Study of Antimicrobial Activities of Antibiotics and Herbal Extracts against Oral Pathogens - *Streptococcus mutans* and *Fusobacterium nucleatum*

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Abstract

Introduction: Oral health is extricably linked to general health and vice versa therefore maintaining healthy mouth is of vital importance for a person. Antibiotics play a very important role in preventing diseases of oral cavity but many studies reveal that many of such oral micro-organism tend to show resistance towards common antibiotics. To overcome this, traditional medicines have been considered as useful alternatives to synthetic drugs. Now a day's trend is to use more of the Herbal products as cosmetics and the same shift is seen in dental products such as mouth rinse and Tooth pastes. To evaluate the efficiency of these extracts of medicinal plants, we conducted the Antimicrobial Susceptibility test of the oral pathogens against herbal extracts, and compared it with routinely used antibiotics.

Material and Method: The antibacterial activities on the bacteria were studied by approaching two different ways of treatments: i) common allopathic antibiotics ii) herbal antibiotic extracts

Two bacteria commonly found in the oral cavity of the patients were chosen for antibiotic susceptibility tests - *Streptococcus mutans* and *Fusobacterium nucleatum*

Four herbal extract - Aloe Vera, Triphala, Neem, and Shyama Tulsi and four antibiotics - Ceftazidime, Gentamycin, Amikacin and Ciprofloxacin were chosen for the study.

Kirby Bauer Disc Diffusion method was employed to check the efficacy of the antibiotics and herbal extracts against the isolated pathogens.

Result: In our study, Aloe Vera, failed to indicate any positive response when treated against pathogenic bacteria; *Fusobacterium nucleatum* , and *Streptococcus mutans* . The herbal extracts of "Triphala" succeeded to develop a satisfactory inhibition zone, compatible to the allopathic drugs. "Neem" was found to be highly potent antibiotic agent against few bacteria;*Steptrococcus mutans.*

Discussion: The gradual increment in resistance against the allopathic medicines and the adverse effects has compelled the dental surgeons and other researchers to look for some novel herbal compounds which could be used for effective treatment of oral diseases

Conclusion: The overall results on antimicrobial susceptibility tests against every isolated oral pathogenic bacterium, showed that the overall performance of allopathic drugs were good as antibiotic agents to check the bacterial growth. However, all the bacteria were found to resist the antibacterial effects of other treated herbal extracts. "Neem" was found to be highly potent antibiotic agent against few bacteria like *Steptrococcus mutans.*

Key words: Antimicrobial, Herbal extracts, pathogens, sensitivity

Introduction:

The oral cavity harbours variety of microbes[1]. The factors which determine the oral microflora include environmental factors like temperature, oxygen content, pH, nutrient availability etc. Some host factors like host tissues and fluids, genetics, diet and microbial factors like adherence, retention and co-aggregation, microbial intra- and interspecies interactions, clonal heterogeneity, virulence mechanisms create a dynamic and complex ecosystem that supports growth and multiplication of micro-organism[1]. Several studies revealed that imbalance may render the host susceptible to infection.

It is well proven that most periodontopathogens are commensals in the oral cavity and express their virulence only in a susceptible host or when changes occur in the oral ecosystem[2]. To fight with oral pathogens there are many traditional systems of medicine to overcome the pathogenic activities of oral microbes.The clinical efficacy of many existing antibiotics is being threatened by rapid emergence of multidrug-resistant pathogens[3]. Thus there is need for research into new analytical methods for standardization of phytomedicines.

Natural products, either as pure compounds or as formulated with measured constituents of plant extracts, provide unlimited opportunities for emergence of new drug. Herbal drugs are now a days very popular among users[4]. They are preferred mainly due to easy availability in market, cheaper as compared to synthetic ones, having least side effects and toxicity. As per a report of the World Health Organization (WHO - 2014) 4 billion people i.e., the 80% of the world population are presently using herbal medicine for some aspect of primary health care.

Materials and Methods[5-13]:

The study was carried out following the proper guidelines framed by the ethical committee of the Institute.

In the present study we selected two common oral pathogenic bacteria commonly found in the oral cavity of infected person. Further, the antibacterial activities on the same bacteria were studied by approaching two different ways of treatments:

i) Common allopathic antibiotics

ii) Herbal antibiotic extracts

Two bacteria commonly found in the oral cavity of the patients were chosen for antibiotic susceptibility tests - *Streptococcus mutans* and *Fusobacterium nucleatum*

Four herbal extract - Aloe Vera, Triphala, Neem, and Shyama Tulsi and four antibiotics - Ceftazidime, Gentamycin, Amikacin and Ciprofloxacin were chosen for the study.

