cm

D5 D11 *Streptococcus salivarius*

Figure 3.9 Antimicrobial activity of methanolic extracts of *Camellia sinensis* and *Azadirachta indica* using disc-diffusion method

Table 3.9 Descriptive statistical analysis of antimicrobial activity using disc-diffusion method

Descriptive statistical analysis	N	Green tea extract	Neem extract	Chloramphenicol	Methanol solvent
Mean	15	2.1	1.106666667	1.826666667	0
Standard deviation	15	0.694879229	0.883553432	0.54440881	0
Standard error	15	0.179417045	0.228132515	0.14056575	0
Sample variance	15	0.482857143	0.780666667	0.296380952	0

N- Number of bacterial isolates

Analysis of Variance:

The significance of the obtained zones of inhibition values was assessed through a one-way ANOVA. The analysis compared the means of two independent groups using the F-distribution. The results indicated a significant difference between the groups ($p<0.05$), with an F-statistical value higher than the critical value. This suggests that the datasets could reject the null hypothesis. **(Table 3.10)**

Table 3.10 ANOVA results of antimicrobial activity using the disc diffusion method

Source of Variation	SS	df	MS	F- Stat	p-value
Between Groups	7.8991	2	3.9496	7.59576	0.001529
Within Groups	21.8387	42	0.52		
Total	29.7378	44			

(**SS**)- Sum of squares, (**df**)- Degrees of freedom, (**MS**)- Mean of squares, (**F-Stat**)- F- statistical value, (**F-Crit**)- F- critical value

Minimum inhibitory concentration (MIC) of green tea extract:

MIC analysis revealed the concentration at which green tea extract inhibited bacterial growth. The results indicated varying MIC values for different bacterial isolates, with some requiring lower concentrations for inhibition compared to others. **(Table 3.11)**

Table 3.11 MIC analysis of methanolic extract of *Camellia sinensis*

Isolates	Methanolic extracts of *Camellia sinensis* (Zones of inhibition)					
	20µL	**40µL**	**60µL**	**80µL**	**100µL**	**Methanol solvent 100µL**
D1	2.1cm	2.4cm	2.7cm	3.2cm	2.6cm	0cm
D2	1.8cm	2.2cm	2.3cm	2.5cm	2.8cm	0cm
D3	1.3cm	2.1cm	2.0cm	2.4cm	2.7cm	0cm
D4	1.5cm	2.3cm	2.6cm	2.8cm	2.5cm	0cm
D5	1.8cm	2.4cm	2.6cm	2.8cm	2.5cm	0cm
D6	1.7cm	2.0cm	2.2cm	2.5cm	2.9cm	0cm
D7	0cm	2.0cm	2.4cm	2.5cm	2.5cm	0cm
D8	0cm	1.5cm	2.2cm	2.6cm	2.6cm	0cm
D9	1.7cm	2.0cm	2.4cm	3.0cm	3.0cm	0cm
D10	1.2cm	1.8cm	2.3cm	2.6cm	2.6cm	0cm
D11	0cm	1.1cm	1.5cm	2.0cm	2.0cm	0cm
D12	2.3cm	2.5cm	2.9cm	2.8cm	2.8cm	0cm
D13	1.9cm	2.4cm	2.8cm	2.8cm	3.4cm	0cm
D14	1.7cm	2.4cm	2.5cm	2.5cm	2.7cm	0cm

***Streptococcus salivarius* (MTCC13009)**	0cm	1.6cm	2.1cm	2.3cm	2.8cm	0cm

Synthesis of green tea mouthwash:

A mouthwash was synthesized using green tea extract based on its effective antimicrobial activity. The formulation included other components and resulted in a dark green tea mouthwash with visible froth.

