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Antimicrobial efficacy of *Acacia nilotica, Murraya koenigii* (L.) *Sprengel, Eucalyptus* hybrid, *Psidium guajava* extracts and their combination on *Streptococcus mutans* and *Lactobacillus acidophilus*

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ABSTRACT

Background: The aim of this *in vitro* study was to assess antimicrobial efficacy of *Acacia nilotica, Murraya koenigii* (L.) *Sprengel, Eucalyptus* hybrid*, Psidium guajava* extracts, and their combination on *Streptococcus mutans* and *Lactobacillus acidophilus*.

Materials and Methods: The branches of four plants were collected, identified, and authenticated by a taxonomist. The plants were rinsed in water, healthy leaves were separated and shade dried over a period of 3–4 weeks. Soxhlet apparatus using ethanol was employed for extraction procedure. The combinations of plant extracts were prepared by mixing equal quantities of 10% solutions of each of these extracts. 0.2% chlorhexidine and dimethyl sulfoxide were used as positive and negative controls, respectively. The antimicrobial efficacy testing was done using agar well-diffusion method under anaerobic conditions. The mean diameter of inhibition zone was computed and compared between different categories using one-way analysis of variance and Tukey's *post-hoc* test. A qualitative assay was carried out to identify the various phytochemical constituents in the plants. The data was assessed by SPSS version 20. The statistical significance was fixed at 0.05.

Results: All the plants extracts and their combinations inhibited *S. mutans and L. acidophilus*. However, the quadruple combination of *A. nilotica* + *M. koenigii* (L.) *Sprengel* + *Eucalyptus* hybrid + *P. guajava* produced the maximum inhibition zone (23.5 ± 2.2 mm) against *S. mutans*. Although, 0.2% chlorhexidine produced the highest inhibition zone against *L. acidophilus* (18.8 ± 1.2 mm), *A. nilotica* extract produced maximum inhibition among the various plant extracts and their combinations (14.1 ± 1.8 mm).

Conclusion: All the individual plant extracts and their combinations were effective against *S. mutans* and *L. acidophilus*. These could be tried as herbal alternates to chlorhexidine. However, these *in vitro* results have to be further evaluated for any toxicity of the polyherbal combinations in animal models and effectiveness has to be assessed using *in vivo* studies on humans.

Key Words: Acacia, nilotica, Eucalyptus, hybrid, Lactobacillus acidophilus, Murraya, koenigii (L.) Sprengel anomaly, Psidium guajava, Streptococcus mutans

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INTRODUCTION

Oral health affects general health by causing considerable pain, suffering, and by influencing what people eat, their speech, their quality of life, and well-being.[1] Despite marked improvements in oral health in many developed countries, the prevalence of dental caries is gradually increasing, and periodontal diseases are among the most common afflictions of mankind.[2-4] Oral diseases are the fourth most expensive diseases to treat in some countries. The treatment of dental caries alone which is estimated at around 3500 USD/1000 children exceeds the total health budget for children in the majority of low-income countries.[1] The situation for adults in developing countries is still worse, as most of them suffer from an accumulation of untreated oral diseases. The approach best suited for control of oral diseases in developing countries such as India is to focus on prevention with innovative strategies.[5]

The use of fluorides in different forms and mechanical oral hygiene combined with appropriate professional maintenance are the recognized practices for prevention of dental caries. Antimicrobial mouth rinses have also been suggested as adjuncts to mechanical plaque control methods.[5,6]

Chlorhexidine, a cationic bisbiguanide with a very broad antimicrobial spectrum is the most commonly used mouth rinse. The major advantage of chlorhexidine over most other compounds is its substantivity. It binds to soft and hard tissues in the mouth, enabling it to act for an extended duration.[7] Therefore, chlorhexidine is used as a positive control in many clinical trials of new mouth rinse formulations and is considered the gold standard. However, chlorhexidine has several side effects, such as staining of teeth, alteration in taste alteration, and besides development of resistant microorganisms which limit its long-term use.[8]

Streptococcus mutans and *Lactobacillus acidophilus* are the principal microorganisms involved in the causation of dental caries.[9] The reduction in the number of these bacteria and their adhesion to the tooth surface will reduce the risk for dental caries.[9] The antimicrobial efficacy studies in the past have assessed the efficacy of plant extracts on *S. mutans*.[5] There exists a need to develop alternate innovative strategies that can inhibit both these bacteria. This will significantly facilitate in the control of dental caries; the most common dental disease afflicting mankind.[5] One such strategy would be to verify the enormous wealth of medicinal plants abundantly available in nature.[5]