Collection of Samples:

The oral samples were taken by rotating the sterile swab. The samples were collected in sterile 5ml of Thioglycollate Broth and subjected for microbiological analysis.

Cultivation

Media used for the study were:

1) For *Fusobacterium nucleatum* -

a) Blood Agar + Neomycin (100 ug / ml) + Vancomycin (7.5 ug / ml)

b) Bile Esculin Agar

2) For *Streptococcus mutans*   –

a) Blood Agar

b) Mitis Salivarius Agar

Incubation:

The inoculated plates were properly labelled and placed inside the McIntosh Filde's Jar using Gas pack which made the environment anaerobic.

Antibiotic used[15-17]:

1) CAZ – Ceftazidime – 30 mcg

2) GEN – Gentamicin – 10 mcg

3) AK – Amikacin – 30 mcg

4) CIP – Ciprofloxacin – 5 mcg

ANTIMICROBIAL SENSITIVITY TESTING AGAINST HERBAL EXTRACTS[15-22]:

Herbal Products used:

1) Aloe Vera

2) Triphala

3) Neem

4) Tulsi

Preparation of Herbal Extracts:

1) **ALOE VERA GEL**: abbreviated as AV on the petriplates.

Fresh leaves of Aloe Vera were washed and their thick epidermis was removed, and thick straw-coloured gel was collected in a sterile container and 100 grams of the gel was mixed in one liter of 2% dimethyl sulfoxide (DMSO) and kept at 40^0C, being used as a stock solution.

2) TRIPHALA: (*Terminalia Chebula* "Harad", *Terminalia Belerica* "Vibhataki",

Phyllantus Embelica "Aamla") abbreviated as T on the petriplates. The Triphala Churna commercially available under brand name of Baidyanath Triphala was used. 1:1 ratio of Triphala powder was dissolved in distilled water. It was then filtered and used as stock solution.

3) NEEM (*Azadiracta indica*), : abbreviated as NE on the petriplates.

The fresh leaves were plucked and washed with water and then cleaned with Phosphate Buffer saline for 2–3 minutes. Further the same was crushed in Phosphate buffer saline in 1:1 w/v ratio. The suspension was filtered with help of muslin cloth and used as stock solution.

4) **SHYAMA TULSI** (*Occimum sanctum* L. Sym O. Tenniflorum) abbreviated as TU on the petriplates.

The fresh leaves were plucked and washed with water and then cleaned with Phosphate Buffer saline for 2–3 minutes. Further the same was crushed in Phosphate buffer saline in 1:1 w/v ratio. The suspension was filtered with help of muslin cloth and used as stock solution.

Preparation of Discs of Herbal Extracts:

The filter paper was cut in 6mm diameter and were dipped in each herbal extract and left overnight for soaking. Next day the discs were kept for drying on the hot plate for about 1-2 hours. These discs were placed on the Mueller Hinton Agar Media inoculated with test bacteria. The plates were subjected for incubation anaerobically inside McIntosh Jar at 37^0C for 24 hours.

Inoculum Preparation:

The inoculum density of bacterial isolate to be tested was standardized with 0.5 McFarland turbidity standards at 625 nm, for the bacterial suspension to have a final inoculum of 1×10^8 cfu /ml.

Placing the Antibiotic Disc:

The Antibiotic Disc, that are commercially available were chosen having the antibiotics that are commonly used by the Dentists and placed on the petriplates and incubated anaerobically at 37^oC for 24 hours.

ANTIMICROBIAL SENSITIVITY TESTING

An in vitro test of the effectiveness of selected antibacterial agents against test bacteria was done by Antibiotic sensitivity tests using Kirby-Bauer Disc Diffusion method.

Media used:

For Antimicrobial sensitivity tests, Mueller Hinton Agar media was used.

Result:

1. Macroscopic (colonies) morphology -

a) *Fusobacterium nucleatum*   -

i) It exhibited Grey coloured colony on Blood Agar supplemented with Neomycin and Vancomycin.

b) Streptococcus mutans   –

i) It developed white mucoid colony on Mitis Salivarius Agar media

ii) It showed Alpha haemolysis on Blood Agar media

2. Microscopic morphology and staining characteristics:

Gram Reaction shown by various microflora

1. *Streptococcus mutans* - Gram Positive Coccus, Small chains

2. *Fusobacterium* nucleatum - Gram Negative Bacillus, Single

3. Biochemical Reactions-

a) Fusobacterium: Indole positive, Bile positive, Gelatin positive, Esculin positive, Starch negative.

b) Streptococcus: Catalase positive, Voges Prausker positive, Glucose positive, Mannitol positive, Raffinose positive, Melbiose positive, Sorbitol positive, Sucrose positive, Lactose positive, Maltose positive, Esculin positive.

