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Original Article

Dentistry Section

Anti Cariogenic Effect of *Terminalia Chebula*

VIDHYA REKHA[1], JAYAMATHI[2], RAMAKRISHNAN[3], DEVAKI VIJAYALAKSHMI[4], PRABU[5], NANDHA KUMAR[6], SUNAYANA MANIPAL[7], KEERTHIDAA[8]

ABSTRACT

Background: *Terminalia chebula* is one of the traditional medicines used in the treatment of many diseases and possesses a wide variety of therapeutic activities. The aim of this study was to determine the antimicrobial properties of *Terminalia chebula* against oral pathogens related to caries.

Materials and Methods: In this study, Antimicrobial activity was tested using Kirby bouer method by streaking. Total phenol and total flavonoid content were analysed. Twenty high caries risk patients were subjected to rinse aqueous extract of *Terminalia chebula*. Salivary samples were collected for pH and microbial screening. Oral pathogens were identified by qualitative biochemical analysis.

Results: The total phenol content of extract was found to be 21.33 ±1.633 (mean ± SD) and total flavonoids was found to be 23.17 ± 2.317 (mean ± SD). There was a gradual increase in pH till 45mts post-rinse when compared to pre-rinse was observed. Antimicrobial effect of *Terminalia Chebula* aganist microbes showed that there was a significant reduction between the pre-rinse and post-rinse samples.

Conclusion: These promising findings suggested the presence of antimicrobial activity of *Terminalia Chebula* against oral pathogens and proven to be an effective alternate antimicrobial agent.

Keywords: Antimicrobial activity, Catalase test, Dental caries, *Terminalia chebula*

INTRODUCTION

Dental caries, is one of the most prevalent infectious diseases, which results in destruction of dental hard tissue. It is the consequence of the interaction among oral microflora, diet, dentition and oral environment. Oral bacteria include *Streptococci*, *Lactobacilli*, *Staphylococci*, *Corynebacteria*, various anaerobes and yeast. Some *Lactobacillus species* have been associated with dental caries although these bacteria are normally symbiotic in humans and are found in the gut flora [1]. *Streptococcus mutans* (*S.mutans*) is the main causative agent, although additional acidogenic microorganisms may be involved. The ability to metabolize carbohydrates, to adhere to and to form bio film on tooth surfaces is associated with the cariogenicity of this pathogen [2]. Many studies on caries related microorganisms indicated that some natural products can affect survival and virulence of *S.mutans* [3]. The inhibitory effects of cranberry, propolis, coffee, wine, cocoa, tea, and some dairy products on *S. mutans*, in vitro and in experimental animal studies, have been reported [4]. Dental caries prevention is preferable to treatment, because treatment might come too late to avoid the loss of tooth. Conventional preventive methods such as the use of alcohol or antibiotics, e.g. chlorhexidine, erythromycin, ampicillin and penicillin, have proven effective in preventing dental caries but these have several undesirable side effects. Thus, there is a need for alternative prevention and treatment options that are safe, effective and economical. The main aetiological agents of dental caries are *S. mutans* and *Lactobacillus species* [5]. A mouth rinse is a chemotherapeutic agent used as an effective home care remedy to enhance oral hygiene and prevent dental caries by targeting the cariogenic bacteria. A variety of synthetic antimicrobial mouthwashes are available to prevent dental caries. However, excessive use of these chemicals has been reported to cause adverse effects [6]. Due to the adverse effects of synthetic drugs considerable attention has been paid to natural remedies which are safe and effective. Today, there is widespread interest in drugs derived from plants, which has led to the screening of several medicinal plants for their potential antimicrobial activity. In our study we have selected one medicinal plant—*Terminalia chebula* (*T.chebula*) to study the efficacy of its extract as an anticariogenic mouth rinse.

