Original Article

# Anti-microbial efficacy of *Vanilla planifolia* leaf extract against common oral micro-biomes: A comparative study of two different antibiotic sensitivity tests

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## Abstract

**Introduction:** Over the past century, several antibiotics have been discovered and used to treat various microbial diseases. However, over the past few decades, with the emergence of anti-microbial resistant strains of microbiomes, it has become increasingly necessary to discover and develop alternative anti-microbial agents. Herbal formulations have shown promising results in the past decade. However, many herbal formulations remain unexplored. The present study aims to explore the anti-microbial properties of a newly prepared *Vanilla planifolia* extract.

**Methodology:** *Vanilla planifolia* leaves were collected, shade-dried, and then powdered. The powdered leaves of *Vanilla planifolia* (100 gm) were extracted by the cold percolation method with 300 ml ethanol at room temperature for 72 hours. The extracts were then tested for its constituent anti-microbial activity by the agar well method and disk diffusion method against different commonly found oral micro-biomes. The zones of inhibition were noted and measured, and the results were derived. Statistical analysis was performed using the Student t-test (P <_ 0.001). Based on the statistical analysis, conclusions were drawn.

**Results:** The ethanolic extracts of *Vanilla planifolia* on the agar plates showed considerable anti-microbial activity in both the test methods against *Staphylococcus aureus, Streptococcus mutans, and Enterococcus*. However, no effect was found against *Candida albicans*. There was no significant difference in the results obtained by the two test methods (P >_ 0.001).

**Conclusions:** This experimental study presents a medicinal plant, an orchid V*anilla planifolia*, which demonstrates the presence of essential anti-microbial agents in it, making it a potent, potential dental biomaterial with a positive and benefitting effect on the oral micro-environment.

**Keywords:** Antibiotic, anti-microbial, ethanol extract, *Vanilla planifolia*

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## INTRODUCTION

The oral environment is said to harbour millions of microorganisms. These microorganisms, commonly referred to as oral micro-biomes, are both pathogenic and commensal in nature. There exists a natural equilibrium by which the pathological and commensal microbes maintain a

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certain homeostasis. In an event where this balance is lost, opportunistic microorganisms proliferate and commence the disease processes.[1] Oral health diseases occur in three inter-connected stages, namely, plaque formation on teeth, progression to dental caries, and associated peri-odontal diseases. Dental plaque is a complex, metabolically inter-connected, highly organised bacterial ecosystem. Around 108 million organisms are present in 10 grams of plaque. The predominant microorganisms are *Streptococcus, Actinomyces, and Veillonella* species, all being cariogenic. This layer of plaque facilitates carious processes when undisturbed. The increase and decrease in pH determines the caries process. A few main opportunistic pathogens of dental caries include *Streptococcus mutans, Escherichia coli,* and *Candida albicans*. Poor maintenance of oral hygiene accounts for the main reason for accumulation of these microbes and their deleterious activities in the oral cavity. The chronic presence of these microbes in the oral cavity leads to severe forms of peri-odontal disease that affect the peri-odontal membrane and the underlying alveolar bone, ultimately resulting in tooth loss.[2,3]

Anti-microbial activity can be defined as a collective term for all active principles (agents) that act by inhibiting the growth of bacteria, prevent the formation of microbial colonies, and destroy harmful microorganisms. In simple terms, anti-microbial activity refers to the process of killing or inhibiting the disease-causing microbes. Various anti-microbial agents are used for this purpose. Anti-microbials could be anti-bacterial, anti-fungal, or anti-viral. All of them possess unique modes of action to suppress the bacterial growth and progression of infection.

As mentioned, the primary etiological factor for dental diseases can be attributed to dental plaque. The process of plaque formation on the tooth surface initially starts from a limited number of pioneer microbial species and progresses to the complex flora, leading to mature dental plaque. This progression is characterised by an initial adherence of oral bacteria to the salivary pellicle and subsequent accumulation by inter-bacterial adherence and their growth. Ultimately, when undisturbed for a period of time, the tooth surface gets coated layer by layer with a dense, complex micro-community that results in a decay by destruction of the hard enamel layer.[4,5] Additionally, the activities of these microbes also contribute to halitosis and other oral diseases.

