Antimicrobial efficacy of the combinations of *Acacia nilotica*, *Murraya koenigii* (Linn.) Sprengel, *Eucalyptus*, and *Psidium guajava* on primary plaque colonizers: An *in vitro* study

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ABSTRACT

**Background:** The rise in disease incidence, increased resistance of pathogenic bacteria to currently used antibiotics and chemotherapeutics, opportunistic infections in immunocompromised individuals, and financial considerations in developing countries necessitates alternate preventive and treatment strategies for oral diseases.

**Objective:** The objective of the study is to assess the antimicrobial efficacy of triple and quadruple combinations of *Acacia nilotica* (AN), *Murraya koenigii* (Linn.) (MKL) Sprengel, *Eucalyptus* (Euca), and *Psidium guajava* (PS) on primary plaque colonizers.

**Materials and Methods:** The phytochemicals in four plants were extracted using Soxhlet apparatus. The dried extracts were diluted with dimethyl sulfoxide (DMSO) to prepare stock solutions (100 mg/ml) of each plant. The triple and quadruple combinations were prepared after mixing equal quantities of stock solutions from each plant extracts. The antimicrobial efficacy testing was done on *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius* using agar well diffusion method. Chlorhexidine of 0.2% composition and DMSO were used as positive and negative controls, respectively. The mean diameter of inhibition zone between different categories was compared using one-way analysis of variance.

**Results:** The combination of AN + MKL Sprengel + Euca + PS produced the highest mean diameter of inhibition zone (23.5 ± 2.17 mm) against S. mutans. The combination of AN + MKL Sprengel + Euca produced the maximum antimicrobial efficacy against S. sanguis (19.83 ± 1.33).

**Conclusion:** All the triple and quadruple combinations of the plant extracts offered antimicrobial benefits either superior or comparable to 0.2% chlorhexidine against *S. mutans*, *S. sanguis*, and *S. salivarius*.

**Key words:** *Acacia nilotica*, antimicrobial efficacy, dental caries, *Eucalyptus*, *Murraya koenigii* (Linn.) *Sprengel*, oral bacteria, *Psidium guajava*

Oral health affects general health by causing substantial pain and suffering and by changing what people eat, their speech, and their quality of life and well-being.¹ Hence, the compartmentalization involved in viewing the mouth separately from the rest of the body must cease. Oral diseases are the most common of the chronic diseases and are important public health problems because of their prevalence, their impact on individuals and society, and the high cost of their treatment.¹ Despite marked advances in oral health in many industrialized countries, populations of dentally disadvantaged individuals do exist in these countries, often indigenous child populations and those people of low socioeconomic status, where oral health is deteriorating.² Dental plaque is a specialized bacterial biofilm that develops on the surface of teeth, dental restorations, prostheses, and

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implants. The colonizing bacteria that make up the biofilm consortium produce organic acids, such as lactic acid, by metabolizing carbohydrates that result in the formation of dental caries. Plaque is also involved in the etiology of periodontal diseases. Dental caries continues to be a significant public health problem in most parts of the world. The prevalence of dental caries is steadily increasing, and periodontal diseases are among the most common afflictions of humankind. Tooth loss, owing to poor periodontal health, affects almost 20% of the adult population worldwide. This can lead to significant morbidity and premature death. The prevalence of dental caries among adults and elderly in an urban settlement colony of New Delhi was 82.4% and 91.9%, respectively. The prevalence among 6–10-year-old children in rural areas of Udaipur was 63.2% and 85.7% of the children needed treatment for dental caries. The prevalence of gingivitis among 5–14-year-old children in rural areas of Udaipur district was 84.4%. Oral diseases are the fourth most expensive diseases to treat in some countries. The treatment of dental caries alone which is estimated at 3513 USD/1000 children, exceeds the total health budget for children in most low-income countries.

The situation for adults in developing countries is worse as they suffer from accumulation of untreated oral diseases. The approach best suited for developing countries such as India is to focus on prevention with innovative approaches. The global need for alternate prevention and treatment strategies for oral diseases that are safe, effective, and cost-effective comes from the increase in disease incidence (particularly in developing countries), resistance of pathogenic bacteria to currently used antibiotics and chemotherapeutics, opportunistic infections in immunocompromised individuals, and financial considerations in developing countries. Despite several agents being commercially available, these chemicals can alter oral microbiota and have undesirable side effects such as vomiting, diarrhea, and tooth staining. The bacterial resistance to most (if not all) of the antibiotics commonly used to treat oral infections (penicillins and cephalosporins, erythromycin, tetracycline and derivatives and metronidazole) has been documented. Other antibacterial agents used in the prevention and treatment of oral diseases, including cetylpyridinium chloride, chlorhexidine, amine fluorides, or products containing such agents, are reported to exhibit toxicity, cause staining of teeth, or in the case of ethanol (commonly found in mouthwashes) have been linked to oral cancer. Hence, the search for alternative products continues and natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals.

