Original Article

Antimicrobial efficacy of the combinations of *Acacia nilotica, Murraya koenigii L.* sprengel, *Eucalyptus* hybrid and *Psidium guajava* on primary plaque colonizers

Abstract

Background: There is an urgent need for innovative strategies to combat the two most common dental diseases of mankind namely dental caries and periodontitis.

Objective: The aim was to assess the antimicrobial efficacy of the double combinations of *Acacia nilotica* (AN), *Murraya koenigii L.* Sprengel (MKL), *Eucalyptus* hybrid *and Psidium guajava* on primary plaque colonizers.

Materials and Methods: The plant extracts of AN, MKL. Sprengel, *Eucalyptus* hybrid and *P. guajava* were prepared using Soxhlet apparatus. The stock solutions of individual plant extracts (100 mg/ml) were prepared. Equal quantities of stock solutions were mixed to obtain six double combinations of herbal extracts. The antimicrobial efficacy testing was done against three primary plaque colonizers using agar well-diffusion method. 0.2% chlorhexidine and dimethyl sulfoxide were used as positive and as negative controls. The mean inhibition zone between the categories was compared using one-way Analysis of Variance and Tukey's *post hoc* test.

Results: The combination of AN and *P. guajava* produced the highest mean diameter of inhibition zone (21.08 mm ± 2.11) against *Streptococcus mutans.* The chlorhexidine produced the least inhibition zone against *S. mutans* (14.50 ± 2.07). The combination of AN and *P. guajava* produced the maximum antimicrobial efficacy against *Streptococcus sanguis* (19.67 ± 1.03) and *Streptococcus salivarius* (20.33 ± 1.86).

Conclusion: All the combinations of plant extracts have the potential to be used as antiplaque and anticaries agents. The combinations of herbal extracts offer enhanced antimicrobial efficacy due to the synergistic effects besides slowing the development of resistance.

Key words:
Acacia nilotica, antimicrobial efficacy, dental caries, dental plaque, Eucalyptus hybrid, Psidium guajava, Murraya koenigii L. Sprengel, Streptococcus mutans

Introduction

Oral diseases are important public health problems worldwide. The major bulk of the world population is afflicted by dental caries and periodontal diseases.[1] It is evident from the literature that the prevalence of dental caries is rising with almost around 90% of the school-aged children and majority of the adults being affected in spite of advances in the overall health status of the population even in developed countries.[1] Oral health being an integral component of general health is related to the quality of life that extends beyond the functions of the craniofacial complex. There is considerable evidence that links poor oral health to chronic conditions namely diabetes, cardiovascular diseases, rheumatoid arthritis, osteoporosis and pregnancy complications (preterm low-birth weight).[2-4] Poor periodontal health leading to tooth loss affects almost 20% of the adult population worldwide and contributes to significant morbidity and premature death.[5,6] The curative dental care consumes approximately 10% of the public health expenditure in many industrialized countries

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thereby reflecting the economic impact of oral diseases.[5] On the contrary, the expenditure on oral health care is very low; access to dental care is very minimal and mostly limited to emergency dental care or pain relief in the majority of the developing countries.[1]

The plaque bacteria are primarily responsible for dental caries and periodontal diseases.[7] Several antibacterial agents such as chlorhexidine, fluorides, and various antibiotics are used as antiplaque and anticaries agents. However, these agents have been reported to exhibit undesirable side-effects that include nausea, vomiting, tooth staining. Hence, there is a continuous search for alternative products that can combat dental caries and periodontal diseases simultaneously.[8]

Ayurveda, an ancient medicinal system is practiced extensively in India for 1000's of years. Worldwide, close to 80% of the population, especially from developing countries use plant medicines for primary health care. Medicinal plants are an important source of raw materials in the manufacture of drugs intended for preventive and curative applications. There is an exponential growth in the academic, public and Government interest in traditional medicine owing to the rising incidence of adverse drug reactions as well as cost considerations. A methodical and orderly evaluation of the plant extracts and their combinations present an ideal approach in the evolution of novel drugs from plants.[9]

