

Evaluation of *Syzygium aromaticum* extract as an antibacterial and antibiofilm agent against dental caries infection

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Abstract

Dental caries is the most prevalent and localized disease of the oral cavity. Many bacterial genera can act as oral pathogens e.g. *Streptococcus mutans*, *Pseudomonas aeruginosa*, and *Lactobacillus casei*. In this study, the antibacterial and antibiofilm potential of *Syzygium aromaticum* (Clove) was evaluated against important dental caries-causing bacterial strains (*S. mutans*, *P. aeruginosa*, and *L. casei*). Three extracts of clove with various solvents i.e., 85% ethanol extract, distilled water, and methanol extract were used and processed to check the Minimum bactericidal concentration (MBC) and Minimum Inhibitory concentration (MIC). The disk diffusion method was performed to obtain the inhibition of bacterial growth. 85% ethanol extract showed the highest zone of inhibition of $16.8 \pm 1\text{mm}$ against *S. mutans*, methanol showed the highest zone of inhibition of $19.1 \pm 1\text{mm}$ against *L. casei* and Distilled H_2O extract showed the highest zone of inhibition of $14.8 \pm 1\text{mm}$ against *P. aeruginosa*. Ampicillin was used as a positive control. *S. mutans* had a 1.212 OD value at 540nm wavelength, which is higher than the other two bacteria. All 3 extracts have less antibiofilm activity against *L. casei* as compared to other bacteria, on the other hand these extracts have high antibiofilm activity against *P. aeruginosa*. This study highlighted the antibacterial and antibiofilm activity of *S. aromaticum* extracts against dental caries and therefore it can be used for medicinal purposes against dental caries infection.

Introduction

Dental caries is a localized infectious disease that is mostly caused due to opportunistic and oral pathogens. Dental pain and tooth loss are commonly caused by dental caries because it causes the demineralization of tooth surfaces which is due to the biofilms' production of dental pathogens. Microbial activity increases acid production through the intake of carbohydrates. The ecological plaque hypothesis

reveals that ecological and bacterial changes can occur by other factors that can change or influence the ecological interference between microbes and hosts (He et al., 2019). Dental caries also causes calcification in teeth and decay. Various Bacterial species e.g. Anaerobic, Gram-negative species are involved in causing periodontal diseases. These include *Actinobacillus spp.*, *Fusobacterium spp.*, *Prevotella*

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spp., and *Porphyromonas gingivalis*. Usually, in the case of periodontal disease, the gingival crevice surroundings are infected, causing several different inflammatory responses. These responses include the cellular response of the gingival as well as connective tissues (Bin et al., 2020).

Dental surfaces are accumulated by Bacterial plaque, which is the primary etiologic agent that contains native oral flora causing dental caries. The way of working of Cariogenic bacteria is in various recognized ways e.g. metabolic exchange, co-aggregation, exchange of genetic material, and cell-cell communication. Bacteria can make dental biofilms and survive for long periods by using these mechanisms and can be difficult therapeutic targets causing dental diseases (Maripandi et al., 2011). Periodontal diseases are considered the most frequent oral infection that causes eventual tooth decay and destruction of tissues that are supporting the tooth. It forms highly pathogenic biofilms that boost inflammatory host response. These biofilms are the main cause of dental caries (Colombo et al., 2016). Dental caries are causing major public health problems around the globe. The World health organization claimed that it is mostly infecting school children about 60% to 90% (Petersen & Ogawa, 2016). The bacterial strain *S. mutans* form plaques by colonizing on the tooth surfaces and readily instigates the plaque-forming process by producing different types of polysaccharides extracellularly using sucrose solution. Additionally, the plaque margin accession of Gram-positive and Gram-negative bacteria was highlighted (Bin et al., 2020). *Lactobacilli* have traditionally been thought of as non-pathogenic and beneficial probiotics for humans, and they offer a wide range of potential benefits, according to studies published in recent years. However, a growing number of findings linking them to human diseases such as septicemia, rheumatic vascular disease, meningitis, lung abscesses, and infective endocarditis have led to the conclusion that they should be regarded as emerging pathogens (Ahirwar et al., 2017).

