10.5005/jp-journals-10024-1528

ORIGINAL RESEARCH

An *in vitro* Study to determine the Effect of *Terminalia chebula* Extract and Its Formulation on *Streptococcus mutans*

[1]Sushma S Nayak, [2]Anil V Ankola, [3]Sharda C Metgud, [4]Uday Kumar Bolmal

ABSTRACT

Aim: Many weapons are available in the arsenal of a dental professional to combat dental caries, which is almost ubiquitously present. From a public health perspective, most of these weapons are far from being an ideal drug. Hence, there is a demand for better and effective antibacterial agents. This factor stimulated the process of the present study. The aim of the study was to determine the effect of ethanol extract of *Terminalia chebula* on *Streptococcus mutans*.

Materials and methods: Dried ripe fruits of *Terminalia chebula* were procured and powdered. Physical tests were done to estimate purity of the fruit powder. Hydroethanolic and aqueous extracts were prepared according to standard procedures. Minimum inhibitory concentration of the extracts was determined by tube dilution method and confirmed by agar dilution method. The effect of the hydroethanolic extract on sucrose induced adhesion, glucan-induced aggregation and on glycolysis of *Streptococcus mutans* was also assessed. Preservative, gelling agent and sweetener were added in suitable quantities to the ethanol extract, and mouthrinse was formulated. Minimum inhibitory concentration of the formulation was also determined.

Results: Yield was better in case of aqueous extract. The Minimum inhibitory concentration of hydroethanolic extract was determined to be 2.5%. Minimum inhibitory concentration of the aqueous extract was determined to be 10%. Hydroethanolic extract of *Terminalia chebula* (2.5%) inhibited sucrose induced adherence and aggregation of *Streptococcus mutans in vitro*.

Conclusion: The mouthrinse formulated from ethanol extract of *Terminalia chebula* demonstrated substantial antibacterial activity and could be used as an effective anticaries agent.

[1]Senior Lecturer and Research Scholar, [2]Professor and Head [3]Professor, [4]Assistant Professor

[1,2]Department of Public Health Dentistry, KLE VK Institute of Dental Sciences, KLE University, Belgaum, Karnataka, India

[3]Department of Microbiology, JN Medical College, KLE University, Belgaum, Karnataka, India

[4]Department of Pharmaceutics, KLE College of Pharmacy Belgaum, Karnataka, India

Corresponding Author: Sushma S Nayak, Senior Lecturer and Research Scholar, Department of Public Health Dentistry KLE VK Institute of Dental Sciences, KLE University Belgaum, Karnataka, India, Phone: 09449289359, e-mail: dr_sushmanayak@yahoo.co.in

Clinical Significance: *Terminalia chebula* mouthrinse can be effectively used in clinical practice as an anticaries mouthrinse with additional benefit being that it is safe and economical.

Keywords: Terminalia chebula, Combretaceae, Dental caries, Herbal mouthrinse, Streptococcus mutans, Hydroethanolic extract.

How to cite this article: Nayak SS, Ankola AV, Metgud SC, Bolmal UK. An *in vitro* Study to determine the Effect of *Terminalia chebula* Extract and Its Formulation on *Streptococcus mutans*. J Contemp Dent Pract 2014;15(3):278-282.

Source of support: Nil

Conflict of interest: None declared

INTRODUCTION

Oral diseases are among the most prevalent diseases, especially in the developing countries of the world. It has also been reported that they are the fourth most expensive disease to treat.[1] Taking these aspects into consideration, prevention of these diseases appears to be the only effective alternative.

Dental caries and periodontal diseases are bacterial infections which are more prevalent as compared to other oral diseases.[1] A number of antibacterial chemicals have been in use for the prevention and treatment of these oral infections. However, most of them lack the properties that an ideal antibacterial mouthrinse should possess. These conventional antimicrobial mouthrinses which are presently available possess certain drawbacks, like irritation, burning sensation, alteration of taste and high cost.[2,3] Thus, there is a need to discover compounds that are effective, safe and economical.

