Evaluation of Antimicrobial Activity of Extracts of Terminalia chebula and Piper nigrum against Streptococcus mutans: An In Vitro Study

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Authors’ contributions

This work was carried out in collaboration among all authors. Author PB designed the study, data analysis, final draft preparation. Authors RKK, AB, AD, GKA wrote the protocol and wrote the first draft of the manuscript, and Literature Search. All authors read and approved the final manuscript.

ABSTRACT

Objective: Dental caries is an infectious disease in which S. mutans plays a key role. Haphazard and irrational use of antibiotics leads to antibiotic resistance and fatal diarrhoeal diseases in children. Antimicrobial potency of Terminalia chebula and Piper nigrum extracts against several bacterial strains have been documented. The aims of this study were to assess and compare the antimicrobial activities of T. chebula and P. nigrum extracts against S. mutans with Ciprofloxacin as the positive control.

Materials and Methods: For this purpose, S. mutans was isolated from plaque samples of people with active caries lesions. Antimicrobial potency of both T. chebula and P. nigrum were tested using agar well diffusion method.

Results: All the tested extracts showed antibacterial activity against S. mutans bacteria. Regarding the two tested herbs extracts, a higher antimicrobial activity was shown by the methanol extract of...
**Introduction**

Antibiotics are recommended for dental patients both for prevention and treatment of infections. Antibiotic prophylaxis was employed to prevent transmission of oral microorganisms to other sites or organs in a dental patient who is at risk [1].

In children, antibiotics were indiscriminately used especially for treating dental and ear infections.[2] T.J. Pallasch reported that, [3] inappropriate use of antibiotics in dentistry occurs either due to their use for extended time periods or due to their prescription in 'irrelevant situations' such as after a dental procedure in apparently healthy patient to prevent an infection which is unlikely to occur [3] In spite of the lack of clinical trials indicating their necessity for antibiotics, their prescription by dental practitioners is continuously increasing. Over uses of antibiotics in children is raising the prevalence of fatal diarrheal cases and extreme modification of gut micro flora.[4] The World Health Organization’s (WHO) theme for year 2011 was on Antibiotic resistance, which explains the magnitude of the problem.[5]

For over a decade, the pace of development of new antimicrobial agents has slowed down while the prevalence of resistance has grown at a massive rate. Antibiotics that are used successfully at present may fail to control the infection subsequently. It is vital to discover new antibacterial agent, to with less resistance, predominantly from plant sources with minimum side effects and high potency to combat many infections.[6] Occasionally the use of antibiotics in combination can produce the additive inhibitory effect which cannot be obtained with single antibiotic. The major benefits of using combination of antibiotics can enhance the antibacterial activity, the therapy time and most importantly prevent antibiotic resistance. [1]

**Terminology chebula** is a deciduous plant found more prevalent in South East Asia. Different types of extracts such as decoction and powder of **T. chebula** fruit is used to treat numerous diseases including dental caries and bleeding gums.[6] **Piper nigrum** commonly known as “Black pepper” has several medicinal uses, due to their antioxidant effects it is used as a remedy for cold and flu.[7]

As reported by Agarwal [8] and Mostafa et al. [9], **Terminalia chebula** posses anti-bacterial activities against both gram-negative and gram-positive bacteria. In a study conducted by Sweta et al. [7], **Piper nigrum** was found to have good antibacterial activity against **S. mutans**. There are no in-vitro studies comparing the antibacterial efficacy of **Terminalia chebula** and **Piper nigrum**. So the present study was designed to compare the antimicrobial efficacy of **Terminalia chebula** and **Piper nigrum** against **Streptococcus mutans**.

**Materials and Methods**

**2.1 Herbal Extract preparation**

The fresh ripe fruits of **T. chebula** and **P. nigrum** were collected from the local herb market in Chennai city, Tamilnadu, India. The samples were washed thoroughly with tap water followed by distilled water to remove dirt and impurities. The samples were left to dry naturally at room temperature for two days and grind to a smooth powder and stored in air tight containers. Five different solvents namely ethanol, methanol, acetone and aqueous hot and cold were used for extraction. Three different concentrations, 1000 µg, 500 µg and 250 µg of ground herbs samples were separately soaked in 100ml of acetone, ethanol, methanol, and cold sterile distilled water for 24 hours. In addition, the same amounts of each sample were mixed in 100ml of hot sterile distilled water (100°C) and kept undisturbed for 24 hours.

All preparations were filtered using sterile filter paper and the filtered preparations were condensed below 40°C under vacuum using rota evaporator. The vacuum dried concentrates were sterilized by ultra violet rays and were stored in sterile air tight sterile containers at 4°C.

