

Evaluating the Antimicrobial Efficacy of an Innovative, Novel Herbal Formulation on Dental Caries and Plaque Microorganisms - A Clinical Research

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<http://dx.doi.org/10.13005/bpj/1794>

(Received: 01 October 2019; accepted: 21 October 2019)

To develop a novel, innovative herbal formulation that can prevent both dental caries and periodontal diseases simultaneously. This clinical research involved *in vitro* studies to determine Minimum Inhibitory Concentration (MIC) of combinations of *Acacia nilotica*, *Murraya koenigii* Linn Sprengel, *Eucalyptus* hybrid, and *Psidium Guajava* on dental caries and plaque bacteria followed by evaluation of phytochemical constituents in these plant extracts. MIC was determined using broth dilution method. Then, an animal experimentation was carried out to determine median lethal dose and adverse drug reactions following single oral administration of polyherbal extract which was followed by human experimentation. A short term double blind randomized study on 34 adult human volunteers was undertaken to assess antiplaque efficacy of a novel polyherbal mouth rinse using 0.2% chlorhexidine as positive control. MIC of the polyherbal combinations on *S. mutans*, *S. sanguis*, *S. salivarius*, *L. acidophilus*, *F. nucleatum* and *P. gingivalis* was found to be 0.25%, 0.05%, 0.05%, 0.1%, 0.25% and 0.25% respectively. Median lethal dose (LD50) for herbal formulation was found to be more than 2000mg/kg body weight. *In vivo* study found that the polyherbal mouth rinse group had lesser plaque buildup, *S. mutans* and total viable count in comparison with saline group with no significant difference in plaque build-up and total viable count between polyherbal rinse group and 0.2% chlorhexidine. Polyherbal mouth rinse containing *Acacia nilotica*, *Murraya koenigii* L. sprengel, *Eucalyptus* hybrid and *Psidium guajava* was effective against dental caries and plaque induced dental diseases.

Keywords: Antiplaque Efficacy; Dental Caries; Median Lethal Dose; Minimum Inhibitory Concentration; Periodontal Diseases.

Oral health is an inseparable part of individual to speak, eat and socialize without general health and a healthy mouth enables an experiencing active disease, discomfort or

embarrassment¹. Health promotion measures are deemed incomplete without promotion of oral health and prevention of many oral diseases that have a major bearing on general health¹. The prevalence of dental caries is progressively increasing and periodontal diseases are one of the most common human afflictions². Loss of teeth attributable to periodontitis often causes discomfort, compromises esthetics and function. Moreover, recent studies suggest an association between chronic low grade infections such as periodontitis with systemic health problems³ including cardiovascular diseases⁴, pre-term low birth weight⁵, diabetes mellitus⁶ and chronic obstructive pulmonary disease³. Treatment of established dental diseases is very expensive and not a realistic option for the poor. There is an immediate need for promoting preventive measures that are acceptable, cost-effective and easily available.

The conventional practices for prevention of dental caries and periodontal diseases are use of fluorides in various forms and mechanical oral hygiene combined with proper professional maintenance respectively. In reality, however, the degree of motivation and dexterity required for optimum oral hygiene is beyond the ability of a large section of the population. Antimicrobial mouth rinses have also been suggested as adjuncts for mechanical plaque control methods. Most commonly used antiplaque agent is chlorhexidine gluconate. The use of chlorhexidine has some potential drawbacks like altered taste sensation, staining of teeth and development of resistant microorganisms that incapacitate their long term use⁷. There exists a need to develop some innovative strategies that act against microorganisms responsible for these highly prevalent oral diseases. One such strategy would be to authenticate the enormous wealth of medicinal plants richly available in natural world.

Our in vitro studies have demonstrated antimicrobial efficacy of plant extracts of *Acacia nilotica*, *Murraya koenigii* L. Sprengel, *Eucalyptus* hybrid and *Psidium Guajava* against dental caries and plaque bacteria⁸⁻¹⁰.

In most studies, individual plant extracts have been tested against various bacteria and fungi. Some studies have tested the antimicrobial efficacy of plant extracts on microorganisms that

caused either dental caries or periodontal diseases. The combination of these plant extracts that can act against both dental caries and periodontal pathogens was never investigated. A methodical and orderly evaluation of plant extracts and their combinations present an ideal approach for evolution of novel drugs from plants¹¹. In this background, the present clinical research was undertaken with the aim of developing a novel, innovative polyherbal formulation that can prevent both dental caries and periodontal diseases.

Objectives

To determine the MIC of the combinations of *Acacia nilotica*, *Murraya koenigii* Linn Sprengel, *Eucalyptus* hybrid, and *Psidium Guajava* on *Streptococcus mutans*, *Lactobacillus acidophilus* (dental caries bacteria), *Streptococcus sanguis*, *Strptococcus salivarius* (primary plaque colonizers), *Fusobacterium nucleatum* (secondary plaque colonizer) and *Porphyromonas gingivalis* (tertiary plaque colonizer).

To undertake a qualitative assay of phytochemical constituents present in the four plant extracts.

To determine the median lethal dose and adverse drug reactions following single oral dosage of the combinations of *Acacia nilotica*, *Murraya koenigii* L. Sprengel, *Eucalyptus* hybrid and *Psidium Guajava* extracts using female Albino Wistar rats.

