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Original Article

Dentistry Section

Antibacterial Activity of Kabasura Kudineer on Periodontal Pathogens: An In-vitro Study

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ABSTRACT

Introduction: Antiseptic agents used in periodontics, such as bis-biguanide derivatives (Chlorhexidine - CHX) or essential oils, are primarily chemical substances. However, the recent rise of herbal derivatives, with their activity against putative periodontal pathogens, has sparked interest. This study delves into a polyherbal formulation, Kabasura Kudineer (KSK), which is a blend of medicinal herbs with known individual pharmacological benefits, to determine its antibacterial efficacy against periodontal pathogens in-vitro.

Aim: To assess the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of KSK extract against pure strains of putative periodontal pathogens, namely *Porphyromonas gingivalis* (American Type Culture Collection - ATCC 33277), *Fusobacterium nucleatum* (ATCC 25586), and *Aggregatibacter actinomycetemcomitans* (ATCC 43718).

Materials and Methods: An in-vitro study was performed in the Department of Periodontics, Sri Ramachandra Dental College and Hospital, Chennai, Tamil Nadu, India, between December 2022 and February 2023. In the study, the aqueous extract of KSK was prepared by maceration and stored under controlled conditions at 4°C. The MIC and MBC were determined against commercially available strains of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* using the broth dilution method and MIC assay method, respectively. The MIC and MBC activities of the KSK extracts were compared with CHX as a standard.

Results: Kabasura kudineer has been proven to possess antibacterial activity against all three tested periodontal pathogens. The MIC value of KSK extract was similar to that of CHX, as assessed by the broth dilution method. The MIC of KSK extract for *P. gingivalis* was 0.4 µg/mL, while for *A. actinomycetemcomitans* and *F. nucleatum*, it was 0.2 µg/mL; the CHX values against all three periodontal pathogens were 0.2 µg/mL. The MBC of KSK extract for *P. gingivalis* was 0.8 µg/mL, and for *A. actinomycetemcomitans* and *F. nucleatum*, it was 0.2 µg/mL, with CHX values against all three periodontal pathogens also being 0.2 µg/mL.

Conclusion: The aqueous extract of KSK demonstrated a noteworthy MIC and MBC, exhibiting effectiveness against the three periodontal pathogens evaluated in this study, thereby demonstrating its potential for use as an adjunct in periodontal therapy.

Keywords: *Aggregatibacter actinomycetemcomitans*, Drug resistance, *Fusobacterium nucleatum*, Microbial activity, Periodontitis, Plant extracts, *Porphyromonas gingivalis*

INTRODUCTION

Periodontitis is a complex multifactorial disease characterised by an interaction between biofilm and the host inflammatory response, resulting in alterations in bone and connective tissue metabolism. Periodontal disease is a significant health problem that has spread worldwide, ranking as the 11th most prevalent condition globally and the 2nd most prevalent among oral conditions [1]. It worsens oral health and adversely affects an individual's systemic health [2]. Persistent dental plaque is the primary etiological agent of periodontitis. Dental plaque is a host-associated biofilm [3], whose microbial composition and sensitivity to antimicrobial agents have been subjects of research interest. Anti-infective agents are used as adjuncts with nonsurgical and surgical procedures for managing periodontal infection [4].

The discovery of antibiotics has substantially changed the approach to treating infectious diseases and has reduced their threat [5]. However, the widespread usage of these drugs has led to antimicrobial resistance. Furthermore, antibiotics can occasionally be associated with adverse effects like immunosuppression and hypersensitivity reactions. The ever-increasing threat from drug-resistant bacteria calls for novel solutions based on natural products from plants selected based on documented ethnomedicinal use [6].

