Comparison between Bacterial Species of Betel Leaf Chewers and Non-Chewers to Evaluate the Percentage of Antibiotic Resistant Bacteria in Mouth: *In Vivo* and *In Vitro*

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Abstract: **Betel leaf is cultivated as cash crop mostly in southern parts of India and is chewed as raw by a large proportion of Indian population. Antibiotic resistant microbe on betel leaf has led to ban on its export and import in few countries. These antibiotic resistant bacteria further replace the normal flora of mouth causing serious consequences for the future. A total of 30 betel leaf samples were collected from local retail shops and wholesale market. Bacterial isolation was done through betel leaf extract by serial dilution technique. The average Total Viable Count (TVC) of local shop samples were $3.14X10^7$ CFU/ml and wholesale market samples were $2.62 X 10^7$ CFU/ml. Mainly three species of bacteria were isolated from samples which were *Streptococcus sp. Staphylococcus sp.* and *Streptobacillus sp.* Antibiotic sensitivity test showed that *Streptococcus sp*. and *Streptobacillus sp*. were resistant to Penicillin and Azithromycin respectively. The effect of betel leaf juice on mice oral microflora was also conducted. The result revealed the change and replacement in oral microflora by antibiotic resistant bacteria. The study highlights the need of implementation of hygiene and safe practices during the cultivation, selling and use of betel leaves to protect the public health.**

***Index Terms*: Betel leaf, Microflora, TVC, Oral cavity, Antibiotic Sensitivity**

I. INTRODUCTION

Scientific name of betel plant is *Piper betle L*. In India it is known as paan. It is very popular in south east Asia and some other European countries such as: Pakistan and Bangladesh. These nations likewise trade betel to Europe, United States and different places of Asia. The betel plants are developed all through India with the exception of the dry North western parts. A very much depleted prolific sandy or sandy topsoil or sandy dirt soil with pH scope of 5.6 – 8.2 is considered reasonable for its development. Nonetheless, in the regions with lower water capacity (1500- 1700 mm) the yield is developed with little and incessant water systems, for example consistently in summer and each 3-4 days in winter, while satisfactory drainage is needed during the blustery season. The harvest is normally developed by people who are traditionally ranchers a great many ages following the conventional strategies. The expense of development of betel plants also called Boroj when grown in very high quantity might be about Rs 1-2 lakh/ha at the beginning during the first year that may come down to about Rs 0.5-0.6 lakh/ha in the resulting years and a base net benefit of Rs 0.5-1.0 lakh/ha/year or more (Rs 5.02 lakh/ha/year) can also be obtained by a established farm. This may establish a gross production of the betel leaves' worth about Rs 9000 million consistently in the country where the yield is become on about 55,000 ha of land. On a normal about 66% of such creation is contributed by the province of West Bengal where it is developed on around 20,000 ha of land. These betel leaves are also known for their antibiotic activity and also used as mouth-freshner.

The surface area of Betel leave can be contaminated with microbial microorganisms by dirtied air, water and soil, during pre-collection stage. Bundling materials utilized for convey and capacity at Betel leaf, dampness content and water utilized for washing of Betel leaf are significant wellsprings of tainting during post-gather stage. It has been assessed that as much as 80 % of illnesses in developing nations are related with water. Scarcely any individuals approach a sufficient amount of well- treated water supply or to a compelling sewage removal framework. These applies to both swarmed metropolitan and the rustic territories and the outcome Is the undeniable degree of fecal matter tainting and related infections Like hookworm, cholera and ongoing loose bowels.

Regularly 'paan' venders don't follow the overall rules of individual tidiness, hence making conditions positive for the transmission and expansion of water borne infections. The carelessness to keep the overall guideline of individual cleanliness isn't completely because of obliviousness, yet in addition because of the customary propensities what's more, traditions of individuals. Because of shortage of sifted water in summer, individuals need to utilize unfiltered water for cleaning purposes. The normal act of washing 'paan' leaves with the tainted water may likewise help in the spread of enteric illnesses among the buyers. Because of the genuine ramifications of devouring tainted 'paan', the present work was planned to lead a pilot review on how these microorganisms can affect our oral health.