Some studies in the past have demonstrated the antimicrobial efficacy of Acacia nilotica (AN), Murraya koenigii (Linn.) Sprengel, and Psidium Guajava (PS). However, these limited studies have assessed the efficacy of individual extracts on oral microbes, and it may be possible to maximize the antimicrobial effect of the plant extracts by using them in combination. The combinations of plant extracts lower the rate of development of resistant strains besides enhancing their potency by the synergistic action of their components. In this background, the present study assessed the antimicrobial efficacy of the triple and quadruple combinations of AN, MKL Sprengel, Euca, and PS on primary plaque colonizers.

**MATERIALS AND METHODS**

**Study design and setting**

This was an in vitro study conducted at the research laboratory, Center for Scientific Research and Development, People’s University, Bhopal, between July and December 2013.

**Plant material**

The leaves of four plants were collected from the surrounding areas, identified, and authenticated by a taxonomist. The leaves were then rinsed in water treated with reverse osmosis and shade dried over a period of 3–4 weeks at room temperature. The coarse powder of dried leaves was obtained by hand crushing individual leaves separately. The fine powder was subsequently prepared using a mixer grinder, stored in airtight plastic bottles, and labeled. The bottles were stored in refrigerator at 4°C till further use.

**Bacteria**

The American Type Culture Collection strains of Streptococcus mutans, Streptococcus sanguis, and Streptococcus salivarius imported from the United States were revived at the research laboratory for further microbiological assay. The bacterial cultures were then maintained on brain heart infusion agar slants with periodic subculturing and stored at 4°C.

**Plant extraction**

Soxhlet apparatus was used for the extraction of plant material. Fifty grams of ground powder from each plant was placed in a porous bag or “thimble” made of strong filter paper and loaded into the main chamber of the Soxhlet extractor. The extractor was subsequently placed into a distillation flask containing the solvent (ethanol). The Soxhlet was then equipped with a condenser, and the solvent was heated to reflux. The warm solvent vapors traveled up the distillation arm and flooded into the chamber housing the thimble. It was automatically emptied by a siphon side arm back down to the distillation flask once the chamber was almost full. This cycle was allowed to repeat many times so that the desired compound gets concentrated in the distillation flask. The solvent containing active compounds...
from each plant was filtered, concentrated under reduced pressure (30 ± 10 mbar) in a rotary evaporator at 30–60°C to a syrupy consistency, and finally dried at room temperature. The weight of the dried mass was recorded and used for experimental studies.[15]

Preparation of combination of extracts
Initially, the stock solutions of the individual plants were prepared by dissolving 100 mg of the dried extract in 1000 µl of dimethyl sulfoxide (DMSO). Two hundred microliters of the stock solution from three plants was then mixed to obtain the triple combinations of plants. Four triple combinations and one quadruple combination of the plant extracts with equal quantity (200 µl) of 10% solution of each plant (100 mg of the dried extract/1000 µl DMSO) were prepared in this way and assessed for antimicrobial efficacy.

Antimicrobial efficacy testing
The antimicrobial efficacy testing of the triple combination of plant extracts (50 µl volume) was done using agar well diffusion method. The diameter of the inhibition zone was measured at three different planes on the under surface of the agar plate using a transparent scale. Chlorhexidine (0.2%) and DMSO were used as positive and negative controls, respectively. The experiment was done in duplicate, and mean inhibition zone was computed using the six readings after subtracting the well diameter (7 mm).

Qualitative assay of phytochemical in plant extracts
Mayer’s reagent and Dragendorff’s reagent were used identify the presence of alkaloids. Anthraquinones were identified using Borntrager's test, terpenoids using Salkowski’s test, saponins using Froth and emulsion test, flavonoids using Shinoda and alkaline reagent tests, tannins using Ferric chloride and lead acetate tests, and cardiac glycosides using Legal test and Keller–Kiliani test.[9]

Data entry and statistical analysis
The statistical analysis was done using SPSS version 20 (IBM, Chicago, IL, USA). The mean diameter of inhibition zone between different categories was compared using one-way analysis of variance and Tukey’s post hoc test. The statistical significance was fixed at 0.05.