Result -

Statistical Analysis:

Two way ANOVA were employed to verify the various drug effects against each bacterium species and the overall results revealed statistically highly significant ($p<0.001$).

Interpretation of the result:-

The zones of inhibition around each of the antibiotic discs were measured to the nearest millimetre with the help of Antibiotic Zone Reader Scale, HiMedia. According to the zone size measured in mm, the allopathic drugs and herbal extracts were graded as sensitive, intermediate and resistant against the bacterial isolates.

Reading / Observation:

The results were interpreted as:

1) Sensitive: This category had isolates that were inhibited by the antimicrobial agent of known dosage. The Inhibition Zone Range was 28 – 34 mm.

2) Resistant: the "resistant" category implies that isolates were not inhibited by the antimicrobials of known concentrations and the inhibition zone diameters that were less than 20 mm in diameter.

3) Intermediate: the "intermediate" category included isolates with Inhibition zone that fall in the range of 20 - 27 mm based on the treatment studies.

The effects of the allopathic antibiotics against each bacterium are as follows:

a) Ceftazidime: 30mcg dose of Ceftazidime affected much against *S.mutans*, but it did not show any statistically significance while compared with the effects revealed against *F. nucleatum*.

b) Gentamicin: 10 mcg of Gentamicin was found to check significantly the growth of most *Fusobacterium nucleatum* and *S. mutans*.

c) Amikacin: 30 mcg dose of Amikacin was found to check *F. nucleatum* and *S. mutans.*

d) Ciprofloxacin: 75 mcg dose of Ciprofloxacin was found to affect more or less equally to all the treated bacteria as the zones of inhibitions developed by it against both bacteria were not found to be differed from each other statistically.

The effects of the herbal extracts as antimicrobial agents against each bacterium are as follows:

a) **Aloe Vera:** No significant inhibition zones were seen against the test bacteria, in other words all the bacteria were found to resist the effect of Aloe Vera as an antimicrobial agent.

b) **Triphala:** Triphala was found to be the effective antimicrobial herbal agent and showed intermediate zone of inhibition against *Streptococcus mutans* .

c) **Neem:** When Neem extract was treated against the pathogenic bacteria, no significant antimicrobial affects were exhibited. In other words all the bacteria were found to resist the antimicrobial property of the Neem.

d) **Tulsi:** When Tulsi extract was treated against the pathogenic bacteria, no significant antimicrobial affects were exhibited. In other words all the bacteria were found to resist the antimicrobial property of the Tulsi. However, its antimicrobial properties was found to be little better while compared with other treated herbal agents like, Neem.

Discussion:

Dental caries develops only due to the Bacteria existing in the dental plaque or biofilm[1]. It is well established that the development of dental caries involves gram-positive bacteria eg, *mutans streptococci, lactobacilli* and *actinomycetes* whereas periodontal diseases have been linked to anaerobic gram-negative bacteria(*Porphyromonas gingivalis, Agregatibacter, Prevotella,* and *Fusobacterium*)[14]. Oral bacteria have been constantly reported to show increased resistance towards common antibiotics such as penicillin, cephalosporin, erythromycin, tetracycline, and metronidazole which have been used therapeutically for the treatment of oral infection In dentistry since long time, phytomedicine has been used as anti-inflammatory, antibiotic, analgesic, sedative and also as endodontic irrigant[15,17]. The gradual increment in resistance against the allopathic medicines and the adverse effects has compelled the dental surgeons and other researchers to look for some novel herbal compounds which could be used for effective treatment of oral diseases[18-22]. Aloe vera has been suggested for a wide variety of ailments but its use in dentistry is limited. However, our results failed to indicate any positive response when treated against oral pathogenic bacteria; *Fusobacterium nucleatum* , and *Streptococcus mutans*   .

Conclusion:

The overall results on antimicrobial susceptibility tests against every isolated oral pathogenic bacterium, showed that the overall performance of allopathic drugs were good as antibiotic agents to check the bacterial growth. However, the herbal extracts of "Triphala" which is indeed a mixture of three different herbal constituents, *Terminalia Chebula* "Harad", *Terminalia Belerica* "Vibhataki", *Phyllantus Embelica* "Aamla" also succeeded to develop a satisfactory inhibition zone, compatible to the allopathic drugs. Nevertheless, all the bacteria were found resist the antibacterial effects of other treated herbal extracts. "Neem" was found to be highly potent antibiotic agent against few bacteria;*Steptrococcus mutans*.