Studies have found *Acacia nilotica* (AN),[8,10] *Murraya koenigii L.* Sprengel (MKL),[11,12] *Eucalyptus* hybrid[13] and *Psidium guajava*[14,15] to possess antimicrobial effect on oral microorganisms. The plant medicines derived using combinations of these extracts may offer significant benefits due to the synergistic action of the components present, which not only enhance the biological activity of the drug, but simultaneously lower the toxic effect. The literature on the antimicrobial efficacy of combination of these herbal extracts on dental caries and plaque bacteria are nonexistent. The present study assessed the antimicrobial efficacy of the double combinations of AN, MKL Sprengel, *Eucalyptus* hybrid *and P. guajava* on primary plaque colonizers.

Materials and Methods

Study design and setting

This *in vitro* study was conducted over a period of six months from July to December 2013 at the research laboratory, Center for Scientific Research and Development, People's University, Bhopal.

Plant material

The leaves of four plants were collected from the surrounding areas, identified and authenticated by a taxonomist. After thorough rinsing with water treated with reverse osmosis, the leaves were shade dried over a period of three-four weeks at room temperature. The dried leaves were hand crushed separately to obtain coarse powder. Subsequently, the fine powder was prepared using a mixer grinder and stored in airtight plastic bottles. The bottles were labeled and stored in the refrigerator at 4°C till further use.

Bacteria

The American Type Culture Collection strains of *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus salivarius* was imported from USA. The bacteria were revived at the research laboratory for further microbiological assay. The bacterial cultures were maintained on BHI agar slants with periodic subculturing and stored at 4°C.

Plant extraction

The extraction process was carried out using Soxhlet apparatus. 50 g 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 onto 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 travelled 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 extracts were 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.[16]

Preparation of combination of extracts

The stock solutions of the individual plants were prepared by dissolving 100 mg of the extract in 1000 µl of dimethyl sulfoxide (DMSO). 200 µl of the stock solution from AN was then mixed with equal quantity of MKL Sprengel to obtain the combination of these two plants.

The following double combinations of plant extracts were prepared by mixing equal quantity of the stock solutions of individual extracts.

- *Acacia nilotica* + *P. guajava*
- *Acacia nilotica* + *Eucalyptus* hybrid
- *Murraya koenigii L. Sprengel* + *P. guajava*
- *Murraya koenigii L. Sprengel* + *Eucalyptus* hybrid
- *Eucalyptus* hybrid + *P. guajava*.

Antimicrobial efficacy testing

The antimicrobial efficacy of the combination of plant extracts (50 µl volume) was assessed using agar well diffusion method. The diameter of the inhibition zone was measured at three different planes on the undersurface of the agar plate using a transparent scale. 0.2% chlorhexidine was used as a positive control and DMSO, as a negative control. The experiment was done in duplicate, and mean inhibition zone was computed using the six readings after accounting for the well diameter (7 mm).

Data entry and statistical analysis

The data analysis was performed using SPSS version 20 (IBM, Chicago 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 details of the four plants and bacteria used for antimicrobial efficacy testing in the present study are denoted in Tables 1 and 2, respectively.

Antimicrobial efficacy of extracts

All the six double combinations of the plant extracts were effective in inhibiting 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

Plant	Botanical name	Family	Weight of dried extract (g)	Yield (%)
Babul	*Acacia Arabica*	Leguminosae	9.48	18.96
Curry	*Murraya koenigii L.* Sprengel	Rutaceae	5.42	10.84
Eucalyptus	*Eucalyptus* hybrid (*E. canaldulensis* × *E. ovata)*	Myrataceae	15.77	31.54
Guava	*Psidium guajava*	Myrataceae	12.57	25.14

E. canaldulensis: Eucalyptus camaldulensis, *E. ovate*: Eucalyptus *ovate*

Table 2: Details of the bacteria used for antimicrobial efficacy testing

Bacteria	ATCC number	Selective media used for revival %	Type of haemolysis on blood agar plate	Media for antimicrobial efficacy testing
S. mutans	25,175	Brain heart infusion agar with 5% sheep blood	Gamma haemolysis	Brain heart infusion agar
S. sanguis	10,556	Brain heart infusion agar with 5% sheep blood	Alpha haemolysis	Brain heart infusion agar
S. salivarius	13,419	Brain heart infusion agar with 5% sheep blood	Gamma haemolysis	Brain heart infusion agar