To assess the feasibility of using this polyherbal formulation as a mouth rinse for daily use.

To evaluate the antiplaque and antimicrobial efficacy of the polyherbal mouth rinse on a group of adults in a randomized control trial.

MATERIALS AND METHOD

This research was carried out over a period of three and half years from March 2013 to September 2016 at the center for Scientific Research and Development, People's University, Bhopal following approval of research protocol by the Institutional Review Board. The study involved in vitro studies to assess antimicrobial efficacy of plant extracts followed by qualitative evaluation of phytochemical constituents in these plant extracts. Then, an animal experimentation was carried out

to determine the median lethal dose and adverse drug reactions following single oral administration of the polyherbal extract. This was followed by a short term randomized controlled trial on human adults.

The branches of four plants with ethno-pharmacological background which were easily available in central India were collected from surrounding areas. The plants were identified and authenticated by a taxonomist. Healthy leaves were separated from these branches, washed and shade dried over a period of three-four weeks at room temperature. The dried leaves were hand crushed and fine powder was prepared using a mixer grinder. Powders were stored in coded airtight plastic bottles in refrigerator at 4°C till further use. Soxhlet apparatus with ethanol as solvent was used for plant extraction process.

Preparation of combination of extracts

Stock solutions of the individual plants were prepared by dissolving 100 mg of the extract in 1000 µl of dimethyl sulfoxide (DMSO). The quadruple combinations of plant extracts were prepared by mixing equal quantities of the stock solutions of individual extracts.

Minimum Inhibitory Concentration (MIC) of the herbal formulation

MIC of the combinations of *Acacia nilotica*, *Murraya koenigii* Linn Sprengel, *Eucalyptus hybrid*, and *Psidium Guajava* on *Streptococcus mutans*, *Lactobacillus acidophilus* (dental caries bacteria), *Streptococcus sanguis*, *Streptococcus salivarius* (primary plaque colonizers), *Fusobacterium nucleatum* (secondary plaque colonizer) and *Porphyromonas gingivalis* (tertiary plaque colonizer) was carried out using broth dilution method. MIC was defined as the minimum concentration of extract that caused 20% inhibition in growth of test microorganism^{12,13}. The percentage of bacterial inhibition by each extract was computed using the following equation

$$\text{Percentage inhibition} = \left[\frac{\text{Optical density in control} - \text{Optical density in test set}}{\text{Optical density in control}} \right] \times 100$$

Phytochemical constituent analysis

A qualitative analysis was carried out to determine the phytochemical constituents in *Acacia nilotica*, *Murraya koenigii* Linn Sprengel, *Eucalyptus hybrid*, and *Psidium Guajava* extracts.

Alkaloids were determined using Mayer's test, Wagner's test and Hager's test. Terpenoids were detected using Salkowski Test, saponins using foam test, flavonoids using Magnesium and hydrochloric acid reduction test, tannins using Ferric chloride test and cardiac glycosides using Legal test and Killer Kiliani test. The presence of anthraquinones was detected by adding 1ml dilute ammonia solution to 2 ml of extract dissolved in distilled water. The development of pink color in ammonia layer following vigorous shaking confirmed the presence of anthraquinones.

Animal experimentation

Median lethal dose and adverse drug reactions following single oral dosage of the combinations of *Acacia nilotica*, *Murraya koenigii* L. Sprengel, *Eucalyptus hybrid* and *Psidium Guajava* extracts was evaluated using female Albino Wistar rats. These rats weighed approximately 150-200g. Animal experiment was conducted at Center for Scientific Research and Development (CSR D), People's University, Bhopal between 1st January 2015 and 30th March 2015 after obtaining ethical clearance from the Institutional Animal Ethics Committee (IAEC), CSR D, Bhopal. Experiment was undertaken according to the OECD (Organization for Economic Co-operation and Development) guidelines-425.^{14,15} Healthy adult female Albino Wistar rats from Central Animal Facility (All India Institute of Medical Sciences, New Delhi), were procured and allowed to get accustomed to the experimental conditions for 5 days. All animals were fed standard dry rat pellet diet, and ad libitum of tap water. 15 rats were assigned into five groups of three rats each. The first group was kept as control while other groups of were fed different doses of the herbal formulation by single oral administration (500, 1000, 1500, 2000 mg /kg body weight). Control group of rats were fed normal saline. 18 h prior to dosing, rats were fasted. The compound was administered orally as a single dose using a 22-gauge oral feeding needle. Animals were observed continuously for first two hours and subsequently, at 4th, 6th and 24 hrs following administration of extract to identify changes in the behavioral responses such as tremors, convulsion, salivation, diarrhoea, lethargy, sleep, coma and monitored for any mortality. The numbers of deceased rats in each group was counted and percentage of mortality

was calculated using graphical method of Miller and Tainter¹⁵. An approximate LD₅₀ was determined by observing the mortality experienced by the rats in different groups at the end of 24 hours. Blood samples from the surviving rats were collected at the end of 24 hours and subjected to liver function tests. Estimation of serum total protein, albumin, total bilirubin, aspartate aminotransferases (AST), alanine aminotransferases (ALT), and alkaline phosphatase (ALP) activities were performed as various biochemical indices of liver function. Liver weight, body weight, feed intake and faecal output were also determined.

