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Evaluation of the Antimicrobial efficacy of a novel polyherbal extract against Streptococcus Mutans: An in-vitro study

Research Article

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Abstract

Aim: The present study was done to assess the antibacterial efficacy of a polyherbal extract against Streptococcus mutans (S. mutans) in-vitro. Materials and methods: In the current study, the ethanolic extracts of *Haritaki, Bhibitak, Amalaki, Yashtimadhu*, Haridra and Vacha were subjected to microbiological assay. The microbial growth inhibitory potential of the polyherbal extract was determined by using the agar disc diffusion method. Results: Mean zone of inhibition of polyherbal extract mix against S. mutans at 48 hours was 24mm. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of polyherbal extract mix on S. mutans was 0.1gm/mL and 0.2gm mg/mL respectively. Conclusion: The polyherbal extract had antimicrobial efficacy against streptococcus mutans. This polyherbal extract could have potential clinical implications. Clinical significance: as many chemical agents used for prevention of dental caries might have long term adverse effects, use of plant based products could be an excellent treatment alternative. This polyherbal extract possesses antibacterial properties against streptococcus mutans and hence could be used for prevention of dental caries. The herbs used in the study are easily available, economically feasible and are being used since time immemorial.

Key Words: Streptococcus mutans, Polyherbal, Antimicrobial, *Triphala, Terminalia Chebula, Terminalia bellerica, Emblica officinalis, Glycyrrhiza glabra, Curcuma longa, Acorus Calamus.*

Introduction

Dental caries is a chronic infectious disease caused by formation of biofilm on tooth surface. Amongst the various bacteria found in the human oral cavity, Streptococcus mutans (S.Mutans) is one of the most virulent cariogenic species.(1,2) Cariogenic factors that enable S.Mutans to predominate in the dental biofilm and induce the development of caries include the ability to synthesize intracellular and extracellular polysaccharides, acidogenic, acidiuric, acidophilic properties, production of cell adhesion proteins and mutacins. (1,3)

A gold standard antimicrobial agent widely used in dentistry is Chlorhexidine. The side-effects like staining and altered taste limit its long term use and acceptability by the patients.(4,5) Despite several over-the-counter therapeutic agents available for the prevention of caries, natural remedies are warranted. The field of Ayurveda and plant-based medicines has been practiced in India since time immemorial and is now practiced globally as alternative medicine. Various herbs like Triphala, Haridra and Vacha are known to have antimicrobial effect.(6,7)

In the Ayurvedic literature, "Sarangdhar Samhita" dated 1300 AD, had highlighted the concept of poly-herbalism. These polyherbal formulations are known to show better effectiveness and attain greater therapeutic efficacy in varied diseases. The beneficial effects of herbs is executed due to various phyto-constituents present in herbs. These effects are further enhanced when compatible herbs are combined together (8,9,10). Polyherbal formulations are a product of the nature, and hence are relatively safer than allopathic medicine, with the added advantage of having fewer adverse effect(11). With the above pharmacological facts under consideration, in the current research, six plant i.e Haritaki, Bhibitak, Amalaki (together are known as *Triphala*), Yashtimadhu, Haridra, and Vacha were selected to formulate a polyherbal extract.

The dried powders of fruits (without seeds) of *Terminalia Chebula* (Haritaki), *Terminalia bellerica* (Bibhitak), and *Embellica Officinalis* (Amalaki) mixed in equal parts formulate *Triphala*. *Triphala* (12,13), root extracts *Glycyrrhiza glabra* (Yashtimadhu) (14,15) *Curcumin longa* (Haridra)(16) and leaf and rhizome of

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Acorus Calamus (Vacha)(17) have demonstrated noteworthy antimicrobial activity against S.Mutans.

The aforementioned herbs individually have an incredible antibacterial and anti-cariogenic effect. With this point in view, the present research was carried out to assess the synergistic antibacterial efficacy of a mix of these herbs (polyherbal mix) against S.Mutans in-vitro.