Oral health is extricably linked to general health and vice versa therefore maintaining healthy mouth is of vital importance for a person's self-esteem and general well-being[1,2]. For the prevention of bacteremia and endocarditits antibiotic administration prior to invasive dental procedure is recommended[3,4]. Antibiotics play a very important role in preventing diseases of oral cavity but many studies reveal that many of such oral micro-organism tend to show resistance towards common antibiotic, many studies also reveal that in most of the cases these antibiotics fails to eliminate the pathogens from oral cavity for which combination of drugs has to be suggested which impacts great adverse effect in the patients'[4,17]. health like hypersensitivity, toxicity, tooth staining and drug resistance, their mental and also financial burden builds up. To overcome this traditional medicines have been considered as useful alternatives to synthetic drugs. Now a day's trend is to use more of the Herbal products as cosmetics and the same shift is seen in dental products such as mouth rinse and Tooth pastes[20-22]. To evaluate the efficiency of these extracts of medicinal plants, we conducted the Antimicrobial Susceptibility test of the oral pathogens against herbal extracts, and compared it with routinely used antibiotics. The role of herbal extracts in the prevention of oral infection can be seen by the appearance of inhibition zones. In Antimicrobial Susceptibility test against antibiotics, we found fusion of inhibition zones in most of the plates, suggestive of combined effect of two antibiotics so single therapy will not be effective against these pathogens and also data shows that particular antibiotic was effective against any one or two of the test isolates. Traditional medicinal plants have an almost maximum ability to synthesise aromatic substances most of which are phenols or their oxygen substituted derivatives. Amongst all the herbal extracts used, Aloevera[15] was found to be least effective against all the isolates, whereas Triphala, Neem and Tulsi were found to be most effective respectively.

References:

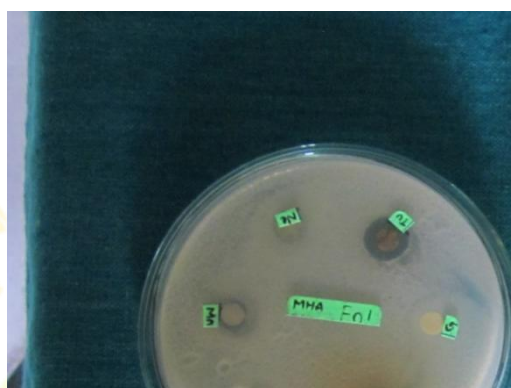1) Socransky S and Haffajee AD.,(2005): Periodontal microbial ecology. Periodontol 2000. 38: 135−87

2) Kulshrestha R, Neral A, Srinivasa TS, Baig SA. (2011): Comparison of oral microflora of Diabetic and non-Diabetic patients with Periodontitis. J Pure Appl Microbiol. 5(2): 883-886

3) Teresa Cristina Costa, Franco E Franco, Patrícia, AMOROSO, José Moacir, Marin Fernando Antonio, de Ávila,(2007):Detection of Streptococcus mutans   and Streptococcus sobrinus in Dental Plaque Samples from Brazilian Preschool Children by Polymerase Chain Reaction. Braz Dent J. 8(4): 329-333.

4) Essam F.A. Al-Jumaily, Nidhal.A.Muhimen, Abd Al-Wahid Al-Shaibany, Nada H.A. Al-Mudallal, (2008): Isolation and identification of mutan's Streptococci bacteria from human dental plaque samples. J Philip D. Marsh,(2000): Role of the Oral Microflora in Health. Microbial Ecology in Health and Disease.12:130–137

5) Bridson EY, Brecker A,(1970): Design &formulation of microbial culture media. Methods in microbiology. Vol.3A, PP 229-295, Ed Norris & Ribbons. Academic Press.

6) Meynell GG, Meynell E, (1965):Book on Theory & practice in experimental bacteriology. Cambridge University Press.

7)   Skyes G, (1956): Book on Constituents of bacteriological culture media. Cambridge University press.

8)   Skinner FA, Shaptom DA, Board RG,(1971): Isolation of anaerobes. The society for applied bacteriology. Academic Press. Technical series 5.