Preparation of the mouth rinse for In vivo study

The mouth rinses were prepared in the department of Pharmacology, in a Pharmacy college. 0.25 gm of each plant extract was accurately weighed into a clean sterile mortar. The required quantity of Tween -80 and menthol were added to obtain a homogenous mixture. This was triturated after adding small quantity (5-10ml) of sterile water to get a homogenous solution which was made up to 1000 ml and packed in coded plastic bottles. Since mouth rinses were prepared without any added preservatives, they were preserved for

Table 1 A. Minimum Inhibitory Concentration of combinations of *Acacia nilotica*, *Murraya koenigii* L.Sprengel, *Eucalyptus hybrid* and *Psidium Guajava* on *S. mutans*, *S. sanguis* and *S. salivarius*.

Concentrations	<i>S. mutans</i> (Mean optical density ± SD)	% inhibition	<i>S. sanguis</i> (Mean optical density ± SD)	% inhibition	<i>S. salivarius</i> (Mean optical density ± SD)	% inhibition
0.05%	0.4±0.01	11.9	0.4±0.02	20.5	0.4±0.02	24.6
0.1%	0.4±0.02	19.4	0.4±0.02	26.6	0.4±0.03	30.3
0.25%	0.4±0.02	30.7	0.4±0.02	33.5	0.4±0.03	34.1
0.5%	0.3±0.01	41.3	0.3±0.01	36.2	0.3±0.01	41.9
0.75%	0.3±0.02	45.6	0.3±0.01	44.4	0.3±0.02	49.2
1.0%	0.3±0.03	50.9	0.3±0.02	53.2	0.2±0.01	57.4
1.25%	0.2±0.02	70.0	0.1±0.02	75.8	0.2±0.01	67.7
1.5%	0.1±0.02	75.5	0.1±0.02	83.6	0.1±0.01	73.4
Chlorhexidine	0.2±0.02	69.3	0.2±0.01	66.5	0.2±0.01	73.1
Control (without extract)	0.5±0.03		0.5±0.03		0.6±0.03	

Percentage inhibition is computed using equation OD control / OD control – OD test X 100

Concentration inhibiting at least 20% of bacterial growth is considered MIC

Table 1 B. Minimum Inhibitory Concentration of combinations of *Acacia nilotica*, *Murraya koenigii* L.Sprengel, *Eucalyptus hybrid* and *Psidium Guajava* on *L. acidophilus*, *F. nucleatum* and *P. gingivalis*.

Concentrations	<i>L. acidophilus</i> (Mean optical density ± SD)	% inhibition	<i>F. nucleatum</i> (Mean optical density ± SD)	% inhibition	<i>P. gingivalis</i> (Mean optical density ± SD)	% inhibition
0.05%	0.5±0.01	16.0	0.6±0.02	4.0	0.5±0.01	13.8
0.1%	0.5±0.02	22.1	0.5±0.03	17.2	0.5±0.02	17.5
0.25%	0.4±0.02	33.5	0.5±0.02	22.0	0.4±0.01	31.2
0.5%	0.4±0.02	37.4	0.4±0.01	29.9	0.4±0.02	37.6
0.75%	0.3±0.02	45.0	0.4±0.01	38.0	0.3±0.02	43.1
1.0%	0.3±0.02	56.6	0.3±0.01	48.5	0.3±0.02	54.9
1.25%	0.2±0.02	63.4	0.3±0.02	55.2	0.2±0.01	65.1
1.5%	0.2±0.02	70.1	0.2±0.02	61.3	0.2±0.02	66.8
Chlorhexidine	0.2±0.02	63.5	0.2±0.02	65.3	0.2±0.01	71.3
Control (without extract)	0.6±0.02		0.6±0.02		0.6±0.01	

Percentage inhibition is computed using equation OD control / OD control – OD test X 100

Concentration inhibiting at least 20% of bacterial growth is considered MIC

seven days following their preparation to observe any changes in colour, odour, and consistency. The mouth rinses were inoculated in nutrient agar plates after seven days of preservation to check for any bacterial contamination. Mouth rinses were simultaneously used by the investigator to test their odor, consistency and side effects over a period of seven days.

In vivo study

A short term parallel arm double blind randomized controlled trial was undertaken to assess the antiplaque and antimicrobial efficacy of the herbal formulation on a group of adults selected from a hostel. Study was conducted among thirty four adult volunteers aged between 18-30 years residing in a hostel. The study was conducted over a period of two weeks in August 2016. After obtaining permission from concerned authorities to conduct the study, written informed consent was obtained from each participant. Principal investigator addressed all prospective participants in a group discussion and clarified their doubts. Ethical clearance was obtained from the

Institutional Ethics Committee (IEC). The research was conducted in accordance with the guidelines of World Medical Association declaration of Helsinki. The null hypothesis for in vivo study was stated as “there is no significant difference in the antiplaque and antimicrobial efficacy of three mouth rinses”. The sample size was estimated based on hypothesis testing between two means using nMaster software. The sample size was estimated to be 11 per group at an assumed mean difference of 0.45, effect size of 1.12, at 5% alpha error and 80% power. However, the sample size was increased to 12 per group to compensate for 10% drop out.