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**Conclusion:** These findings confirm the Antimicrobial potency of **T. chebula** which can be used as an alternative antibiotic and/or in combination with allopathic antibiotics to prevent the antibiotic resistance.

**Keywords:** Antibiotic resistance; Dental caries; Herbal extract; **Piper nigrum**; **S. Mutans**; **Terminalia chebula**.
2.2 Tested Microorganisms

*Streptococcus mutans* used as the test organism in this study which was isolated from plaque samples. The plaque samples were collected from caries lesions using sterile toothpicks. The wooden toothpicks were immediately dipped in 1 ml of sterile phosphate-buffered saline and stored at 4°C, to distribute the plaque sample homogeneously throughout the solution, saline with plaque samples was vortexed for 60 seconds. Hundred fold dilutions of the both herbal samples was made in 1 fold concentrated sterile phosphate-buffered saline and transferred to *Mitis Salivarius Bacitracin* (MSB) agar plates. The MSB agar was supplemented with 1% agar 15% sucrose, 0.0001% potassium tellurite and 0.2U of bacitracin per ml. Then the plates were incubated at 37°C anaerobically for 48 hrs.

After 48 hours, colony identification was done using colony morphology. The colonies sample plates were then transferred to plates containing brain–heart infusion broth (HiMedia, India) which were incubated for 18 hours at 37°C. After the incubation period, the cultures were streaked on MSB agar and incubated at 37°C anaerobically for 48 hrs. All the bacterial isolates were subjected to morphotyping and *S. mutans* strains were characterized by phylogenetic analysis and biotyping.

2.3 Screening for Antimicrobial Activity

The acetone, methanol, ethanol, cold and hot water extracts of *T. chebula* fruit and pepper were used for screening the antimicrobial activity by agar well diffusion method. Pure isolate of the microorganisms were subcultured on specific media recommended for that particular microorganism at 37°C for 24 hours. A petri plate containing *S. mutans* was stroked with a sterile loop and transferred to normal saline of 0.85% concentration under sterile conditions. Density of the microorganism in saline suspension was adjusted to 106 cells/ml and this suspension was used as inoculums in the agar well diffusion assay. 100µl of the *S. mutans* inoculum was spread on the agar plates and the plates were allowed to dry. Wells of 8mm diameter were made on the agar plate with a borer and lower portions of the wells were sealed with molten agar. The herbal extracts were reconstituted for the bioassay analysis using 20% Dimethyl Sulfoxide (DMSO).

100µl of each herbal extract was dispensed directly into the wells of the inoculated agar plates. The plates were left for 10 min to allow the diffusion of the extract and then incubated at 37°C for 24 hours. Sterile DMSO acted as the negative control while 10 µl (10 µg) Ciprofloxacin served as the positive control. Zone of inhibition around the inoculated agar wells, which indicates the antimicrobial activity was recorded if it was greater than 8mm. The experiment was performed thrice and the mean value of the zone of inhibition was calculated.

2.4 Determination of Minimum Inhibitory Concentration (MIC)

The lowest concentration of an extract that completely inhibits the growth of the microorganism in 24 hours is the Minimum Inhibitory concentration (MIC) [10]. The MIC for the herbal extracts in the present study was determined by using modified agar well diffusion method [11]. A twofold serial dilution of each extract was reconstituted by mixing the powder in 20% DMSO followed by diluting with in sterile distilled water to achieve twofold serial dilution. The concentration thus achieved was in the range of range of 50mg/ml and 0.39 mg/ml. A 100µl volume of each dilution was introduced into agar wells already reloaded with 100µl of standardized inoculum of *S. mutans*. All test plates were incubated at 37°C for 24 hrs aerobically and observed for the zone of inhibition. The lowest concentration of each herbal extract showing zone of inhibition was considered as the MIC.

The collected data were coded and analysed using “Statistical Package for Social Sciences” (SPSS) software version 12. T-test was used to analyse the difference in antibacterial efficacy of *T. chebula* and *P. nigrum* with different extracts.

3. RESULTS AND DISCUSSION

Independent sample T-test showed antimicrobial activity of *T. chebula* and *P. nigrum* extracts varied in different organic and aqueous extracts (p<0.05). Ciprofloxacin, which was the positive control, produced inhibition zones with significant size against the test bacteria (Graph 1).

The minimum inhibitory concentration of all 5 extracts of both the herbs (Table 1). The antimicrobial activity of the 5 different extracts of *T. chebula* and *P. nigrum* showed that, all the five extracts of the both herbs showed varied antimicrobial activity against *S. mutans* (Graph 1). Larger zone of inhibition was produced by Ciprofloxacin (27.02±0.62), while DMSO showed
Herbal therapy is very common form of alternative medicine practiced all over the world [12]. It has also became a part of dentistry as a regular ingredient in tooth pastes, mouth rinses and gum paints etc. Evidence suggests that herbal extracts have used to treat gum and tooth problems from many centuries. In many countries, twigs of plants with antimicrobial qualities are commonly called chew sticks are used often to brush the teeth. Herbal extracts show medicinal effect as they interact with chemical receptors within the body and especially plant extracts that control inflammation and bleeding are of significant importance to dental professionals [13,14,15].

