Comparative Evaluation of Antimicrobial Effects of Herbal Extracts Incorporated in Dentifrices among Adults: an In-vitro Study and a Double Blinded Randomized Control Trial

ABSTRACT

Introduction: A positive correlation has been observed between Streptococcus mutans, Candida albicans and periodontal diseases. Several substances including synthetic chemicals and natural agents have shown promising antimicrobial activity against the S. mutans- C. albicans complex Extracts of Aloe Vera, Triphala, Tulsi, Pomegranate, and Acacia catechu have established therapeutic effects in medicine. The aim of the study was to evaluate the antimicrobial activity and the Minimum Inhibitory Concentration (MIC) of five herbal extracts in-vitro and to compare the antibacterial effects of 5 dentifrices containing the herbal extracts and a commercially available dentifrice on the plaque sample counts of S. mutans and C. albicans in-vivo

Methodology: Extracts were obtained using a standardized method. Minimum inhibitory concentration was calculated for both the microorganisms using modified agar-well diffusion and macro broth dilution technique and the inhibition zone diameter was calculated. Dentifrices containing the extracts were formulated and the in vivo antimicrobial activity was evaluated by studying the CFU count from plaque samples of 420 participants.

Results: Highest antimicrobial activity against S Mutans and C albicans was shown by Acacia catechu and Tulsi respectively and least was by pomegranate. Mean reduction in microbial counts (CFU/ml) by inoculating nutrient broth from baseline to 60 days was highest in Acacia catechu and Triphala for S Mutans and C albicans respectively. All herbal extracts performed better than the commercially available toothpaste

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Conclusion: Dentifrices with herbal extracts of Aloe Vera, Triphala, Tulsi, Pomegranate, and Acacia catechu could serve as an alternative to commercially available dentifrices for treating oral diseases whose causative organisms are S. mutans and C. albicans

Key Words:

Herbal medicine, Streptococcus mutans, Candida albicans, Anti-Bacterial Agents, Dentifrices

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1 INTRODUCTION

Global burden of diseases (2016) reported periodontal diseases as the eleventh most common disease in the world affecting almost 20-50% of the population of the world [1,2]. Recent studies have suggested that when periodontitis goes untreated, the recovery rate of *S. mutans* is high from surfaces such as the dorsum of the tongue, buccal mucosa, supra gingival and sub gingival plaque and saliva [3–5] demonstrating a positive correlation with chronic periodontitis. [6]. *Candida albicans* is one of the most predominant fungal species and is most often seen in association with *S. mutans*. Both synthetic chemicals and natural agents have shown promising antimicrobial activity against the *S. mutans*- *C. albicans* complex [7]. However, the chemical agents used in the form of mouth rinses and dentifrices are associated with some concerns regarding their untoward effects. [8]. The history of herbal medicine can be rooted from ancient civilization which has well documented evidence of their use as traditional medicine. Herbal extracts of Aloe vera, Triphala, *Ocimum sanctum* (Tulsi), *Punica granatum Linn* (Pomegranate), and *Acacia catechu* (AC) have therapeutic properties which have been well demonstrated in herbal medicine. Their antimicrobial properties have been studied in-vitro [9–12] and clinically in the form of extracts, mouthwashes [13], gels [14], and dentifrices [15] in randomised controlled trials which have been proven to be effective against both *S. mutans* [11,16–19]and *C. albicans* [10,19,20]. However, there is paucity of literature in the comparative analysis of these agents together. The rationale of the study was to evaluate the antimicrobial activity and the Minimum Inhibitory Concentration (MIC) of five herbal extracts in-vitro and to compare the antibacterial, antiplaque and antigingivitis effects of 5 dentifrices containing the herbal extracts and a commercially available dentifrice on the plaque sample counts of *S. mutans* and *C. albicans* in-vivo

2 MATERIALS AND METHOD

2.1 Study Design

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The study was divided into two phases.

Phase I: In-vitro study to assess the zone of inhibition and minimum inhibitory concentration (MIC) of the five herbal extracts.

Phase II- Six group parallel arm double blinded randomised controlled trial

2.2 Ethical Approval

Ethical clearance was obtained from the Institutional review board (IRB) and ethics committee of Dr G.D. Pol Foundation's YMT Dental College and Hospital, Navi Mumbai.

2.3 Study Groups

Group I Aloe Vera based dentifrices,

Group II- Triphala based dentifrice

Group III- *Ocimum sanctum* (Tulsi) based dentifrice,

Group IV- *Punica granatum Linn* (Pomegranate) based dentifrice

Group V- *Acacia catechu* (AC) based dentifrice

Group VI- A commercially available dentifrice

2.4 Procurement of the Material

Aloevera leaves, Punica granatum fruits and Tulsi leaves were obtained from the botanical garden of Ayurvedic college of YMT group of institutions. Triphala which is a herbal formula composed of the powder of three fruits: amalaki, bibhitaki and haritaki, was made as a powder. Acacia catechu (AC) was obtained from the botanical garden of Ayurvedic college of YMT group of institutions. All plant specimens were identified by a botanist for their authenticity at the Agarkar Research Institute, Pune (Gov. of Maharashtra, letter no. 1-6/485/2019) and a voucher specimen was deposited in the departmental herbarium.