The participants offering informed consent with eligible criteria cited beneath were recruited

Inclusion Criteria

Adult participants aged between 18-30 years
Free from systemic diseases
Free from physical and mental disabilities
Willing to offer informed consent
Minimum 20 natural permanent teeth

Table 2. Summary of qualitative assay of phytochemical constituents in plant extracts derived using hot and cold extraction methods

Phytochemical constituents and test used	Extraction method	<i>Acacia Arabica</i>	<i>Murraya koenigii L.Sprengel</i>	<i>Eucalyptus hybrid</i>	<i>Psidium Guajava</i>
Alkaloids (using Mayer’s reagent)	Cold	-	+	-	+
	Hot	-	-	-	-
Alkaloids (using Dragendorff’s reagent)	Cold	-	+	-	+
	Hot	-	-	-	-
Anthraquinones (Borntrager’s test)	Cold	+	-	-	+
	Hot	+	-	-	+
Terpenoids (Salkowski’s test)	Cold	-	+	+	+
	Hot	-	-	+	+
Saponins (Froth and emulsion test)	Cold	+	-	+	+
	Hot	-	-	+	-
Flavanoids (Shinoda test)	Cold	+	-	+	+
	Hot	+	-	+	+
Flavanoids (alkaline reagent test)	Cold	+	-	+	+
	Hot	+	-	+	+
Tannins (Ferric chloride test)	Cold	+	+	+	+
	Hot	+	+	+	+
Tannins (lead acetate test)	Cold	+	+	+	+
	Hot	+	-	+	+
Cardiac glycosides (Legal test)	Cold	+	+	+	+
	Hot	+	+	+	+
Cardiac glycosoids (Killer – kiliani test)	Cold	+	+	+	+
	Hot	+	+	+	+

+: positive, -: negative

Exclusion Criteria

Gross oral defects that interfered with chewing
 History of use of antibiotics in the last three months
 Any deleterious and parafunctional habits
 Presence of removable / fixed appliances

Forty eight participants residing in the hostel were considered for initial screening. However, twelve participants were excluded. Six participants were not recruited as they expressed non availability, three participants admitted that they have some deleterious habit, two participants had unilateral mastication owing to grossly decayed teeth and one participant had used amoxicillin for five days within the last 20 days for severe throat infection.

Pre-intervention prophylaxis

Scaling and polishing was offered to all eligible thirty six participants using a portable ultra sonic scaler to reduce plaque score to zero. This was confirmed by a trained and calibrated clinical evaluator using Turesky's modification of Quigley Hein Plaque Index¹⁶. Cronbach's alpha value was 0.9 for intra-examiner reliability indicating good correlation between the scores during test – retest method.

Randomization and Group allocation

The eligible participants were randomly

assigned to three different categories. Thirty six participants were requested to stand in a line and were asked to loudly spell out numbers 1, 2, 3 in an order. All participants calling out number 1 were assigned to group 1 while participants calling out 2 and 3 were assigned to group 2 and 3 respectively. The randomization and group allocation was done by the coordinator and information on group allocation was kept confidential till the research was over.

Intervention

Each participant was given a oral hygiene kit consisting of a medium bristled tooth brush, a 200 gram non-fluoridated tooth paste and a 150 ml coded mouth rinse bottle. The mouth rinses were coded as A, B and C.

Group 1: Saline Mouth rinse

Group 2: Mouth rinse containing *Acacia nilotica*, *Murraya koenigii* Linn Sprengel, *Eucalyptus* hybrid, and *Psidium Guajava* extracts at 1%.

Group 3: 0.2% chlorhexidine diluted to 50% with distilled water.

Each participant was distributed a 150 ml mouth rinse bottle. The participants were instructed to use 10 ml of mouth rinse once in the morning before breakfast and once in the night after dinner for one minute. The participants were directed to

Table 3. Summary of acute toxicity results on experimental rats

Parameter	Control	500 mg/kg B.W	1000 mg/kg B.W	1500mg/kg	2000mg/kg	Statistics
Number	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	Total (n=15)
Body weight (gms)	276.9±11.0	289±16.6	292.1±3.8	291.5±10.6	288.5±1.0	F value: 1.091 p: 0.41
Total Proteins (g%)	7.6±0.0	7.5±0.2	7.5±0.1	7.6±0.1	7.6±0.1	F value: 0.665 p: 0.63
Albumin (g%)	3.4±0.0	3.4±0.2	3.5±0.1	3.5±0.2	3.6±0.0	F value: 1.279 p: 0.34
Bilirubin (mg%)	0.6±0.1	0.7±0.0	0.7±0.1	0.7±0.0	0.7±0.0	F value: 2.203 p: 0.14
AST (IU/L)	61.2±1.4	64.3±2.1	60.0±1.3	60.2±1.8	61.7±2.6	F value: 2.375 p: 0.12
ALT (IU/L)	40.3±1.0	41.6±0.5	40.9±0.9	40.6±0.7	40.4±1.5	F value: 0.860 p: 0.52
ALP (IU/L)	65.4±1.1	65.1±0.5	64.7±0.3	64.2±0.5	66.4±2.1	F value: 1.681 p: 0.23
Liver weight (gms)	6.2±0.2	6.1±0.1	6.8±0.4	6.7±0.1	6.3±0.6	F value: 2.89 p: 0.08

No mortality was observed in any of these groups

avoid intake of any solid and liquid food items for half an hour following mouth rinsing. All participants refrained from using any other oral hygiene aids other than tooth brush and paste provided to them. A medium bristled brush and a 200 gram non-fluoridated tooth paste was given to each of these participants. Directions for the use of mouth rinse in local language were written on the bottles. Contact details of investigator were also written on the bottle and participants were informed to report any adverse events such as altered taste sensation, burning, nausea, vomiting etc. The mouth rinse bottles were collected back at the end of one week and the second set of mouth rinse bottles were distributed on day 7. This ensured that the participants were scrutinized for compliance with regard to use of mouth rinse.