Drug-resistant bacteria are a very challenging threat to the cure of many very common infections. The need of the hour is to introduce novel and natural antimicrobial agents to combat these infections. Natural plant products can be used for the long term for oral diseases and these plants make sure that secondary effects will not come. Herbal plant extracts have a significant role to prevent the oral cavity from dental caries and these are being used as antimicrobial agents in traditional medicines (Vashistha et al., 2018). *S. aromaticum*, often known as clove, belongs to the Myrtaceae family. Clove oil has therapeutic properties and is commonly used in special sauces, past flavors, and gravy. Clove is commonly known for its antibiotic characteristics, including antifungal, antibacterial, antioxidant, and

insecticidal properties (He et al., 2019). Cloves are the dried scented unopened floral buds of an evergreen tree that grows 10-20 meters tall and is native to India, Indonesia, Zanzibar, Mauritius, and Ceylon. They are valued as a flavoring agent and are used as a scenting spice, chewing tobacco, and a component of betel chew. Cloves are used to treat a variety of ailments, including nausea and vomiting, cough, diarrhea, dyspepsia, flatulence, stomach distension, and gastrointestinal spasms, as well as pain relief, uterine contractions, and nerve stimulation. Cloves are also antimicrobial, anti-mutagenic, anti-inflammatory, antioxidant, antiulcerogenic, antithrombotic, antifungal, antiviral, and antiparasitic (Aneja & Joshi, 2010).

This study aims to show the antibacterial and antibiofilm activity of *S. aromaticum* extracts against dental caries-causing bacteria (*S. mutans*, *L. casei* and *P. aeruginosa*).

Materials and Methods

Sample Collection

Samples of patients suffering from dental caries (n=20) were collected in peptone water cork-fit sterile test tubes from Allied Hospital Faisalabad. After transportation samples were further processed in the Institute of Microbiology, University of Agriculture, Faisalabad.

Sample Processing and Culturing

Samples were inoculated on nutrient agar plates by spread plate method and incubated at 37°C for 24 hrs. On the next day, colonies were observed and streaked on Brain Heart Infusion (BHI) agar plates separately and further incubated for 24hrs at 37°C. The bacterial colonies were observed on the very next day and Gram's staining was performed to identify the bacterial species microscopically. For biochemical characterization, tests were performed and results were noted.

P. aeruginosa was streaked on cetrimide agar plates and incubated for 24 hrs and observed for greenish coloration of media around the bacterial colonies. After the colony morphological identification, the gram's staining was performed and observed under the microscope, which shows gram-negative rods (pink color). Biochemical tests were performed for the confirmation of *P. aeruginosa*. *L. casei* were streaked on a nutrient agar plate and observed for colony morphology and morphological characteristics. Gram's staining indicated the gram-positive rods (purple color) under microscope. Colonies of *S. mutans* were streaked on blood agar plates, which showed alpha hemolysis (alpha hemolytic bacteria). Then gram staining showed gram-positive cocci (purple color) chains indicating it is *S. mutans*.

Biochemical confirmation

For *P. aeruginosa*, catalase test was performed and there was clear bubble formation showing that it is catalase positive bacteria. Citrate test was performed to check if the bacteria utilized citrate as an energy source; it showed blueish color (citrate positive). *L. casei* fermented glucose sugar shown via color change of the reagent. For *S. mutans*, biochemical tests like the voges prosker test and mannitol fermentation tests were performed. The pink color in voges prosker test indicated the VP-positive bacteria. Mannitol fermentation was observed for *S. mutans*, i.e., it changed the color of the indicator from red to yellow.

Clove Extract Preparations

Clove buds were crushed and ground to form a homogeneous powder. 20 gm clove powder was weighed and dissolved in 3 extraction solutions separately and brought up to 200 ml in the sterile flasks. Three extracts of clove buds were prepared with 85% ethanol, Methanol, and Distilled H₂O. These extracts were stirred for 15 days, and filtered through filtration assembly. The extracts were stored in falcon tubes for further use (El-Maati et al., 2016).