2.5 Preparation of Extracts

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Aloe vera [21]

Fresh aloe vera leaf gel was dried, powdered, soaked in methanol and ethanol for 24 hours. This was then filtered and dried. This dried extract was further powdered and then dissolved in distilled water. Acetone extract was prepared in a similar manner except that the extracted powder was dissolved in 0.15N NaOH and was further neutralized with 0.15N HCl

Triphala [22]

The ingredients used in the Triphala Churna were Amlaki (Embellica officinalis), Bibhitaka (Terminalia bellerica), Haritaki (Terminalia chebula). They were cleaned, dried, sieved, powdered and mixed in the ratio of 1:1:1. Ethanolic and aqueous extracts were concentrated in a water bath (kokate; sofawora 1993). Extracts were dried under reduced pressure using Rotary Vacuum Evaporator (Equitron® Roteva) and were preserved in the desiccator until further use.

Tulsi

The whole plant was weighed, cleaned, air-dried and weighed again. This was then crushed and mixed with ethanol for the process of maceration. Filtration was done to separate the filtrate and residue which were further processed and subjected to evaporation at 70°C. Extracts were then dried and a dark green residue was obtained. These were refrigerated until further use. Dried extracts were also subjected to phytochemical screening.

Pomegranate

Pomegranate fruits were washed with distilled water and cut manually to separate the arils and peel. The juice was manually extracted and subjected to drying ± 5ºC for 6 hrs or till its moisture content reached ~5-6 %. Seed powder was separately blended with distilled water or 80% methanol followed by filtration, centrifugation to obtain extracts which were kept at -20°C prior to analysis.

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Acacia Catechu [23]

It was extracted from heartwood by drying at room temperature, boiling heartwood chips with 10% hydro-alcoholic solution. The specimen was processed for pharmacognostic standardisation.

2.6 Preparation of Microbial Strains

Typed pure strains of *S. mutan* (ATCC 890) and *C. albican* (ATCC) were obtained from MTCC Chandigarh. Clinical Laboratory, Department of Microbiology. All test strains were re-isolated three successive times on Mueller Hinton agar (MHA) for purification and the identity was confirmed by standard bacteriological methods.

2.7 Preparation of dentifrices

Laboratory preparation of toothpaste was done by trituration method. A liquid base was prepared first with humectants, preservatives and water. To this base a binder was added, triturated well and kept aside for 15 min to allow the binding agent to swell. Next, powder ingredients except detergent were sifted together and were added gradually to the aqueous mucilaginous mixture with slow but continuous stirring. After addition of all powders, a flavoring agent was added. Surface active agent was added at the end and mixed slowly and thoroughly to prevent aeration or foaming. Mixing was continued till all constituents were evenly distributed. The finished product thus obtained was allowed to stand for 24 h. The paste was finally filled into collapsible tubes, stored and used for further studies to determine hard and sharp edged abrasive particles, the paste was extruded about 15 to 20 cm length from the collapsible tube of each sample on a butter paper. Then all the samples were tested by pressing it along its entire length by a finger for the presence of hard and sharp edged abrasive particles. The five different formulations of herbal toothpastes were prepared by using fine powder of calcium carbonate.

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All the formulations differed only with respect to the presence of the active ingredient. MIC% values for both *S. mutans* and *C. albicans* were measured for each extract and the higher of the two concentrations (i.e. MIC% *S. mutans* or *C. albicans*) for a particular extract was added in the dentifrice, in order to have its effect on both *S. mutans* and *C. albicans*. The prepared dentifrices were then transferred to laminate tubes.

2.8 Outcome Measures

Determination of the minimal inhibitory concentration (MIC)

The MIC was determined by two test methods, a modified agar-well diffusion method (Rios et al., 1988; [24]) and the macrobroth dilution technique (NCCLS, 1993), respectively.

In the agar-well diffusion technique, a two-fold serial dilution of each extract in 50% DMSO was prepared to obtain a 0.1–100 mg/ml concentration range. 100-ul volume of bacterial cells was inoculated on the MHA plates. All the plates were incubated at 37 °C for each dilution that was introduced in triplicate wells in the pre-inoculated MHA plates. The least concentration of each extract or control drug showing a clear zone of inhibition was taken as the MIC.

For the macrobroth dilution antibacterial assay, twofold serial dilutions of the extracts and control drug were prepared in tubes with MHB as diluent. Each dilution was seeded with the test organism to the standard concentration (5×105 cfu/ml). The MIC was taken as the last dilution showing no noticeable growth (turbidity).

Using the values of MIC determined for each extract by the agar-well diffusion and macrobroth dilution techniques, respectively, the MIC index was calculated for each test organism.

Zone of inhibition

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This was done using the Kirby buer method or disc diffusion. A piece of filter paper impregnated with the extract was placed onto the surface of MH agar impregnated with the inoculum of test microorganism. The extract diffused into agar after which it's concentration radially decreased from the spot at which the antibiotic was applied outwards. If the extract was effective against the bacteria at a particular concentration, the bacteria will not grow when the concentration of the agar at that point is more than the effective concentration. This region of no bacterial growth is called the Zone of Inhibition. It was measured in millimeters as the clear zone surrounding the well containing the extract, using vernier callipers. All the zones of inhibition were measured by two different methods of illumination (illuminated by reflected light and by transmitted light) as follows:

(i) Reflected light. The test plates were placed, medium side up, on a black, nonreflecting surface and were illuminated with reflected light from a desk lamp. The test plates were held 2 to 3 inches (ca. 5.1 to 7.6 cm) above the black surface to expedite zone measurement. The zones were then measured by holding a vernier calliper against the back of the petri plate.

