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Screening for Anti-Caries Activity of Selected Medicinal Plants

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Abstract: Greater proportion of the world population, especially the emerging countries rely on the old-fashioned scheme of medicines. The use of herbs in medicine is getting popularized because of its natural origin with no or minor side effects. In the contemporary investigation six medicinal plants *Terminalia chebula* L *Coleus vettiveroides* L, *Acmella oleraceae* L, *Vitex negundo* L, *Acacia catechu* W and *Schleichera oleosa* L were ended. Effective inhibition of dental pathogens by solvent extract of selected medicinal plants was evidenced. Among the selected plants dried fruits of *Terminalia chebula* L showed marked anti-caries activity against *Streptococcus mutans* (MTCC 497) and *Streptococcus oralis* (MTCC 2696) in comparison with antibiotic. It was found to significantly inhibit biofilm formation by *Streptococci* sp., an oral pathogen. This erudition affords some scientific rationale for the use of this plant for the treatment of dental diseases.

Key words: Dental Pathogen · Medicinal Plants · Anti-Caries Activity · Anti-Biofilm Activity

INTRODUCTION

Dental caries is a pandemic disease that affects the individuals of virtually all aged groups. It is a multifactorial, irreversible microbial disease of the calcified tissues of the teeth, characterized by demineralization of the inorganic portion and destruction of the organic substances of the tooth which often leads to the cavitation [1-2]. The development of dental caries involves acidogenic and aciduric Gram positive bacteria (Mutant *Streptococci* sp*., Lactobacillus* sp., etc) [3]. Although a large number of organisms have been isolated from dental caries, a few genera are commonly preponderate which are *S. mitis, S. sanguinis, S. salivarius, S. oralis, S. sobrinus* and *S. mutans* found in occlusal and smooth surface caries [4].

Dental caries prevention is preferable to treatment, because treatment might come too late to avoid the loss of the tooth. Conventional preventive methods such as the use of antibiotics, e.g. chlorhexidine, erythromycin, ampicillin and penicillin, have proven effective in preventing dental caries. However, unwarranted employ of these chemicals has been testified to alter the oral and intestinal flora, staining of the teeth and restorations, taste of food and a burning sensation at the tip of the tongue [5]. Natural products have shown to be high-quality alternative headed for synthetic chemical substances for caries prevention [6].

Herbal remedies have a long history of use for gum and tooth problems such as dental caries [7]. It is well known that our ancestors have used unrefined sea salt, neem seed oil, twigs of babul, mango or neem tree to clean their teeth [8]. Many popular toothpastes contained extracts of herbal medicinal plants which include Clove, Menthol, Neem and Triphala [9]. The contemporary research focused on the anti-caries activity of particular indigenous medicinal plants against three dental caries causing microorganisms.

MATERIALS AND METHODS

Assortment of the Plant Material: Medicinal plants were collected from Palakkad District and identified at Botanical Survey of India, Tami Nadu Agricultural University, Coimbatore, Tamil Nadu, India, powdered and stored at 4°C throughout the investigation. The dried fruits of *Terminalia chebula* L and roots of *Coleus vettiveroides* L, leaves of *Acmella oleraceae* L and *Vitex negundo* L, bark of *Acacia catechu* W and *Schleichera oleosa* L were used to prepare extract. Ethnobotanical realities of the selected medicinal plants have been given in Table 1.

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Table 1: Ethnobotanical facts of medicinal plants used in this study

Botanical Name	Family Name	Common Name	Parts Used
Terminalia chebula L	*Combretaceae*	Kadukkai	Dried fruit
Coleus vettiveroides L.	*Lamiaceae*	Vettiver	Root
Acmella oleracea L	*Asteraceae*	Aakravu	Leaf
Vitex negundo L.	*Lamiaceae*	Nochi	Leaf
Acacia catechu W.	*Fabaceae*	Karungali	Bark
Schleichera oleosa L.	*Sapindaceae*	Poovatham	Bark

Preparation of Plant Extract: Plant samples were shadow dried and crumpled thoroughly to acquire the coarse powder with a mechanical grinder. The solvent extraction was equipped by using Soxhlet apparatus. 100 g of the powdered plant materials were loaded in Soxhlet assembly and extracted using ethanol for 72 hrs. At the end, the extract was passed through Whatman filter paper No 40. The filtrate was concentrated by using vacuum evaporator under pressure and temperature 30°C. The crude extract was prepared by dissolving known quantity of the dry extract in DMSO (Dimethly sulfoxide), to have a stock solution of 100 mg/mL.