In the recent years, the focus of research is on the use of herbs in the treatment of various diseases. It has been reported that 80% of drug molecules are natural products or inspired from natural compounds. Research on herbal products is gaining attention, mainly because they are economical, effective and exert minimal adverse effects if any. The rich biodiversity of India has remained untouched as far as discovery of new chemical entities are concerned.[4] Herbs are finding application in the treatment and prevention of virtually all diseases including oral diseases.

Among the vast treasure of herbs available, *Terminalia chebula* is one such herb which has been commonly used for

prevention and treatment of various diseases from ancient times. It is widely available in various parts of South Asian countries. *Terminalia chebula* is known as 'Haritaki', 'Alalekayi' or 'Harad'. Studies conducted regarding *Terminalia chebula* have shown that it exerts wide range of activities like anti-bacterial, antiviral, antifungal, antioxidant, cytoprotective, hepatoprotective and radioprotective activities.[5] Thus, it would be of interest to study the effect of such a beneficial herb as a mouthrinse.

Various studies have been conducted to assess the antimicrobial efficacy of *Terminalia chebula* on various microorganisms.[5] However, only three studies have assessed the effect of aqueous extract of *Terminalia chebula* on *Streptococcus mutans*.[6-8] But, none of the studies conducted till now have assessed the effect of ethanol extract of *Terminalia chebula* on *Streptococcus mutans*. Since *in vitro* studies are the preliminary studies done to establish the efficacy of any antibacterial agent, an *in vitro* study was conducted with an aim of determining the effect of *Terminalia chebula* extract prepared using two different solvents, on *Streptococcus mutans*. Another objective of this study was to formulate a mouthrinse of *Terminalia chebula*.

MATERIALS AND METHODS

The present study was an *in vitro* experimental study.

Collection of Fruits

Dried ripe fruits of *Terminalia chebula* were collected from an ayurvedic pharmacy. They were authenticated by a Professor from Botany Department, RLS College, Belgaum. The reference number of the authentication certificate is RLSI/BOT/Consul-65.

The fruits were visually inspected and appropriate ones were selected. The seeds were removed and the fruit was ground into coarse powder using a domestic grinder. The powder was then passed through a sieve.

Preparation of the Ethanol Extract

One hundred gram of powder was weighed and then 70% ethanol was added. Soxhlet method of extraction was used to prepare the extract.[9] The mass obtained was subjected to distillation to recover alcohol. It was then kept on water bath to remove water content from the extract. The extract was then stored in an air tight sterile glass container.

Preparation of Aqueous Extract

Aqueous extract was prepared by maceration process as follows: 100 gm of *Terminalia chebula* powder was weighed and put in a conical flask. Distilled water was added. Few drops of chloroform were added. The mix was kept in the conical flask for a period of 7 days with intermittent shaking of the mix. After 7 days, the mass obtained was filtered through a muslin cloth. The filtrate obtained was then placed in a China dish on a water bath with a conical flask inverted over it, and the mass was then dehydrated. The opening of the conical flask was plugged with cotton to avoid external contamination. A semisolid mass was obtained, which was stored in an air tight container.[9]

Analysis of the Extract

Phytochemical analysis of *Terminalia chebula* fruit powder was done. Total ash value and acid soluble ash value were determined to determine the purity of the powder. Alcohol extractive values were also estimated to determine the yield.[9]

Determination of Minimum Inhibitory Concentration

Streptococcus mutans culture MTCC 497 was procured from IMTECH, Chandigarh. Brain Heart Infusion broth was used for culturing the MTCC 497 *Streptococcus mutans* strain. Minimum inhibition concentration of aqueous extract and ethanol extract was determined using tube dilution method as per the standard protocol. Standardization of inoculum was done by using 0.5 Mc Farland turbidity standard which is equivalent to 105 CFU/ml. The dilutions used were 1:10, 1:5, 1:2.5, 1:1.25 and 1:0.625. Equal volumes of standardized *Streptococcus mutans* suspension was added to all the tubes. Equal volume of Brain Heart Infusion broth and *Streptococcus mutans* suspension was used as control. It was incubated at 37°C for 24 hours anaerobically. The readings were taken in comparison with the control tube. The final dilution where no turbidity was observed and considered as the minimum inhibitory concentration (MIC value) of the antimicrobial agent. MIC was also confirmed with agar dilution method.[10]