The best method to cease this chain of events is by mechanically and chemically disturbing the plaque accumulation on a daily basis. This may be achieved by the addition of active agents such as anti-microbial agents in dentifrices that are intended for daily or routine usage. The routine usage of such dentifrices keeps the oral bacterial load at a suppressed level, therefore avoiding any undue pathogenic activity over time. Therefore, the need to include anti-microbial agents in dentifrices to maintain oral organisms to a level consistent with oral health has been stressed.[6] Many experiments have been performed with various agents to evaluate their efficacy as anti-microbial agents when added to dentifrices. Some of the key ingredients used are chlorhexidine gluconate, tricolsan, and fluoride. One such anti-microbial agent is *Vanilla planifolia*, an orchid that has been studied extensively and proved to have superior anti-microbial properties. Extracts from various parts of the orchid, such as leaves and stems, have shown to contain alkaloid substances and phyto-compounds that are potent anti-microbials. However, this plant has not been scientifically validated for its anti-microbial activity. Therefore, the present study aimed to explore the anti-microbial properties of the newly prepared *Vanilla planifolia* extract.

## MATERIALS AND METHOD

The study was an *in vitro* study and was conducted at the Department of Microbiology at Saveetha Dental College and Hospitals, Chennai. The required ethical clearance was obtained well in advance from the Student Research Board, Saveetha Dental College, Chennai (Protocol number: IHEC/SDC/ENDO-1807/21/255).

### Preparation of the *Vanilla planifolia* extract

The plant's leaves were harvested from commercial plantations situated at Coorg, South India. The leaves were then washed with plain water and then cut into smaller pieces before they were shade-dried and then ground into a coarse powder. The ground powder (100 gram) was then subjected to an extraction process with 300 ml of ethanol at room temperature for 72 hours using a cold percolation technique. The filtrates were concentrated at 40°C under reduced pressure and stored in a refrigerator at 2–8°C for further investigations [Figures 1 and 2].

### Micro-biological analysis

The yield was tested for its anti-microbial activity against *S aureus, S mutans, Enterococcus, and C albicans* [Figure 3]. The strains were further sub-cultured from pure strains under standard micro-biological isolation protocols. Agar plates were prepared using Muller Hinton agar (as described in NCCLS-2000 and by Al-Waili and Saloom[7,8]), and the agar was allowed to sit for 24 hours. The bacteria were then smeared on the surface of the set agar, and the plates were inoculated at 37 degree Celsius for 24 hours in an inoculation chamber.

**Figure 1:** *Vanilla planifolia* extraction

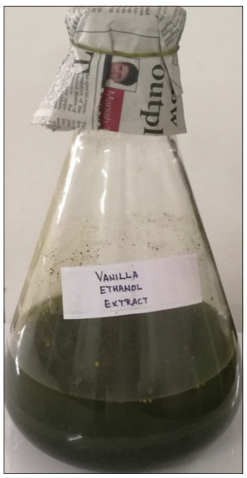


**Figure 2:** *Vanilla planifolia* ethanol extract

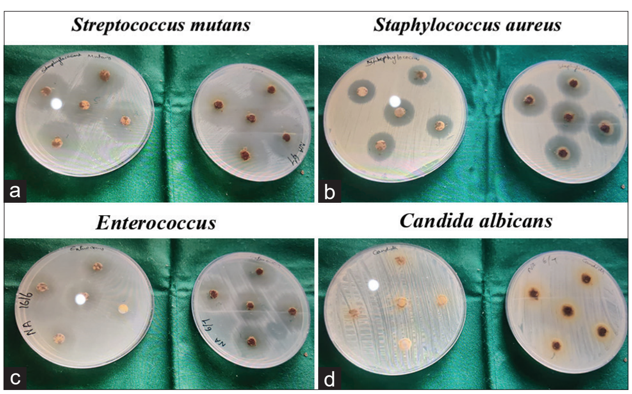


**Figure 3:** Anti-microbial activity of the extract using the disk diffusion method and agar well diffusion method. (a) *Streptococcus mutans*. (b) *Staphylococcus aureus*. (c) *Enterococcus*. (d) *Candida albicans*

To determine the anti-microbial activity of the extract, two different tests were employed, namely, the disk diffusion method and the agar well diffusion method. For the disk diffusion method, standard filter paper disks were first autoclaved and then soaked/immersed in a solution of the vanilla extract and were air-dried. They were then placed on the surface of agar plates that contained the respective microorganisms. For the agar well diffusion method, a pre-made template was used to cut wells in the agar plate of a specific dimension and then these wells were filled with a bulk of the *Vanilla planifolia* extract and the plates inoculated by the same methodology as mentioned above.