T. chebula in the family Combretaceae has been extensively used in Ayurveda, Unani and Homoeopathic medicine. The fruit is rich in easily hydrolysable tannins, anthraquinone, flavonol, carbohydrates, glucose and sorbitol [7]. Several studies revealed that the fruit contains flavonol, high phenolic content, glycosides, triterpenoids, coumarin conjugated with gallic acids called chebulin as well as other phenolic compounds [6,8]. *T.chebula* exhibited anti-bacterial activity against number of Gram-positive and Gram-negative human pathogenic bacterial species. It also exhibits anti-fungal and anti-viral properties [9]. The dried ripe fruit of *Terminalia chebula* has traditionally been used in the treatment of asthma, sore throat, vomiting, hiccough, diarrhoea, bleeding piles, gout and heart and bladder disease [10]. Both in vitro and in vivo studies on *T. chebula* found out the therapeutic evaluations of the fruit, for example, antimutagenic [11], antidiabetic [12], antiproliferative [13], antioxidant [14], antimicrobial [15], and hepatoprotective activities [14]. However, only limited toxicity data of this plant has been reported [16]. Very few studies were carried out to establish the anti cariogenic effect of *T. chebula* against *Streptococcus mutans*. This study is the first attempt to strengthen the previous studies to show the therapeutic effect on other caries causing bacteria, like *Lactobacillus acidophilus* and *Candida* albicans along with *Streptococcus mutans*. The extract of *T. chebula* when used as an anticariogenic mouth rinse against three dental caries causing bacteria *S. mutans, Lactobacillus* and

Phyto constituents	Mean ± SD
Total phenol content (mg/g of gallic acid)	21.33 ±1.633
Total phenolic content (mg/g of quercetin)	23.17 ± 2.317

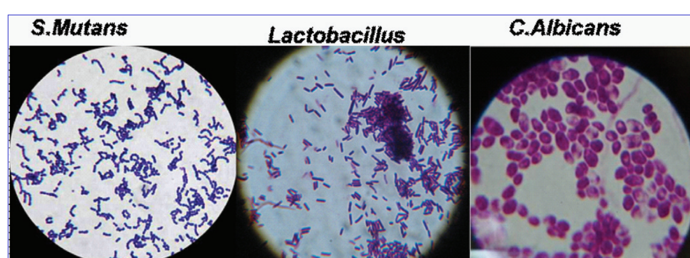
[Table/Fig-1]: Quantitative analysis of phytocnstituents of *T.Chebula* Extract

S.No	Time (min)	Mean ±SE
01	Pre rinse-0	6.423 ±0.0156
02	Post rinse- 15	7.330 ± 0.300©
03	Post rinse- 30	7.381 ± 0.0225©
04	Post rinse- 45	7.100 ± 0.0188©
05	Post rinse- 60	6.720 ±0.0479©

[Table/Fig-2]: Effect of *T. Chebula* salivary pH analysis
All values are Mean ± SE of 20 samples. ©p < 0.001 as compared to Group –1 by POST HOC Tukey HSD

S.No	Species	Reduction in %
01	*S.Mutans*	50.71
2	Lactobaillus	74.81
03	*C.albicans*	68.57

[Table/Fig-3]: Antimicrobial effect of *T.Chebula* aganist microbes



[Table/Fig-4]: Gram staining of oral pathogens

Strain	Concentration of extract				
	400µg / ml	200 µg / ml	100 µg / ml	50 µg / ml	25 µg / ml
S.mutans	No growth	No growth	No growth	No growth	Turbidity
Lactobacilli	No growth	No growth	No growth	Turbidity	Turbidity
Candida albicans	No growth	No growth	No growth	Turbidity	Turbidity

[Table/Fig-5]: Antimicrobial activity of *T.Chebula*

Candida. This study also focused on the total phenol content, total flavanoid content to ensure the anti bacterial effect of *T. Chebula*.

MATERIALS AND METHODS

Preparation of the extract

The extract was prepared according to Jagtap et al., [17] with slight modification. The dried ripe fruit of *T. chebula* was obtained and ground into a fine powder. A 10g amount of powdered fruit was separately soaked in 100ml of hot sterile distilled water (100°C) and allowed to stand for 30min on a water bath with occasional shaking, and kept undisturbed for 24h. Each preparation was filtered through a sterilized Whatman No.1 filter paper and the filtered extract was concentrated under vacuum evaporator. The dried extract thus obtained was exposed to UV rays for 24h and checked for sterility on nutrient agar plates and stored in labelled sterile bottles in a freezer at 4°C until further use. This concentrate was then diluted with sterile distilled water to get a mouth rinse of 10% (w/v) concentration [17].