Phytochemical Screening of Extracts: Phytochemical screening was ended by the methods of Harborne [10] for examining the secondary metabolites responsible for curing ailments.

Tested Microorganisms: Freeze dried form of human dental caries pathogens, *Streptococcus mutans* (MTCC 497), *Streptococcus oralis* (MTCC 2696) and *Lactobacillus acidophilus* (MTCC 10307) were obtained from Microbial Type Culture Collection, Chandigarh, India.

Media Used: Brain heart infusion agar (BHI) and Lactobacillus MRS agar are the transport media used to maintain clinical dental caries sample in viable condition. Muller Hinton agar (Hi media Pvt. Ltd., Mumbai, India) for conducting antibacterial tests and Brain heart infusion broth for biofilm assay was used.

Antimicrobial Susceptibility Testing: Agar well diffusion method prescribed by National Committee for Clinical Laboratory Standards (NCCLS 2006) was employed in antimicrobial susceptibility testing for the different solvent extract concentrations of each plant [11]. Muller Hinton Agar (100 mL) was sterilized in separate conical flasks, cooled and inoculated with 1 mL of the respective test bacterial suspension. After thorough mixing, the inoculated medium was transferred into sterilized Petri dishes and on solidification of agar medium, wells of about 6 mm diameter were punched into it with a sterilized cork borer. A 100 ul of the extracts ranging from 25, 50 and 75ug/mL were added in the respective wells. A well containing 25 µg of Tetracycline was used as positive and 20% of DMSO applied to other well served as negative control. The plates were incubated at 37°C and the diameter of inhibition zone was measured after 24 h of incubation [12]. The experiments were performed in triplicates and the mean values of the diameter of inhibition zones with ± standard deviation were tabulated.

Determination of Minimum Inhibitory Concentration (MIC) : Different concentrations of plant extracts (0.5 to 2.0 mL) was prepared and dispensed into test tubes and made up to 5 mL with sterile Muller Hinton broth. One drop of an overnight broth culture of dental caries causing microorganisms was inoculated and the tubes were incubated for 24 - 48 h at 25°C to determine the MIC. Controls were also maintained simultaneously inoculated medium without plant extract. The MIC value was reserved as the lowest concentration of compound at which there is no visible growth of the test organisms after 24 - 48 h of inoculation at 25°C [13].

Biofilm Inhibition Assay: Brain Heart Infusion (BHI) broth containing 1% D-glucose was prepared and 3 mL of the broth was transferred into screw cap tubes. The broth was then sterilized by autoclaving at 121°C, 15lbs, for 15 min. The screw cap tubes were then inoculated with 30 µl of overnight grown culture of *S. mutans* and *S. oralis* were served as a control. The plant extracts were added in to the screw cap tubes containing broth culture at a concentration of 50 µL from stock were served as a Test. The tubes were then tilted at an angle of 30°C and incubated at 37°C for 18 h. Screw cap tubes inoculated with test organism alone were taken as the control. After incubation the supernatant (Non adherent cells) was carefully decanted without disturbing the adhering cells and the pH was noted at this time. Washed the tube containing biofilm with saline (0.85% NaCl), added 3 mL saline and mixed well to separate the cells which adhered with glass surface. Then the changes in O.D. were recorded at 550 nm [14].