Material and Methods

The present study was performed in-vitro at a private laboratory between March and October 2019. The research protocol was approved by Institutional Ethics Committee, VSPM Dental College and Research Center, Nagpur.

Identification and collection of plant material

The samples of *Terminalia Chebula* Retz ((Haritaki), *Terminalia bellerica* Roxb (Bibhitak), *Embellica Officinalis* Gaertn (Amalaki), *Glycyrrhiza glabra* Linn (Yesthimadhu), *Curcuma Longa* Linn (Haridra), *Acorus Calamus* Linn (Vacha) were collected from field, identified, and validated by a taxonomist (Table 1)

Washing and drying of plant material

The plant parts were hand washed in running water followed by distilled water and then air-dried in dark at room temperature for 6-8 weeks. Air drying helps to preserve the heat labile components and drying in dark reduces the loss of light sensitive active ingredients in the plants.

Grinding and size reduction

The dried plant material was ground to a fine powder in a sterile mixer as per the guidelines given by Melaku.(18) This is an important pre-preparation step for an ideal extraction. Homogenized and smaller particle sizes lead to better and increased surface contact with the extraction solvent resulting in efficient extraction. The powdered plant parts were stored in air-tight containers and refrigerated at 4º Celsius till further use.

Preparation of Extract

25 grams of the powdered herb was filled individually in the thimble and extracted successively using 150 ml ethanol. The solvent extraction was done using a Soxhlet Extractor for 48 hours. The extracts were further concentrated by means of a rotary flash evaporator. After complete solvent evaporation, the extract was weighed and stored in sealed bottles. The stock solution was prepared by mixing 100 mg of extract in 1000µl dimethyl sulfoxide.

Preparation of polyherbal extract

The polyherbal combination of plant extract was prepared using the measured quantity of stock solution of individual extracts. All the six plant extracts i.e. Haritaki, Bibhiktaki, Amalaki, Yashtimadhu, Haridra, Vacha were mixed in the proportion of 1:1:1:1:1:0.5 respectively.

Preparation of Micro-organism and growth conditions

Streptococcus Mutans strains (MTCC no. 497) were purchased from CSIR Institute of Microbial Technology, Chandigarh. Bacteria were sub-cultured on Nutrient Agar at 37ºC prior to being cultured in Brain Heart Infusion (BHI) broth for 24 h in an anaerobic chamber with 5% CO_2. Bacterial cells were collected by centrifugation at 3000 rpm for 15 minutes, washed twice, and re-suspended in 0.1% peptone water. Turbidity was adjusted to match the same of 0.5 McFarland standards (McFarland, 1907).

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The lowest concentration that inhibited visible growth of Streptococcus Mutans was recorded as the Minimum Inhibitory Concentration (MIC). The lowest concentration of the extract that did not yield any colony growth of Streptococcus Mutans after the incubation period was regarded as Minimum Bactericidal Concentration (MBC).

The microbial growth inhibitory potential of the polyherbal extract was determined by using the agar disc diffusion method as defined by Jorgensen et al.(19, 20) The polyherbal extract was subsequently two-fold serially diluted with BHI broth. Concentration used for MIC and MBC was ranging from 0.05µgm to 0.5gm. Several microbial colonies of cultured bacteria were mixed with sterile ringer solution to prepare the inoculum with turbidity comparable with the 0.5 McFarland standardized solution equal to 106–108 CFU / ml. 50ul Streptococcus mutans were inoculated into the Brain Heart Infusion Medium (BHI). Filter paper discs containing 20 µl (2mg/ disc) polyherbal extract at varying concentrations were placed on the agar surface. The petri dishes were incubated under 37 °C for 48 h. Zone of Inhibition in mm was recorded at 48 h. The test was tested in duplicate and the mean of the zone of inhibition were considered.

