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Antimicrobial activity of formulated herbal extract against Dental Plaque isolates

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Abstract- An increase in the drug resistance attributes of bacteria as well as negative changes in oral mucosa in regards to the extensive use of chemical based broad spectrum antiseptics against dental caries has resulted in rise of the demand of potential, safe and inexpensive herbal medicines. Therefore the present study was conducted to evaluate and compare the antimicrobial activity of fruit extract of three plants Amalaki (*Phyllanthus emblica*) Bibhitaki (*Terminalia bellirica*) and Haritaki (*Terminalia chebula*) either alone or in combined formulation (Triphala) against cryogenic pathogen combined with dental plaque. To check antimicrobial property, Zone of Inhibition and Minimum Inhibitory Concentration (MIC) of these extracts were calculated. The herbal formulation reduces > 55% in vitro bacterial colony forming units (CFU) causing dental plaque over a period of 2 weeks. Median Lethal Dose (L_{50}) of herbal formulation was found to be 500µg/ml. After reckoning all the antimicrobial activities, Triphala formulation was found better than the individual extract and also comparable to most commonly used antiplaque agent Chlorhexidine. Therefore to reduce the risks associated with the use of synthetic antiplaque agents Triphala formulation can be used as a potent substitute for antiseptics.

Keywords : colony forming units, median lethal dose, minimum inhibitory concentration, zone of inhibition

INTRODUCTION

Dental caries and gingivitis are both associated with the accumulation of bacterial plaque on and around the teeth. Therefore prevalence of these two diseases should be affected by methods aimed at preventing plaque formation or removing plaque from the teeth.[1] Self-performed mechanical plaque removal is an unquestioned method of plaque control.[2] Tooth brushing with chemical based antimicrobial drugs is most widely practiced form of oral hygiene in most countries.[3] However prolonged use of antimicrobial agents result in increased bacterial resistance[4] as well as several adverse effects on oral mucosa. Hence awareness among public is increasing to adapt traditional or indigenous medicines which is not only safe but also has an enhanced anti-inflammatory properties.[5] Now a days, traditional medicine is becoming popular due to the factors such as availability, affordability, cultural familiarity and family influence.[6] In light of these facts, dental healthcare approach is directed towards holistic method like Ayurveda in managing diseases and conditions related to dental plaque.[7] Ayurvedic herbs have nature's own power of remedies. Formulated herbal toothpowder and paste are available that are effective and

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safe for number of diseases. These ingredients are combined in such a way that helps in cleaning of teeth, prevention from various dental problems such as bleeding gums, sensitive tooth, formation of tartar etc.[7] With this knowledge in background the present study was designed with the aim to evaluate and compare the effects of three plant's fruit extracts Amalaki (*Phyllanthus emblica*), Bibhitaki (*Terminalia bellirica)* and Haritaki (*Terminalia chebula*) alone as well as in formulation (Triphala) on four most common clinical isolates causing dental plaque and gingivitis as well as on MTCC strain. These local plants were selected on the basis of their medicinal properties against dental plaque and gingivitis as reported in various literatures.[8]

MATERIALS & METHODS

Sample Collection and Bacterial Enumeration:

Dental plaque samples were collected from twenty volunteers. Volunteers exhibiting mild to moderate supragingival plaque and no evidence of gingival inflammation and strains were selected. Supragingival plaque was collected and inoculated in Nutrient broth (HiMedia, India). For viable cell enumeration broth culture was serial diluted with autoclaved PBS up to 10-0 to 10-5 and 100ul of each dilution was spread on nutrient agar plate and incubated overnight at 37°C. Numbers of viable colonies were calculated using the following method.

$$\text{CFU/ml original sample} = \frac{\text{no of colonies}}{\text{plate}} \text{x (1/ml aliquot plated) x dilution factor}$$

Preparation of Plant extract and Triphala-formulation:

Fresh fruits (taken without seed) of Amalaki (*Phyllanthus emblica*), Bibhitaki (*Terminalia bellirica*) and Haritaki (*Terminalia chebula*) were purchased from local market in Patna, Bihar, India and authenticated by Botany Department, Patna University, Patna. Plant materials (fruits) were shade dried. The dried fruits were crushed to coarse powder consistency and aqueous extract was prepared (1g/ml) in DD water separately.[9] The filtrate of each extract was used either alone or in combination. For Triphala-formulation dry fruits of all the three medicinal plants were finely powdered in equal amounts and stirred with boiling distilled water for about 90 min.[9] The filtrate was concentrated under reduced pressure. The concentrated liquid was spray-dried to get the dry powder of "Triphala-formulation"(yield ~40%).