Antibacterial activity of Clove extracts

Mueller Hinton agar was used to perform the disk diffusion method to check the antibacterial activity of all 3 extracts of clove. 6 disks were placed in each petri plate and ampicillin was used as a positive control (Jelani et al., 2023).

Filter paper disks of 8mm were prepared and dipped into each extract separately and allowed to dry for 6 h. 0.5 MacFarland standard was prepared, and each bacterial suspension was compared with it and inoculated on the MH agar plates; then disks were placed on each plate at a distance of 24 mm (Garba et al., 2019).

Determination of Minimum Inhibitory Concentrations

96 well plate was used to obtain MIC values of different extracts of clove. Different dilutions of extracts were used

from 50 µl/ 50 µl to 3.375 µl/ 50 µl clove extracts/ 10% Dimethyl sulfoxide (DMSO) solution from the 1st to 6th well. 7th and 8th wells were used as positive and negative control respectively. Then OD value was observed in the spectrophotometer at 540 nm wavelength and the solution of less than 0.5 OD value was poured on Nutrient agar plates and colonies were observed. The wells that have OD less than MIC value were also poured on nutrient agar plates to check the MBC value of the extracts.

Antibiofilm activity

96 well plate was used to check the antimicrobial activity of different extracts against different bacterial species. 3 extracts were poured with the concentrations of 50 µl/ 50 µl to 3.375 µl/ 50 µl of clove extracts / 10% DMSO solution from 1st to 6th well. 7th and 8th wells were used as a positive and negative control. After 48 hrs the solutions were discarded and 50 µl of crystal violet stain is poured into those wells and washed after 15 minutes. Then added ethanol into wells and mix well. OD values were observed via spectrophotometer at 540 nm wavelength.

Results

Confirmation of Bacterial Species

Three bacteria *P. aeruginosa*, *L. casei*, and *S. mutans* were isolated and confirmed via morphological characteristics, biochemical tests and molecular identification (data not shown).

Antibacterial activity of Clove

Different zones of inhibition were observed for each extract of clove. 85% ethanol extract showed the highest zone of inhibition of 16.8±1.1mm against *S. mutans*, methanol showed the highest zone of inhibition of 19.1±1.189mm against *L. casei* and distilled H₂O extract showed the highest zone of 14.8±0.871mm against *P. aeruginosa* (Table 1). Ampicillin was used as a positive control.

Table 1. Zone of inhibition of *S. aromaticum* extracts against dental caries causing bacteria

<i>S. aromaticum</i> Extracts	Dental caries causing bacteria		
	<i>L. casei</i>	<i>S. mutans</i>	<i>P. aeruginosa</i>
85% Ethanol Extract	12.5 ± 1.05 mm	16.8±1.1 mm	13.5±1 mm
Methanol Extract	19.1± 1.189 mm	17.3±0.91 mm	16.8±0.991 mm
Distilled H ₂ O Extract	12.0 ±1.08 mm	13.3±0.981 mm	14.8±0.871 mm

Ampicillin	13.0±1.01 mm	16.0±1.098 mm	15.0±1.09 mm
Control	-----	-----	-----

MIC and MBC activity

The OD values were noted for different concentrations of

clove extracts using different solvents and are presented in the table 2.