The mouth is the entryway of the body to the outer world and addresses perhaps the most organically mind boggling and huge locales in the body. The human oral pit contains unique sort of microbes. There are various territories including the teeth, gingival sulcus, cheeks and tonsils. The oral pit, or mouth, incorporates a few microbial territories, like teeth, appended gingiva, tongue,

lips. Adjoining with the oral pit are the tonsils, pharynx, throat, Eustachian tube, center ear, windpipe, lungs, nasal entries, and sinuses. Studies have shown that diverse oral designs and tissues are colonized by particular microbial networks. The biological properties of the mouth make it unique in relation to any remaining surfaces of the body. Nonetheless, the mouth should not be viewed as a uniform climate. It comprises of a few very different living spaces every one of which will uphold the development of a trademark microbial local area. The territories of the oral cavity that give clearly unique natural conditions to colonization and development incorporate the lips, cheek, sense of taste, tongue, gums and teeth.

There are many kind of microorganisms which can proliferate and colonize in the human oral cavity. Anaerobic microbes which can be present in oral microflora include: *Actinomyces, Arachnia, Bacteroids, Bifidobacterium, Eubacterium, Fusobacterium, Lactobacillus, Peptococcus, Peptostreptococcus, Selenomonas, Treponema.* Many fungal organisms can also colonize in the oral cavity such as: *Aspergillus, Alternaria, Penicillium, Fusarium* and *Candida.*

Oral diseases also called as oral contaminations or infections, are a group of diseases that happen around the oral cavity. They incorporate dental disease, dental boil and Ludwig's angina. In a grown up, billions of microbes, infections and parasite dwell inside the oral pit and address in excess of 500 distinct species. They are all in all known as the oral microbiome.

In a healthy person, the oral microbiome is in powerful balance with the all microorganisms that none of the microbes or gathering of creatures overwhelms. Notwithstanding, certain circumstances, similar to a rotting tooth root or penetrating a puncture from a fish bone, can create a climate that disrupts the typical oral microbiome and encourage and support the development of pathogenic microorganisms.

II. MATERIAL AND METHODS

2.1 Sample collection

Betel leaves were collected from the whole seller and local shop in Bhopal. The samples are taken and contained into a sterile container. The samples were taken cautiously to the Bacteriology lab for bacteriological investigation.

2.2 Juice extraction from betel leaves

The betel leaf samples were washed with phosphate buffered saline (PBS). Then the leaves were crushed with distilled water with the help of pestle and mortar. Betel leaf juice was filtered with filter paper for further use.

2.3 In vitro analysis

0.1 ml solution of ten fold diluted (10^{-1}-10^{-6}) betel leaf juice was inoculated onto the nutrient agar medium and incubated for 24-48 hours at 37˚C. Morphological characteristics of isolated colonies was recorded. Gram staining and other biochemical tests were also performed for the identification of isolated microorganisms.

After the identification of isolated microorganisms the antibiotic sensitive test was performed in which agar diffusion assays were performed in petri dishes containing nutrient agar medium. The agar plate was flooded with betel leaf juice and antibiotics were poured in the well situated in the center of the petri dish. The plates were then incubated overnight at 37˚C. The antibiotic sensitivity of that particular microbe was interpret letter by the presence of zone of inhibition.

2.4 In vivo analysis

Seven days before infection (Pre- treatment)- To assess the possible effect of the sample, the animals (n= 4 per group) were pre-treated with the saline sample with the help of oral dosing for 7 days. After 7 days saliva sample was collected by swabbing using sterile Cotton bud in 5ml PBS solution and plated on NAM aseptically and incubated at 37°C for 24 hours following the identification and antibiotic sensitivity test of microorganism.

The day of infection- Betel leaves juice was dripped into the mouth with the help of the syringe according to OECD guidelines. After 24 hours, the saliva sample was collected in 5 ml of PBS solution and plated on Nutrient agar medium and incubated at 37°C for 24 hours following the identification and antibiotic sensitivity test of microorganism.