Physical characteristic and phytochemical screening of extracts:

The physical characters of the extract were noted and the percentage yield of each extract was calculated. The fruit extracts were screened for the presence or absence of secondary metabolites using standard procedures. The analysis was conducted as per standard methods.[10,11]

Assay of antibacterial activity:

Screening of plant extracts for their antibacterial activity was conducted using the agar well diffusion method.[12] About 100 µl of test compound was introduced into the well and the culture plates were kept in refrigerator for diffusion for 30 min and then incubated overnight at 37°C. The antimicrobial activity was interpreted by measuring the diameter of zone of inhibition in mm.

Determination of Minimum Inhibitory Concentration (MIC):

To measure the MIC values, micro-broth dilution method was used. The reconstituted extracts were assayed against clinical isolates and MTCC890 in BHI medium. Equal volume of the various concentrations of each extract and BHI broth were mixed in micro-tubes and McFarland standard of the organism suspension was added to each tube. The tubes were incubated aerobically at 37°C for 24 hours. The MIC was determined by sub culturing the test dilution on BHI agar and further incubated for 24 hours. The highest dilute on that yielded no single bacterial colony was taken as the Minimum inhibitory concentration or Minimum bactericidal concentration.

Preparation of herbal formulation as mouthwash:

This study was conducted to measure the decrease in total viable count of oral cavity after using 10% herbal formulation as mouthwash at the end of 15 days, twice a day usage and to compare the same with 0.2% chlorhexidine.[13] All dried powder equally mixed and dissolved in distilled water to form 10% of the extract. To improve the patient compliance, 2ml of glycerine (sweetening agent) was added to the solution. The solution was brought to boil for 10 min, cooled and filtered.

***In vitro* Study Design:**

A total of 60 volunteer subjects (for various treatments) with their age ranging between 15-50 years were selected from the Outpatient Department of Periodontics, Faculty of Dental Sciences, Patna Dental College and Hospital, Patna, Bihar, India. The criteria for the selection of subjects were as follows:

Inclusive criteria: No missing teeth, No development anomalies, No cervical abrasion and erosion on enamel surface.

Exclusive criteria: Medically compromised, emotionally/mentally disturbed and pregnant females, those subjects with history of drug induces gingival enlargement, pregnancy gingival enlargement, pubertal gingival enlargement and fibrotic gingival enlargement.

The efficacy of plaque removal by different extracts and its formulation was tested during 2-3 week periods of supervised and controlled tooth brushing. Average baseline plaque score and experimental plaque score were recorded on a Performa. Plaque accumulation was recorded using Turesky-Gilmore-Glickman modification (1970) of Quigley-Hein Plaque Index System.

Cytotoxic assay:

Cytotoxic assay of each plant extract was determined by using *Artemia salina* (Brine shrimp) for lethal dosage (LD_{50}).[14] Each plant extract was prepared by diluting to 1 ppm concentration from different dilutions and were analyzed for the lethal dosage against *Artemia salina* on watch glasses.1ml of different dilutions of concentration 5, 50 and 500 ppm were added to three watch glasses and incubated at room temperature for 24 hours. The lethal dosage is calculated by comparing the live ones against dead ones and percentage of live *Artemia* were calculated.

Statistical Analysis:

Statistical analysis was performed using analysis of variance (ANOVA) to determine the differences among products tested. In the presence of significant differences, pair wise comparisons were made *via* Tukey Honestly Significant Difference (HSD) test. The Tukey HSD test was used as the post hoc test to control the possibility of alpha-error owing to smaller sample size. The confidence level of the study was kept at 95%; hence, a '*p*' value < 0.05 indicated statistically significant differences.

RESULTS AND DISCUSSION

The percentage yield of each of the aqueous extract of *Terminalia bellirica* fruit was found to be maximum (~23%) with dark greencolour and forest belly like odour while *Phyllanthus emblica* has minimum yield (10%) with dark brown colour and aromatic odour. The percentage yield of *Terminalia chebula* was found to be 14% having brown colour and aromatic order (Table 1). It has been reported that the difference in percentage yield of any plant extract depends on solvent used, the solubility of various ingredients, methods and types of extraction used. The qualitative determination of active phytochemicals in aqueous extracts of dried fruit of each plant was found positive for alkaloids, saponins, flavonoids, glycosides, fixed oils and fats, tannin and phenolic compounds while negative for proteins and amino acids (Table 2). This indicates that these active phytochemicals are responsible for antimicrobial activity. Results obtained are in agreement of other coworkers who demonstrated that free radical scavenging property and anti-plaque activity of these active phytoconstituents may be important as an effective agent to treat patients with dental caries and to prevent formation of dental plaque in near future.[9]

