

Original Research Article

<https://doi.org/10.20546/ijcmas.2026.sp13.024>

Isolation of Anaerobic Bacteria (*Actinobacillus actinomycetemcomitans*) and *Porphyromonas gingivalis*) from Subgingival Plaque and their *invitro* Susceptibility to Standard Drugs, Seitz Filtered Lyophilized Ethanolic Extract of *Terminalia catappa* L. and *Terminalia chebula* L.

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ABSTRACT

Periodontitis affects 65 % Of the Indian population which leads to coronary heart diseases. The aim of the present study was to isolate anaerobic bacteria – *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* which are strongly associated with periodontitis. After careful removal of supragingival plaque, a pooled sample was taken using sterile paper point and was inoculated in to a test tube containing 1.5ml BHI broth kept at 4°C until the processing procedure was carried out. Tryptic Soy-serum in Bacitracin Vancomycin Agar (TSBV) for *Actinobacillus actinomycetemcomitans* and Blood agar supplemented with hemin and vitamin are the enriched and selective medium used for *Porphyromonas gingivalis*. Anaerobic condition was maintained inside the anaerobic jar by placing a Gas pack in it. Indicator tablet was placed inside the jar. After 5-7 days colony morphology was observed. Further characterization by biochemical tests and antibiogram showed that these anaerobic bacteria were highly sensitive to Norfloxacin followed by novobiocin, amoxicillin and gentamycin. Besides these the antibacterial activity of Seitz filtered lyophilized ethanolic extract of *Terminalia chebula* (Fruit rind) and *Terminalia catappa* – unripe (Fruit rind) was assayed by two fold Microdilution assay. After 4 days of anaerobic incubations MIC of plant extracts was estimated as 400µg/ml for both the bacteria. The present report seems to be the first successful clinical isolation of the anaerobic bacteria (*Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*) from India and the positive efficacy of lyophilized ethanolic extract of two medicinal plants with their MIC value and the sensitivity profile of the isolates to standard antibiotics.

Keywords

Periodontal pathogens,
Porphyromonas gingivalis,
Terminalia extracts,
In vitro susceptibility

Article Info

Received:
15 August 2026
Accepted:
22 September 2026
Available Online:
10 October 2026

Introduction

Oral cavity is invaded by millions of bacteria. The proximity of the bacteria to the tooth structure renders more pathogenicity of the bacteria. These bacteria organize and interact among themselves and form a community like set up. Gingivitis and Periodontitis are common disease involving these structures.

The inflammation of gingival progress into periodontitis. Ingar *et al.*, in 1999 studied the Taxonomical Characteristics of these bacteria causing infection. This progression is brought by changes in bacterial population in the plaque. Initial colonization by gram positive aerobic bacteria followed by anaerobic bacteria and these play a major role in the periodontal pathogenicity.

Actinobacillus actinomycetemcomitans and *Porphyromonas gingivalis* are found in large number under the conditions of Gingivitis and Periodontitis. J. Slots, 1982 reported TSBV agar as selective media for isolation of *A.actinomycetemcomita*. P. L. Derry *et al.*, 1982 studied the use of the anaerobic incubator for the isolation of anaerobes from clinical sample. Hence the aim of the present study was to isolate the anaerobic bacteria – *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* from subgingival plaque and periodontal lesions and to estimate the MIC value by IN VITRO susceptibility against Seitz filtered Lyophilized Ethanolic extracts of *Terminalia catappa* and *Terminalia chebula*.

Materials and Methods

Samples were collected from patients suffering from chronic generalized Periodontitis and chronic generalized gingivitis. After careful removal of supragingival plaque, a pooled subgingival plaque sample was taken from these pockets using 2 sterile paper points per pocket. The paper point was inserted in the depth of the subgingival pocket and kept in position for 60 seconds. The collected paper points (sterile) were inserted into Transport media, BHI broth. The tube containing BHI broth and the sample were then sealed using paraffin wax to maintain the anaerobic (condition) atmosphere for the survival of the anaerobic bacteria. The clinical sample stored in the anaerobic atmosphere was transported to the laboratory within 2 hours of collection. The sample was kept in the incubator without disturbing the anaerobic atmosphere for 24 hours. Turbidity was observed after 24 hours confirming the growth of the organism.

Petriplates of Blood agar supplemented with hemin and vitamin and TSBV agar were prepared and kept for sterility check, the incubated clinical sample was streaked on to the prepared sterilized media plates by quadrant streak.

The plates were then sealed with paraffin tape (adhesive tape) to avoid contamination of air microbes. Anaerobic jar with gas pack and indicator tablets were kept ready. The anaerobic jar was then kept in the incubator, kept under 37 deg C for 5-7 days; undisturbed.

The plates were now ready for the observation of colonies and further biochemical and antibiogram tests. Results obtained were recorded and the isolated colonies were sub cultured for further reference

Assay Method

The antimicrobial assay of the extract was carried out by Micro plate dilution technique. The MIC value by IN VITRO susceptibility against Seitz filtered Lyophilized Ethanolic extract of *Terminalia catappa* and *Terminalia chebula*.

The principle of dilution tests was to measure the MIC of the extract for the bacteria by detecting the concentration of the extract by doubling dilution in the series that prevented the development of visible growth of the test organisms. After incubation, the tubes were adjusted to obtain turbidity comparable to that of McFarland Standard. In the case of rapid growers, sterile broth was added to the test tube containing the organisms to make the turbidity as that of that standard. The test tubes which were less turbid were further incubated and then compared with the standard.