KSK is a classic polyherbal Siddha formulation consisting of 15 medicinal herbs mixed in equal quantities. The ingredients include *Zingiber officinale*, *Piper longum*, *Syzygium aromaticum*, *Tragia involucrata*, *Anacyclus pyrethrum*, *Hygrophila auriculata*, *Terminalia chebula*, *Adhatoda vasica*, *Clerodendrum serratum*, *Coleus amboinicus*, *Tinospora cordifolia*, *Saussurea lappa*, *Andrographis paniculata*, *Sida acuta*, and *Cyperus rotundus* [Table/Fig-1] [7]. According to Siddha medicine, 'Kapacuram' refers to upper and lower respiratory tract fever, and this formulation has been used to treat fever and respiratory diseases [8]. Research has shown that each ingredient of KSK is reported to possess antimicrobial, antibacterial, anti-inflammatory, anticancer, antiviral, antioxidant, antifungal, and antipyretic properties [8-12].

The phytochemical constituents of KSK ingredients, including adhavasinone, ar-curcumine, carvacrol, cirsimaritin, cyperene [13], dehydrocostus lactone [14], evofolin, gingerdiol, guaiol [15], oxygenated flavones [16], piperine, p-cymene, quercetin, rutin, salvigenin, serratogenic acid, thymol [17], vasicine, α-pinene, β-phellandrene, β-selinene, γ-terpinene, and myristic acid [18], have been proven to possess antibacterial and antimicrobial properties.

In a 2013 study, Soumaya KJ et al., explored the analgesic, anti-inflammatory, and genotoxic activities of one of KSK's ingredients, *Cyperus rotundus*, which contains potent flavonoids with potential immune cell-modulating and therapeutic effects [19]. Thus, KSK extract, with its proven antimicrobial properties, can be tested against periodontopathic pathogens. This may represent an economical natural antimicrobial agent with minimal side effects and maximum benefits.

Ingredients	Common name/local name
Zingiber officinale	Ginger/Sukku
Piper longum	Long pepper/Thippili
Syzygium aromaticum	Clove/Kirambu
Tragiainvolucrata	Indian stinging nettle/Dusprasha/Sirukanchoriver
Anacyclus pyrethrum	Mount Atlas Daisy/Akkirakaram
Hygrophila auriculata	Swamp weed/Kokilaksha/Neermulliver/Kulekhara
Terminalia chebula	Chebulic Myrobalan Harde whole/Haritaki
Adhathodavasica	Malabar nut/Aadathodai
Clerodendrum serratum	Blue flower red glory tree/Bharangi
Coleus amboinicus	Mexican mint/Indian borage/Karpooravalli
Tinosporia cordifolia	Guduchi/Seendhil
Saussurealappa	Costus root/Koshtam
Andrographis paniculata	Bitterweed/Creat/Nilavembu
Sida acuta	Common Wireweed/Vattathiruppiver/Raja pata
Cyperus rotundus	Musta

[Table/Fig-1]: The contents of Kabasura kudineer (KSK).

The aim of the present study was to assess the MIC and MBC of KSK solvent extract against pure strains of putative periodontal pathogens: *Porphyromonas gingivalis* (ATCC 33277), *Fusobacterium nucleatum* (ATCC 25586), and *Aggregatibacter actinomycetemcomitans* (ATCC 43718). The null hypothesis of the present study is that the KSK extract would not exhibit antimicrobial effects against the chosen periodontal pathogens. Conversely, the alternative hypothesis posits that the KSK extract would exhibit antimicrobial effects against the chosen periodontal pathogens.

MATERIALS AND METHODS

An in-vitro study was conducted in the Department of Periodontics, Sri Ramachandra Dental College and Hospital, Chennai, Tamil Nadu, India, between December 2022 and February 2023. Ethics approval for the study was obtained from the Institutional Ethics Committee of Sri Ramachandra Institute of Higher Education and Research (CSP/22/MAR/106/92).