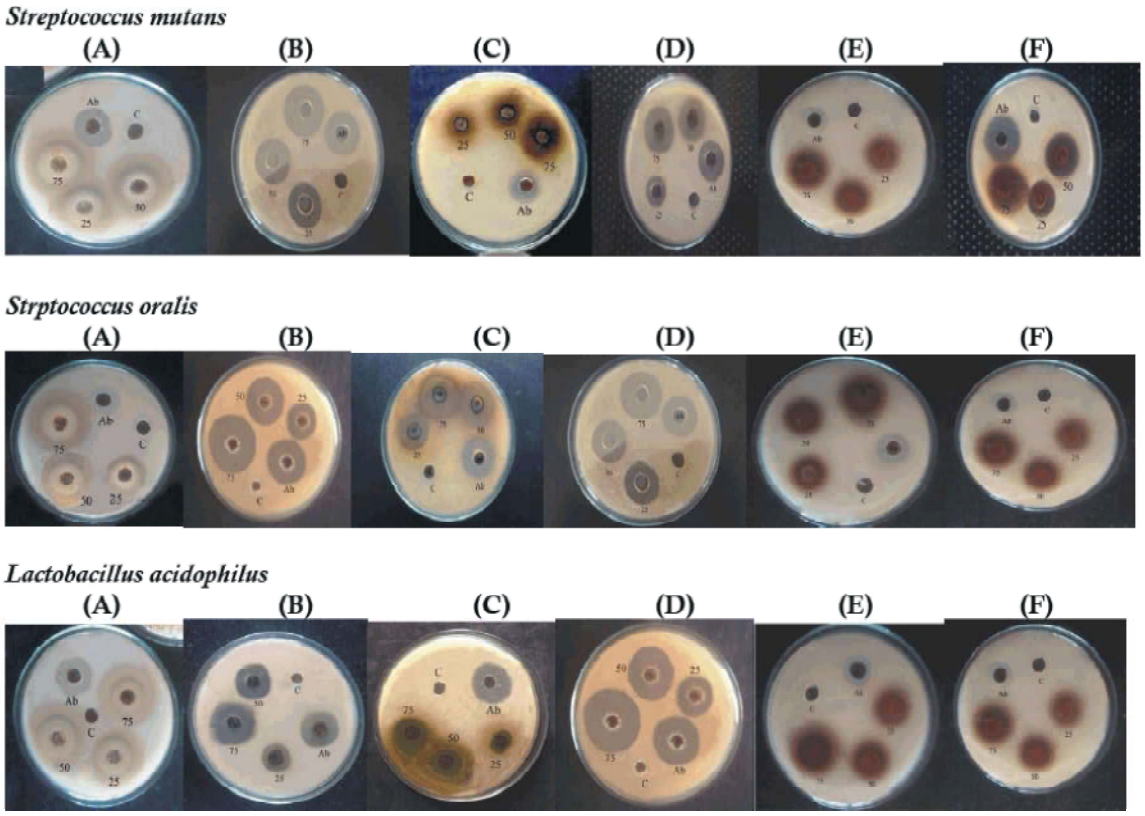

Plate 1: Antimicrobial activity of ethanol extract of medicinal plants against dental caries causing microorganisms (A) *Terminalia chebula* L; (B) *Coleus vettiveroides* L; (C) *Vitex negundo* L; (D) *Acmella oleraceae* L; (E) *Accacia catechu* L; (F) *Schleichera oleosa* L at different concentration (25µg/ml; 50µg/ml; 75 µg/ml); (Ab) Antibiotic – Tetrachycline and (C) Control.

RESULTS AND DISCUSSIONS

The medicinal plants were acquired from Palakkad District, Kerala, India; shade dried at 25°C for 5 days. Powdered well and subjected to successive solvent extraction.

Phytochemical Screening of the Collected Medicinal Plants: The solvent (Ethanol) extracts of the collected medicinal plants were analyzed for the presence of phytochemicals. The results of the phytochemical screening were tabulated in Table 2. The ethanolic extract of all the six medicinal plants showed the presence of Alkaloids, Saponin, Flavonoids, Tannin and phenolic compounds, Steroids Carbohydrates, Cardioglycosides and Terpenoids. Whereas Chaiya *et al*. [13] reported the presence of tannins and flavonoids and phenolic compound in extracts of *Glycyrrhiza glabra* L, *Terminalia chebula* Retz var. *chebula, Terminalia bellirica* (Gaertn.) Roxb., *Phyllanthus emblica* L. Phytochemical screening showed alkaloids, carbohydrates, saponins, tannins, flavonoids, anthraquinones and cardiac glycosides in the ethanol and petroleum ether extract of stem bark of *Acacia nilotica* whereas cardiac glycosides while proteins, amino acids, fixed fats and oils were absent in it [15,16]. Similarly the plant extract of *Acacia arabica, Glycyrrhiza glabra, Achyranthes aspera* rich in glycosides, alkaloids, flavonoids, phenol, tannin and saponin has been reported [17].