(ii) Transmitted light. The test plates were held in front of a desk lamp, and the zones were measured with a vernier calliper held against the back of the petri plate. In either situation, the diameters of the zones of inhibited growth were measured to the nearest whole millimeter, including the diameter of the 0.25-inch (6.35-mm) disk. If there was no inhibition, a zone of 6 mm was recorded. The endpoint was to be taken as the area showing no obvious growth that could be detected with the unaided eye, not including a faint haze of growth or tiny colonies which can be detected only with difficulty at the edge of the zone of inhibited growth. If a few large colonies appeared within an otherwise clear zone of inhibition, contamination was suspected, and the test was repeated after checking for purity.

Microbial growth in saliva samples (in-vivo study):

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Samples of saliva were taken at baseline, 30 days and 60 days. They were inoculated in the respective nutrient broth and colony forming units (CFU/ml) were counted for each of the six groups.

2.9 Sample Size Calculation

Sample size was calculated using the least mean difference as 0.15 & 0.3 as SEM from a study by Nair and Malaiappan, 2016.[8] the formula used is as follows:

n=2 Zα+Zβd2

n=2 1.96+0.84 0.30.15

= 62.72

= 63

Adding 10% for attrition

Where,

Zα is determined from table values

is standard deviation; d=x1-x2= 0.15

n= sample size

420 participants were recruited in to six groups.

2.10 Inclusion and Exclusion criteria

Subjects with informed consent, subjects having Gingival Bleeding Index (GBI) score >40%, subjects with at least 20 natural teeth present were included in the study.

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Subjects with medical disorders or probing depth >3 mm, individuals under antimicrobial therapy at least 1 month prior to the study and using mouth rinses or dentifrices containing substances with anti-inflammatory properties, smokers and pregnant women were excluded from the study.

2.11 Collection of plaque sample

On the day of collection, the participants were instructed not to eat or drink anything for at least one hour before the collection of saliva samples. The collected samples were transferred into 5ml. sterile disposable vials and carried in a vaccine carrier with freezing mixture to the laboratory, where analysis of the samples were done on the same day. Samples were collected at baseline, at the end of 1 month, 2 months after the use of assigned dentifrice at baseline by a single, previously trained & calibrated examiner

All subjects were asked to Maintain and fill a compliance sheet and get the used toothpaste packs at every visit which was weighed and assessed for compliance.

Frequent reminder phone calls as a part of motivation

2.12 Randomization

Participants were randomly allocated 6 study groups with 70 subjects in each group.

Allocation sequence was generated using the lottery method by graph pad (https://www.graphpad.com/quickcalcs/randomize2/).

2.13 Allocation concealment

Allocation concealment was done using the SNOSE (sequentially numbered, opaque, sealed envelopes) technique which concealed the sequence until interventions were assigned. Allocation ratio was 1:1.

2.14 Blinding

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All laminate tubes procured were without any labels. Labelling was done by wrapping a paper which had letters A, B, C, D, E, F. Double blinding was done, i.e. primary investigator and subjects were not aware of the intervention.

2.15 Statistical analysis

Data obtained was compiled on a MS Office Excel Sheet (2016). Data was subjected to statistical analysis using the Statistical package for social sciences (SPSS v 23.0, IBM). Descriptive statistics like frequencies and percentage for categorical data, Mean & SD for numerical data have been depicted. Normality of data was checked using Shapiro-Wilk test & Kolmogorov-Smirnov test and it was found that data did not follow a normal curve. Inter group comparison for microbial counts were done using Kruskal-Wallis Analysis of Variance followed by Mann-Whitney U test for group wise comparison. Intra group comparison for microbial counts was done using Friedman's test followed by pair wise comparison using Wilcoxons Signed rank test. For all the statistical tests, $P < .05$ was considered to be statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%. For MIC, means and standard deviations were calculated.

3 RESULTS

3.1 Antimicrobial activity of extracts at 48 hours :(Fig. 1)

The antimicrobial activity of all the extracts at 48 hours was more than the negative control. For *S. mutans*, antimicrobial activity among groups was in the following order: AC>Triphala>Aloe-Vera>Tulsi>Pomegranate. For *C. albicans*, it was as follows: Tulsi>Triphala>AC>Aloe-Vera>Pomegranate.

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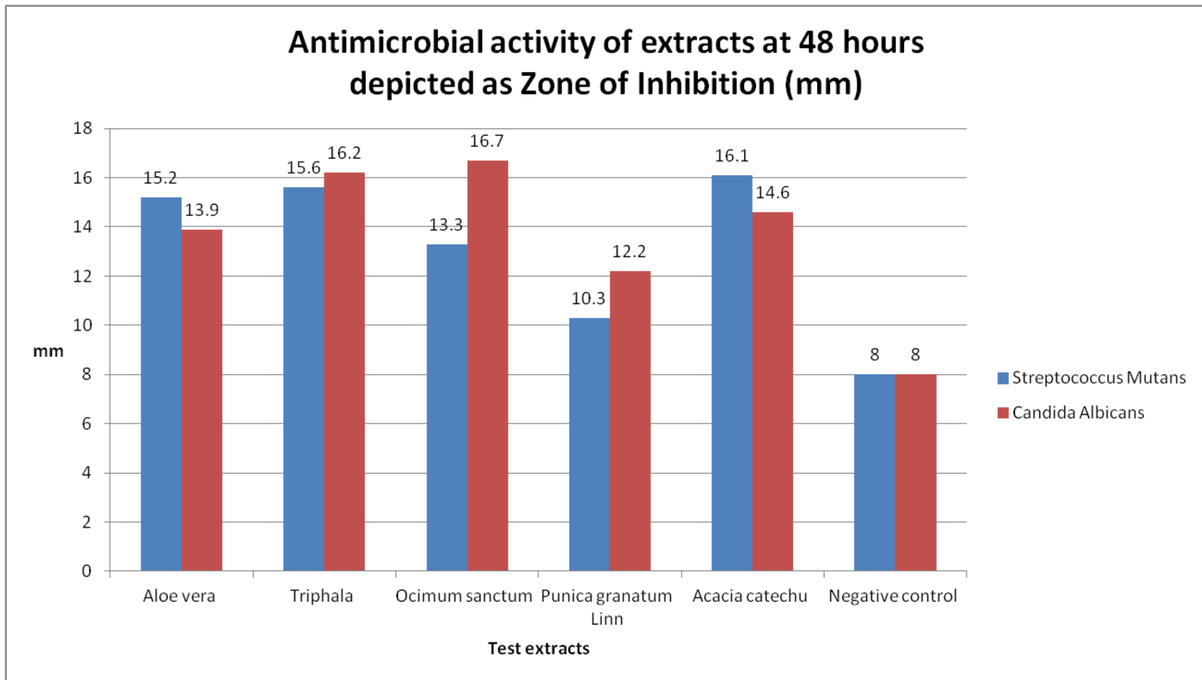