RESULTS
The taxonomic details of the four plants and their yield using Soxhlet extraction process are provided in Table 1. The details of the three bacteria used for antimicrobial efficacy testing are presented in Table 2.

Antimicrobial efficacy of extracts
All the triple and quadruple combinations of plant extracts containing active compounds derived using Soxhlet extraction inhibited the growth of *S. mutans*, *S. sanguis*, and *S. salivarius*.

| Table 1: The plant profile and yield of the four herbal extracts assessed in the present study |
| Botanical name | Common name | Family | Weight of dried extract (g) | Yield (%) |
|----------------|-------------|--------|-----------------------------|-----------|
| AN | Babul | Leguminosae | 0.081 | 4.55 |
| MKL | Curry | Rutaceae | 0.101 | 5.05 |
| Euca | Euca | Myrtaceae | 0.198 | 9.9 |
| PS | Guava | Myrtaceae | 0.139 | 6.95 |

| Table 2: Details of the bacteria used for antimicrobial efficacy testing |
| Bacteria | ATCC number | Selective media used for revival | Type of hemolysis on blood agar plate | Media for antimicrobial efficacy testing |
|-----------|--------------|-------------------------------|---------------------------------|-----------------------------------|
| S. mutans | 25175 | BHI agar with 5% sheep blood | Gamma hemolysis | BHI agar |
| S. sanguis | 10556 | BHI agar with 5% sheep blood | Alpha hemolysis | BHI agar |
| S. salivarius | 13419 | BHI agar with 5% sheep blood | Gamma hemolysis | BHI agar |

The combination of AN + MKL *Sprengel* + Euca + PS produced the highest mean diameter of inhibition zone (23.5 ± 2.17 mm) (mean ± standard deviation) against *S. mutans* followed by the combinations of AN + Euca + PS (22.25 ± 0.61), MKL *Sprengel* + Euca + PS (20.67 ± 1.63), AN + MKL *Sprengel* + PS (20.50 ± 3.15), and AN + MKL *Sprengel* + Euca (20.33 ± 2.94) in the descending order. Chlorhexidine (0.2%) produced an inhibition zone of 14.5 ± 2.07 mm. The difference between various categories was statistically significant [P = 0.001, Table 3]. However, the multiple pairwise comparisons between the different categories found no significant difference against *S. mutans* [Table 4].

The combinations of AN + MKL *Sprengel* + Euca produced the maximum antimicrobial efficacy against *S. sanguis* (19.83 ± 1.33) followed by the combinations of AN + MKL *Sprengel* + Euca + PS (18.92 ± 0.67), AN + MKL *Sprengel* + PS (18.67 ± 2.25), AN + Euca + PS (18.58 ± 2.06) and MKL *Sprengel* + Euca + PS (17.0 ± 1.1) in the descending order. There was a significant difference in the mean diameter of inhibition zone between different categories with the least efficacy observed with 0.2% chlorhexidine (13.17 ± 1.47) [P = 0.001, Table 3]. The multiple pair-wise comparisons found all the combinations to offer significantly higher mean inhibition zone against *S. sanguis* compared to 0.2% chlorhexidine with no difference between different plants extract combinations [Table 4].

Although all the combinations of plant extracts inhibited the growth of *S. salivarius*, there was no statistically significant difference in the mean diameter of inhibition
zone between different herbal combinations and 0.2% chlorhexidine \( [P = 0.119, \text{Table 3}]. \) DMSO failed to inhibit the growth of these bacteria and hence was not considered for analysis.

It can be inferred from the results of this study that the triple and quadruple combinations of the plant extracts offered antimicrobial benefits either superior or comparable to 0.2% chlorhexidine against \( S. \) mutans, \( S. \) sanguis, and \( S. \) salivarius.

The summary of phytochemical constituents identified using qualitative assay of four plants is presented in Table 5.

**Table 3: Antimicrobial efficacy of triple combinations of four plant extracts against \( S. \) mutans, \( S. \) sanguis, and \( S. \) salivarius**

| Extracts in combination   | Mean diameter of inhibition zone (SD) |
|---------------------------|---------------------------------------|
|                           | \( S. \) mutans | \( S. \) sanguis | \( S. \) salivarius |
| AN + MKL + PS             | 20.50 (3.15)    | 18.67 (2.25)    | 19.17 (1.33)      |
| AN + MKL + euca           | 20.33 (2.94)    | 19.83 (1.33)    | 17.67 (1.21)      |
| AN + euca + PS            | 22.25 (0.61)    | 18.58 (2.06)    | 20.00 (1.10)      |
| MKL + euca + PS           | 20.67 (1.63)    | 17.00 (1.10)    | 18.67 (1.21)      |
| AN + MKL + euca + PS      | 23.50 (2.17)    | 18.92 (0.67)    | 18.42 (0.67)      |
| Chlorhexidine             | 14.50 (2.07)    | 13.17 (1.47)    | 17.83 (2.79)      |