Activity of Powdered Extracts of Medicinal Plants Against Dental Caries: The ethanolic extract of the collected medicinal plants were tested for their efficacy against dental caries instigating organisms. The concentrations of the extract used are 25, 50 & 75µg/mL and that of the antibiotic of 20µg/mL. The antimicrobial activity of the plant extracts was revealed in Table 3.

Table 2: Phytochemical screening of medicinal plants

Plant Name	AL	SA	FL	TA & PH	ST	CHO & CG	Oils & Fats	TE	AA & Proteins	Gums & Mucilages
Terminalia chebula L.	-	+	-	+	-	+	-	-	-	-
Coleus vettiveroides L.	+	+	-	-	+	+	-	+	-	-
Acmella oleraceae L.	+	+	+/-	+	-	+/-	-	+	-	+
Vitex negundo L.	+	+	+	-	+	+	-	+	-	-
Acacia catechu W.	-	-	-	+	+	+	-	+	-	-
Schleichera oleosa L.	+	+	+	-	+	+	-	+	-	-

AL – Alkaloids, SA-Saponin, FL- Flavonoids, TA- Tannin & PH - phenolic compounds, ST- Steroids, CHO- Carbohydrates & CG-Cardioglycosides, Oils and Fats, TE-Terpenoids, AA- Amino acids and Proteins, Gums and mucilages; '+' =Present; '-'=Absent

Table 3: Effect of medicinal plants against organisms causing dental caries

		Mean Diameter of Zone of Inhibition (cm)		
		Organism Name		
Plant Name	Conc. (µg/mL)	*Streptococcus mutans*	*Streptococcus oralis*	*Lactobacillus acidophilus*
Terminalia chebula L.	Ab	1.23 ± 0.25	1.3 ± 0.15	1.2 ± 0.1
	25	0.1 ± 0.1	0.2 ± 0.1	0.8 ± 0.2
	50	1.3 ± 0.1	1.2 ± 0.2	0.7 ± 0.4
	75	1.6 ± 0.3	1.6 ± 0.3	1.6 ± 0.3
Coleus Vettiveroides L.	Ab	1.3 ± 0.1	1.53 ± 0.15	1.53 ± 0.25
	25	0.3 ± 0.2	0.6 ± 0.3	0.2 ± 0.1
	50	0.9 ± 0.3	1.1 ± 0.4	0.9 ± 0.4
	75	1.5 ± 0.1	1.7 ± 0.3	1.3 ± 0.3
Vitex negundo L.	Ab	0.8±0.1	0.7±0.2	0.8±0.15
	25	0.7±0.1	0.7±0.2	1.1±0.2
	50	0.9±0.1	0.9±0.1	1.4±0.1
	75	1.2±0.2	1.4±0.1	1.6±0.1
Acmella oleracea L.	Ab	0.8±0.1	0.7±0.2	0.8±0.15
	25	0.6±0.5	0.8±0.1	1±0.1
	50	1.0±0.2	1.1±0.4	1.3±0.2
	75	1.5±0.1	1.7±0.3	1.5±0.1
Acacia catechu W.	Ab	0.8±0.1	0.7±0.2	0.8±0.15
	25	0.9±0.1	0.7±0.2	1.1±0.2
	50	1.1±0.1	0.9±0.1	1.4±0.1
	75	1.3±0.1	1.4±0.1	1.6±0.1
Schleichera oleosa L.	Ab	0.6±0.15	0.7±0.2	0.8±0.1
	25	0.7 ± 0.1	0.9 ± 0.6	0.9 ± 0.1
	50	1.3 ± 1.5	1.2 ± 0.2	0.7 ± 0.4
	75	1.6 ± 0.3	1.6 ± 0.3	1.6 ± 0.3