Results

This study was undertaken to evaluate the antimicrobial activity of a polyherbal extract containing Triphala, Yashtimadhu, Haridra, Vacha extracts against Streptococcus Mutans using the agar disc diffusion method. The concentration range of the polyherbal extract used for MIC and MBC was 0.05 µgm to 0.5 gm.

The polyherbal extract showed a MIC value of 0.1gm/ml. The zone of inhibition was recorded at 24mm. (Fig:1) The MBC was valued at 0.2gm/ml. (Table 2) Increasing the concentration beyond this did not produce any significant increase in the zone of inhibition. Lower concentrations of the polyherbal mix did not demonstrate any valid antibacterial activity on S.Mutans.

Table 1: Components of Polyherbal extract and its quantity

S.No	Name of Drug	Botanical Name	Family	Plant part	Quantity
1	*Haritaki*	*Terminalia chebula Retz*	Combretaceae	Fruit rind	1 part
2	Bibhitaki	*Terminalia bellerica Roxb*	Combretaceae	Fruit rind	1 part
3	Amalki	*Embellica officinalis Gaertn*	Phyllanthaceae	Fruit rind	1 part
4	*Yashtimadhu*	*Glycyrrhiza glabra Linn*	Fabaceae	Root	1 part
5	*Haridra*	*Curcuma longa Linn*	Zingiberaceae	Rhizome	1 part
6	*Vacha*	*Acorus calamus Linn*	Acoraceae	Rhizome	1 /2 part

Table 2: Anti-microbial activity of Polyhebal extract mix for S.mutans

S No	Test parameter	Method	Micro-organism	Growth Medium	Incubation Temperature	Incubation Time	Test result
1	Minimum Inhibitory Concentration (MIC)	Agar Disc Diffusion	Streptococcus mutans	BHI Agar	36ºC	48 hrs	0.1gm/ml
2	Minimum Bactericidal Concentration (MBC)		Streptococcus mutans	BHI Agar	36ºC	48hrs	0.2gm/ml

Discussion

The current in-vitro experimental study was an innovative attempt to explore the antimicrobial effectiveness of a polyherbal extract (conflate of six plant part extracts) on caries pathogen, S.Mutans. The polyherbal extract inhibited the complete growth of S.Mutans at 0.2gm/ml. The MIC of the mix was 0.1gm/ml and the antimicrobial efficacy was found suitable against S mutans. Thus, conflate of these herbs with antimicrobial activity could prove to be essential therapeutic agents to prevent and treat dental caries.

The individual components of this conflate have already been proved by various studies. Nayak et al. proposed that, T. *chebula* (Haritaki) increases the salivary pH and inhibits S.Mutans.(21) Mizan et al proved strong antimicrobial activity of methanolic extracts of T. *Bellerica* (Bhibitak) fruits against S.Mutans with a zone of inhibition of 12.5 mm.(22) Tannic acid is one of the major constituents of the ripe fruit of *Terminalia Chebula* and T. *Bellerica*. It is considered to be bacteriostatic and bactericidal. The anti-microbial action is associated with its ability to destroy microbial adhesins, enzymes and cell envelope transport proteins.(7) At very low levels of 50 µg/mL, Triphala has excellent bacteriostatic and bactericidal activity. Approximately 5% *Triphala* solution prevents S.Mutans by 83.7% (23) MIC of Triphala against S.Mutans was found to be 12.5%. (24) 0.6% *Triphala* mouthwash is known to have considerable anti-cariogenic action, comparable to that of chlorhexidine but with least disadvantages and at a lesser cost. (23, 25)

Ann et al observed that deglyzirrhized G. *glabra* (Yashtimadhu) significantly inhibited S.Mutans biofilm formation at concentrations over 4 µg/ml for glucose and 16 µg/ml for sucrose, respectively.(15) Ethanolic extracts from roots of G glabra were shown to have antibacterial activity against S.Mutans with 0.195mg/mL MIC and 3.12 mg/mL MBC. (26) The antibacterial activity of Yashtimadhu is attributed to glycyrrhizin, which dose-dependently prevents the glucosyltransferase activity of mutans streptococci, which is involved in the formation of insoluble glucans required in biofilm formation.(26,27)