S. mutans: Streptococcus mutans, S. sanguis: Streptococcus sanguis, S. salivarius: Streptococcus salivarius, ATCC: American Type Culture Collection

The combination of AN and *P. guajava* produced the highest mean diameter of inhibition zone (21.1 mm ± 2.1) (mean ± standard deviation) against *S. mutans* and then the combinations of *Eucalyptus* hybrid and *P. guajava* (19.7 ± 2.6), AN and *Eucalyptus* hybrid (18.9 mm ± 0.8 mm), AN *and* MKL Sprengel (17.7 ± 1.2), MKL Sprengel and *P. guajava* (16.3 ± 1.9) and the combination of MKL Sprengel with *Eucalyptus* hybrid (16.1 ± 1.0) in the descending order. The chlorhexidine produced the least inhibition zone against *S. mutans* (14.5 ± 2.1). The difference between different categories was statistically significant [P = 0.001, Table 3].

The combination of AN and *P. guajava* produced the maximum antimicrobial efficacy against *S. sanguis* (19.7 ± 1.0) followed by the combinations of AN and MKL Sprengel (18.3 ± 0.7), AN and *Eucalyptus* hybrid (17.8 ± 2.1), *Eucalyptus* hybrid and *P. guajava* (17.7 ± 0.8), MKL Sprengel and *Eucalyptus* hybrid (15.5 ± 2.1) and MKL Sprengel with *P. guajava* (14.3 ± 1.5) 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 [P = 0.001, Table 3].

The combination of AN *and P. guajava* produced the highest mean diameter of inhibition zone against *S. salivarius* (20.3 ± 1.9), followed by the combinations of AN and *Eucalyptus* hybrid (20.0 ± 2.3), AN *and* MKL Sprengel (19.4 ± 1.6), *Eucalyptus* hybrid and *P. guajava* (18.8 ± 1.3), MKL Sprengel + *Eucalyptus* hybrid (16.8 ± 1.7) and MKL Sprengel with *P. guajava* (14.8 ± 1.9) in the descending order. A statistically significant difference in the mean diameter of inhibition zone between different herbal combinations and 0.2% chlorhexidine (17.8 ± 2.8) was found against *S. salivarius* [P = 0.001, Table 3]. DMSO failed to inhibit the growth of these bacteria and hence, was not considered for analysis.

The results of the multiple pair wise comparisons between different categories against *S. mutans, S. sanguis* and *S. salivarius* is denoted in Table 4. It can be inferred from the results of the present study that the double combinations of the four plant extracts offered antimicrobial benefits either superior or comparable to 0.2% chlorhexidine against the three

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

Extracts in combination	Mean diameter of inhibition zone±SD* (95% CI)*		
	S. mutans	*S. sanguis*	*S. salivarius*
A. nilotica + *Murraya koenigii L.* Sprengel	17.7±1.2 (16.4-18.9)	18.3±0.7 (17.5-19.0)	19.4±1.6 (17.8-21.1)
A. nilotica + *Psidium guajava*	21.1±2.1 (18.9-23.3)	19. 7±1.0 (18.6-20.8)	20.3±1.9 (18.4-22.3)
A. nilotica + *Eucalyptus* hybrid	18.9±0.8 (18.1-19.8)	17.8±2.1 (15.6-20.1)	20.0±2.3 (17.6-22.4)
Murraya koenigii L. Sprengel + *Psidium guajava*	16.3±1.9 (14.2-18.3)	14.3±1.5 (12.8-15.9)	14.8±1.9 (12.7-16.8)
Murraya koenigii L. Sprengel + *Eucalyptus* hybrid	16.1±1.0 (15.0-17.2)	15.5±2.1 (13.3-17.7)	16.8±1.7 (15.0-18.6)
Eucalyptus hybrid + *Psidium guajava*	19.7±2.6 (17.0-22.4)	17. 7±0.8 (16.8-18.5)	18.8±1.3 (17.4-20.2)
Chlorhexidine	14.50±2.7 (12.3-16.7)	13.2±1.5 (11.6-14.7)	17.8±2.8 (14.9-20.8)
Statistical inference	*F*: 9.949	*F*: 14.762	*F*: 6.014
	df: 6	df: 6	df: 6
	P: 0.001	*P*: 0.001	*P*: 0.001