Assessment criteria

On 15th day after intervention, plaque buildup in each participant was assessed by one blinded investigator using Turesky's modification of Quigley Hein Plaque Index. Along with clinical evaluation, supragingival plaque samples were collected from the buccal surface of maxillary left first permanent molar in each participant. Salivary

flow before and 90 minutes after mouth rinsing was assessed on day fifteen. The unstimulated saliva samples were collected before and 90 minutes after mouth rinsing in each participant. Microbial evaluation was undertaken using plaque samples while saliva samples were used for assessing the change in salivary pH before and after rinsing the mouth.

Microbial evaluation

S mutans and total viable count in plaque samples was evaluated using spread plate technique in a microbiology laboratory at Mysuru. The plaque samples also were coded as A, B and C to ensure blinding.

Estimation of salivary parameters

The salivary flow before and after mouth rinsing was evaluated besides saliva samples were estimated for pH. Salivary pH was determined using pH meter (Systronics, Ahmedabad, MK – 6). The CONSORT (Consolidated Standards of Reporting Trials) flow diagram for the randomized trial is presented as figure 1.

Statistical methods

The statistical analysis was done using Statistical Package for Social Sciences (SPSS)

Table 4. Distribution of study participants in different intervention groups

Group	Number of participants N (%)	Mean age \pm Standard deviation (years)
Group 1 (Placebo)	12 (35.2)	19.4 \pm 1.4
Group 2 (polyherbal mouth rinse)	11 (32.4)	20.3 \pm 2.3
Group 3 (0.2% chlorhexidine)	11 (32.4)	19.3 \pm 2.0
Total	34 (100)	19.7 \pm 1.9
Statistical inference	F value: 0.72, p value: 0.06	

Table 5. Comparison of mean plaque build up between different intervention groups on day 15 following intervention

Group	Number of participants N (%)	Mean plaque score \pm Standard deviation
Group 1 (Placebo)	12 (35.2)	1.82 \pm 0.12
Group 2 (polyherbal mouth rinse)	11 (32.4)	0.36 \pm 0.02
Group 3 (0.2% chlorhexidine)	11 (32.4)	0.28 \pm 0.01
Total	34 (100)	0.76 \pm 0.7
Statistical inference	F value: 1721.41, p value < 0.001	
Post hoc comparison	Effect size (r)	p value
Group 1 Vs Group 2	0.92	< 0.001
Group 1 Vs Group 3	0.93	< 0.001
Group 2 Vs Group 3	0.22	0.93

version 20 (IBM, Chicago USA). One way Analysis of Variance (ANOVA) and Tukey's post hoc test, independent sample t-test and Pearson's chi square test were used. The statistical significance was fixed at 0.05.

RESULTS

In vitro study

MIC of the combinations of *Acacia nilotica*, *Murraya koenigii* L.Sprengel, *Eucalyptus* hybrid and *Psidium Guajava* on *S. mutans*, *S. sanguis*, *S. salivarius*, *L. acidophilus*, *F. nucleatum* and *P. gingivalis* was found to be 0.25%, 0.05%, 0.05%, 0.1%, 0.25% and 0.25% respectively (table 1 A and B). The summary of qualitative assay of

phytochemical constituents in four plant extracts derived using hot and cold extraction methods is presented in table 2.

Acute toxicity

Median lethal dose (LD₅₀) for the combinations of *A.nilotica*, *Murraya koenigii* L.Sprengel, *Eucalyptus* hybrid and *Psidium Guajava* extracts was found to be more than 2000mg/kg body weight as there was no mortality in any groups. There was no statistically significant difference in the body weight, liver weight, and various parameters of liver function between rats that received different doses of herbal formulation in comparison with control group (table 3).

In vivo study

Thirty four participants were available

Table 6. Comparison of mean *S. mutans* and total viable count between different intervention groups on day 15 following intervention.

Group	Number of participants N (%)	Mean <i>S. mutans</i> CFU (10 ⁻²) ± Standard deviation	Mean total viable count CFU (10 ⁻²) ± Standard deviation	
Group 1 (Placebo)	12 (35.2)	282.4 ± 22.2	328.4 ± 28.6	
Group 2 (polyherbal mouth rinse)	11 (32.4)	195.3 ± 9.7	232.5 ± 18.6	
Group 3 (0.2% chlorhexidine)	11 (32.4)	158.3 ± 12.2	212.5 ± 14.3	
Total	34 (100)	208.4 ± 48.2	251.4.3 ± 56.261.5	
Statistical inference	F value: 123.2 p value < 0.001		F value: 175.3, p value < 0.001	
Post hoc comparison	Effect size (r)	p value	Effect size (r)	p value
Group 1 Vs Group 2	0.9	<0.001	0.9	<0.001
Group 1 Vs Group 3	0.9	<0.001	0.9	<0.001
Group 2 Vs Group 3	0.8	<0.001	0.1	0.91