In this background, the present study was undertaken to assess antimicrobial efficacy of *Acacia nilotica, Murraya koenigii* (L.) *Sprengel, Eucalyptus* hybrid*, Psidium guajava* extracts and their combination on *S. mutans* and *L. acidophilus.*

MATERIALS AND METHODS

Study design and setting

This was an *in vitro* study conducted over a period of 6 months from December 2013 to May 2014 at the research laboratory, Center for Scientific Research and Development, People's University, Bhopal. The approval for the study was obtained from the Research Advisory Committee, Pacific Academy of Higher Education and Research University, Udaipur. The branches of four plants abundantly available in Central India were collected in November 2014. The plants were identified and authenticated by a taxonomist. The branches were thoroughly rinsed in water treated with reverse osmosis and then, the healthy leaves were separated from these branches. The leaves were shade dried over a period of 3–4 weeks at room temperature, hand crushed separately to obtain a coarse powder of the leaves. Subsequently, the fine powder was prepared, stored in airtight plastic bottles, labeled, and preserved in a refrigerator at 4°C until further use.

Soxhlet apparatus using ethanol as a solvent was employed for the extraction process. The dried leaf extract was mixed with the required quantity of dimethyl sulfoxide (DMSO) to obtain the working concentration of the extract (i.e., 100 mg/ml). The combinations of the four plant extracts were prepared by combining an equal quantity of 10% solution of each of the four plant extracts.

The antimicrobial efficacy testing of the plant extracts and their combinations on *S. mutans* (American Type Culture Collection number 25175) was done using agar well-diffusion method on brain heart infusion agar. Columbia agar base supplemented with 5% sheep blood cell were used for antimicrobial efficacy testing on *L. acidophilus* (ATCC 314) under anaerobic conditions. The freshly prepared inoculum for one bacterium was evenly spread all over the surface of each agar plate first in horizontal and then in the vertical direction using sterile cotton swabs under aseptic conditions. The plates were left undisturbed for 1 h to facilitate the complete absorption of the inoculum. Then, three wells of 7 mm diameter were made on the surface of seeded plates using sterile cork borer. 50 µl of each extract was loaded in the respective labeled well. All the plates were then incubated in a bacteriological incubator at 37 ± 2°C for 24–48 h. The diameter of the inhibition zone was measured on the undersurface of the agar plate at three different planes using a transparent scale after accounting for the well diameter. 0.2% chlorhexidine and DMSO were used as positive and negative controls, respectively. The DMSO was used to make sure that it had no effect on bacterial growth. 50 µl of ethanol which is used as a solvent in extraction process was added to one of the wells to check the inhibitory effect.

The entire experiment was done in duplicate sets and the mean diameter of inhibition zones for each extracts combinations against these bacteria was computed.

Photochemical constituents assay

The various phytochemical constituents such as alkaloids (using Mayer's reagent and Dragendorff's reagent), anthraquinones (Borntrager's test), terpenoids (Salkowski's test), saponins (Froth and emulsion test), flavonoids (Shinoda and alkaline reagent tests), tannins (Ferric chloride and lead acetate tests), and cardiac glycosides (Legal test and Keller–killani test) were identified using a qualitative assay.[6]

Data entry and statistical analysis

The data on antimicrobial activity was assessed by 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 four plants used for antimicrobial efficacy testing in the present study, their yield for 50grams of leaf powder are denoted in Table 1. The mean diameter of inhibition zone produced by each plant extract and their double, triple, and quadruple combinations against *S. mutans* and *L. acidophilus* are denoted in Table 2. DMSO and ethanol failed to inhibit the growth of *S. mutans* and *L. acidophilus*.