Fig 1: Graphical representation of the antimicrobial activity of 5 herbal extracts against *S. mutans* (blue) and *C. albicans* (Red) in vitro. X axis represents the groups and Y axis represents the mean values of the zone of inhibition in mm.

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3.2 Minimum Inhibitory concentration (MIC) of extracts:

The MIC% of the extracts for *S. mutans*, were as follows: AC (0.78%), Aloe-Vera and Triphala (1.56%), Tulsi (3.12%) and Pomegranate (12.5%).

The MIC% of the extracts for *C. albicans*, were as follows: AC (3.12%), Triphala and Tulsi (6.25%), Aloe-Vera (12.5%) and Pomegranate (25%)

3.3 Baseline demographic data

A total of 420 subjects were recruited in the study with a distribution of 70 subjects in each group. The mean age of the subjects was 38.72(±2.62), with female preponderance.

3.4 Intergroup (Table 1) and pair wise (Table 2) comparison of colony forming units of *S. mutans* at baseline, 1 month and 2 months follow up:

S Mutans: A significant difference was observed for the values between the groups (P =.008) for *S. mutans* at baseline. The CFU values were in the following descending order group 2 (Triphala) > group 4 (Pomegranate) > group 6 (Commercially available dentifrice) > group 3 (Tulsi) > 5 (*Acacia catechu)* > group 1 (Aloe Vera). This difference was significant when comparing group 2 with the other groups, with the values of group 2 being higher. However, the difference in the CFU count was not statistically significant between other pairs of groups.

At 1 month there was a statistically significant difference (P<.001) observed between the CFU counts in the following descending order group 6 > group 4 > group 3 > group 5 > group 2 > group 1 . The difference was significant between all the pairs of groups except when comparing group 1 (Aloe vera) with group 2 (Triphala) and group 1 with group 5 (*Acacia catechu)* where no significant difference was observed.

At two months, there was a statistically significant difference (P<.001) observed between the CFU counts in the following descending order the counts were higher in group 6 > group 4 > group 5 > group 3 > group 2 > group 1. The difference was significant between all the pairs of groups, except group 4 (Pomegranate) vs. group 5 (*Acacia catechu*) where no statistically significant difference was observed.

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Table 1: Intergroup comparison of CFU count of *S. mutans* using the Kruskal Wallis test

	Group	Mean	Standard Deviation	Median	Mean Rank	P value
Baseline	1	25.37	1.905	26	189.14	0.008

	2	26.91	2.575	28	258.48	
	3	25.57	2.619	26	195.63	
	4	25.94	2.581	26	215.16	
	5	25.56	2.857	24	195.23	
	6	25.86	2.611	25.5	209.36	
1 month	1	21.11	1.798	22	119.01	0.000
	2	21.37	2.168	21	132.44	
	3	23.51	1.640	24	225.22	
	4	24.71	1.942	25	285.11	
	5	21.74	3.670	22	169.18	
	6	25.74	1.612	26	332.05	
2 month	1	18.29	1.364	18	73.26	0.000
	2	18.70	.922	19	87.54	
	3	21.80	1.691	22	207.00	
	4	23.91	1.847	24	281.27	
	5	23.60	3.445	24	260.29	

	6	26.41	1.952	26	353.64	

Table 2: Pair wise comparison of CFU count of S. mutans between the groups at baseline, 1 and 2 month follow up showing statistically significant difference using the Mann-Whitney U test

Group	vs. group	Mann-Whitney U value	P value of Mann-Whitney Test
Baseline			
1	2	1505.500	0.000
2	3	1757.500	0.004
2	4	1909.500	0.023
2	5	1828.500	0.009
2	6	1890.500	0.019
1 Month Follow up			
1	3	912.000	0.000
1	4	519.000	0.000
1	6	54.000	0.000
2	3	1114.000	0.000
2	4	648.500	0.000
2	6	199.500	0.000
3	4	1535.500	0.000

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3	5	1758.500	0.004
3	6	829.500	0.000
4	5	1265.000	0.000
4	6	1840.500	0.009
5	6	818.000	0.000
2 Month Follow up			
1	2	1968.500	0.037
1	3	324.500	0.000
1	4	18.000	0.000
1	5	332.000	0.000
1	6	0.000	0.000
2	3	352.500	0.000
2	4	0.000	0.000
2	5	359.000	0.000
2	6	0.000	0.000
3	4	1023.000	0.000
3	5	1657.000	0.001
3	6	202.000	0.000
4	6	867.000	0.000
5	6	1161.000	0.000

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3.5 Intergroup (Table 3) and pair wise (Table 4) comparison of colony forming units of C. albicans at baseline, 1 month and 2 months follow up:

At baseline: A significant difference was observed for the values between the groups (P =.010). The CFU counts were in the following descending order group 5 > group 6 > group 4 > group 1 > group 2 > group 3. When performing a pair wise comparison there was a highly significant difference (p<0.01) seen when comparing group 5 (*Acacia Catechu*) with other groups whereas no significant difference was observed between the pairs of other groups (p>0.05).