CFU = Colony Forming Units

Table 7. Comparison of salivary parameters before and 90 minutes after mouth rinsing in different intervention groups

Group	Salivary flow rate before mouth rinsing in ml/minute (Mean ± SD)	Salivary flow rate after mouth rinsing in ml/minute (Mean ± SD)	Statistical inference	Salivary pH before mouth rinsing	Salivary pH after mouth rinsing	Statistical inference
Group 1 (Placebo)	0.36 ± 0.1	0.36 ± 0.03	t value: 0.40 p = 0.70	6.8 ± 0.13	6.8 ± 0.2	t value: -0.23 p = 0.82
Group 2 (polyherbal mouth rinse)	0.4 ± 0.03	0.4 ± 0.04	t value: - 1.09 p = 0.30	6.9 ± 0.13	6.9 ± 0.2	t value: -1.13 p = 0.52
Group 3 (0.2% chlorhexidine)	0.4 ± 0.04	0.4 ± 0.05	t value: - 1.19 p = 0.30	6.8 ± 0.11	6.9 ± 0.12	t value: - 1.39 p = 0.25
Statistical inference	F value: 1.23 p value: 0.31	F value: 2.01 p value: 0.15		F value: 2.4 p value: 0.11	F value: 3.3 p value: 0.06	

for follow up examination on day 15 and one participant each from group 2 and 3 were dropped out. The dropped out participant in group 2 used an antimicrobial drug that was prescribed for fever during the intervention period while participant in group 3 stopped using the assigned mouth rinse due to unpleasant taste. Mean age of study participants was 19.7 ± 1.9 years. There was no significant difference in the mean age of study participants between different intervention groups ($p = 0.06$, table 4). The mean plaque build-up was highest in group 1 that used placebo (1.82 ± 0.12) compared to other groups. The difference in mean plaque score between different categories was statistically significant ($p < 0.001$, table 5). The post hoc test

found a significant difference between group 1 against groups 2 and 3. However, the difference in the mean plaque scores between group 2 (0.36 ± 0.02) that used 1% polyherbal mouth rinse and group 3 (0.28 ± 0.01) that used 0.2% chlorhexidine with 50% dilution was not statistically significant ($p = 0.93$, table 5). Mean *S. mutans* colony count (CFU/ml) was highest in group 1 (282.4 ± 22.2) followed by group 2 (195.3 ± 9.7). The least count was found in group 3 (158.3 ± 12.2). The difference in the mean *S. mutans* count between different categories was statistically significant ($p < 0.001$, table 6). The post hoc revealed a significant difference between group 1 with other groups as well as between group 2 and 3. The mean total

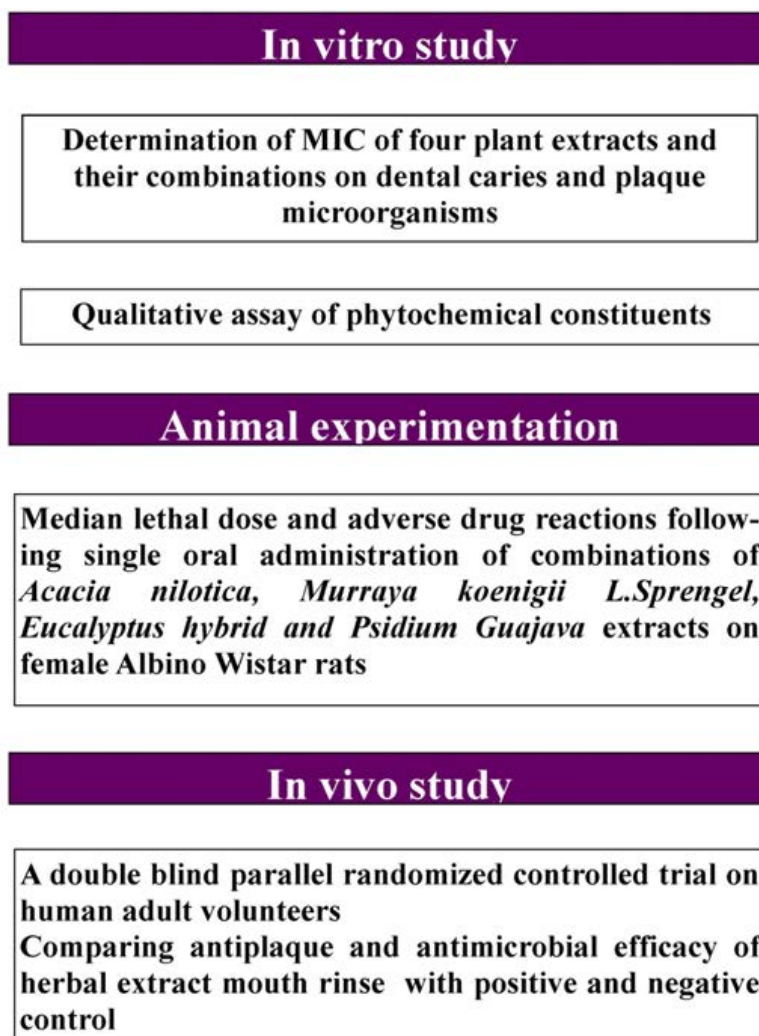


Fig. 1. Diagrammatic representation of clinical research

viable count (CFU/ml) was highest in group 1 (328.4 ± 28.6) followed by group 2 (232.5 ± 18.6). The least count was found in group 3 (212.5 ± 14.3). The difference in mean total viable count between different categories was statistically significant ($p < 0.001$, table 6). The post hoc test revealed a significant difference between group 1 with others. However, the difference between group

2 and 3 was not statistically significant ($p = 0.91$, table 6). There was no significant difference in the mean salivary flow rate and pH between different groups at baseline and post-intervention as well as between baseline and 90 minutes after mouth rinsing in each group (Table 7). No significant differences were observed in compliance and reported adverse effects between three groups.