Adherence Inhibition

The effect of ethanol extract of *Terminalia chebula* was assessed using Brain Heart Infusion broth containing 2% sucrose and 0.1 ml of the extract, which was inoculated with the overnight culture of *Streptococcus mutans*. The concentrations tested include 10, 5, 2.5, 1.25 and 0.625% of the ethanol extract of *Terminalia chebula*. Control consisted of cells grown in BHI broth containing sucrose and 0.1 ml PEG 400. The tubes were inclined at 30° and incubated at 37°C for 24 hours. The adherent and nonadherent bacteria were quantified spectrophotometrically.[6]

Inhibition of Glycolysis

Two milliliter of saliva was collected, and 0.1 ml 5% glucose and 0.1 ml of various concentrations of the extracts ranging from 0.625 to 10% were added. Saliva/glucose mixes containing 0.1 ml PEG 400 was used as control. The pH

of the saliva/glucose mixes was recorded immediately, at 60-minute intervals.[6]

Inhibition of Glucan-induced Aggregation

Streptococcus mutans cells were incubated with from 0.625 to 10% of the extract for 1 hour at 37° and then dextran was added. *Streptococcus mutans* cells were incubated for 1 hour with PEG 400 and this acted as control. The culture fluid was then scored for cell aggregation.[6]

Formulation of Mouthrinse

Based on MIC value, formulation of mouthrinse was done. Sodium carboxymethylcellulose, methyl paraben and mannitol were used in suitable quantities and the mouthrinse was prepared. MIC was determined again. Same quantities of sodium carboxymethylcellulose, methyl paraben and mannitol were added to distilled water and its' effect on MTCC 497 was determined.

RESULTS

Phytochemical analysis revealed that the fruits contained tannins, glycosides, amino acids. The yield was high when water was used as solvent. Ash value was found to be within the limits specified by API (Table 1).

Minimum inhibitory concentration was 10% for aqueous extract and 2.5% for ethanol extract of *Terminalia chebula* fruit. Minimum inhibitory concentration of the formulated mouthrinse was 2.5% .

Maximum inhibition of adherence (45%) as compared to control was observed at a concentration of 2.5% of ethanol extract (*see* Table 1).

Maximum inhibitory effect on glycolysis of *Streptococcus mutans* was observed at concentration of 1.25% of ethanol extract (Table 2).

Maximum inhibitory effect on aggregation of *Streptococcus mutans* was observed at concentration of 2.5% of ethanol extract (Table 3).

A total of 1.25% ethanol extract showed maximum inhibition of glycolysis of *Streptococcus mutans* immediately after contact with extract and 60 minutes after contact (Table 4).

A total of 2.5% of the ethanol extract had a strong effect on inhibition of aggregation of *Streptococcus mutans*. There were minute clumps of cells of *Streptococcus mutans*, when 2.5% of the extract was used. When 5, 1.25 and 0.62% of the extract was used easily visible clumps of cells of *Streptococcus mutans* were formed (Table 5).