Seven days post infection (Post- treatment)- The rats were again treated with the Betel leaves juice samples for 7 days regular intervals according to OECD guidelines. On last day the saliva samples were collected in 5 ml of PBS solution by swabbing using sterile cotton bud and plated on NAM and incubated at 37°C for 24 hours following the identification and antibiotic sensitivity test of microorganism.

III. Results-

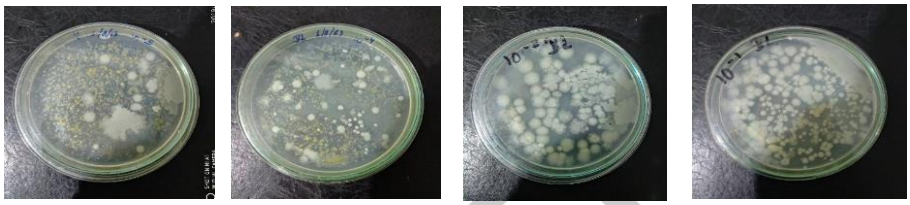
3.1 In vitro analysis

The betel leaf juice was used according to the 1mg/ml stock solution concentration and the results are following-

Table 1- Total viable count of betel leaf and identification of microorganism.

Betel leaf source	Sample	TVC CFU/ml	Microorganism identified
Whole seller	Sample 1	2.15×10^8	*Streptococcus sp.*
	Sample 2	2.62×10^7	*Streptococcus sp.*
Local shop	Sample 3	2.20×10^8	*Streptobacillus sp.*
	Sample 4	3.14×10^7	*Streptobacillus sp.*

Fig.- Sample 1 Sample 2 Sample 3 Sample 4



Antibiotic sensitivity test

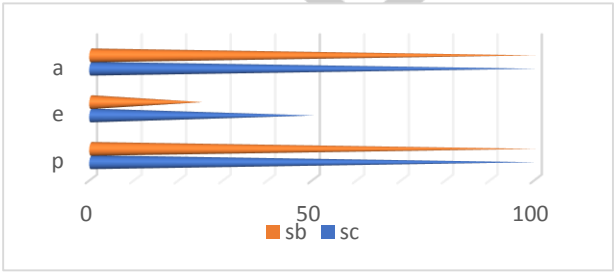
The isolated bacterial strains were subjected to antibiotic sensitivity test to evaluate their antibiotic resistance and following results are obtained-

Table 2 – Antibiotic sensitivity test results

Antibiotic	Bacteria	
	Streptococcus	***Streptobacillus***
Penicillin	Resistant	Resistant
Erythromycin	Sensitive	Sensitive
Azithromycin	Resistant	Resistant

Fig.- Penicillin plate Azithromycin plate

Antibiogram profile of bacteria's



- Description of Graph :- a = azithromycin sb = *streptobacillus*
  e = erythromycin sc = *streptococcus*
  p = penicillin

- This graph shows the percentage of Antibiotic sensitivity of bacterial samples. There are 3 antibiotics in y axis – penicillin, erythromycin, azithromycin respectively and percentage resistance is on x axis.
- Both the bacterial sample *Streptococcus* and *Streptobacillus* are resistant to antibiotics Penicillin & Azithromycin on the other hand they are sensitive to erythromycin at 25% and 50% respectively.

3.2 In vivo analysis

Saliva samples were collected from the pre- treated and post treated group of rats and a dilution of 10-4 is plated on agar plates. The quantity of oral microflora was low in pre- treated groups and the post treated groups showed variance in the species of bacteria as well as their quantity also increased with the betel leaf juice uptake.

Table 3- CFU of pre- treatment group samples

Sample No.	CFU	Identified M.O.
Sample 1	4.40×10^7	*Monococcus sp.*
Sample 2	4.73×10^7	*Monococcus sp.*
Sample 3	3.95×10^7	*Monococcus sp.*
Sample 4	3.44×10^7	*Monococcus sp.*

Table 4- CFU of post treatment group samples 4

Sample No.	CFU	Identified M.O.
Sample 1	6.20×10^7	*Streptococcus sp.*
Sample 2	7.51×10^7	*Streptococcus sp.*
Sample 3	6.83×10^7	*Streptococcus sp.*
Sample 4	6.57×10^7	*Streptococcus sp.*