All the plants extracts and their combinations inhibited the growth of *S. mutans*. However, the quadruple combination of *A. nilotica* + *M. koenigii* (L.) *Sprengel* + *Eucalyptus* hybrid + *P. guajava* produced the maximum inhibition zone (23.5 ± 2.2 mm) followed by the triple combinations of *A. nilotica* + *Eucalyptus* hybrid + *P. guajava* (22.3 ± 0.6 mm). 0.2% chlorhexidine produced an inhibition zone of 14.5 ± 2.1 mm. The difference in the mean diameter of inhibition zone between the various categories

Table 1: Details of four plants used in the present study

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

P. guajava: *Psidium guajava*; *M. koenigii*: *Murraya koenigii*; *A. nilotica*: *Acacia nilotica*; *E. canaldulensis*: *Eucalyptus canaldulensis*; *E. ovate*: *Eucalyptus ovata*

Table 2: Antimicrobial efficacy of plant extracts and their combinations against *Streptococcus mutans* and *Lactobacillus acidophilus*

Plant extract	Mean diameter of inhibition zone in mm (SD)	
	S. mutans	*L. acidophilus*
A. nilotica	22.0 (0.6)	14.1 (1.8)
M. koenigii (L.) *Sprengel*	11.3 (0.5)	13.8 (0.4)
Eucalyptus hybrid	21.5 (2.2)	13.9 (1.4)
P. guajava	20.6 (1.3)	11.7 (4.0)
A. nilotica + *M. koenigii* (L.) *Sprengel*	17.7 (1.2)	11.6 (0.7)
A. nilotica + *P. guajava*	21.1 (2.1)	12.5 (0.5)
A. nilotica + *Eucalyptus* hybrid	18.9 (0.8)	12.8 (1.0)
M. koenigii (L.) *Sprengel* + *P. guajava*	16.3 (1.9)	11.7 (0.4)
M. koenigii (L.) *Sprengel* + *Eucalyptus* hybrid	16.1 (1.0)	11.8 (1.6)
Eucalyptus hybrid + *P. guajava*	19.7 (2.6)	12.3 (0.8)
A. nilotica + *M. koenigii* (L.) *Sprengel* + *P. guajava*	20.5 (3.2)	12.4 (0.5)
A. nilotica + *M. koenigii* (L.) *Sprengel* + *Eucalyptus* hybrid	20.3 (2.9)	12.4 (0.5)
A. nilotica + *Eucalyptus* hybrid + *P. guajava*	22.3 (0.6)	13.4 (0.4)
M. koenigii (L.) *Sprengel* + *Eucalyptus* hybrid + *P. guajava*	20.7 (1.6)	12.4 (0.5)
A. nilotica + *M. koenigii* (L.) *Sprengel* + *Eucalyptus* hybrid + *P. guajava*	23.5 (2.2)	13.3 (1.2)
Chlorhexidine	14.5 (2.1)	18.8 (1.2)
Statistical inference		
F	18.0	9.4
df	15	15
P	0.001	0.001

P. guajava: *Psidium guajava*; *M. koenigii*: *Murraya koenigii*; *A. nilotica*: *Acacia nilotica*; *L. acidophilus*: *Lactobacillus acidophilus*; SD: Standard deviation was statistically significant (P = 0.001, Table 2). The *post-hoc* test revealed a significant difference between different plants extracts combinations and 0.2% chlorhexidine suggesting that any of the plants extracts combinations may be tried as effective herbal alternates to chlorhexidine to inhibit the growth of *S. mutans*.

All the individual plant extracts were found to inhibit the growth of *L. acidophilus*. The highest zone of inhibition among the plant extracts was produced by *A. nilotica* (14.1 ± 1.8 mm) followed by *Eucalyptus* hybrid (13.9 ± 1.4 mm). However, 0.2% chlorhexidine produced a significantly higher mean diameter of inhibition zone (18.8 ± 1.2 mm) compared to all the individual plant extracts ($P < 0.001$, Table 2). The *post-hoc* tests revealed a significant difference between chlorhexidine and all the plant extracts while the difference between different plant extracts was not statistically significant.

The results of the qualitative assay of phytochemical constituents in the four plant extracts derived by soxhlet extraction process using ethanol as solvents is summarized in Table 3. *A. nilotica* demonstrated the presence of anthraquinones, flavonoids, tannins, and cardiac glycosides while *M. koenigii* (L.) *Sprengel* contained tannins and cardiac glycosides. *Eucalyptus* hybrid contained terpenoids, flavonoids, tannins, and cardiac glycosides while *P. guajava* revealed the presence of anthraquinones, flavonoids, tannins, and cardiac glycosides.