T. chebula is an herb with high medicinal values that has been frequently used in Indian traditional medicine. Several authors like Chattopadhyay et al. [16] and Bag et al. [17] have proved the antimicrobial activity of T. chebula extracts. Its efficacy against H. Pylori was reported by Zaidi et al. [18]. It was also found to have an inhibitory effect on Xanthomonas campestris pv. citri, and Salmonella typhi as observed by Cheema et al. [19] and Gupta et al. [20] respectively. Gupta et al. [20] also identified antiviral activity of T. chebula against HSV-1, HIV-1, as well as Cytomegalovirus. Tannins constitute about 30% of the T. chebula extract, which are responsible for their remarkable anticaries activity.

Pepper is a most commonly used spice. Pepper extracts are known as anticancer, anti-hypertensive, and detoxifying properties due to the presence of volatile oils, alkaloids, terpenes and flavones, which also responsible for preventing food spoilage and inhibition of food pathogens. [21-23]

Pradhan et al. [24] in their study has demonstrated that, phenolic compounds present in pepper exhibit antimicrobial properties against Salmonella typhimurium, Staphylococcus aureus and Escherichia coli. In the present study, 5 solvents each of T. chebula and Piper nigrum were evaluated for their antimicrobial activity. All of them have demonstrated different grade of antimicrobial activity.

### Table 1. Minimum Inhibitory Concentration (MIC) of T. chebula and P. Nigrum

| Herbal Extract     | MIC (mg/ml) | T. chebula | P. nigrum |
|--------------------|-------------|------------|-----------|
| Hot aqueous        | 25          | 12.5       |           |
| Cold aqueous       | 25          | 12.5       |           |
| Acetone            | 25          | 12.5       |           |
| Methanol           | 25          | 12.5       |           |
| Ethanol            | 25          | 12.5       |           |

Graph 1. Comparison of antibacterial activity of five extracts of T. chebula and P. nigrum against S. mutans along with the positive control (Ciprofloxacin)

\[ p-value=0.000724 \]
Test microorganism chosen for the present study was \textit{S. mutans} as it is the initial colonizer in dental caries and pulpal infections.[25] Dental caries is an infectious disease in which \textit{S. mutans} plays a key role. Like in other infections, pathogen should colonize before the disease occurrence and \textit{S. mutans} is regarded as the chief causative agent of dental caries [26].

Results from this in-vitro study revealed that all the extracts of \textit{T. chebula} and \textit{P. nigrum} showed growth inhibition of \textit{S. mutans}. It is also observed that the methanol extract of \textit{T. chebula} was more effective against \textit{S. mutans} compared to the other extracts tested (Graph 1). This result was consistent with a similar study conducted by Aneja KR [27]. As signified by the result of our study, herbal extract with an organic solvent namely methanol and acetone showed better antimicrobial action[28,29] This finding is significant, because the decoction of the herb prepared by boiling it in water is most commonly used for treating any infection. The result also showed that antibacterial efficacy of the plant compounds was not affected by boiling water which denotes that the herbal materials contain heat stable compounds. In a similar study to evaluate the antibacterial efficacy of \textit{T. chebula}, Nayak SS [30] has demonstrated that, ethanol extract of the herbal mouth rinse showed sustained inhibitory action on salivary \textit{Streptococcus mutans}. Antibacterial effect of \textit{Piper nigrum} against \textit{Staphylococcus aureus}, which is an important gram positive bacteria associated with dental caries and dental implant infections [31] was studied by Anju TR [32]. In their study, \textit{Piper nigrum} exhibited inhibitory effect on \textit{Staphylococcus aureus} with a zone of inhibition of 2.5cm. Previous research by Karsha et al. [33] proved that acetone extract of \textit{Piper nigrum} showed antimicrobial activity against gram positive bacteria.

Future clinical trails focusing on the potential adverse effects of \textit{T. Chebula} and \textit{P. nigrum} should be designed along with testing different synergistic combinations with other antimicrobial agents.

4. CONCLUSION

This study clearly demonstrated the antibiotic activity of \textit{Terminalia chebula} against \textit{Streptococcus mutans}. It can be effectively used as an independent antimicrobial agent or as a synergistic agent along with other previously antimicrobial agents against resistant microorganisms.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Sathyabama Dental College and Hospital/IHEC/Study No 055

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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