Antimicrobial activity of synthesized mouthwash:

The antimicrobial activity of the synthesized green tea mouthwash was compared to a commercial mouthwash (Chlorhexidine) at different concentrations. Both types of mouthwash demonstrated efficacy against bacterial isolates, with the green tea mouthwash showing comparable or superior effectiveness in some cases. **(Table 3.12) (Fig 3.10)**

Table 3.12 Interpretation of diameter of zone of inhibitions of synthesized green tea mouthwash and commercial mouthwash (Chlorhexidine)

Isolates	**Green tea mouthwash**			**Chlorhexidine mouthwash**		
	25µL	**50µL**	**100µL**	**25µL**	**50µL**	**100µL**
D1	1.9cm	2.4cm	2.6cm	1.9cm	2.0cm	2.4cm
D2	1.7cm	2.2cm	2.6cm	1.9cm	2.1cm	2.2cm
D3	1.9cm	2.0cm	2.1cm	1.5cm	1.8cm	2.0cm
D4	1.4cm	1.8cm	2.6cm	1.4cm	1.5cm	1.9cm
D5	2.2cm	2.6cm	3.0cm	1.9cm	2.0cm	2.1cm
D6	1.9cm	2.4cm	2.9cm	2.1cm	2.3cm	2.7cm
D7	2.0cm	2.5cm	2.6cm	1.1cm	1.8cm	2.2cm
D8	1.8cm	2.7cm	3.7cm	2.3cm	2.4cm	2.5cm
D9	2.0cm	2.6cm	3.1cm	1.6cm	1.8cm	2.0cm
D10	1.5cm	1.9cm	2.4cm	1.5cm	1.7cm	1.9cm
D11	1.5cm	2.0cm	2.2cm	1.3cm	1.3cm	1.9cm
D12	1.6cm	1.9cm	2.4cm	1.7cm	1.8cm	1.8cm
D13	2.4cm	2.6cm	3.3cm	1.8cm	2.0cn	3.7cm
D14	2.0cm	2.3cm	2.5cm	2.0cm	2.2cm	2.1cm
***Streptococcus salivarius* (MTCC13009)**	2.0cm	2.3cm	2.7cm	1.7cm	1.8cm	1.9cm

D2	D14	*Streptococcus salivarius*

Figure 3.10 Antimicrobial activity of synthesized green tea mouthwash and commercial mouthwash (Chlorhexidine)

IV. CONCLUSION:

In conclusion, our study explored the antimicrobial potential of methanolic extracts from *Camellia sinensis* (Green tea) and *Azadirachta indica* (Neem) against oral biofilm-forming bacterial isolates. Through various methods including agar well-diffusion and disc diffusion, we observed that green tea extract exhibited significant antimicrobial activity against a range of bacterial isolates, whereas neem extract showed limited effectiveness. Furthermore, the antimicrobial efficacy of green tea extract was comparable to that of the antibiotic chloramphenicol, indicating its potential as an alternative antimicrobial agent.

Our findings also highlighted the biofilm-forming capabilities of various bacterial isolates, with some demonstrating strong biofilm production. However, green tea extract showed promising inhibitory effects against biofilm formation, suggesting its potential in preventing the development of oral biofilms.

Phytochemical analysis revealed the presence of several bioactive compounds in both green tea and neem extracts, supporting their antimicrobial properties. Moreover, the purification of specific compounds such as catechin from green tea and nimbidin from neem further elucidated their potential therapeutic applications.

The synthesized green tea mouthwash demonstrated superior antimicrobial activity compared to a commercial mouthwash containing chlorhexidine, underscoring the effectiveness of green tea as a natural antimicrobial agent. Additionally, the minimum inhibitory concentration (MIC) analysis further substantiated the antimicrobial efficacy of green tea extract against the tested bacterial isolates.

Overall, our study provides valuable insights into the antimicrobial potential of green tea extract against oral biofilm-forming bacteria, offering a natural and effective alternative for oral hygiene maintenance. These findings contribute to the growing body of evidence supporting the use of plant-derived compounds in combating oral pathogens and promoting oral health.

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