Triphala was used to evaluate antimicrobial activity either alone or in formulation against four clinical isolates named *Streptococcus mutants* (SP2), *Stephylococcus aureus*, (SP6), *Streptococcus mitis* (SP68) and *Streptococcus oralis* (SP69) as well as MTCC strain. The antimicrobial activity of Triphala was compared with commercially available chlrohexidine and distilled water. The antimicrobial activity was established by using agar gel diffusion and broth dilution methods. It was found that Amalaki inhibited effectively the clinical and MTCC strain with an inhibition zone of 12mm (Table 3). It also inhibits *Stephylococcus aureus*, (SP6), *Streptococcus mitis* (SP68) and *Streptococcus oralis* (SP69) and with an inhibition zone of 11mm, 11mm, and 10mm respectively (Table 3).

Table 1: Physical characteristics and percentage yield of different plant extracts.

Plant extract	Consistency	Colour	Order	Dry powder weight (g)	Dry Extract weight. (g)	Yield (%)
Emblica officinalis	Semi Solid	Dark brown	Aromatic	242.200	24.80	10.23
Terminalia bellerica	Semi Solid	Dark Green	Forest Belly like	217.06	49.60	22.79
Terminalia chebula	Semi Solid	Brown	Aromatic	300.00	43.40	14.49

Table 2: Qualitative determination of active ingredients in aqueous extracts of dried fruits of different plants.

Plant constituents	Plant Extract		
	Emblica officinalis	*Terminalia bellerica*	*Terminalia chebula*
Alkaloids	+	+	+
Saponins	+	+	+
Flavonoids	+	+	+
Glycosides	+	+	+
Carbohydrate	+	+	+
Fixedoilsandfats	–	+	+
Tannins and phenolic compounds	+	+	+
Proteins and amino acids	–	–	–

Bibhitaki showed an inhibition zone of 16mm each for clinical and MTCC strain of *Streptococcus mutants* as well as *Streptococcus mitis* while an inhibition zone of 13mm and 12mm against *Stephylococcus aureus* (SP6), *Streptococcus oralis* (SP69) respectively. Haritaki showed maximum inhibition zone of 17mm each for clinical and MTCC strain of *Streptococcus mutants* as well as *Streptococcus mitis* while an inhibition zone of 15mm and 11mm against *Stephylococcus aureus* (SP6), *Streptococcus oralis* (SP69) respectively (Table 3). Other researchers (Jagtap and karkera) had also reported that the extracts of *Terminalia chebula* strongly inhibit the growth and adherence of *S. mutants*. Oral rinsing with extract of *Terminalia chebula* significantly reduced both total bacterial counts and *Streptococcal* counts in saliva samples. Contrarily, the Triphala formulation inhibited all the four clinical isolates as well as the MTCC strain more effectively (Table 3) when compared to the individual extract as well as chlorhexidine (Table 3). Triphala inhibited the four strains namely *Streptococcus mutants* (SP2), *Stephylococus aureus* (SP6), *Streptococcus mitis* (SP68), *Streptococcus oralis* (SP69) with an inhibition zone of 18m, 14mm, 19mm, and 13mm respectively. It inhibited MTCC strain with an inhibition zone of 18mm. Our results show that Triphala formulation has more antibacterial activity on clinical isolates and MTCC strains and also when compared to chlorhexidine, it demonstrates more efficacies on these strains. Our report hence corelates with similar study exhibiting antimicrobial activity in relation to Triphala (Jagdish *et al*., 2009; Biradar*et al*., 2008). They also concluded that Triphala had potent antioxidant and antimicrobial activity and inhibited the growth of *S. mutants* and gram positive *cocci*, involved in plaque formation.

The MIC results (Table 4) showed that Haritaki is a potent inhibitor of SP2 and MTCC890 strain with MIC of 6.25% while Bibhitaki intoxicated SP6, SP68 and SP69 with MIC of 12.5%, 6.25% and 12.5% respectively. Amalaki intoxicated SP2 and MTCC890 strain with MIC of 25% while SP6, SP68 and SP69 with MIC of 50% which reflects that Amalaki alone is not an effective inhibitor of pathogenic bacteria. In contrast Triphala formulation showed better inhibitory effect than any of the other three extracts. It showed an MIC value of 3.125% against SP2 and MTCC strain while an MIC of 6.25% against SP6, SP68 and SP69. In our result Triphala was found better that chlorhexidine (Table 3). This suggests that the anti-oral-streptococci efficacy of Triphala is better than that of Chlorhexidine.