The commercially available powdered version of KSK was procured and used for the investigation. Three bacterial strains—*Porphyromonas gingivalis* (ATCC-33277), *Fusobacterium nucleatum* (ATCC-25586), and *Aggregatibacter actinomycetemcomitans* (ATCC-43718)—were obtained from Maratha Mandal's Central Research Laboratory in Belgaum. Maceration was employed to prepare the Kabasura kudineer aqueous extract [20]. Coarsely powdered KSK was placed inside a container, and the menstruum was poured on top until it completely covered the powder. The container was then kept for at least three days, with periodic shaking to ensure complete extraction. After extraction, the micelle was separated from the menstruum by evaporation over a water bath [20]. The extracts were subsequently stored in airtight jars under controlled conditions for future use [Table/Fig-2] [20].

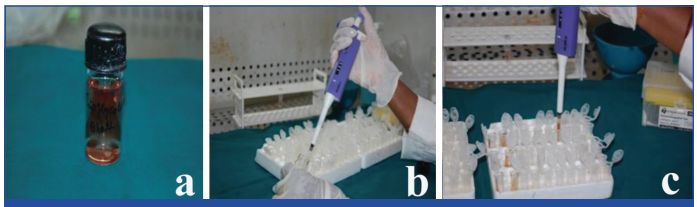
[Table/Fig-2]: Preparation of aqueous extract of KSK: a) Preparation of KSK extract by maceration using a rotatory evaporator; b) Extracts placed in airtight jars; c) Prepared KSK extract used for antimicrobial assay.

Study Procedure

Stock preparation was conducted using 10 mg of the extract, which was then dissolved in 10% Dimethyl Sulfoxide (DMSO). The MIC of the plant extract was evaluated following the protocol outlined in the published literature, which is described briefly below [21].

To determine the MIC, 10 serial dilutions of the KSK extract were prepared using Thioglycollate broth. Initially, 20 µL of KSK was added to 380 µL of Thioglycollate broth. For subsequent dilutions, 200 µL of Thioglycollate broth was added to each of the next nine tubes. From the initial tube, 200 µL was transferred to the first tube containing 200 µL of broth to create a 10^{-1} dilution, and this process was repeated up to a 10^{-10} dilution. From the maintained stock cultures of the required organisms, 5 µL was taken and added to 2 mL of Thioglycollate broth. Next, 200 µL of this culture suspension was added to each serially diluted tube. The tubes were incubated for 48-72 hours in an anaerobic jar at 37°C and were observed for turbidity. Two control tubes were used: one for broth control and the other for growth control (broth + chosen organism). The results were read after 72 hours to determine the lowest concentration of the KSK extract that inhibits bacterial growth.

The bacterial strains *P. gingivalis* (ATCC-33277), *F. nucleatum* (ATCC-25586), and *A. actinomycetemcomitans* (ATCC-43718) were added to Thioglycollate medium supplemented with hemin and vitamin K (which support the growth of the chosen bacterial species; hemin aids cellular respiration and metabolism, while vitamin K acts as a cofactor for metabolic processes), alongside the aqueous extract of KSK at ten different dilutions ranging from 0.2 to 100 µg/mL. Three test groups were established: Group 1 (Broth+KSK extract+*P. gingivalis*/P. g), Group 2 (Broth+KSK extract+F. n), and Group 3 (Broth+KSK extract+A. a). The positive control used was CHX+broth+organism (P. g/F. n/A. a, depending on the experiment). The tubes were then incubated in an anaerobic jar for 48-72 hours at 37°C and observed for turbidity [Table/Fig-3]. The experiment was repeated in triplicate.



[Table/Fig-3]: Determination of Minimum Inhibitory Concentration (MIC) of aqueous extract of Kabasurakudineer (KSK) using thioglycolate medium: a) Thioglycolate broth; (b) Addition of Chlorhexidine (CHX) to the Eppendorf tube containing the broth with the bacteria; (c) Addition of KSK extract to the Eppendorf tube containing the broth with the bacteria.