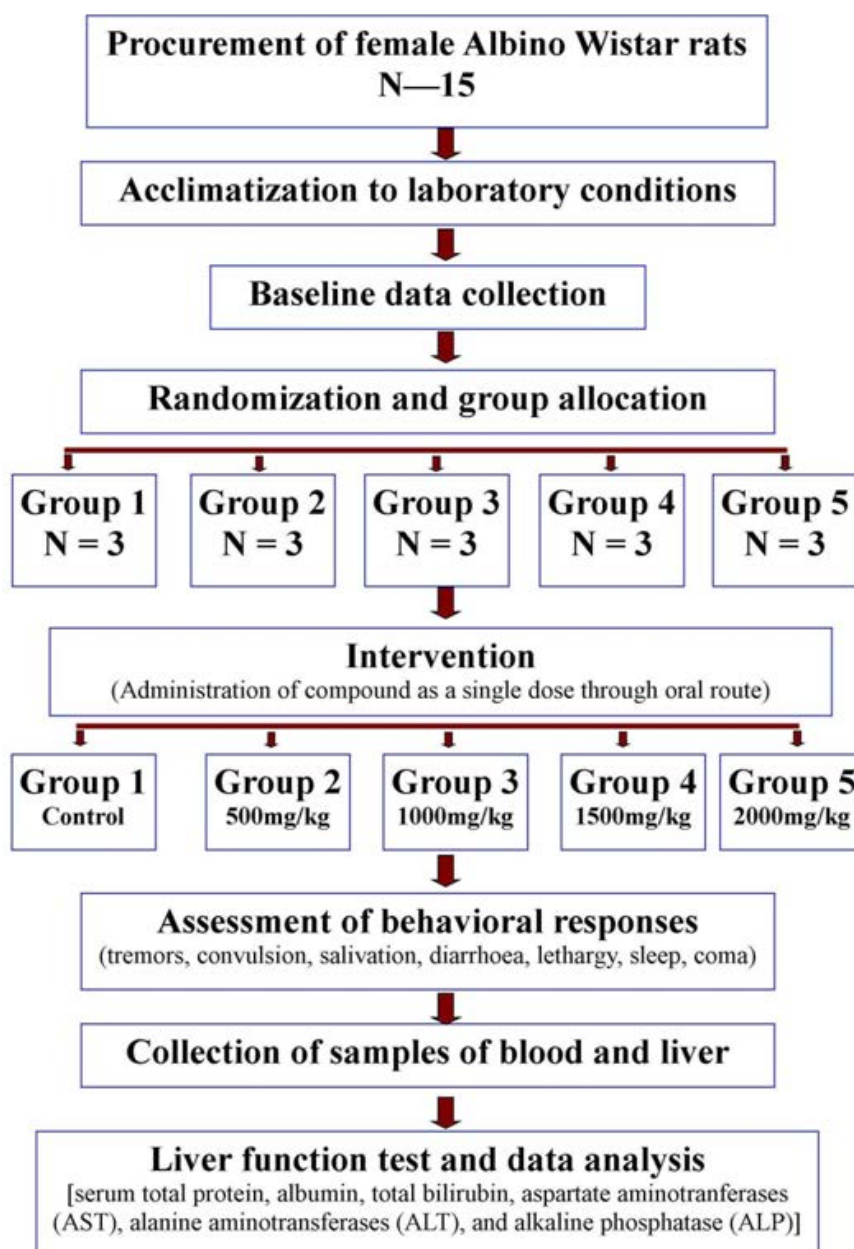


Fig. 2. Flow diagram of animal experimentation on rats

DISCUSSION

The increasing trends in dental caries and gingival periodontal diseases amongst the populations in developing countries, the increasing costs of treating these diseases, and the potential side effects associated with conventional antiplaque agents call for alternate strategies. Herbs are one of remedial agents that have been used in traditional medicine for several thousand years.

The combinations of *Acacia nilotica*, *Murraya koenigii* L.Sprengel, *Eucalyptus hybrid* and *Psidium Guajava* inhibited the growth of *S. mutans*, *S. sanguis*, *S. salivarius*, *L. acidophilus*, *F. nucleatum* and *P. gingivalis* with MIC of 0.25%, 0.05%, 0.05%, 0.1%, 0.25% and 0.25% respectively. MIC of the combinations of plant extracts against these bacteria was varying from 0.05% to 0.25% and its antimicrobial efficacy was found to be good.¹⁷ This was the first of its kind where MIC of combinations of *Acacia nilotica*, *Murraya koenigii* L.Sprengel, *Eucalyptus hybrid* and *Psidium Guajava* was assessed on oral bacteria. Hence, our results could not be compared with previous published literature. The study found no significant differences in the behaviors as well as hepatic function of rats at different doses in comparison with control group of rats. None of