DISCUSSION

Terminalia chebula belongs to the family *Combretaceae*. It has been considered to be 'King of medicines'.[5] Since it is

Table 1: Extractive and ash values of *Terminalia chebula* fruit powder

Extractive values		
a	Alcohol soluble extractive value	48% w/w
b	Water soluble extractive value	64% w/w
Ash value		
a	Total ash value	3% w/w
b	Acid insoluble ash value	3.5% w/w
c	Water soluble ash value	4% w/w

Table 2: Minimum inhibitory concentration of aqueous, hydro-ethanol extract and mouthrinse formulated from *Terminalia chebula*

	Extract/mouthrinse	*Minimum inhibitory concentration*
a	Aqueous extract	10%
b	Hydroethanol extract	2.5%
c	Formulated mouthrinse	2.5%

Table 3: Inhibition of adherence of *Streptococcus mutans* to glass surface, by ethanol extract of *Terminalia chebula* (mean value of 3 readings in percentage

Concentration	*Inhibition of adherence*
Control	0
10% extract	33.4 ± 2.2
5% extract	37.51 ± 1.8
2.5% extract	45.85 ± 2.3
1.25% extract	20.85 ± 1.4
0.625% extract	22.93 ± 1.8

Table 4: Effect of ethanol extract of *Terminalia chebula* on glycolysis of *Streptococcus mutans* (mean pH of 3 readings)

Concentration	*Before addition of extract*	*Immediately after addition of extract*	*60 minutes after addition of extract*
Control	7.5 ± 0.02	7.5 ± 0.01	7.5 ± 0.01
10% ethanol extract	7.5 ± 0.03	7.5 ± 0.01	7.5 ± 0.01
5% ethanol extract	8.0 ± 0.01	8.0 ± 0.01	8.0 ± 0.02
2.5% ethanol extract	8.0 ± 0.02	8.5 ± 0.01	8.5 ± 0.01
1.25% ethanol extract	8.0 ± 0.03	9.0 ± 0.02	9.0 ± 0.02
0.625% ethanol extract	8.5 ± 0.01	8.5 ± 0.01	8.0 ± 0.01

Table 5: Effect of ethanol extract of *Terminalia chebula* on aggregation of *Streptococcus mutans*

Concentration	*Extent of aggregation*
Control	Very large flocculent clumps in clear supernatant fluid
10% ethanol extract	Well-defined clumps of cells in clear supernatant fluid
5% ethanol extract	Easily visible small clumps of cells in turbid fluid
2.5% ethanol extract	Visible minute clumps of cells in turbid fluid
1.25% ethanol extract	Easily visible small clumps of cells in turbid fluid
0.625% ethanol extract	Easily visible small clumps of cells in turbid fluid

believed to have originated from ambrosa, it is known to possess immense medicinal power. It is a moderate sized or large tree found throughout India, chiefly in deciduous forests and areas of light rainfall, but occasionally also in slightly moist forests, up to about 1500 m elevation; through-out India, flowers appear from April to August, and fruits ripen from October to January. Its leaf, bark and fruit have been widely used for medicinal purpose. The only contraindication is its systemic intake in pregnancy, dehydration, weak digestion and heat stroke conditions.[5,12]

The contents of the fruit are tannins, anthraquinones and polyphenolic compounds.[12] Tannins have been assigned a number of activities including stimulation of phagocytic cells, host mediated tumor activity and a wide range of anti-infective actions. They have been traditionally used against inflammatory conditions of the oral cavity.[11]

Terminalia chebula fruit was selected for the study as it is rich in tannins (containing about 30-40% tannins). It has been reported that tannins demonstrate considerable antibacterial activity.

The most commonly used solvents in investigation of antimicrobial activity of plants are methanol, ethanol and water.[13] However, methanol is toxic and hence was not used in the present study. In the present study, two solvents were compared, water and 70% ethanol. Ethanol was used as solvent as most of the tannins are soluble in ethanol. Thirty percent water was added to ethanol so that hydrolyzable tannins are also dissolved into it. Combination of solvents helps to obtain the best solvent system.[14] It has also been reported that plant extracts from organic solvent give more consistent antimicrobial activity as compared to water extracts.[13]

The yield of *Terminalia chebula* using ethanol and water as solvent was compared by determining the extractive values. Yield is estimated by weighing a fixed small quantity of fruit powder and solvent and preparing the extract. The amount of extract obtained is weighed. The extract obtained by both methods is then compared. Extractive values are used as a means of evaluating drugs, the constituents of which are not readily estimated by other means.[9] In the present study, it was observed that the yield was more when water was used as solvent. The total ash value, acid insoluble ash value and water soluble ash value of the drug were in the range recommended by API thus indicating that the purity of the drug used was in acceptable limit.