Table 2. Concentrations of clove extracts (per 50µl in 10% DMSO) used for MIC and MBC determination

Clove Extracts	Bacteria	OD values of different Concentrations of clove extracts						+ve Control	-ve Control
		50µl	25µl	12.5µl	6.25µl	3.125µl	1.562µl		
85% Ethanol	<i>S. mutans</i>	0.017	0.123	0.405	0.623	0.834	0.901	-----	1.321
	<i>P. aeruginosa</i>	0.113	0.177	0.443	0.698	0.887	0.906	-----	1.041
	<i>L. casei</i>	0.016	0.105	0.312	0.475	0.668	0.721	-----	0.812
Methanol	<i>S. mutans</i>	0.021	0.109	0.354	0.570	0.776	0.927		
	<i>P. aeruginosa</i>	0.077	0.121	0.409	0.598	0.877	0.913		
	<i>L. casei</i>	0.008	0.087	0.297	0.397	0.564	0.697		
Distilled H ₂ O	<i>S. mutans</i>	0.125	0.341	0.567	0.666	0.856	0.976		
	<i>P. aeruginosa</i>	0.176	0.377	0.634	0.785	0.907	0.902		
	<i>L. casei</i>	0.117	0.254	0.342	0.553	0.664	0.710		

Antibiofilm activity

In antibiofilm activity assays, *S. mutans* showed greater ability to form biofilm than *P. aeruginosa* and *L. casei*, that had a 1.212 OD value at 540nm wavelength, which is

higher than other 2 bacteria (Table 3). All 3 extracts have less antibiofilm activity against *L. casei* as compared to other bacteria. On the other hand, these extracts have high antibiofilm activity against *P. aeruginosa*.

Table 3. Antibiofilm activity of different clove extracts

Clove Extracts	Bacteria	OD values of different Concentrations of clove extracts						+ve Control	-ve Control
		50μl	25μl	12.5μl	6.25μl	3.125μl	1.562μl		
85% Ethanol	<i>S. mutans</i>	0.018	0.117	0.345	0.667	0.867	0.987	--	1.212
	<i>P. aeruginosa</i>	0.119	0.167	0.453	0.702	0.897	0.976	--	1.054
	<i>L. casei</i>	0.006	0.094	0.256	0.480	0.668	0.707	--	0.781
Methanol	<i>S. mutans</i>	0.009	0.109	0.305	0.567	0.784	0.920	--	--
	<i>P. aeruginosa</i>	0.087	0.125	0.409	0.650	0.870	0.909	--	--
	<i>L. casei</i>	0.002	0.078	0.284	0.390	0.547	0.687	--	--
Distilled H ₂ O	<i>S. mutans</i>	0.125	0.315	0.560	0.698	0.867	0.965	--	--
	<i>P. aeruginosa</i>	0.178	0.367	0.625	0.780	0.898	0.976	--	--
	<i>L. casei</i>	0.117	0.214	0.345	0.523	0.634	0.708	--	--

Figure 1 showed concentrations of clove 85% ethanol extracts on the left side and the OD values on the right side. The results have shown a significant decrease in the OD values of 3 bacterial strains *S. mutans*, *P. aeruginosa* and *L. casei*, indicating the significant effect of the increasing concentrations of 85% ethanol extract. It was found that *L. casei* is more sensitive to the extract as compared to the other 2 bacterial species.

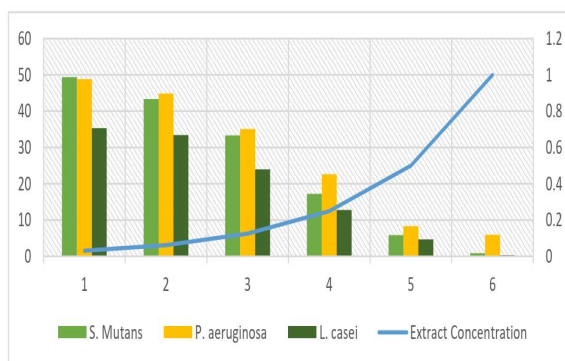


Figure 1. Antibiofilm activity of 85% ethanol extract of *S. aromaticum* (clove)

In the figure 2, the concentrations of clove Methanol extracts are shown on the left side, and on the right side, the OD values have shown a significant decrease in the OD values of 3 bacterial strains *S. mutans*, *P. aeruginosa* and *L. casei*. The figure describes that *L. casei* is more sensitive to the extract as compared to the other 2 bacterial species (Figure 2).