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. The literature suggests *A. nilotica* to contain alkaloids, saponins, cardiac glycosides, tannins, flavonoids, and anthraquinones.[10] The antimicrobial efficacy of *A. nilotica* is mainly attributed these phytochemical constituents. Deshpande and Kadam[6] while evaluating the antimicrobial efficacy of *A. nilotica* on *S. mutans* found it to be effective (mean diameter of the inhibition zone was 31 mm ± 0.7). Lakshmi and Krishanan[11] and Geetha *et al.*[12] in their *in vitro* studies have found the methanolic and acetone extracts of *A. nilotica* to inhibit the growth of *L. acidophilus* similar to the findings of this study.

M. koenigii (L.) *Sprengel* was found to contain sterols, alkaloids, and flavonoids.[13] Ramesh *et al.*[14] and Prabhakar *et al.*[15] have found curry leaves mouthwashes to be effective against cariogenic bacteria and suggested it to be a cheaper alternative to allopathic mouthwashes similar to our results.

Antimicrobial efficacy of *eucalyptus* was attributed to its phytochemical constituents such as alkaloids, steroids, tannins, flavonoids, saponins, phenolics, glycosides and macrocarpals A, B and C. Nagata *et al.*,[16] Saxena *et al.*[17] and Takarada *et al.*[18] in their studies have found the *eucalyptus* extracts to possess antibacterial activity against *S. mutans*. The inhibitory effect was proposed to be mediated by preventing the adhesion of *S. mutans*. These findings are similar to our results.

Antimicrobial potential of *P. guajava* is due to the presence of various essential oil constituents such as monoterpenes, 1.8-cineol, and acetate of α-terpenil along with guajaverin and psidiolic acid.[19,20]

The results of the antimicrobial efficacy of combinations of extracts on *S. mutans* were in

Table 3: Phytochemical constituents in plant extracts derived using hot extraction methods

Phytochemical constituents and test used	*A. nilotica*	*M. koenigii* (L.) *Sprengel*	*Eucalyptus* hybrid	*P. guajava*
Alkaloids (using Mayer's reagent)	–	–	–	–
Alkaloids (using Dragendorff's reagent)	–	–	–	–
Anthraquinones (Borntrager's test)	+	–	–	+
Terpenoids (Salkowski's test)	–	–	+	+
Saponins (Froth and emulsion test)	–	–	+	–
Flavonoids (Shinoda test)	+	–	+	+
Flavonoids (alkaline reagent test)	+	–	+	+
Tannins (Ferric chloride test)	+	+	+	+
Tannins (lead acetate test)	+	–	+	+
Cardiac glycosides (Legal test)	+	+	+	+
Cardiac glycosides (Killer–kiliani test)	+	+	+	+

+: Positive; –: Negative; *P. guajava*: *Psidium guajava*; *M. koenigii*: *Murraya koenigii* agreement with our previous study.[21] 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.[22-24] Moreover, the plant extracts have been reported to have minimal side effects. However, the literature on the antimicrobial efficacy of these plant extracts in combinations against *L. acidophilus* was nonexistent. This was the first of its kind and hence, could not compare our results of combinations of plant extracts with other studies.

Limitations

The present study used crude extracts of the plants rather than purified compounds on *S. mutans* and *L. acidophilus*. The experiment is done in duplicate sets using the plant extracts obtained in one particular season in a year. The phytochemical constituents vary with seasons. The quantitative assay of each of these extracts will highlight the bioactive compound present in high concentrations. In this background, the results of this study are only preliminary and further *in vitro* studies using plant extracts in different seasons, purified compounds of these plants is the need of the hour. The evaluation of these extracts on secondary and tertiary plaque colonizers would enable us to evolve a new strategy that can simultaneously inhibit both dental caries and plaque microorganisms. The efficacy of these extracts in the form of mouth rinse under *in vivo* conditions is required to validate the results of this study.

CONCLUSION

All the individual plant extracts, their double, triple, and quadruple combinations have been found to be effective against *S. mutans* and *L. acidophilus*. These results demonstrate that any of these plant extracts and their combinations could be tried as herbal alternates to chlorhexidine. However, these *in vitro* results have to be further evaluated for any toxicity of the polyherbal combinations in animal models before their effectiveness is assessed using *in vivo* studies on humans.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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