QUANTITATIVE PHYTOCHEMICAL ANALYSIS

Estimation of Total Phenolic Content

Total Phenolic Content was determined according to the method of Sadasivam & Manickam [18]. The largest group of plant secondary metabolites belongs to phenolic group. They range from simple structures with only one benzene ring to larger molecules such as tannins, anthraquinones, flavonoid and coumarins. They are defined as compounds that have at least one hydroxyl group attached to a benzene ring. Phenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects including antioxidant activity. Total phenolic content is determined in extracts by using the Folin-Ciocalteu method. This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation a green blue complex measurable at 750 nm. is formed. The total phenol content of extract is being related to the antioxidant activity shown by it. The total phenol content is expressed as gallic acid equivalent in mg/g or % w/w of the extracts.

Estimation of Total Flavonoid Content

Flavonoids are water-soluble polyphenolic compounds which are extremely common and wide spread in the plant kingdom as their glycosides. It consists of a single benzene ring joined to a benzo-gamma-pyrone structure. They are able to complex metal ions, acts as antioxidants and bind to proteins such as enzymes and structural proteins. The different classes within the group are distinguished by additional oxygen containing heterocyclic rings and hydroxyl groups. These include the catechins, leuco anthocyanidins, flavonones, flavones, anthocyanidins, flavonols , chalcones, aurones and isoflavones. Total flavonol is determined by aluminum chloride colorimetric method.

0.5 ml of the extract is separately mixed with1.5ml ethanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30min, the absorbance of the reaction mixture is measured at 415nm. Using the standard curve the total flavonol content of the extracts are calculated. The total flavonol content is expressed as quercetin equivalent in mg/g or % w/w of the extracts [19].

Sample collection

A total of 20 patients, who satisfied the selection criteria of high caries risk group were included in the study. The patients subjected for the study were informed about study protocols and written consent was obtained. They were not allowed to eat for at least 2 hr. Unstimulated salivary samples were collected, using sterile syringes from every patient before they were subjected for rinsing with extract(Pre-rinse). Then they were allowed to gargle and to be held in the mouth for 15mts, 30mts, 45mts and 60mts. Salivary samples were collected after 15min, 30min, 45min and 60min (post-rinse). A salivary sample from each patient was collected for pH, buffering capacity before (pre-rinse) and after rinsing (post-rinse) at repeated intervals of 15, 30, 45 and 60 mts and for microbial screening, saliva was collected only after 60min.

Salivary pH analysis

Salivary samples from the 20 patients were taken and the pH analysed using a chair-side kit (GC saliva check) with the use of pH sensitive test strips.

Microbial screening

Pre-rinse and post-rinse salivary samples (after 60mts) were collected for microbial analysis. Muller Hinton agar medium was used. The technique used for assessing microbial analysis was the Kirby Boaver technique by streking. The salivary samples were diluted with sterile saline in the ratio of 1:100 and streaked on the medium for 48h at 37°C. After incubation, the colony forming units were recorded by colony counter by counting chamber technique and mean percentage of reduction (PR) was calculated.

Biochemical analysis

The isolated bacteria were identified by qualitative analysis.

Catalase test

The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. To perform this test, a single isolated colony was streaked on a glass slide and one drop of 3% hydrogen peroxide (Merck, Germany) was added on to it and observed.

Germ tube test

GT-7 is single use vial intended for the preliminary identification of *Candida* albicans by germ tube formation (GT70002-0613). The GT-7 tube contains pure lyophilized Fetal Bovine Serum in a single test quantity.

Gram staining test

The presumptive colonies were examined through Gram-staining technique. The isolated bacteria were examined using gram staining kit (Becton, Dickinson and Company, USA) according to Collins and colleagues technique and were observed under light microscope with a magnification of 100x.