**Statistical inference**

\( F = 11.258, \) \( df = 5, \) \( P = 0.001 \)

\( *SD= \) Standard deviation. \( S. \) mutans = \( S. \) mutans, \( S. \) sanguis = \( S. \) sanguis, \( S. \) salivarius = \( S. \) salivarius, AN = \( A. \) nilotica, MKL = \( M. \) koenigii \( ( \text{Linn.} ) \) \( S. \)prengel, Euca = \( E. \) cuplyptus, PS = \( P. \) guajava

**DISCUSSION**

The natural products derived from medicinal plants are an abundant source of biologically active compounds, many of which form the basis for the development of new lead chemicals for pharmaceuticals. With respect to diseases caused by microorganisms, the increasing resistance of many common pathogens to currently used therapeutic agents, such as antibiotics and antiviral agents, has led to renewed interest in the discovery of novel anti-infective compounds. As there are approximately 500 000 plant species occurring worldwide, of which only 1% has been phytochemically investigated, there is great potential for discovering novel bioactive compounds. In an attempt to explore the possibility of enhancing the potential of individual plant extracts by using them in combinations, this study assessed the antimicrobial efficacy of the triple and quadruple combinations of four plant extracts.

We found the antimicrobial efficacy of the triple and quadruple combinations of AN, MKL \( S. \)prengel, Euca, and PS to be either superior or comparable to 0.2% chlorhexidine against \( S. \) mutans, \( S. \) sanguis, and \( S. \) salivarius. The results of our study could not be compared with any previously published literature as this study is the first of its kind comparing the antimicrobial efficacy of combinations of plant extracts on oral bacteria. Qualitative assay in our study revealed the presence of anthraquinones, flavonoids, tannins, and cardiac glycosides in the leaf extract of AN. However, the literature suggests bark extract of AN to contain

**Table 4: Multiple pair wise comparisons with regard to antimicrobial efficacy of triple combination of four plant extracts \( S. \) mutans, \( S. \) sanguis, and \( S. \) salivarius**

| Plant extract in combinations | \( S. \) mutans | \( S. \) sanguis | \( S. \) salivarius |
|-----------------------------|-----------------|-----------------|-----------------|
| AN + MKL + PS versus AN + MKL + euca | 0.994 | 0.995 | 0.994 |
| AN + MKL + PS versus AN + euca + PS | 1.000 | 1.000 | 1.000 |
| AN + MKL + PS versus MKL + euca + PS | 1.000 | 0.890 | 1.000 |
| AN + MKL + PS versus AN + MKL + euca + PS | 1.000 | 1.000 | 1.000 |
| AN + MKL + PS versus MKL + euca | 0.998 | 0.001 | 0.998 |
| AN + MKL + euca versus AN + MKL + PS | 0.994 | 0.995 | 0.994 |
| AN + MKL + euca versus AN + euca + PS | 0.794 | 0.990 | 0.794 |
| AN + MKL + euca versus MKL + euca + PS | 1.000 | 0.139 | 1.000 |
| AN + MKL + euca versus AN + MKL + euca + PS | 1.000 | 1.000 | 1.000 |
| AN + euca + PS versus AN + MKL + euca | 0.998 | 0.924 | 0.998 |
| AN + euca + PS versus AN + MKL + euca + PS | 0.990 | 1.000 | 0.990 |
| AN + euca + PS versus AN + MKL + euca + PS | 1.000 | 0.001 | 0.870 |
| AN + euca + PS versus MKL + euca + PS | 0.870 | 0.001 | 0.870 |
| AN + euca + PS versus MKL + euca | 1.000 | 0.890 | 1.000 |
| AN + MKL + euca + PS versus AN + MKL + euca | 1.000 | 0.139 | 1.000 |
| AN + MKL + euca + PS versus AN + euca + PS | 0.998 | 0.924 | 0.998 |
| AN + MKL + euca + PS versus MKL + euca | 1.000 | 0.742 | 1.000 |
| AN + MKL + euca + PS versus MKL + euca | 1.000 | 0.005 | 1.000 |
| AN + MKL + euca + PS versus MKL + euca + PS | 1.000 | 0.001 | 1.000 |