Each plate of agar consisted of five disks or wells containing the *Vanilla planifolia* extract. This was performed so as to obtain multiple results of the zone of inhibition so as to obtain reliable results. The zone of inhibition was observed after the 24 hour inoculation period, and then, they were measured using a standard caliper instrument. The measurement of the diameter of the zone of inhibition at the widest portion of the zones was performed and noted in millimetres. The means of the zone of inhibition were derived, and a final mean zone of inhibition for each microorganism under study was determined. To determine a base value, chlorhexidine (CHX) was used as a control sample. The sample protocols of sample preparation and culture media preparation, inoculation, and measurement were followed for the control specimen too.

## RESULTS

Data related to the zone of inhibitions were collected and tabulated using the Microsoft Excel program. These data were analysed using descriptive statistics first. Inferential statistics was performed to compare the means of zones of inhibition in each method using Student's t-test. Statistical analyses of data were performed using SPSS software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

Both the test and control groups (0.2% CHX) showed considerable anti-microbial properties. The ethanolic extracts of *Vanilla planifolia* on the agar plates showed anti-microbial activity in both the test methods against *S aureus, S mutans, and Enterococcus*, whereas no activity was found against *C albicans*. The zone of inhibition in each method has been tabulated [Tables 1 and 2]. The means of the zones of inhibition obtained using the disk diffusion method and agar well method have been calculated [Tables 3 and 4], and the mean comparisons of both the antibiotic sensitivity tests have been performed by employing a t-test [Table 5].

## DISCUSSION

A majority of dental problems are caused by plaque formation and accumulation, and these can be treated by achieving plaque control. For this purpose, anti-microbial agents that act by disrupting the plaque-forming bacteria are required to be included in routinely used oral hygiene products.

**Table 1: Anti-bacterial activity assessed using the disk diffusion method**

| Zone of inhibition(in mm) | 1 | 2 | 3 | 4 | 5 | CHX (0.2%) |
|---|---|---|---|---|---|---|
| *Staphylococcusaureus* | 18 | 18 | 21 | 20 | 18 | 22 |
| *Streptococcus mutans* | 19 | 18 | 19 | 18 | 20 | 24 |
| *Enterococcus* | 20 | 19 | 19 | 18 | 21 | 21 |
| *Candida albicans* | | | Nil | | | 25 |

**Table 2: Anti-bacterial activity assessed using the agar well diffusion method**

| Zone of inhibition(in mm) | 1 | 2 | 3 | 4 | 5 | CHX (0.2%) |
|---|---|---|---|---|---|---|
| *Staphylococcusaureus* | 26 | 25 | 26 | 26 | 24 | 22 |
| *Streptococcus mutans* | 31 | 33 | 32 | 34 | 34 | 24 |
| *Enterococcus* | 28 | 27 | 27 | 29 | 27 | 21 |
| *Candida albicans* | | | Nil | | | 25 |

**Table 3: Means and standard deviations of the zone of inhibition of organisms in the disk diffusion method**

| Group | Mean | Std. Deviation | $P$ ($P \leq 0.001$) |
|---|---|---|---|
| *Staphylococcus aureus* | 19.00 | 1.414 | 0.001 |
| *Streptococcus mutans* | 18.80 | 0.837 | 0.001 |
| *Enterococcus* | 19.40 | 1.140 | 0.001 |
| CHX (0.2%) | 22 | 0.000 | 0.000 |

**Table 4: Means and standard deviation of the zone of inhibition of organisms in the agar well diffusion method**

| Group | Mean | Std. Deviation | $P$ ($P \leq 0.001$) |
|---|---|---|---|
| *Staphylococcus aureus* | 25.40 | 0.894 | 0.001 |
| *Streptococcus mutans* | 32.80 | 1.304 | 0.001 |
| *Enterococcus* | 27.60 | 0.894 | 0.001 |
| CHX (0.2%) | 22 | 0.000 | 0.000 |

**Table 5: Comparative statistics between the two test methods**

| Methodology | Mean | Std. Deviation | ($P \leq 0.001$) |
|---|---|---|---|
| Agar disk diffusion method | 27.02 | 1.232 | 0.005 |
| Agar well diffusion method | 28.07 | 1.431 | 0.002 |

The present study was an attempt to prove the anti-microbial properties of the plant extracts of *Vanilla planifolia*, a prospective dental biomaterial. *Vanilla planifolia* is known to be first cultivated by the ancient Totonac Indians of Mexico. Until the mid-19th century, Mexico was the main producer of vanilla. Later in 1819, beans of this plant were shipped to the Réunion and Mauritius islands by the French. Today, these plants are mainly grown in Madagascar, Indonesia, Mexico, Tahiti, and India. In India, *Vanilla planifolia* plants are grown only in the southern states of the country. Cultivation of these plants on South Indian lands has significantly stretched to about 24,000 hectares with a turnover of about 700 tonnes of vanilla pods. *Vanilla planifolia* is grown by hand pollination of its flower.[9]