Antimicrobial activity

Antimicrobial activity was examined by determining the minimal inhibitory concentration (MIC) using macro-dilution broth technique [20]. *T. chebula* showed antimicrobial activity against *S. mutans*, *Lactobacillus* and *C.albicans* strains and optimal MIC was at 50µg/ml, 100µg/ml and 100µg/ml respectively. After 24h incubation period, the inhibition zone was recorded.

STATISTICAL ANALYSIS

All values used in analysis represented as mean ± SE. Multiple comparison of POST HOC Tukey's test was applied to find out the statistically significant groups and Independent t-test was applied to compare groups.

RESULTS

Quantitative analysis of phytoconsituents of *T. chebula*

Phenolic compounds are defined as compounds that have at least one hydroxyl group attached to a benzene ring. The total phenol content of extract is being related to the antioxidant activity shown by it. It was found to be 21.33 ±1.633 (mean ± SD). Flavonoids are water-soluble poly phenolic compounds which acts as antioxidants and the result was found to be 23.17 ± 2.317 (mean ± SD) [Table/Fig-1].

pH analysis

[Table/Fig-2] shows the results of pH analysis. All the groups showed a significant difference between the pre-rinse and post-rinse groups. On comparing the groups at various time intervals, at 15, 30, 45 and 60mts, there was no significant difference among groups (p> 0.001). There was a gradual increase in pH till 45mts post rinse when compared to pre-rinse. At 60mts, post-rinse the pH values are almost close to pre-rinse pH values . Among the post rinse groups (15 min, 30min and 45min) there is no significant difference at various time intervals.

Microbial screening

Qualitative Biochemical analysis

Catalase Test

Most cytochrome containing organisms produce a catalase enzyme which breaks down hydrogen peroxide into oxygen and water. When a small amount of a catalase producing organism like *S.mutans* was introduced into hydrogen peroxide, effervescence of oxygen form as a result of the enzyme's activity. Immediate evolution of gas bubbles indicating a positive test and conforms *S.mutans* strains.

In performing catalase test, no effervescence was observed indicating that the isolated bacterium is catalase negative and could not mediate the decomposition of H_2O_2 to produce O_2. It was well known that *Lactobacillus acidophilus* is catalase negative. Formation of rare bubbles after 20 to 30 sec is considered a negative catalase test.

Germ tubes formed within 2h indicated the *Candida* albicans as oral pathogen.

[Table/Fig-3] shows antimicrobial effect of *T.Chebula* aganist microbes. There was a significant reduction between the pre-rinse and post-rinse samples. *S. mutans* showed highly significant antibacterial activity (50.71%) of *T. chebula* against *S. mutans*, *Lactobacillus* showed 74.81% and *C.albicans* showed 68.57% of reduction.

Identification of microbial strains

The isolated bacteria were observed by light microscope. It is clear that the bacteria was gram positive, rod shaped coccobacilli, occurring singly or in chains. The gram staining results indicated that the isolated bacteria could be identified as *lactobacilli*, gram positive *S.mutans* and budding yeast cells [Table/Fig-4].

Anti microbial activity

Antimicrobial activity was examined by determining the minimal inhibitory concentration (MIC). *T. chebula* showed antimicrobial activity against *S. mutans*, *Lactobacillus* and *C.albicans* strains and optimal MIC was at 50µg / ml, 100µg / ml and 100µg / ml respectively [Table/Fig-5].

DISCUSSION

T. chebula possesses a wide variety of activities like antibacterial, antifungal, anti-viral, anti-carcinogenic, anti-oxidant, adaptogenic and anti-anaphylactic, hypolipidemic, hepatoprotective, cardio protective, antidiabetic, wound healing, immunomodulatory and chemo preventive [21].

Natural antioxidants such as flavonoids and polyphenols are believed to possess antioxidant properties due to their reducing and chelating capabilities. Flavonoids and polyphenols are secondary plant metabolites that are widely distributed in fruits, leaves, bark, and other parts in plants with free radical scavenging abilities [22]. Terminalia species, which are a rich source of flavonoids, tannins, and many phenolic derivatives, which exerts antioxidant capabilities [23]. The antioxidant property of phenolic compounds is attributed to their ability to absorb and neutralize free radicals. It was well known that *T.Chebula* exhibited antimicrobial activity in the treatment of caries.