In *S. mutans* higher inhibition was observed by *T. chebula* L and *S. oleosa* L extracts whereas *A. catechu* W and *V. negundo* L showed the minimum inhibition at the higher concentration of tested. Ethanol extract of *C. Vettiveroides* L and *A. oleracea* L showed the highest inhibition of 1.7 ± 0.3 against *S. oralis* when used in 75 µg/mL concentrations, whereas inferior activity was observed by *V. negundo* L. and *A. catechu* W at the same concentration. In case of *L. acidophilus,* expressed more sensitivity towards all the plant extracts tested. The ethanolic extracts of the medicinal plants at higher concentration completely inhibited the growth of the dental pathogens.

Using antibiotics against pathogens causing dental caries is very common practice. But the side effects are enormous. Use of plant and plant products are good alternative against the antibiotics [18]. Anti-caries studies were performed by making the aqueous extract and ethanolic extract of *Psidium guajava, Mimusops elengi* and *Achyranthes aspera* and ethanolic extract produced higher inhibitory results than water extract against *S. mutans* and *S. oralis* [6]. Added research showed that the results of antimicrobial assay of four different leaf extracts of *Syzygium cumini* as well as the positive control ciprofloxacin (For bacteria) produced the zone of inhibition against all the oral bacteria [19].

The antibacterial activity of the ethanolic extract of *Acacia nilotica* exhibited high degree of activity than petroleum ether extract against *S. mutans.* This might be due to less amount of active compound extracted in petroleum ether extract was reported [15]. Whereas *Coriandrum sativum, Mentha arvensis* plant extracts showed antimicrobial activity against *S. mutans* only which corresponds to 11mm and 10mm zone respectively [20]. The antibacterial screening of aqueous and methanol extract carried out *in vitro* on the following bacteria viz., *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Streptococcus faecalis* and *Yersinia enterocolitica*. It has been showed that the methanol extracts had wider range of activity on these organisms. This study supported, the traditional medicines (herbal extracts) to cure many diseases like diarrhea, intestinal tract, throat, ear infections, fever, dental and skin diseases [21].

Mic of Selected Medicinal Plants: Minimum inhibitory concentration was ended against dental caries causing organisms using the extracts of medicinal plants by means of the liquid dilution method [20]. Cutting-edge ethanolic extract of *T. chebula* L showed least inhibitory against *L. acidophilus* whereas *S. mutans* and *S. oralis* was inhibited as a higher rate (Table 4). Added learning exhibited that crude ethanol extracts of *Terminalia bellirica*, *Glycyrrhiza glabra* and *Syzygium aromaticum* moderately inhibited the growth of *S. mutans*, showing inhibition zones from 8-15 mm and MIC less than 12.5mg/mL [13]. The ethanolic extract of *V. amygdalina* has the lowest minimum inhibitory concentration on *S. mutans* at 25 mg/mL and *S. aureus* at 40 mg/mL [22]. In disparity, MIC of ethanol extracts of *Glycyrrhiza glabra* L, *Terminalia chebula* Retz var. *chebula, Terminalia bellirica* (Gaertn.) Roxb., *Phyllanthus emblica* L was low as compared to petroleum ether extract was reported [15].

Anti-Biofilm Activity of Selected Medicinal Plants Against Dental Caries: The OD (Optical density) at 550 nm was measured and High OD denotes the ability of microorganism to form the biofilm. *S. mutans* and *S. oralis* were found to form biofilm on the glass surface after 18 h of incubation and a pH drop in culture broth was observed. It was found that *S. mutans* showed maximum turbidity (0.45) and pH (5.89) on incubation without the plant extract. The OD value (0.34) shown by *S. oralis* indicate that these have less adhesion ability than *S. mutans* and it shown a pH of 6.