Lee et al evaluated the effect of essential oil in *Curcumin longa* (Haridra) plant to inhibit the cariogenic property of Streptococcus mutans and concluded that at concentrations from 0.5% to 4% inhibited the growth and acid production of S.mutans. (28) Haridra may inhibit the biofilm activity of S.Mutans through inhibition of gtfB, gtfD, and gtfC gene (glucosyltransferases) and ftf gene (fructosyltransferase) involved in biofilm formation (29). Tambur et al listed MIC of A. *calamus* (Vacha) ethanol extract for S mutans at 300 µg/ml.(8) The alpha and beta asarones in the rhizomes and leaves of A. *calamus* are responsible for the antimicrobial activities. (17)

As mentioned above, even though the active phytochemical constituents of single plant have been well recognized, they are sometimes unable to achieve the desired therapeutic effects. To overcome this problem, plants of varying potency may be combined together to produce an enhanced therapeutic effect as compared to individual plant. This phenomenon of positive herb-herb interaction is known pharmacodynamics synergism. (11, 30) In the current research, the polyherbal extract may have presented with the synergistic effect when the individual active constituents with similar therapeutic activity targeted S.mutans by diverse mechanism of action. This polyherbal mix will also demonstrate least adverse effects as the herbs have been independently used in various traditional remedies.

In the present study, Dimethyl sulfoxide was used as a solvent due to its specific properties like being highly polar. It is an aprotic solvent and is beneficial in bringing out the pure properties of all the components of the herb being dissolved. (31) Soxhlet, apparatus is ideal for extraction of dry, finely divided solid, which was attained with fine grinding. The Soxhlet was used for extraction as this method requires smaller quantity of solvent. It causes displacement of transfer equilibrium by repeatedly bringing fresh solvent in contact with the solid matrix. Soxhlet extractor also maintains high extraction temperature with heat from the distillation flask. Filtration is also not required for the extract with this process(20).

The agar disc diffusion susceptibility technique was used to assess MIC and MBC in the current study. It is simple, useful and well-standardized procedure. The zone diameters of the polyherbal extract were interpreted using the scoring published by Clinical and Laboratory Standards Institute (CLSI) (32). The advantages of the disc diffusion method is its simplicity and it does not require special equipments. The results can also be easily interpreted by clinicians. Its results are "qualitative," and allows the researcher to interpret MIC conveniently and also provide flexibility in selection of discs for testing drug efficacy against the said micro-organism. It is the least expensive of all susceptibility methods.(33)

The Zone of Inhibition reached 24 mm for this polyherbal formulation, indicating the high potential of this polyherbal formulation against S.mutans. The combination of using plant extracts has many advantages, such as higher potency associated with the synergistic effect of phytochemicals, and a lower rate of development of resistance, as they are multifaceted mixes that can disrupt the adaptability of microorganisms.(34) The present results are promising and may augment the use of natural products instead of chemical therapeutics or could also be used as adjunctive therapy. Clinical applications of this polyherbal extract include when preparing mouthwashes, toothpastes, herbal oral wipes etc. for daily use.

The limitations of the present study are that, this polyherbal extract was tested in-vitro and the actual time these herbs come in contact with oral fluids might influence its efficacy. Further studies should be carried out with the above polyherbal extract as mouthwash or other forms to evaluate its antimicrobial activity.

Conclusion

Efficacy of polyherbal extract provides a cumulative effect of all the herbs hence could provide better efficacy. The observations of this study suggest that this polyherbal extract had anti-microbial efficacy against streptococcus mutans, which is the most common cariogenic bacteria. Thus, it could have multiple clinical applications.

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