Leaf extracts of *Vanilla planifolia* have conclusively demonstrated anti-microbial and anti-fungal effects on *E coli, Pseudomonas aeruginosa, Bacillus subtilis, and Aspergillus niger,* with a few of these being main pathogens responsible for oral diseases.[10] It also displays anti-spasmodic, anti-inflammatory, and analgesic activity, which can be used to reduce toothache and inflammation caused because of infections.[11] Vanillin's anti-microbial properties against yeasts and other microorganisms have been evaluated.[12,13] However, no research has been performed to check the efficacy of this plant on the oral micro-environment, making the use of *Vanilla planifolia* in dentistry unexplored. Literature search revealed no studies that evaluated the effect of this plant on dental caries or dental pain.

In the present study, extracts of *Vanilla planifolia* have demonstrated anti-microbial effects against *S aureus, S mutans,* and *Enterococcus*, the most commonly occurring oral commensals. *S aureus* causes a number of distinct oral infections such as angular cheilitis,[14] parotitis,[15] staphylococcal mucositis,[16] and so forth. It has also been suggested that *S aureus* may have a role in dental implant failure.[17,18] It is very likely that oral infections caused by *S aureus* are persistent with infections caused by the organism at various other body sites. The risk of such cross infections from a variety of sources has been strongly suggested by existing scientific evidence.[19-21] Twenty-five species of oral streptococci inhabit the human oral cavity and represent about 20% of the total oral bacteria.[22] *S. mutans* hold a crucial role in fermenting carbohydrates, leading to acid production and resulting in the demineralisation of the enamel layer of the tooth surface. *S. mutans* have a central role in the etiology of dental caries because these can adhere to the enamel salivary pellicle and to other plaque bacteria.[23,24] *S Mutans* are strong acid producers and hence cause an acidic environment creating the risk for cavities.[25,26] *Enterococci* are normal commensal Gram-positive cocci that inhabit the gastro-intestinal tract and the human oral cavity.[27] Existing scientific data on oral prevalence of *enterococci* vary widely in different studies[28] and range from 0 to 50% depending on the oral source of the tested specimens (saliva, root canals, plaque) and the studied populations.[29] *Enterococcus* was shown to reside within different layers of the oral biofilm, leading to failure of endodontic therapy.[30] By standard protocols and evidence-based studies, it is conclusive that one of the two microbial sensitivity tests mentioned above are sufficient to scientifically conclude the anti-microbial efficacy of an herbal extract under testing. However, there are studies which have shown that sustained or continuous exposure to an anti-microbial agent over a duration of time has an increased efficacy against microorganisms. The exposure to an anti-microbial agent in its crude or bulky quantity has proved to be equally or more effective on several occasions. This evidence may be taken advantage of in the field of dentistry as it is often possible to directly deliver the anti-microbial agent in a topical manner. For instance, the anti-microbial agents may be loaded in fluoride gels and incremental continuous exposures can be devised, or the agent may be incorporated along with a disinfection agent such as the ones used in root canal disinfection and exposure can be prolonged from a few hours to a few days. It is this evidence that leads to our hypothesis that a larger bulk of the microbial agent may cause a greater effect on the micro-biomes. However, the present study proved that it would only make a negligible difference from the quantity of the anti-microbial agent and rather the actual potency and sensitivity was the only factor that made a difference. We may conclude stating that *Vanilla planifolia* has the presence of anti-microbial properties against oral microbes that are known to have a role in dental caries and plaque accumulation, thereby leading to severe gingival and peri-odontal conditions, and it makes *Vanilla planifolia* an anti-microbial agent that can be used as a potent dental biomaterial for the oral environment.

## CONCLUSIONS

When consumers and dentists adopt herbal plants incorporated into oral hygiene products, the side effects of oral care products containing synthetic compounds will be safeguarded and also reduce the cost of treatment. This experimental study presents one such medicinal plant, an orchid *Vanilla planifolia*, which demonstrates the presence of essential anti-microbial agents in it, making it a potent dental biomaterial with a positive and benefitting effect on the oral microenvironment.

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### Conflicts of interest
There are no conflicts of interest.

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