*Standard deviation. *S. mutans: Streptococcus mutans, S. sanguis: Streptococcus sanguis, S. salivarius: Streptococcus salivarius*, SD: Standard deviation, CI: Confidence interval, *A. nilotica: Acacia nilotica*

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

Plant extract in combinations	Bacteria (*P*)		
	S. mutans	*S. sanguis*	*S. salivarius*
AN + MKL versus AN + PS	0.130	0.969	1.000
AN + MKL versus AN + Euca	0.998	1.000	1.000
AN + MKL versus MKL + PS	0.994	0.004	0.009
AN + MKL versus MKL + Euca	0.983	0.172	0.652
AN + MKL versus Euca + PS	0.887	1.000	1.000
AN + MKL versus chlorhexidine	0.219	0.001	0.990
AN + PS versus AN + MKL	1.000	0.969	1.000
AN + PS versus AN + Euca	1.000	0.798	1.000
AN + PS versus MKL + PS	0.001	0.001	0.001
AN + PS versus MKL + Euca	0.167	0.001	0.167
AN + PS versus Euca + PS	0.994	0.681	0.994
AN + PS versus Chlorhexidine	0.702	0.001	0.702
AN + Euca versus AN + MKL	0.998	1.000	1.000
AN + Euca versus AN + PS	0.811	0.798	1.000
AN + Euca versus MKL + PS	0.499	0.018	0.002
AN + Euca versus MKL + Euca	0.393	0.420	0.306
AN + Euca versus Euca + PS	1.000	1.000	1.000
AN + Euca versus chlorhexidine	0.009	0.001	0.870
MKL + PS versus AN + MKL	0.009	0.004	0.009
MKL + PS versus AN + PS	0.001	0.001	0.001
MKL + PS versus AN + Euca	0.002	0.018	0.002
MKL + PS versus MKL + Euca	0.900	0.995	0.900
MKL + PS versus Euca + PS	0.045	0.031	0.045
MKL + PS versus Chlorhexidine	0.350	0.649	0.350
MKL + Euca versus AN + MKL	0.652	0.172	0.652
MKL + Euca versus AN + PS	0.167	0.001	0.167
MKL + Euca versus AN + Euca	0.306	0.420	0.306
MKL + Euca versus MKL + PS	0.900	0.995	0.900
MKL + Euca versus Euca + PS	0.926	0.550	0.926
MKL + Euca versus Chlorhexidine	1.000	0.420	1.000
Euca + PS versus AN + MKL	1.000	1.000	1.000
Euca + PS versus AN + PS	0.994	0.681	0.994
Euca + PS versus AN + Euca	1.000	1.000	1.000
Euca + PS versus MKL + PS	0.045	0.031	0.045
Euca + PS versus MKL + Euca	0.926	0.550	0.926
Euca + PS versus chlorhexidine	1.000	0.001	1.000

S. mutans: Streptococcus mutans, S. sanguis: Streptococcus sanguis, S. salivarius: Streptococcus salivarius, AN: *Acacia nilotica*, MKL: *Murraya koenigii L*. Sprengel, Euca: *Eucalyptus* hybrid, PS: *Psidium guajava*. *Tukey's posthoc test applied

primary plaque colonizers. Among the various combinations of plant extracts, the combination of AN and *P. guajava* offered the maximum benefits against all the three bacteria tested in this study. However, the combinations of AN *and Eucalyptus* offered a significantly higher zone of inhibition against *S. mutans and S. sanguis* compared to 0.2% chlorhexidine.