Various procedures of extraction exist which include maceration with agitation, percolation or continuous extraction (e.g. in Soxhlet extractor). Soxhlet extraction method was used to prepare extract in the present study. This extraction method has been reported to be more efficient as the crude drug sample is continually exposed to fresh solvent.[11]

The minimum inhibitory concentration of the extracts was determined using dilution method as it has been reported to be more sensitive and is able to distinguish between bacteriostatic and bacteriocidal effects and is used for quantitative determination.[10,11]

Phytochemical analysis of the fruit powder in the present study revealed the presence of constituents like tannins, glycosides and amino acids. The antibacterial activity of the extract and the formulated mouthrinse may be attributed to tannins.

In the present study, the MIC of aqueous extract determined was 10%, whereas the previous studies have reported MIC of 6%.[6,7] The difference could be due to the variation in the fruit used in the studies or variation in the extraction preparation procedures. The MIC of ethanolic extract in the present study was 2.5%. To the best of author's knowledge, this is the only study which has determined the effect of ethanolic extract on *Streptococcus mutans*. Since ethanol which is a more polar compound was used as solvent, higher quantities of tannin would have dissolved and this could have led to inhibition of the micro-organism at lower concentration. It has also been various variables that influence the quality of herb which include climatic conditions under which the plant grows, choice of extraction methods and antimicrobial test reported that there are methods[14,15] Variation in extraction procedures like length of extraction period, solvent used, pH, temperature, particle size and solvent-to-sample ratio also influences the quality of the extract. These factors also could have led to variation in the results of the present study and previous researches.

In the present study, mannitol was used as sweetner in the formulation of *Terminalia chebula* mouthrinse. This was chosen as other sweetners, like xylitol, sorbitol exert anticariogenic effect by acting on *Streptococcus mutans*.[16] Methyl paraben was used as preservative and sodium carboxymethylcellulose was used to provide viscosity to the mouthrinse. These agents were tested for their effect on *Streptococcus mutans* and it was observed that these agents did not inhibit the growth of *Streptococcus mutans* thus attributing the antimicrobial activity of the formulation to *Terminalia chebula*.

Mannitol, sodium carboxymethylcellulose and methyl paraben was added to distilled water and its effect on *Streptococcus mutans* was assessed. The growth of *Streptococcus mutans* was not inhibited by this mixture, thus showing that the effect exerted by the formulated mouthrinse is solely due to the herb. *Terminalia chebula* exerts a bactericidal effect on *Streptococcus mutans*.

It has been reported that *Streptococcus mutans* cause dental caries by metabolizing fermentable sugars by glyco-

lysis. The end product of glycolysis is acids. Acid helps in demineralizing the tooth structure. *Streptococcus mutans* produce extracellular polysaccharide or glucans which help in adherence to tooth structure and also in aggregation.[17] This is a major requisite in caries process otherwise the bacteria would be swept away by salivary flow. Hence, the effect of the extract on these three major factors have been studied. Ethanolic extract of *Terminalia chebula* inhibited sucrose mediated adherence of *Streptococcus mutans* to glass surface and also on their aggregation. Maximum inhibition of adherence and aggregation was observed at the minimum inhibitory concentration; however, there was a reduction in the effect as the concentration decreased below the minimum inhibitory concentration. Concentrations of extract above the minimum inhibitory concentration also demonstrated inhibition of adherence and aggregation of *Streptococcus mutans*; however, it was lower than that observed at the minimum inhibitory concentration, but higher than concentrations below the minimum inhibitory concentration. The reason could be that the concentration of 2.5% may be bactericidal whereas the other concentrations might be bacteriostatic. The effect of the extract on glycolysis of *Streptococcus mutans* was also assessed. It was observed that there was higher increase in pH which remained so even after 1 hour at a concentration of 1.25%. Minimum inhibitory concentration showed relatively lesser inhibition of glycolysis than concentration of 1.25%, however, the difference was less. Inhibition of glycolysis could be due to inhibitory effect of tannin on glucosyl transferase enzyme in *Streptococcus mutans*.[18]