Results and Discussion

Seven samples collected from subgingival plaque and periodontal pockets were processed. The colonies on both selective media – TSBV and Blood agar supplemented with hemin and vitamin were observed and following results were observed. Colony morphology on TSBV agar was observed as circular, convex, translucent, glistening 0.5-1 mm diameter pinpoint, with central 4-6 pointed star like configuration with a tinch of orange pigment which is non diffusible. On gram staining, gram negative coccobacill were observed. The organisms were found to be non-motile, capinophilic, catalase positive and oxidase negative.

Table.1 Antibiotic Sensitivity Test [Kirby Bauer Method]

Selective media	TSBV agar	Blood agar
Amoxycillin	19 mm – S	18 mm – S
Penicillin	6 mm – R	8 mm – R
Gentamycin	22 mm – S	23 mm – S
Norfloxacin	27 mm – S	27 mm – S
Metronidazole	6 mm – R	6 mm – R
Novobiocin	23 mm – S	24 mm – S
Nalidixic Acid	20 mm – S	18 mm – I
Vancomycin	15 mm – S	14 mm – S

R – Resistance; S – Sensitive

Table.2 *In Vitro* assay by micro dilution method for aqueous Seitz filtered & lyophilized extract of *Terminalia chebula* and *Terminalia cattappa*.

Sl. No.	Organisms	Inhibitory effects Lyophilized extract of <i>Terminalia chebula</i> and <i>Terminalia cattappa</i> . (µg/ml)						
		800	400	200	100	50	25	12.5
1.	Actinobacillus actinomycetemcomitans	+	+	-	-	-	-	-
2.	<i>A.niger</i>	+	+	-	-	-	-	-

(+) Presence of Inhibition.

(-) Absence of Inhibition

Further biochemical tests and sugar fermentation results confirmed that the isolated colony on the selective media TSBV agar is *Actinobacillus actinomycetemcomitans* and for *Prophyromonas gingivalis*. The colony morphology on the selective media Blood agar supplement with hemin and vitamin was found to be minute, opaque, and adhesive, circular. The colonies observed were black pigmented. The black pigment was a non-diffusible pigment. On gram-staining Gram-negative rods were observed. The organism was observed to be non-motile, non-spore forming anaerobic rods, catalase negative and oxidase negative.

The antibiogram of both anaerobic bacteria was found to be resistant to Penicillin and metronidazole [6 mm] and was found sensitive to Norfloxacin followed by other antibiotics like Amoxycillin, Gentamycin, Novobiocin, Nalidixic acid, Vancomycin [Table. 1].

the antibacterial activity of Seitz filtered lyophilized ethanolic extract of *Terminalia chebula* (Fruit rind) and *Terminalia catappa* – unripe (Fruit rind) was assayed by two fold Microdilution assay [Table. 2]. After 4 days of

anaerobic incubations MIC of plant extracts was estimated as 400µg/ml for both the bacteria.

It's cleared that both the bacteria were successfully isolated by appropriate anaerobic methods. This present study is done with improved method than the earlier work done by Beena *et al.*, in 1996 in which candle jar was used for creating anaerobic environment but here anaerobic gas pack was used which resulted in isolation of *Actinobacillus actinomycetemcomitans* and for *Prophyromonas gingivalis*. *In vitro* studies with standard drugs by Kirby bauer method showed that these bacteria were sensitive to drugs like Norfloxacin, Amoxycillin, Gentamycin, Novobiocin, Nalidixic acid, Vancomycin and resistant to Penicillin and Metronidazole.

In addition to standard drugs, antibacterial activity of Seitz filtered lyophilized ethanolic extract of *Terminalia chebula* (Fruit rind) and *Terminalia catappa* – unripe (Fruit rind) was assayed by two fold Microdilution assay which showed an MIC as 400µg/ml for both the bacteria. In conclusion, *Actinobacillus actinomycetemcomitans* and *Prophyromonas gingivalis* have been isolated and

characterized from the sub-gingival lesions adopting the current methodology for the first time in India. In addition their susceptibility to administered antibiotics and other standard antibiotics have been estimated and found that norfloxacin, novobiocin, amoxicillin and gentamycin are the ideal drugs.

Lastly two herbal extracts *Terminalia catappa* and *Terminalia chebula* have found to possess appreciable inhibitory activity in this bacteria and the MIC value have been estimated as 400 µg/ml.

Author Contributions

Y. Bakthasingh Lazarus: Designed the model and the computational framework and analysed the data. S. Rajarajan: Conceived the original idea and designed the model and wrote the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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How to cite this article:

Bakthasingh Lazarus Y. and Rajarajan S. 2026. Isolation of Anaerobic Bacteria (*Actinobacillus actinomycetemcomitans*) and *Porphyromonas gingivalis* from Subgingival Plaque and Their *in vitro* Susceptibility to Standard Drugs, Seitz Filtered Lyophilized Ethanolic Extract of *Terminalia catappa* L. and *Terminalia chebula* L. Int.J.Curr.Microbiol.App.Sci. Special Issue-13: 209-212. doi: <https://doi.org/10.20546/jcmas.2026.sp13.024>