\( AN= \) \( A. \) nilotica, \( MKL= \) \( M. \) koenigii \( ( \text{Linn.} ) \) \( S. \)prengel, Euca = \( E. \) cuplyptus, PS = \( P. \) guajava, \( S. \) mutans = \( S. \) mutans, \( S. \) sanguis = \( S. \) sanguis, \( S. \) salivarius = \( S. \) salivarius

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The antimicrobial efficacy of AN has been studied in various in vitro and in vivo models. Some studies have found that the ethanolic extracts of AN inhibited the growth of S. mutans (31 ± 0.7 mm). Leaf extract of MKL Sprengel in the present study contained tannins and cardiac glycosides while Vats et al. found the presence of sterols, alkaloids, and flavonoids. Ramesh et al. have recommended curry leaf extracts to be used as home remedies. This recommendation was made following an in vivo study that involved chewing of curry leaves. Chewing the leaves of MKL Sprengel resulted in the creation of an unfavorable oral environment for caries microorganisms. Sunitha et al. have found both the alcoholic and aqueous extracts of MKL Sprengel to be effective against S. mutans. The mean diameter of inhibition zone using alcoholic and aqueous extracts in their study was 16 and 13.05 mm, respectively, at the end of 48 h. The antimicrobial efficacy of Euca was attributed to terpenoids, saponins, flavonoids, tannins, and cardiac glycosides. Literature indicates the presence of phytochemical constituents such as alkaloids, steroids, tannins, flavonoids, saponins, phenolics, glycosides, and macrocarpals A, B, and C in Euca. Some studies have found Euca extracts to possess antibacterial activity against periodontopathic bacteria such as Porphyromonas gingivalis. Another study by Takarada et al. demonstrated the inhibitory effect of Euca oil on S. mutans, Streptococcus sobrinus, P. gingivalis, Actinobacillus actinomycetemcomitans, and Fusobacterium nucleatum. Some studies have found P. guajava to possess antimicrobial efficacy. Leaf extract of P. guajava in our study revealed the presence of anthraquinones, terpenoids, flavonoids, tannins, and cardiac glycosides. Hema et al. and Andrade-Neto et al. in their study attributed the antimicrobial potential of P. guajava to guajaverin, psidiolic acid, and other essential oil constituents such as monoterpenes, 1,8-cineol, p-cimen, and acetate of α-terpenen.

The increase of microbial resistance to antibiotics threatens public health on a global scale as it reduces the effectiveness of treatments and increases morbidity, mortality, and health care costs. Evolution of highly resistant bacterial strains has compromised the use of newer generations of antibiotics. The use of plant extracts in combinations has many advantages such as increased potency owing to the synergistic action of phytochemical constituents, lowered rate of development of resistance because they are complex mixtures and make microbial adaptability very difficult. It has been reported in the literature that the development of bacterial resistance to synergistic drug combinations, such as those found in plants, is slower than for single drug therapies. Moreover, the plant extracts have been reported to possess minimal side effects. The results of the present study demonstrate that any of these combinations of plant extracts could be tried as herbal alternates to chlorhexidine. The major advantage of using plant extracts in combinations over chlorhexidine could be minimal side effects and a slower rate of development of resistance. The combinations of plant extracts, on the other hand, may result in the development of some intermediate compounds that may be toxic to the host tissues. However, this needs to be evaluated in future studies. The assessment of the antimicrobial efficacy of these combinations of plant extracts on other important anaerobic oral bacteria such as Lactobacillus acidophilus, secondary and tertiary plaque colonizers will help in the development of a polyherbal combination that can serve as antiplaque mouth rinse that is effective against dental caries and periodontal diseases.

**CONCLUSION**

All triple and quadruple combinations of AN, MKL Sprengel, Euca, and PS are effective against S. mutans, S. sanguis, and S. salivarius. Their antimicrobial efficacy was either comparable or slightly superior to 0.2% chlorhexidine. Each of these combinations could hence be considered as potential antiplaque agents. There is a need for extensive research to assess antimicrobial efficacy of each of these plant combinations on a wide variety of dental caries and periodontal pathogens, their adverse effects if any on topical use, and in vivo studies demonstrating the potential benefits before they are considered safe and effective alternates to existing therapies. The development of such innovative, novel polyherbal formulations that can be prepared indigenously by rural folk is the need of hour. These kinds of preventive strategies that are easily available, cost-effective, socially and culturally acceptable will aid in the promotion of oral health among rural populations in developing countries.

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Conflicts of interest
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