the rats experienced mortality confirming that a dose of 2000 mg / kg body weight of this herbal formulation is safe and did not produce any symptoms of acute toxicity. These results were consistent with the findings of a study by Guta *et al* (2007)¹⁸ who while evaluating oral acute toxicity of ethyl acetate fruit fraction of *A. nilotica* found the LD₅₀ value to be 7393.4 mg/kg (95% CI, 6019.5-9207.9 mg/kg). Tanko *et al* (2014)¹⁹ while assessing the methanol crude leaves extract and aqueous fraction of *Acacia nilotica* found the median lethal dose (LD₅₀) in rats to be 2,154 mg/kg body weight for both crude and aqueous fractions similar to the results of the present study. The in vivo study found the polyherbal mouth rinse group to have lesser plaque build up, *S. mutans* and total viable count compared to saline group. The lack of significant difference in plaque build-up and total viable count between polyherbal rinse group and 0.2% chlorhexidine suggest that this herbal formulation could be evaluated further as an effective herbal alternate to chlorhexidine. The antiplaque and antimicrobial efficacy of herbal mouth rinse was attributed to various phytochemical constituents present in *A. nilotica*, *Murraya koenigii* L.sprengel, *Eucalyptus hybrid* and *Psidium guajava* leaf extracts. Bhadbhade *et al* (2011)²⁰, Parwani *et al* (2013)²¹, Biswas *et al* (2014)²², have all found

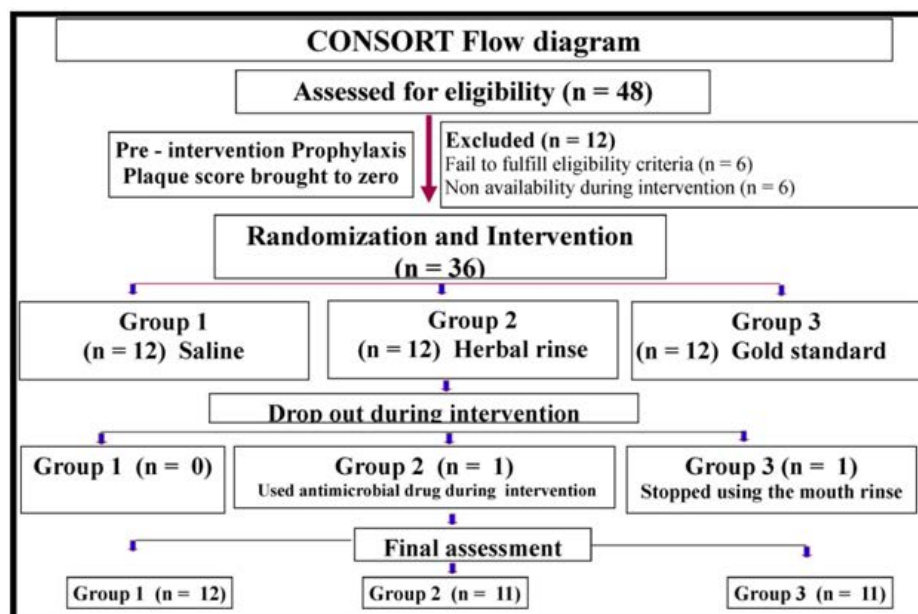


Fig. 3. CONSORT flow diagram of randomized control trial on human adults

different herbal mouth rinses to be effective as antiplaque agents. Phatak *et al* (2011)²³ while assessing the efficacy of a polyherbal mouth rinse found the total viable count in the plaque samples to reduce in 85% of the participants using the polyherbal mouth rinse similar to our findings. The study found no significant differences in the reported side effects, and willingness to use these mouth rinses on a regular basis if given along with tooth brushing while some studies^{22, 24, 25} reported minimal side effects with polyherbal mouth rinse compared to chlorhexidine.

Strengths and limitations

This was the first of its kind where plant extract combinations have been assessed on microorganisms involved in two most prevalent dental diseases. The use of plant extracts in combinations offer increased potency due to synergistic action of phytochemicals, slower rate of development of resistance as they are complex mixtures which can make microbial adaptability difficult²⁶.

The *in vitro* antimicrobial efficacy testing was assessed on important dental caries and plaque microorganisms while assessment on other secondary and tertiary plaque colonizers could offer a more comprehensive assessment. The quantitative evaluation using high pressure liquid chromatography rather than qualitative assay could highlight the concentration of active ingredients in these plant extracts. The polyherbal mouth rinse in the present study was free from any preservatives. The concerns related to shelf life, compelled us to undertake short term study using freshly prepared mouth rinses on healthy adult human volunteers. The long term studies using mouth rinses containing preservatives having a longer shelf life on participants preferably with high risk for dental caries and periodontal diseases such as differently abled participants are essential to provide external validity to the study.

CONCLUSION

The polyherbal mouth rinse containing *Acacia nilotica*, *Murraya koenigii* L.sprengel, *Eucalyptus hybrid* and *Psidium guajava* is feasible to be used as a mouth rinse. It was effective against most common microorganisms involved in dental caries and periodontal diseases. Hence,

this polyherbal mouth rinse may be considered as a simple and effective alternate to chlorhexidine for preventing and controlling dental caries and plaque induced dental diseases. However, future research using purified extracts of these plants and their combinations are recommended to validate our results.

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