The MBC was determined using the MIC dilution plate method. After incubation, the microorganisms were subcultured onto agar plates, and the number of colonies on the agar plates was counted 24 to 48 hours after incubation in an anaerobic jar at 37°C. The bacteriostatic or bactericidal effects of the KSK extract were determined based on the absence or presence of the organism's growth. It can be considered bacteriostatic when microorganisms are growing on the agar plates. Conversely, it can be deemed bactericidal when no colonies are observed [Table/Fig-4].

[Table/Fig-4]: Determination of Minimum Bactericidal Concentration (MBC) of kabasurakudineer (KSK): a) MBC determination from MIC dilution tubes; b) microorganisms subcultured to agar plates; c) Assessment of MBC at different concentrations of KSK extract after 48 hours of incubation.

STATISTICAL ANALYSIS

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS) version 20.0 software. Pearson's Chi-square test was employed to calculate the differences in the MIC and MBC of the KSK as compared to CHX of the KSK extract against pure strains of periodontal pathogens, respectively. Values were deemed significantly different if the p-value was ≤0.05.

RESULTS

The MIC was determined for *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* (P. g, A. a, F. n) species, treated with different concentrations of the KSK extract using the broth dilution method. The MIC was conducted in triplicate for each group. The MIC of KSK extract for P. g was 0.4 µg/mL, while for A. a and F. n, it was 0.2 µg/mL, as indicated by the absence of bacterial growth at these concentrations. The MIC of CHX against all three periodontal pathogens was 0.2 µg/mL. The Chi-square test was used to assess the MIC values between the KSK extract and CHX against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* (P. g, A. a, F. n) species. When comparing both interventions, statistical significance could not be demonstrated, with p-values for *Porphyromonas gingivalis* being 0.453, *Aggregatibacter actinomycetemcomitans* being p=1.000, and *Fusobacterium nucleatum* being p=1.000 [Table/Fig-5,6].

The MBC of KSK extract for *P. g* was 0.8 µg/mL, while for A. a and F. n, it was 0.2 µg/mL. The CHX values against all three periodontal pathogens were also 0.2 µg/mL. When comparing both interventions, statistical significance was found for *Porphyromonas gingivalis*, with a p-value of 0.05, while the p-values for *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* were both 1.000 [Table/Fig-7].

DISCUSSION

Periodontitis is an inflammatory disease of the oral cavity that results in bone and tooth loss. The fundamental cause of periodontitis is bacterial biofilm formation [22]. To limit bacterial resistance to antibiotics, antimicrobial agents derived from plant extracts are emerging as a potential method to control the progression of periodontitis, as they demonstrate significant abilities to reduce the virulence of colonising microorganisms [23]. Despite CHX being the standard antiseptic agent in periodontal treatment regimens, it can cause side effects when used over an extended period [24]. Plant extracts with antimicrobial properties have been increasingly utilised globally. In 2002, the World Health Organisation (WHO) recommended the use of medicinal plants to obtain a variety of drugs. Some studies have demonstrated the effectiveness and safety of using medicinal plant extracts in dentistry as antibacterial and antibiofilm agents [25], serving as alternative options to synthetic compounds, which may have potential long-term side effects [26].

KSK is a polyherbal formulation scientifically proven to provide various pharmacological benefits [8]. Kiran G et al., performed in silico pharmacokinetic and toxicity predictions of KSK by screening 37 compounds, all of which were found to have good oral bioavailability and were free of tumourigenic and carcinogenic properties [27]. A study by Jose SP et al., demonstrated modulation of lipopolysaccharide (LPS)-induced inflammatory response in RAW-264.7 macrophage cells through the blockage of TLR-