The present study indicates that ethanolic extract of *Terminalia chebula* shows considerable antibacterial activity and thus may prove to be an effective anticaries mouthrinse. Semisynthetic modifications or combination of herbal and conventional antimicrobial mouthrinse of the herb can also be attempted.

Further *in vivo* studies need to be conducted to determine the effectiveness of ethanolic extract of *Terminalia chebula* in human subjects. Also, studies are needed to compare the effect of *Terminalia chebula* with standard antimicrobials like fluoride and chlorhexidine.

REFERENCES

1. Erik PP, Denis B, Hiroshi O, Estupinan-Day S, Charlotte N. The global burden of oral diseases and risks to oral health. Bull World Health Organ [serial on the Internet] 2005 Sep [cited 2011 Oct 21];83(9):661-669. Available at: http://www.scielosp.org/scielo.php?script=sci_arttext&pid=S0042-96862005000900011&lng=en. http://dx.doi.org/10.1590/S0042-96862005000900011.
2. Mc Roy LC, Rich SE, Miller. Adverse events associated with chlorhexidine use. J Am Dent Assoc 2008;139(2):178-183.
3. Gagari E, Kabani S. Adverse effects of mouthwash use. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995 Oct;80(4):432-439.
4. Bhutani KK, Gohil MV. Natural products drug discovery research in India: status and appraisal. Indian J Experiment Biol 2010;48:199-207.
5. Chattopadhyay RR, Bhattacharya SK. Terminalia chebula: an update. Pharmacogonosy Reviews 2007;19(1):151-156.
6. Jagtap AG, Karkera SG. Potential of the aqueous extract of Terminalia chebula as an anticaries agent. J Ethnopharmacol 1999 Dec 15;68(1-3):299-306.
7. Carounanidy U, Satyanarayanan R, Velmurugan A. Use of an aqueous extract of Terminalia chebula as an anticaries agent: a clinical study. Indian J Dent Res 2007 Oct-Dec;18(4):152-156.
8. Nayak SS, Kumar BR, Ankola AV, Hebbal M. The efficacy of Terminalia chebula rinse on Streptococcus mutans count in saliva and its effect on salivary pH. Oral Health Prev Dent 2010; 8(1):55-58.
9. Evans WC. Trese and Evans. Phytochemistry. 15th ed. In Pharmacognosy: WB Saunders Publishers p. 137-150.
10. Koneman EW, et al. Koneman's colour atlas and textbook of microbiology. 6th ed; Lippincott Williams and Wilkin 2005.
11. Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African J Biotechnol 2008;7(12):1797-1806.
12. The Ayurvedic Pharmacopoeia of India. Available at: www.ayurveda.hu/api/API-Vol-1.pdf. Accessed on: 20-07-11.
13. Das K, Tiwari RKS, Shrivatsav DK. Techniques for evaluation of medicinal plant products as antimicrobial agent: current methods and future trends. J Med Plants Res 2010;4(2):104-111.
14. Nostro A, Germanò MP, D'angelo V, Marino A, Cannatelli MA. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett Appl Microbiol 2000 May;30(5):379-384.
15. Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol 1999; 86(6):985.
16. Deshpande A, Jadad AR. The impact of polsyol-containing chewing gums on dental caries. J Am Dent Assoc 2008 Dec; 139(12):1602-1614.
17. Loesche WJ. Role of Streptococcus mutans in dental decay. Microbiol Rev 1986 Dec;50(4):353-380.
18. Schee A. Modes of action of currently known chemical antiplaque agents other than chlorhexidine. J Dent Res 1989;68: 1609-1616.