At 1 month: A significant difference (P<.001) was observed for the values between the groups. The CFU counts were in the following descending order group 6 > group 3 > group 5 > group 4 > group 2 > group 1. The difference was significant between all the pairs of groups except when comparing group 1 vs. 2, 2 vs. 4, 3 vs. 5 & 5 vs. 6, where there was a no statistically significant difference observed.

At 2 months: A significant difference (P<.001) was observed for the values between the groups. The CFU counts were in the following descending order group 6 > group 3 > group 5 > group 4 > group 2 > group 1. The difference observed was significant between all the pairs of the groups except between group 1 vs. 2, 1 vs. 4, 1 vs. 5 & 2 vs. 5 where there was no significant difference observed (p>0.05).

Table 3: Intergroup comparison of CFU count of *C. albicans* using the Kruskal Wallis test

	Group	**Mean**	**Standard Deviation**	**Median**	**Mean Rank**	**P value**
Baseline	1	7.47	1.032	7.5	200.32	0.010

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	2	7.34	2.371	7	192.91	
	3	7.23	1.426	7	189.71	
	4	7.41	2.753	8	206.54	
	5	8.36	2.252	9	257.14	
	6	7.51	1.902	8	216.39	
1 month	1	5.49	1.073	5	128.56	
	2	5.64	1.130	5	142.29	
	3	7.17	1.262	7	261.14	
	4	6.14	1.535	6	179.94	
	5	7.13	1.560	8	258.01	
	6	7.63	1.505	8	293.06	0.000
2 months	1	5.09	1.176	5	156.43	
	2	5.44	.958	5	177.77	
	3	6.87	1.239	6	297.00	
	4	4.63	1.321	5	126.79	
	5	5.30	1.108	5	163.23	
	6	7.40	.939	8	341.78	0.000

Table 4: Pair wise comparison of CFU count of *C. albicans* between the groups at baseline, 1 and 2 month follow up showing statistically significant difference using the Mann-Whitney U test

Group	vs. group	Mann-Whitney U value	P value of Mann-Whitney Test
Baseline			
1	5	1723.000	0.002
2	5	1809.000	0.007
3	5	1621.000	0.000
4	5	1931.500	0.029
5	6	1901.000	0.020
1 Month Follow up			
1	3	821.500	0.000
1	4	1874.500	0.014
1	5	1007.500	0.000
1	6	592.500	0.000
2	3	950.000	0.000
2	5	1116.500	0.000
2	6	675.000	0.000
3	4	1477.000	0.000
3	6	1952.500	0.033
4	5	1603.000	0.000
4	6	1157.500	0.000

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2 Month Follow up			
1	3	739.000	0.000
1	6	275.500	0.000
2	3	906.500	0.000
2	4	1720.000	0.001
2	6	423.500	0.000
3	4	551.500	0.000
3	5	845.500	0.000
3	6	1747.500	0.002
4	5	1920.500	0.022
4	6	179.500	0.000
5	6	434.500	0.000

3.6 Intragroup comparison of CFU at different time interval

S. Mutans- There was a statistically highly significant difference seen for the values between the time intervals with higher CFU counts observed at baseline and the least count observed at 2 months for group 1,2,3 and 4 while there was an increase seen at 2 months for group 5 and 6 (Table 5).

Table 5: Intragroup comparison of the CFU count for *S. mutans* in the groups at different time intervals

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Time Interval	Median	Mean rank	P value of Friedman Test
Group 1			
Baseline	26.00	2.96	0.000
1 month	22.00	1.95	
2 month	18.00	1.09	
Group 2			
Baseline	28.00	2.88	0.000
1 month	21.00	2.01	
2 month	19.00	1.11	
Group 3			
Baseline	26.00	2.62	0.000
1 month	24.00	2.01	
2 month	22.00	1.36	
Group 4			
Baseline	26.00	2.34	0.002
1 month	25.00	1.86	
2 month	24.00	1.81	
Group 5			
Baseline	24.00	2.52	0.000
1 month	22.00	1.51	
2 month	24.00	1.97	

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Group 6			
Baseline	25.50	1.89	0.000
1 month	26.00	1.82	
2 month	26.00	2.29	

C. albicans- There was a statistically significant difference seen for the CFU values between the time intervals with higher values at baseline and least at 2 months (Table 6).