S. No.	Broth+organism+varied concentrations of KSK extract/CHX	100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL	3.12 µg/mL	1.6 µg/mL	0.8 µg/mL	0.4 µg/mL	0.2 µg/mL
P.g											
1	KSK 01	S	S	S	S	S	S	S	S	S	R
2	KSK 02	S	S	S	S	S	S	S	S	S	R
3	KSK 03	S	S	S	S	S	S	S	S	S	S
4	CHX	S	S	S	S	S	S	S	S	S	S
F.n											
1	KSK 01	S	S	S	S	S	S	S	S	S	S
2	KSK 02	S	S	S	S	S	S	S	S	S	S
3	KSK 03	S	S	S	S	S	S	S	S	S	S
4	CHX	S	S	S	S	S	S	S	S	S	S
A.a											
1	KSK 01	S	S	S	S	S	S	S	S	S	S
2	KSK 02	S	S	S	S	S	S	S	S	S	S
3	KSK 03	S	S	S	S	S	S	S	S	S	S
4	CHX	S	S	S	S	S	S	S	S	S	S

[Table/Fig-5]: Antibacterial efficacy of varied concentrations of Kabasurakudineer (KSK) aqueous extract (in triplicates) against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* with Chlorhexidine (CHX) as a positive control.
*A.a: *Aggregatibacter actinomycetemcomitans*; F.n: *Fusobacterium nucleatum*; KSK: Aqueous extract of Kabasurakudineer; P.g: *Porphyromonas gingivalis*; S: Sensitive; R: Resistant; CHX: Chlorhexidine

Putative strain	Minimum Inhibitory Concentration (MIC) for test group (µg/mL)	Minimum inhibitory concentration for control group Chlorhexidine (CHX) (µg/mL)	p-value
Porphyromonas gingivalis	0.4	0.2	0.453
Fusobacterium nucleatum	0.2	0.2	1.000
Aggregatibacter actinomycetemcomitans	0.2	0.2	1.000

[Table/Fig-6]: Tabular presentation of Minimum Inhibitory Concentration (MIC) of Kabasurakudineer (KSK) extract against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* as compared to Chlorhexidine (CHX).

Putative strain	Minimum Inhibitory Concentration (MIC) for test group (µg/mL)	Minimum Inhibitory Concentration (MIC) for control group Chlorhexidine (CHX) (µg/mL)	p-value
Porphyromonas gingivalis	0.8	0.2	0.05*
Fusobacterium nucleatum	0.2	0.2	1.000
Aggregatibacter actinomycetemcomitans	0.2	0.2	1.000

[Table/Fig-7]: Tabular presentation of Minimum Bactericidal Concentration (MBC) of Kabasurakudineer (KSK) extract against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* as compared to Chlorhexidine (CHX).
*p>0.05 is statistically significant

mediated NF-κB signal transduction pathways [28]. Another study by Lokhande PD assessed *Piper longum*, an ingredient of KSK, which showed varying degrees of antibacterial activity, with the MIC value of piperine against *Bacillus cereus* and *Escherichia coli* found to be 12.5 µg/mL [29]. Another component of KSK, *Clerodendrum serratum*, was analysed for its antifungal and antibacterial activity, wherein new phenylpropenes were identified, with the dihydroxylated precolpuchol showing the strongest antifungal and antibacterial activity against *Cladosporium herbarum* and *Staphylococcus aureus*, respectively [30].

KSK has been demonstrated to possess immunomodulatory and antiviral activities [31,32]. Parameswaran S et al., analysed the immunomodulatory and thrombolytic potential of KSK. The authors concluded that KSK, at concentrations of 12.5, 25, 50, and 100 µg/mL, showed percentages of immune stimulation of 12.40%, 20.81%, 33.53%, and 43.20% in the phagocytosis assay involving *Candida albicans* assay [31]. A recent publication by Shree Devi MS et al., demonstrated that KSK significantly inhibited SARS-CoV-2 replication in Vero E6 cells, indicating its potential in the treatment of COVID-19 [32]. Muthuramu T et al., assessed the acute toxicity of KSK on laboratory animals. The authors concluded that KSK showed no acute adverse effects; however, in chronic toxicity studies over 90 days, a dosage of 1.5 mL/kg resulted in a transient rise in uric acid, albumin, Serum Glutamic-Oxaloacetic Transaminase(SGOT), and lymphocyte levels [33].