Table 4: MIC of selected medicinal plants against organisms causing dental caries

		OD at 660 nm			
		Concentrations			
Medicinal plants used	Test organisms	0.5 mL	1 mL	1.5 mL	2 mL
Terminalia chebula L.	*S. mutans*	1.68	1.54	1.51	1.2
	S. oralis	1.79	1.72	1.58	1.26
	L. acidophilus	0.90	0.88	0.62	0.51
Coleus Vettiveroides L.	*S. mutans*	1.14	1.08	0.65	0.45
	S. oralis	1.15	0.98	0.92	0.80
	L. acidophilus	0.71	0.68	0.52	0.43
Acmella oleraceae L.	*S. mutans*	1.74	1.4	0.8	0.6
	S. oralis	1.1	1.0	0.9	0.72
	L. acidophilus	0.67	0.65	.0.58	0.38
Vitex negundo L.	*S. mutans*	1.3	0.9	0.6	0.4
	S. oralis	1.73	1.41	1.13	0.91
	L. acidophilus	1.4	1.2	0.74	0.63
Acacia catechu W	*S. mutans*	1.3	0.9	0.6	0.4
	S. oralis	1.73	1.41	1.13	0.91
	L. acidophilus	1.4	1.2	0.74	0.63
Schleichera oleosa L	*S. mutans*	1.18	0.95	0.85	0.84
	S. oralis	0.40	0.30	0.20	0.19
	L. acidophilus	0.53	0.32	0.29	0.23

Table 5: Anti-biofilm activity of selected medicinal plants

Medicinal plants used	Test organisms	Fraction	pH	OD
Terminalia chebula L.	*S. mutans*	Control	5.9	0.45
		Ethanolic Extract	6.9	0.15
	S. oralis	Control	6.1	0.34
		Ethanolic Extract	6.8	0.24
Coleus Vettiveroides L.	*S. mutans*	Control	5.9	0.45
		Ethanolic Extract	6.1	0.29
	S. oralis	Control	6.1	0.34
		Ethanolic Extract	7.1	0.26
Acmella oleraceae L.	*S. mutans*	Control	5.9	0.45
		Ethanolic Extract	6.03	0.30
	S. oralis	Control	6.1	0.34
		Ethanolic Extract	6.12	0.29
Vitex negundo L.	*S. mutans*	Control	5.9	0.45
		Ethanolic Extract	6	0.43
	S. oralis	Control	6.1	0.34
		Ethanolic Extract	7	0.30
Acacia catechu W.	*S. mutans*	Control	7.20	0.35
		Ethanolic Extract	5.89	0.45
	S. oralis	Control	6.67	0.25
		Ethanolic Extract	6.08	0.34
Schleichera oleosa L.	*S. mutans*	Control	6.34	0.34
		Ethanolic Extract	5.89	0.45
	S. oralis	Control	7.52	0.12
		Ethanolic Extract	6.08	0.34

Table 5 showed that the anti-biofilm activity of ethanol extract of all six medicinal plants against the dental caries causing microorganisms. Among the tested medicinal plants *S. mutans* showed a pH of 6.9 and OD value of 0.15 on incubation with ethanol extract of *T. chebula* L showed that the aptitude of the plant to avert the biofilm formation. Similar results were observed against *S. oralis.* The plants were able to significantly disrupt the adhesion of the early plaque colonies to the glass surface. Similar observation has also been reported by the study of Yadav *et al*. [14] which showed that all strains of *S. mutans* displayed maximum biofilm formation and proved that the plant extract *Terminalia chebula* L was inhibited the biofilm formed by *S. mutans* at a greater extend. In connection with this line, ethanolic extract of *C. grandis* leaves has exhibited highest activity against biofilm producing Uropathogenic *E. coli* [23].

CONCLUSIONS

The consequences of the present study disclosed that *Terminalia chebula* L is rich in phytochemicals among all the others. The phytochemical screening can serve as the basis for preparation of herbal monograph for proper identification and authentication of drug. A marked anti-caries activity of *Terminalia chebula* L was found against *Streptococcus mutans* affords some scientific rationale for the use of this plant for the treatment of dental diseases. This result leads to development of better treatment with herbal medicines to overcome the side effects caused by antibiotics against dental caries. The biofilms formed by all clinical isolates were inhibited by *Terminalia chebula* L indicated the possible benefits of this herbal preparation for the inhibition of biofilm formation by *Streptococci* sp., an oral pathogen. Further purification and toxicological studies of the medicinal plant and *in vivo* trials should be carried out for the development of a phytomedicine to act against dental caries causing microbes.

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