Several studies have reported that *S. mutans*, *Lactobacillus species* and *C.albicans* as a main etiological event [24]. *S. mutans* were considered to be the main etiological microorganisms in caries, with *Lactobacilli* and other microorganisms participating in the disease progression. Occasionally, some other microorganisms have been traced as initiator microorganisms. The *S.mutans* ferment many different sugars and they appear to metabolize sucrose to lactic acid more rapidly than other oral bacteria. This was thought to be related to the multitude of enzyme systems catalysing the reactions of transport and metabolism of sucrose expressed by these organisms [25]. It is evident in this study that *T.Chebula* showed a definite reduction in the microbial activity and an increase in the pH resulting in marked anticariogenic effect. Increase in pH was an essential pre-requisite for an ideal mouth rinse. The findings of the present study confirmed the positive relationship with the previous report [24,26].

T. chebula (aqueous extract) strongly inhibited the microbial growth, sucrose induced adherence and glucan induced aggregation of *S.mutans*. Mouth rinsing with a 10% solution of the extract inhibited microbial count [27]. *T. chebula* demonstrated preventive action on dental caries has shown that owning to the microbial reduction may be attributed to the presence of tannins and flavonoid [28].

Chlorhexidine gluconate is a cationic biguanide with broad-spectrum antimicrobial action, whose effectiveness in decreasing the formation of dental biofilm (plaque) and gingivitis has been demonstrated in several clinical studies. Its mechanism of action is that the cationic molecule binds to the negatively-charged cell walls of the microbes, destabilizing their osmotic balance [28]. The literature supported that the anti-bacterial role of polyphenols were effective anticariogenic compounds, interact with microbial membrane proteins and inhibiting adherence of bacterial cells to the tooth surface. It also inhibits glucosyl transferase and amylase, a food derived acids that can damage tooth enamel, and inhibits tooth demineralization by interactions with the organic matrixes. Since commonly available mouthwashes against oral pathogens exert adverse effects, the aqueous extract of *T. chebula* reduced the microbial quantity and proved to be an effective anti-cariogenic agent [29].

CONCLUSION

T. chebula is one of the most versatile plants having a wide spectrum of pharmacological and medicinal activities types of compounds having diverse chemical structure. Though it has a number of pharmacological activities due to the presence of various types of bioactive compounds, very little work has been done on the plausible medicinal applications of this plant against the diseases particularly on multidrug resistant bacterial pathogens. Hence extensive investigation is needed to exploit their therapeutic ability to combat diseases including drug resistant infections. As the global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, a drug development programme should be undertaken to develop modern drugs with the compounds isolated from *T. chebula* effective against different types of diseases.

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PARTICULARS OF CONTRIBUTORS:

1. Post Graduate Student, Department of Public Health Dentistry, SRM Dental College, Chennai, Tamil Nadu, India.
2. Professor and HOD, Department of Biochemistry, Meenakshi Ammal Dental College, Chennai, Tamil Nadu, India.
3. Professor, Department of Periodontics, Meenakshi Ammal Dental College, Chennai, Tamil Nadu, India.
4. Professor and HOD, Department of Orthodontics, Meenakshi Ammal Dental College, Chennai, Tamil Nadu, India.
5. Professor and HOD, Department of Public Health Dentistry, SRM Dental College, Chennai, Tamil Nadu, India.
6. Professor and HOD, Department of Orthodontics, Indira Gandhi Institute of Dental Sciences, Pondicherry, India.
7. Reader, Department of Public Health Dentistry, SRM Dental College, Chennai, Tamil Nadu, India.
8. Under Graduate Student, Meenakshi Ammal Dental College, Chennai, Tamil Nadu, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Vidhya Rekha,
NO 1, Bank Street,
B-2," Sri Bala Enclave", 2nd Floor, Kilpauk, Chennai, India.
Phone: 9176997111, E-mail: vidh_dr@yahoo.co.in

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