Discussion

The potential therapeutic application of many traditional medicinal plants has been assessed in the past. Many of these studies have evaluated the activity of individual plant extracts and products against certain specific oral microbes, while others have focused on the ability of the products to inhibit the formation of dental biofilms by reducing the adhesion of microbial pathogens to the tooth surface, since the formation of plaque is a primary event, which in turn leads to progression to tooth decay and periodontal diseases. The authors of these studies have suggested that it may be possible to maximize the antimicrobial effect of the plant extracts by using them in combination. The combinations of plant extracts may yield significant benefits owing to the synergistic action of components present in them. A combination of plant extracts has dual benefits as it may enhance the biological activity and at the same time lower the toxic effect as well. We assessed the antimicrobial efficacy of the double combinations of AN, MKL Sprengel, *Eucalyptus* hybrid *and P. guajava* on primary plaque colonizers. These plants were selected in view of their easy availability in India. Moreover, some literature indicates these plants to possess antimicrobial effect.

The study found that all the six combinations were effective in inhibiting the growth of *S. mutans, S. sanguis and S. salivarius*. The effectiveness of many of these combinations was relatively superior to that offered by 0.2% chlorhexidine against *S. mutans and S. sanguis*. *Acacia nilotica* contains alkakoids, saponins, cardiac glycosides, tannins, flavonoids and anthraquinones. These phytochemical constituents may be responsible for the antimicrobial and antifungal action of plant extract.[10] Deshpande and Kadam[8] found the ethanolic extracts of AN to inhibit the growth of *S. mutans* (31 mm ± 0.7 mm). MKL Sprengel is found to contain sterols, alkaloids and flavonoids, which may be responsible for antimicrobial action.[17] Sunitha *et al*.[11] demonstrated that the alcoholic extracts of Curry leaf inhibited dental caries microorganisms. Ramesh *et al*.[12] recommended curry leaf extracts as home remedies that create an oral environment unfavorable for microbes. Eucalyptus is found to contain alkaloids, steroids, tannins, flavonoids, saponins, phenolics, glycosides and macrocarpals A, B and C, which exhibit antibacterial activity against periodontopathic bacteria.[18,19] Takarada *et al*.[13] found eucalyptus oil to exert an inhibitory effect on various oral bacteria that included *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, S. mutans*, and *Streptococcus sobrinus*. *P. guajava* contain guajaverin, psidiolic acid and other essential oil constituents such as monoterpenes, 1.8-cineol, ρ-cimen and acetate of α-terpenil, which may offer antimicrobial benefits.[20] Hema *et al*.[14] found *P. guajava* to possess inhibitory effect against *Pseudomonas lundensis, Aspergillus niger* and *Aspergillus flavus*.

"Herbal shotgun" or "synergistic multi-target effects" refers to the use of plant extracts in combinations. The use of herbals and drugs in a multi-targeted approach offer potential benefits in an agonistic synergistic way. The process of additive or synergistic effects is often critical to bioactivity of plant extracts. Development of bacterial resistance to synergistic drug combinations, such as those found in plants, may be slower than for single drug therapies.[21]

This study was the first of its kind assessing the antimicrobial efficacy of the double combinations of AN, MKL Sprengel,

Eucalyptus hybrid *and P. guajava* on oral bacteria. Hence, these results could not be compared with other studies.

The efficacy of these combinations of extracts needs to be further evaluated against the secondary and tertiary plaque colonizers. There is a need to assess the efficacy of triple combinations of these extracts on the primary, secondary and tertiary plaque colonizers.

Conclusions

The study found each combination of herbal extracts to be effective in inhibiting the growth of *S. mutans, S. sanguis and S. salivarius*. The efficacy of the combinations of AN *and Eucalyptus* against *S. mutans and S. sanguis* was significantly higher compared to 0.2% chlorhexidine. All these combinations have the potential to be used as antiplaque and anticaries agents. The combinations of herbal extracts offer enhanced antimicrobial efficacy due to the synergistic effects besides slowing the development of resistance. Further research focusing on the antimicrobial efficacy of the double and triple combinations of these plant extracts against dental caries and plaque microorganisms (that include primary, secondary and tertiary colonizers) in comparison with individual extracts may help in the evolution of an herbal formulation that can inhibit both dental caries and plaque bacteria simultaneously.

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