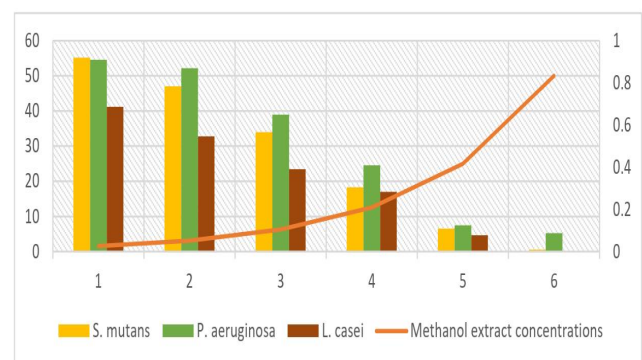


Figure 2. Antibiofilm activity of methanol extract of *S. aromaticum* (clove)

Figure 3 showed concentrations of clove Distilled H₂O extracts on the left side and the OD values on the right side. The figure showed significant decrease in the OD values of 3 bacterial strains *S. mutans*, *P. aeruginosa* and *L. casei*, indicating the significant effect of the increasing concentrations of distilled H₂O extract. It describes that *L. casei* is more sensitive to the extract as compared to the other 2 bacterial species.

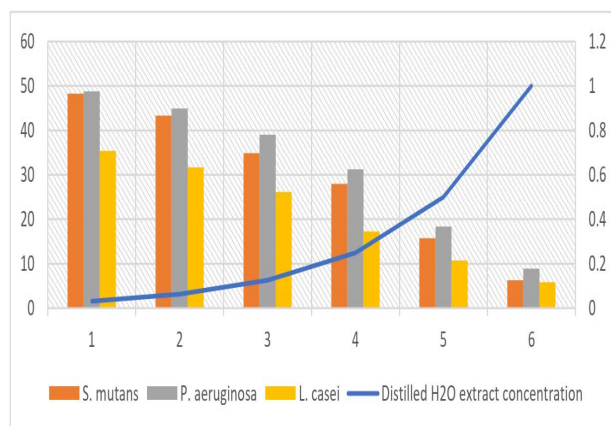


Figure 3. Antibiofilm activity of distilled H₂O extract of *S. aromaticum* (clove)

Discussion

Dental caries is mainly the destruction of localized dental hard tissues as a susceptible host of bacteria. It is due to carbohydrates and their acidic by-products which are produced after fermentation by bacterial strains. The hard dental tissues show the main signs of the demineralization of teeth, but bacteria make biofilms to initiate the process of disease, and biofilms (dental plaque) completely cover the surface of teeth (Mosaddad et al., 2019). The herbal medicine and exploration of herbal remedies are also an alternative that offer good oral health as well as mitigating potential side effects of other medicines. This knowledge along with scientific understanding enable evidence-based decision-making in dental care. The exploration of herbal remedies offers a promising alternative, potentially mitigating some of these side effects while promoting oral health in a more natural and holistic manner.

It was experimented that, the common contaminants of dental caries are *S. mutans*, *L. casei*, and *P. aeruginosa* which can be residential microbiota and opportunistic also, they can cause acidification of the dental cavity and cause dental decay. They are highly pathogenic and biofilm-forming bacteria. *S. mutans* is a cariogenic bacterium that can cause dental caries as well as mouth cancer. *P.*

aeruginosa is an opportunistic bacterium that can attack unhygienic and less cared teeth. *L. casei* is the residential bacteria that cause acidification of the oral cavity, that facilitates the contaminants to cause dental caries. Studies have shown natural compounds like apigenin, carvacrol, cinnamaldehyde, curcumin, catechin, epigallocatechin gallate, eugenol, farnesol, inulin, menthol, myricetin, oleanolic acid, thymol, and resveratrol possess antibacterial and antimicrobial properties and can effectively prevent the growth of *S. mutans*. NCs can damage cell membrane integrity, thereby leading to cell death, and possess the capability to inhibit acid production, a key factor in caries development. NCs also interfere with bacterial adhesion to surfaces, including teeth. The attachment inhibition is achieved by decreasing the expression of adhesion factors such as fruA, ftf, gtfs, and gbpB. The compounds derived from plant source (aqueous or ethanolic extracts) demonstrate antibacterial activity against microorganisms related to dental caries. Some compound extracts exhibit efficacy comparable to chlorhexidine. There are *in vitro* studies demonstrating bactericidal and bacteriostatic activity of plant-based compounds against dental caries causing bacteria including *S. mutans* (Tzimas et al., 2024). There are also *in vivo* studies in which plant based compounds are given along with food derivatives i-e, lollipops, that significantly reduced *S. mutans* in children having higher risk of caries.