In the present study, key periodontal pathogens such as *Porphyromonas gingivalis* (ATCC 33277), *Aggregatibacter actinomycetemcomitans* (ATCC 43718), and the bridging organism *Fusobacterium nucleatum* (ATCC 25586) were selected from amongst the various putative periodontal pathogens. Commonly used methods for extracting medicinal plants and their formulations include maceration, infusion, digestion, decoction, percolation, Soxhlet extraction, microwave-assisted extraction, and ultrasound-assisted extraction [20]. An aqueous extract of powdered Kabasura kudineer was prepared using the maceration technique [20]. The maceration technique is a convenient and simple process that is very suitable for thermolabile plant material and can be performed at low temperatures [20]. Therefore, this technique was employed to prepare the aqueous extract of KSK.

The antimicrobial activities of the prepared KSK extract were analysed by assessing MIC and MBC after exposing the selected periodontal pathogens in the broth to Kabasura kudineer aqueous extract at various dilutions. The broth dilution method was adopted for the MIC assessment. This method was used to test the susceptibility of microorganisms to the prepared extract. It involves serial dilutions of the extract in a liquid growth medium inoculated with a standardised number of bacterial strains. This method can help determine the appropriate treatment for infections.

The results of the present study have shown that the MIC of KSK extract against *A. actinomycetemcomitans* and *F. nucleatum* was similar to CHX in concentrations ranging from 100 µg/mL to 0.2 µg/mL, and it was found to be effective against *P. gingivalis* at concentrations ranging from 100 µg/mL to 0.4 µg/mL. In comparison with the available literature, a study by Saravanan J et al., demonstrated the antibacterial efficacy of the aqueous extract of KSK against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, with an MIC of 250 µg/mL being identified. The authors also suggested the significant antipyretic effect of KSK, which may be related to the inhibition of prostaglandin synthesis [34].

Furthermore, the MBC of an extract is determined by subculturing the MIC tubes onto the growth medium and examining them for bacterial growth [35]. The results of the present study have shown that the MBC of KSK against *A. actinomycetemcomitans* and *F. nucleatum* was the same as that of the control CHX, which is 0.2 µg/mL, and it was effective against *P. gingivalis* strains with a value of 0.8 µg/mL.

Our study is the first of its kind to assess the MIC and MBC of aqueous KSK extract against periodontal pathogens, namely *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum*, providing evidence for the efficacy of KSK's aqueous extract. The clinical implications of the study observations indicate that KSK extracts can be incorporated into mouthwashes and dentifrices for use by patients as part of a plaque control regime. KSK may be an effective alternative to synthetic antimicrobials.

Limitation(s)

As this is an in-vitro study, additional research is required to apply the findings to in-vivo conditions. Since periodontitis is a polymicrobial disease, further detailed investigations should aim to understand the effectiveness of the KSK extract in a multispecies community.

CONCLUSION(S)

In conclusion, KSK demonstrated a noteworthy MIC, showing effectiveness at a low concentration of 0.2 µg/mL against *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* in three repeated trials. Furthermore, this extract exhibited significant inhibitory effects using MBC of periodontal pathogens *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*, suggesting its potential as an alternative to conventional chemical antimicrobials at low concentrations. Additionally, these results are the first of their kind in elucidating the role of the well-known polyherbal formulation KSK against periodontal pathogens. However, further research is necessary to develop formulations containing KSK extracts and to conduct human clinical trials to assess the efficacy of reducing biofilm formation by periodontal pathogens.

Conflict of interest: The kabasura kudineer extract was procured from Earth India Naturals, Tamil Nadu. It is declared that the present study is conducted for Research purpose only and not for any commercial interest in this product.

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