The average baseline plaque score for anterior, posterior, facial, lingual, upper and lower part decreased significantly by treating with all the three extracts as well as Triphala formulation as depicted in Table 5. In contrast to the effect of herbal extract alone, the Triphala formulation showed more percentage reduction of dental plaque. When the subjects were treated with Triphala the average basal scores were reduced significantly by approximately 60%, 56%, 65%, 57%, 54%, and 58%

Table 3: Antibacterial activity of different plant extracts and chlorhexidine.

Bacterial Strain	Activity (Inhibition zone in mm)				
	Amalaki (*Emblica officinalis)*	Bibhitaki (*Terminalia bellerica)*	Haritaki (*Terminalia chebula)*	Triphala-formulation	Chlorhexidine
SP2	12	16	17	18	18
SP6	11	13	15	15	14
SP68	11	16	17	19	19
SP69	10	12	11	14	13
MTCC890	12	16	17	19	18

Table 4: Minimum Inhibitory Concentration of each extracts and Triphala

Bacterial Strain	Comparision of MIC				
	Amalaki	Bibhitaki	Haritaki	Triphala	Chlorhexidine
SP2	25%	25%	6.25%	3.125%	3.125%
SP6	50%	12.5%	50%	6.25%	6.25%
SP68	50%	6.25%	25%	6.25%	12.5%
SP69	50%	12.5%	12.5%	6.25%	6.25%
MTCC890	25%	6.25%	6.25%	3.125%	3.125%

respectively which is comparable to chlorhexidine (Table 5). Control subjects demonstrated almost null reduction in dental plaque. This could be attributed to the antibacterial property of Triphala as demonstrated by Khorana et al., (1959). Subjects in Group III (treated with chlorhexidine) showed significant statistical reduction at the end of the study (Table 5). This result has also been supported by the study of Emilson, (1994) where it was found that chlorhexidine treatment reduces *S. mutants* count during test period. In Group I (Control group) the majority of the samples showed bacterial growth in the range of 191±22 CFU/ml (Table 6). In a similar study conducted by Olmezet al., (1998) it was found that when distilled water was used as a mouthwash in the control group, there was no significant reduction in *S.mutants* count as observed in this study. The cytotoxicity of all extracts were measured by the brine shrimp larval mortality assay which is widely accepted as a convenient probe for potential cytotoxicity and pharmacological activity in plants (Meyer et al., 1982; McLaughlin et al., 1998). In all the three groups of brine shrimp LC_{50} assay, no cytotoxic activity was detected up to 500µg/ml of aqueous extract of the three plant's fruit extract (Table 7).

Table 5: Efficacy of each extracts and Triphala on reduction of different segments of dental plaque.

Segment	Percentage Dental Plaque reduction				
	Amalaki	Bibhitaki	Haritaki	Triphala	Chlorhexidine
Anterior	50.00	48.21	46.55	59.32	58.21
Posterior	49.33	47.22	50.00	56.25	57.29
Facial	50.63	43.04	37.33	65.82	59.01
Lingual	46.00	52.70	53.25	57.89	55.00
Upper	47.14	56.52	54.79	54.93	46.66
Lower	46.43	46.43	40.68	58.93	49.12

Table 6: Efficacy of each extract and Triphala on reduction of CFU bacteria causing dental plaque.

Formulation	No. of Samples	CFU in pre-treatment	CFU Reduction in post treatment
Amalaki	10	188±34	110.5±23
Bibhitaki	10	189±27	109±24
Haritaki	10	190±33	107±35
Triphala	10	191±35	90.2±36
Chlorhexidine	10	193±25	90.6±27
Control	10	191±35	191±22

Table 7: LC_{50} evaluation.

Treatment	Number used / Number dead
Group I (5 µg/ml)	10/0
Group II (50 µg/ml)	10/0
Group III (500 µg/ml)	10/0

CONCLUSION

Ayurveda system of medicine has a long history of therapeutic potential and can reduce the side effects caused by the use of antibiotics. The traditional natural products such as Triphala which shows a scientific proof of its superior antimicrobial potential. The herbal formulation reduces > 55% *in vitro* bacterial colony forming units (CFU) causing plaque over a period of 2 weeks, showing no any cytotoxicity at a concentration as proposed in the present invention and is useful in forms of lotion cream, mouthwash and mouth rinse etc. Hence Triphala can be used as a potential alternative of chlorhexidine (anti-plaque agent).

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