Usually cloves are referred to for its antibiotic activities such as antifungal, antibacterial, antioxidant, and insecticidal properties. Clove extract as a main component of toothpaste/tooth powder/ mouthwash versus 'usual care (toothpaste or tooth powder)'. The Kirby-Bauer disk diffusion method was used to determine the inhibition zones (Hudzicki, 2009). Different zones of inhibition are observed; 85% ethanol extract showed the highest zone of inhibition of 16.8±1.1mm against *S. mutans*, methanol showed the highest zone of inhibition of 19.1±1.189mm against *L. casei* (Service et al., 1996) and distilled H₂O extract showed the highest zone of 14.8±0.871mm against *P. aeruginosa*. Ampicillin was used as a positive control. It is found in a research that the inhibition zones of clove extracts can be ranged from 7mm to 20 mm (Ökmen et al., 2018).

In Antibiofilm activity, *S. mutans* have more ability to form biofilm than *P. aeruginosa* and *L. casei* as is indicated in the results had a 1.212 OD value at 540nm wavelength which is higher than other 2 bacteria. All 3 extracts have strong antibiofilm activity against *L. casei* as compared to other bacteria, on the other hand these extracts have less antibiofilm activity against *P. aeruginosa*. Other scientists also evaluated the antibacterial activity of *S. aromaticum*.

Scientific literature highlights the antibacterial properties of medicinal plants and their potential applications in dentistry, with a particular focus on their activity against a broad range of bacteria and microorganisms involved in oral diseases. Numerous plant extracts and bioactive compounds—including polyphenols, flavonoids, and essential oils—have demonstrated antimicrobial, anti-inflammatory, and antioxidant properties that contribute to maintaining oral health. Although *in vitro* and *in vivo* studies support their therapeutic potential, clinical trials assessing long-term efficacy and safety remain scarce. Clove showed antibacterial effect against all gram-negative isolates, although had the strongest antibacterial effect against *Proteus* species, with a 19 mm zone of inhibition, 0.39 mg/ml minimum inhibitory concentration (MIC), and 0.19 mg/ml minimum bactericidal concentration (MBC) (Faizi & Kazmi, 2017). One more study from Iraq showed that clove essential oil had antibacterial activity little higher than of rosemary oil, MICs ranged from 0.312% (v/v) to 1.25% (v/v) for all tested bacteria (*Acinetobacter baumannii*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*) while MICs for rosemary oil ranged from 0.312% (v/v) to 5 % (v/v) (Abdullah et al., 2015). This research also indicated that clove has a strong antibacterial effect against gram-positive and gram-negative bacterial species.

This study showed the antibacterial and antibiofilm activity of *S. aromaticum* extracts against dental caries-causing

bacteria (*S. mutans*, *L. casei* and *P. aeruginosa*). It has a significant effect and can be used for medicinal purpose against dental caries infection.

Author Contributions

Conceptualization, Shahid Mehmood; Writing—original draft preparation; Khizar Rehman, Muhammad Zulqarnain, Zain Ul Abedein, Aleeha Batool; Writing—review and editing, Shahid Mehmood, Khizar Rehman, Muhammad Zulqarnain, Zain ul Abedein, Aleeha Batool; Supervision, Shahid Mehmood; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Not applicable

Conflicts of Interest

The authors declare no conflict of interest.

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