Table 6: Intragroup comparison of the CFU count for *C. albicans* in the groups at different time intervals

Time interval	Median	Mean rank	P value of Friedman Test
Group 1			
Baseline	7.50	2.75	0.000
1 month	5.00	1.69	
2 month	5.00	1.56	
Group 2			
Baseline	7.00	2.36	0.000
1 month	5.00	1.89	
2 month	5.00	1.75	
Group 3			
Baseline	7.00	2.15	

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1 month	7.00	2.02	0.10
2 month	6.00	1.83	
Group 4			
Baseline	8.00	2.29	0.000
1 month	6.00	2.24	
2 month	5.00	1.48	
Group 5			
Baseline	9.00	2.60	0.000
1 month	8.00	2.09	
2 month	5.00	1.31	
Group 6			
Baseline	8.00	2.16	0.041
1 month	8.00	2.05	
2 month	8.00	1.79	

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3.7 Intragroup Pair-wise comparison of CFU of different time intervals (table 7)

When comparing the CFU counts of *S. mutans* at baseline vs. 1 month and baseline vs. 2 months, a significant reduction in the count was observed for groups 1, 2, 3, 4 and 5. For *C albicans,* when comparing the CFU at baseline vs. 1 month and baseline vs. 2 months, a significant reduction in the count was observed for all the groups except group 3 and 6 where

as between 1 month and 2 months only group 4 and 5 showed a significant reduction in the CFU count.

Table 7: Pair wise comparison of CFU counts between different time intervals

Groups	Time pairs	P value of Wilcoxon Signed Ranks Test
S. mutans		
Group 1	Baseline vs. 1 month	0.000
	Baseline vs. 2 month	0.000
	1 month vs. 2 month	0.000
Group 2	Baseline vs. 1 month	0.000
	Baseline vs. 2 month	0.000
	1 month vs. 2 month	0.000
Group 3	Baseline vs. 1 month	0.000
	Baseline vs. 2 month	0.000
	1 month vs. 2 month	0.000
Group 4	Baseline vs. 1 month	0.001
	Baseline vs. 2 month	0.000
	1 month vs. 2 month	0.009
Group 5	Baseline vs. 1 month	0.000
	Baseline vs. 2 month	0.002
	1 month vs. 2 month	0.008
C. albicans		

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Group 1	Baseline vs. 1 month	0.000
	Baseline vs. 2 month	0.000
Group 2	Baseline vs. 1 month	0.000
	Baseline vs. 2 month	0.000
Group 4	Baseline vs. 1 month	0.007
	Baseline vs. 2 month	0.000
	1 month vs. 2 month	0.000
Group 5	Baseline vs. 1 month	0.000
	Baseline vs. 2 month	0.000
	1 month vs. 2 month	0.000

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4 DISCUSSION

In the in vivo study, on using the herbal extracts in the form of a dentifrice, a reduction in the microbial count of *S. mutans* and *C. albicans* was observed over a period of 2 months which was greater than that of the commercial dentifrice. Triphala and aloe Vera were observed to be consistently highly effective against *S. mutans* and *C. albicans* when compared to the other herbal extracts. This is a novel study which has compared the efficacy of all 5 herbal extracts when used in a dentifrice.

In the in vitro studies, Acacia catechu demonstrated highest antimicrobial activity against *S. mutans* while also proving to be the third most efficacious agent against *C. albicans.* Acacia catechu formulations in various concentrations when tested along with other herbal products have shown significant antimicrobial effects against both gram positive and negative organisms and similar efficacy as chlorhexidine against gingival and periodontal infections. The results of the present study are in accordance with the observations made by Negi and Dave [17]. It has been extensively used in various ayurvedic formulations for many years

including mouthwashes to treat sore throat, various oral diseases including caries and gingival diseases. The antimicrobial properties of AC have been attributed to the active ingredients present in it which include catechins, gallic acid and tannins [25,26]. Although very few studies are available in literature demonstrating its applications in dentistry, it has demonstrated no side effects when used long term for its anti-inflammatory benefits [27].

The second highest antimicrobial activity in vitro was that of Triphala which was also the most effective extract against S Mutans in vivo. Studies of its antimicrobial effects have been performed in various aspects in vitro such as to evaluate its efficacy as an endodontic irrigant, for orthodontic wires [28] and in cultures. When compared with sodium hypochlorite triphala showed promising results [29,30] Active ingredients like tannins, which restrict adhesion; quinines which provide free radicals and cause inactivation of microbial proteins and flavones, flavonoids, and flavonols, which disrupt microbial membrane and cell walls contribute to its antimicrobial its properties[26].

Aloe-vera demonstrated the third highest antimicrobial properties against *S. mutans* and fourth highest against *C. albicans* in vitro. Similar effect has been demonstrated in previous In-vitro studies with aloe-vera [31]. Its antimicrobial properties have been attributed to lignin, vitamins, minerals, salicylic acids, amino acids, and saponins. Aloe vera also contains a latex compound that has been found to be bacteriostatic in nature [32]. In clinical studies Aloe-vera Mouthwash has demonstrated significant antimicrobial effects [13]. Upon its incorporation into dentifrices, aloe-vera was as efficacious as other commercially available dentifrices [15,33].

In the In vitro evaluation, Tulsi demonstrated fourth highest efficacy against *S. mutans* and was the most effective against *C. albicans*. Previous In-vitro studies have also demonstrated significant antimicrobial effects of Tulsi against *S. Mutans* [12] as well as *C. albicans* [11].

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Clinical study, involving chewing of Tulsi leaves in children showed significant reduction in *S. mutans* count [34]. Similarly Mouthwashes have also shown similar results. Tulsi mouthwashes in Candidiasis, demonstrated significant reduction in counts of *C. albicans* and relief in clinical symptoms [35]. Antimicrobial properties of tulsi can be attributed to the presence of eugenol (1-hydroxy-2-methoxy-4-allylbenzene), ursolic acid and carvacrol[32].