9) Mukherjee, K.L. Medical Laboratory,(2006): A Procedure Manual For Routine Diagnostic Tests, New Delhi, Tata- McGraw-Hill Publishing Company Limited;

10) Mackie & McCartney,(2007): Practical Medical Microbiology; 14th edition, Churchill Livingstone, Pg 151-178

11) Koneman's,(2006): Color Atlas and Textbook of Diagnostic Microbiology, 6th Edition, Lippincott Williams & Wilkins, Pg 945-1021

12) Bailey and Scotts,(2007): Diagnostic Microbiology, International Edition- 12th Edition, Mosby Elsevier, 172-215

13) Prafful B. Godkar, Darshan   P Godkar,(2206): Textbook of Medical Laboratory technology, Second Edition, Bhalani Publishing House, Mumbai, India, Pg 583-584

14) David Greenwood, Richard C.B. Slack, John F. Peutherer,(2003): Medical   Microbiology- A guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and control, 7th Edition, Churchill Livingstone, Pg46-60

15) Collins CE,(1935): Aloevera gel as a therapeutic agent in the treatment of roentgen and radium burns. Radiol Rev Chicago Med Rec. 57:137-138.

16) Krinsky DL, Hawkins EB, Pelton R, Willis NA, Lavalle JB., (2003):Natural therapeutics pocket guide, ed. 2. Cleveland: Lexi-Comp. Inc. 379.

17) Levi, M.E. and Eusterman, V.D. (2011): Oral infections and antibiotic therapy. Otolaryngologic Clinics of North America.44: 57-78.

18) Adhvaryu MR, Reddy N and Parabia MH, (2007): Effects of four Indian medicinal herbs on Isoniazid-,Rifampicin- and Pyrazinamide-induced hepatic injury and immunosuppression in guinea pigs.World J Gastroenterol. 13:3199-3205.

19) Ahmad I and Beg AZ, (2001): Antimicrobial and Phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethanopharmacology.74:113–123

20) Fatima S, Farooqi AH, Kumar R and Khanuja SP, (2000): Antibacterial activity possessed by medicinal plants used in tooth powder. J Med Arom Pl Sci. 22: 187-9.

21) Peck M T, Africa CWJ, Stephen LXG, Marnewick J and Majeed A,(2011): An in-vitro analysis of the antimicrobial efficacy of herbal toothpastes on selected primary plaque colonizers. Int. Journal of Clinical Dental Science.2(3): 28-32

22) Prasanth M, (2011): Antimicrobial Efficacy of Different Toothpastes and Mouth rinses: An in Vitro Study. Dental Res. J. 8(2): 85-94.

Zones of inhibition values for Fusobacterium nucleatum   and Streptococcus mutans

Name of Herbs	Fusobacterium nucleatum					Average	Streptococcus mutans					Average
	Disc 1	Disc 2	Disc3	Disc4	Disc5		Disc6	Disc7	Disc8	Disc9	Disc 10	
Triphala	7.8 mm	7.2mm	7.9mm	8.0mm	7.1mm	7.6mm	7.7mm	8.1mm	7.7mm	7.1mm	7.9mm	7.7mm
Neem	4.8mm	4.6mm	4.7mm	4.2mm	4.5mm	4.56mm	4.4mm	4.1mm	4.7mm	4.5mm	4.1mm	4.36mm
Tulsi	2.4mm	2.1mm	2.2mm	2.2mm	2.7mm	2.32mm	1.9mm	2.7mm	2.4mm	2.4mm	2.2mm	2.32mm
Aloe Vera	1.7mm	2.6mm	2.4mm	2.4mm	2.2mm	2.26mm	1.5mm	2.7mm	2.6mm	2.4mm	2.2mm	2.28mm

Zones of inhibition values for Fusobacterium nucleatum   and Streptococcus mutans

Name of Allopathic Drug	Fusobacterium nucleatum					Average	Streptococcus mutans					Average
	Disc 1	Disc 2	Disc 3	Disc 4	Disc 5		Disc 6	Disc 7	Disc 8	Disc 9	Disc10	
Ceftazidime	7.5mm	8.8 mm	8.1 mm	7.9 mm	7.1mm	7.8mm	7.4mm	7.6mm	7.6mm	7.5mm	7.3mm	7.5mm
Gentamicin	5.8mm	5.3 mm	5.5 mm	5.2 mm	4.5mm	5.26mm	5.7mm	5.2mm	5.4mm	5.4mm	5.9mm	5.52mm
Amikacin	5.5mm	5.8mm	5.9mm	5mm	5.2mm	5.48mm	4.9mm	5.5mm	5.3mm	5.6mm	5.1mm	5.28mm
Ciprofloxacin	3.3mm	3.5mm	3.6mm	3.7mm	3.1mm	3.44mm	2.8mm	3.7mm	3.3mm	3.1mm	2.7mm	3.12mm