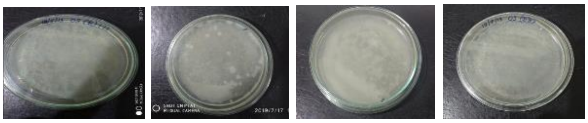
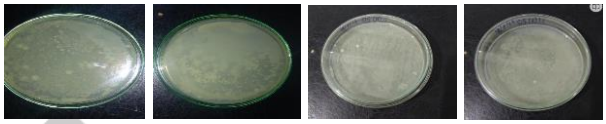


Fig.- Sample 1 Sample 2 Sample 3 Sample 4



Sample 1 Sample 2 Sample 3 Sample 4

Antibiotic sensitivity test

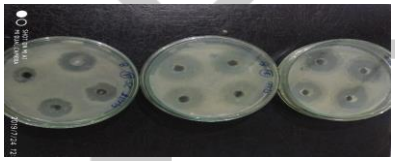
For this experiment we have used three antibiotics- Penicillin, Erythromycin and Azithromycin. In the pre- treatment group, all the bacterial isolates were sensitive to all of these three antibiotics but in the post- treatment group, the bacterial isolates showed complete resistance to penicillin antibiotic and moderate resistance to other two antibiotics.

Table 5- Pre- treatment group sample

Antibiotic	Bacteria
	Monococcus
Erythromycin	Sensitive
Penicillin	Sensitive
Azithromycin	Sensitive

Table 6- Post-treatment group sample

Antibiotic	Bacteria
	Streptococcus
Erythromycin	Sensitive
Penicillin	Resistant
Azithromycin	Sensitive



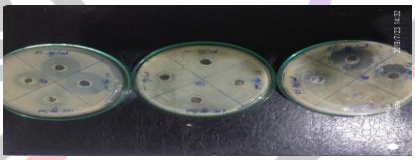


Fig.- Pre- treatment group sample Post- treatment group sample

IV. Discussion-

The highest TVC count was from local shop samples indicating that there are numerous factors which affect the quality of betel leaves and contaminate them while transportation from farms to their whole sellers and local vendors. When we uptake these contaminated betel leaves which have multiple drug resistant bacteria it can affect our oral hygiene. We have seen from our results that these multi- drug resistant bacteria can replace the resident microflora of oral cavity and proliferate there causing serious oral health problems.

The significant increase in the quantity of microbes in oral samples suggests that the intake of betel leaves can influence our oral microflora. Further investigations are expected to decide whether day by day utilization of betel leaves can cause more serious issues. Further exploration ought to be completed to survey the Impact of antibiotic resistant microbes present in betel leaf at our body.

The in vitro activity suggests that there are numerous Antibiotic resistant bacteria present in Betel leaves and have the capability of causing numerous medical conditions. Mainly two types of bacteria's were isolated from the betel leaf juice – *Streptococcus sp.* And *Streptobacillus sp.* The microbes are resistant to numerous antibiotics so they can undoubtedly colonize in human mouth and can influence the oral microflora. The most occuring bacteria in Betel leaves is *Streptococcus spp*. Which is responsible for many sorts of dental infection that can additionally influence our body framework. *Streptococcus mutans* are the main reason behind major periodontal infections.

In vivo movement shows that the microbes present in Betel leaves juice supplant the oral microflora of Have and colonize on their oral cavity. The number of inhabitants in bacterial species increments completely with the take-up of betel leaves juice which implies they can undoubtedly colonize in host's oral pit Furthermore, skirts through have invulnerable framework and cause issues.

This investigation shows that the anti-infection safe microorganisms can supplant the previous microbes in Human oral microflora and colonize there, which can prompt numerous sort of illnesses and wellbeing Issues.

V. Conclusion

The use of Betel leaves as a edible product is harmful because the Betel leaf sold at local market harbors multidrug-resistant food borne bacteria's, which can affect our oral health and lead to health problems.

The Streptococcus spp. are also leading cause of tooth decay and other periodontal diseases and if these bacteria's are Antibiotic resistant than they can easily colonize in human mouth and cause various problems, so the intake of Betel leaf is not totally safe.

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