Extract to demonstrate least in vitro antimicrobial activity in our study against *S. Mutans* and *C. albicans* was Punica granatum linn (pomegranate). However, it was second most efficacious in vivo against *C. Albicans* but still the least efficacious agent against *S. mutans* invivo. However a previous in vitro study has demonstrated significant reduction in bacterial adherence of *S. Mutans* and reduction in counts of *C. Albicans* with Pomegranate gels[14] A previous Invivo study has reported significant reduction in S Mutans counts when the extract was used as a mouth rinse [36]. Although there were not many studies evaluating its efficacy in the form of a dentifrice reported in literature, one unani study highlighted better significant antimicrobial properties against both gram negative and positive organisms [37]. The antimicrobial activity can be attributed to the presence of flavonoids and tannins [38],

5 CONCLUSION

Within the limitations of the studies it can be concluded that all herbal extracts when introduced in the form of dentifrices were effective in reducing the microbial load of *S. mutans* and *C. albicans* with Triphala and Aloe Vera showing the highest efficacy. Dentifrices consisting of these herbal extracts can be considered as alternatives to commercial toothpaste which target oral diseases caused by *S. mutans* and *C. albicans*. However, further studies using standard protocol and concentrations in order to enable commercial incorporation of these extracts need to be conducted.

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6 REFERENCES

[1] GBD 2016 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 2017;390:1211–59.

[2] Fernández-Avilés F. European Workshop on the Link Between Periodontal Health and Cardiovascular Disease. 2010.

[3] Preza D, Olsen I, Aas JA, Willumsen T, Grinde B, Paster BJ. Bacterial profiles of root caries in elderly patients. J Clin Microbiol 2008;46:2015–21.

[4] Van der Reijden WA, Dellemijn-Kippuw N, Stijne-van Nes AM, de Soet JJ, van Winkelhoff AJ. Mutans streptococci in subgingival plaque of treated and untreated patients with periodontitis. J Clin Periodontol 2001;28:686–91.

[5] Loesche WJ, Syed SA, Schmidt E, Morrison EC. Bacterial profiles of subgingival plaques in periodontitis. J Periodontol 1985;56:447–56.

[6] Dani S, Prabhu A, Chaitra KR, Desai NC, Patil SR, Rajeev R. Assessment of in healthy versus gingivitis and chronic periodontitis: A clinico-microbiological study. Contemp Clin Dent 2016;7:529–34.

[7] Salehi B, Kregiel D, Mahady G, Sharifi-Rad J, Martins N, Rodrigues CF. Management of Streptococcus mutans-Candida spp. Oral Biofilms' Infections: Paving the Way for Effective Clinical Interventions. Journal of Clinical Medicine 2020;9:517. https://doi.org/10.3390/jcm9020517.

[8] Ledder RG, Latimer J, Humphreys GJ, Sreenivasan PK, McBain AJ. Bacteriological effects of dentifrices with and without active ingredients of natural origin. Appl Environ Microbiol 2014;80:6490–8.

UNDER PEER REVIEW

[9] Mehta VV, Rajesh G, Rao A, Shenoy R, B H MP. Antimicrobial Efficacy of Punica granatum mesocarp, Nelumbo nucifera Leaf, Psidium guajava Leaf and Coffea Canephora Extract on Common Oral Pathogens: An In-vitro Study. J Clin Diagn Res 2014;8:ZC65–8.

[10] Prabhakar J, Balagopal S, Priya MS, Selvi S, Senthilkumar M. Evaluation of antimicrobial efficacy of Triphala (an Indian Ayurvedic herbal formulation) and 0.2% chlorhexidine against Streptococcus mutans biofilm formed on tooth substrate: An in vitro study. Indian Journal of Dental Research 2014;25:475. https://doi.org/10.4103/0970-9290.142539.

[11] Sivareddy B, Reginald B, Sireesha D, Samatha M, Reddy K, Subrahamanyam G. Antifungal activity of solvent extracts of Piper betle and Ocimum sanctum Linn on Candida albicans: An in vitro comparative study. Journal of Oral and Maxillofacial Pathology 2019;23:333. https://doi.org/10.4103/jomfp.jomfp_167_19.

[12] Agarwal P, Nagesh L, Murlikrishnan. Evaluation of the antimicrobial activity of various concentrations of Tulsi (Ocimum sanctum) extract against Streptococcus mutans: an in vitro study. Indian J Dent Res 2010;21:357–9.

[13] Kamath NP, Tandon S, Nayak R, Naidu S, Anand PS, Kamath YS. The effect of aloe vera and tea tree oil mouthwashes on the oral health of school children. Eur Arch Paediatr Dent 2020;21:61–6.

[14] Vasconcelos LC de S, de Souza Vasconcelos LC, Sampaio FC, Sampaio MCC, do Socorro Vieira Pereira M, Higino JS, et al. Minimum inhibitory concentration of adherence of Punica granatum Linn (pomegranate) gel against S. mutans, S. mitis and C. albicans. Brazilian Dental Journal 2006;17:223–7. https://doi.org/10.1590/s0103-64402006000300009.

[15] Bhati N, Jaidka S, Somani R. Evaluation of antimicrobial efficacy of Aloe vera and Meswak containing dentifrices with fluoridated dentifrice: An in vivo study. Journal of International Society of Preventive and Community Dentistry 2015;5:394. https://doi.org/10.4103/2231-0762.165924.

UNDER PEER REVIEW

[16] Dashtdar M, Dashtdar MR, Dashtdar B, Shirazi MK, Khan SA. In-Vitro, Anti-Bacterial Activities of Aqueous Extracts of Acacia catechu (L.F.)Willd, Castanea sativa, Ephedra sinica stapf and shilajita mumiyo Against Gram Positive and Gram Negative Bacteria. Journal of Pharmacopuncture 2013;16:15–22. https://doi.org/10.3831/kpi.2013.16.014.

[17] Negi BS, Dave BP. In Vitro Antimicrobial Activity of Acacia catechu and Its Phytochemical Analysis. Indian J Microbiol 2010;50:369–74.

[18] Gupta S, Padiyar N, Padiyar B. Comparative Evaluation of Effects of Triphala, Garlic Extracts, and Chlorhexidine Mouthwashes on Salivary Streptococcus mutans Counts and Oral Hygiene Status. International Journal of Clinical Pediatric Dentistry 2018;11:299–306. https://doi.org/10.5005/jp-journals-10005-1530.

[19] Pandita V, Patthi B, Singh S, Singla A, Vashishtha V, Malhi R. Dentistry meets nature-role of herbs in periodontal care: A systematic review. J Indian Assoc Public Health Dent 2014;12:148.

[20] Chandu GN, Pai MH, Prashant GM, Murlikrishna KS, Shivakumar KM. Antifungal efficacy of Punica granatum, Acacia nilotica, Cuminum cyminum and Foeniculum vulgare on Candida albicans: An in vitro study. Indian Journal of Dental Research 2010;21:334. https://doi.org/10.4103/0970-9290.70792.

[21] Lawrence R, Tripathi P, Jeyakumar E. Isolation, Purification and Evaluation of Antibacterial Agents from Aloe vera. Braz J Microbiol 2009;40:906–15.

[22] Venkateswarlu G, Ganapaty S, Sudhakar AMS. Preparation of triphala churna using the ingredients obtained from local market and comparative standardization. Pharmacogn J 2019;11:102–11.

[23] Sharma P. Investigation on photodecomposition of standardised ethyl acetate fraction of Katha. Pharmacogn J 2020;12:815–20.

UNDER PEER REVIEW

[24] Okeke MI, Iroegbu CU, Eze EN, Okoli AS, Esimone CO. Evaluation of extracts of the root of Landolphia owerrience for antibacterial activity. J Ethnopharmacol 2001;78:119–27.

[25] Final report of the safety assessment of Acacia catechu gum, Acacia concinna fruit extract, Acacia dealbata leaf extract, Acacia dealbata leaf wax, Acacia decurrens extract, Acacia farnesiana extract, Acacia farnesiana flower wax, Acacia farnesiana gum, Acacia senegal extract, Acacia senegal gum, and Acacia senegal gum extract. Int J Toxicol 2005;24 Suppl 3:75–118.

[26] Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564–82.

[27] Stohs SJ, Bagchi D. Antioxidant, Anti-inflammatory, and Chemoprotective Properties of Acacia catechu Heartwood Extracts. Phytother Res 2015;29:818–24.

[28] Mishra S, Routray S, Kumar Sahu S, Bhusan Nanda S, Charan Sahu K. The role and efficacy of herbal antimicrobial agents in orthodontic treatment. J Clin Diagn Res 2014;8:ZC12–4.

[29] Prabhakar J, Senthilkumar M, Priya MS, Mahalakshmi K, Sehgal PK, Sukumaran VG. Evaluation of antimicrobial efficacy of herbal alternatives (Triphala and green tea polyphenols), MTAD, and 5% sodium hypochlorite against Enterococcus faecalis biofilm formed on tooth substrate: an in vitro study. J Endod 2010;36:83–6.

[30] Choudhary E, Indushekar KR, Saraf BG, Sheoran N, Sardana D, Shekhar A. Exploring the role of and juice in root canal irrigation: An study. J Conserv Dent 2018;21:443–9.

[31] Fani M, Kohanteb J. Inhibitory activity of Aloe vera gel on some clinically isolated cariogenic and periodontopathic bacteria. J Oral Sci 2012;54:15–21.

[32] Chandra Shekar BR, Nagarajappa R, Suma S, Thakur R. Herbal extracts in oral health care - A review of the current scenario and its future needs. Pharmacogn Rev 2015;9:87–92.

[33] Pradeep AR, Agarwal E, Naik SB. Clinical and microbiologic effects of commercially available dentifrice containing aloe vera: a randomized controlled clinical trial. J Periodontol 2012;83:797–804.

UNDER PEER REVIEW

[34] Ahrestani F, Sankaran M. The Ecology of Large Herbivores in South and Southeast Asia. Springer; 2016.

[35] Annigeri RG, Mangala GK, Thimmasetty J, Sharma N, Kanjani V, Sravya G. Evaluation of tulasi extract mouthwash in the management of oral candidiasis. Journal of Advanced Clinical & Research Insights 2018;5:30–4. https://doi.org/10.15713/ins.jcri.203.

[36] Bansal A, Marwah N, Nigam AG, Goenka P, Goel D. Effect of Achyranthes aspera, 0.2% Aqueous Chlorhexidine Gluconate and Punica granatum Oral Rinse on the Levels of Salivary Streptococcus mutans in 8 to 12 Years Old Children. J Contemp Dent Pract 2015;16:903–9.

[37] Parveen A, Ahmad QZ, Rashid M, Rahman AU, Rehman S. Study of antimicrobial activity of Unani poly herbal toothpaste . Heliyon 2021;7:e06249.

[38] Elshafie HS, Caputo L, De Martino L, Sakr SH, De Feo V, Camele I. Study of Bio-Pharmaceutical and Antimicrobial Properties of Pomegranate (Punica granatum L.) Leathery Exocarp Extract. Plants 2021;10:153. https://doi.org/10.3390/plants10010153.

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