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Research Article

ANTIMICROBIAL EFFECTS OF *PSIDIUM GUAJAVA, SYZYGIUM CUMINI, FERULA ASAFOETIDA* AND *PIPER BETLE* EXTRACTS AGAINST DENTAL CARIES BACTERIA

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ABSTRACT

Background: For thousands of years plants have been used to enhance health and solve medical issues but still there is abundant medicinal flora which is unrevealed through research. The present study was conducted to elucidate the *in vitro* antimicrobial potential of Guava leaves, Jamun tree bark, Heeng powder and Betel leaves extracts with a view of searching a novel extract as a remedy for dental caries pathogens. Material and Methods: Bacteria were isolated and identified from oral swabs taken from dental caries of four patients. Extracts prepared from Guava leaves, Jamun tree bark, Heeng powder and Betel leaves were screened for in vitro antimicrobial activity against these dental isolates, using agar well diffusion method. Result: Six bacteria identified by MALDI-TOF belonged mainly to the genus *Bacillus* and one to *Brevundimonas*. Guava, Jamun tree bark and Betel leaves extract effectively inhibited the growth of all dental caries isolates, but three of them showed resistance to Heeng powder extract. Conclusion: Our results suggest that these plants synthesize natural phytochemicals which have inhibitory effect on isolated oral bacteria. Furthermore, purification and molecular characterization of the active component would enable us to formulate a sustainable oral hygiene product.

Key words: dental caries, antimicrobial activity, MALDI-TOF, *Psidium guajava, Syzygium cumini, Ferula asafoetida* and *Piper betle*

INTRODUCTION

Infections of the oral cavity result from the loss of equilibrium between the hosts' immune response and virulence factors of the indigenous microbiota.[1,2] Irrespective of the advancements in medicine, these infections continue to pose a threat to public health, and put a heavy burden on health care services globally. This is particularly true in developing countries.[3,4] Despite general advances in the overall health, including oral and dental health of the people living in industrialized countries, the prevalence of dental caries in school aged children is close to 90% and majority of adults are also affected.[5] The link between oral infections and the activities of microbial species that form part of the microbiota of the oral cavity is well established.[6] Around 750 bacterial species colonize the oral cavity, out of which 50% are yet to be identified. Interestingly many of these bacteria are associated with oral diseases. The progression of dental caries is governed by acidogenic and aciduric gram-positive bacteria like *S. mutans*, *lactobacilli* and *actinomycetes*, which convert sucrose to organic acids, particularly lactic acid, that dissolve the calcium phosphate present in teeth and eventually lead to decalcification and tooth decay.[7] Several agents are available that can alter the profile of oral microflora but can cause undesirable contraindications such as vomiting, diarrhoea and staining.[8,9] Since ancient times, medicinal plants have been utilized for oral hygiene. Scientific evaluation of several herbs has been undertaken against oral pathogens.

Psidium guajava L popularly known as guava (family *Myrtaceae*), is used to treat dental issues, respiratory and gastrointestinal disorders, and as an antispasmodic, anti-inflammatory, as a cough sedative, anti-diarrheic, etc.[10]

Assafoetida (Ferula asafoetida) is a species of Ferula commonly known as Heeng or Devils dung and is traditionally used for the treatment of different diseases, such as whooping cough, toothache, asthma, ulcer, epilepsy, stomachache, flatulence, bronchitis, intestinal parasites, antispasmodic, weak digestion and influenza.[11]

Syzygium cumini (L.) *Skeels* a polyembryonic species (family-*Myrtaceae*), commonly known as Jamun, is used to strengthen the teeth and gums, to treat leucorrhoea, stomachalgia, fever, gastropathy, strangury, dermopathy, constipation, and to inhibit blood discharges in the faeces.[12]

Piper betle L. Piperaceae (betel leaves) leaves is widely used as a mouth freshener after meal and various researches show that betel extract and betel oil exhibit antimicrobial and antioxidant activities in model systems.[13]

Since all these herbs are known to be associated with oral hygiene and issues, current work was proposed to elucidate their antibacterial against the oral isolates. Thus, with this background the objectives of our study were (i) to isolate organisms from dental caries (ii) to characterize these isolates using enzymatic and/or biochemical methods and MALDI-TOF (iii) to determine their susceptibility to various herbal antimicrobial agents like guava leaves, jamun tree bark, heeng and betel leaf extract.

MATERIAL AND METHODS

Collection of sample

Samples were collected from four patients with dental carries attending Dr. D. Y. Patil Dental hospital, Pimpri, Pune by using sterile cotton swab.

Isolation of organism

The swabs with the sample were inoculated at nutrient broth for 24 hrs for enrichment of the organisms present in it. The enriched broth was then serially diluted in saline tube up to 10^{-6}. Last two dilutions were spread on sterile nutrient agar plate and incubated for 24 hrs at 37^0C.Different colonies obtained on these plates were then used for further studies.

Identification of bacterial isolates

Identification of the isolates was carried out by Gram staining, colony morphology, biochemical tests and MALDI TOF (matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass fingerprinting.

Extraction of herbal ingredients

Preparation of Jamun and Guava extracts

Guava leaves and Jamun tree bark were soaked in saline solution for half an hour. Soaked Guava leaves and Jamun tree bark were grind to paste. 100g of paste was added to 100ml methanol, mixed well and then added to separating funnel. Mixture was thoroughly mixed intermittently after every 15-25 min for uniform mixing and incubated at room temperature for 24 hrs. After 24 hours the aqueous layer was collected and filtered through muslin cloth. This extract was stored in refrigerator for further assay.

Preparation of heeng extract

Raw heeng was used to prepare the extract. 2g of raw heeng was ground using mortar and pestle and to this powdered heeng 10 ml of distilled water was added. This mixture kept on shaker for 24 hrs and then filtered through muslin cloth. This extract was stored in refrigerator for further assay.

Preparation of betel leaves extract

Betel leaves were procured from the local market and dried for 2-3 days. Dried leaves were crushed to powder. 10g of crushed leaves powder was added to 10 ml of ethanol and incubated overnight. This mixture was then filtered through muslin cloth and the filtrate was evaporated in oven. The extracted components were resuspended in 1 ml DMSO and stored in refrigerator for further assays.

Antibacterial activity of herbal extracts against isolated organisms

Pure colony of each isolate was inoculated in 2ml of nutrient broth. The inoculated broth was incubated for 18-20 hours at 37^oC. The turbidity of the suspension was adjusted to 0.5 McFarland standards. The antibacterial activity was checked by well diffusion method.[14] Briefly, overnight grown bacterial cultures were spread on sterile Mueller-Hinton agar plates, wells were bored and 100µl of extract samples were added to the respective well. Plates were incubated for 15min at 4^0C for pre-diffusion and then incubated at 37^0C for 18-20 hr. Zone of inhibition of the test organism were measured to indicate the antimicrobial activity of the extract.

RESULTS

Isolation and identification of oral organism

Total thirteen different colonies were obtained from the swabs collected from four patients that attended the dental clinic. Out of these six isolates were used for further identification by Gram characters (table 1), biochemical characters (table 2) and MALDI TOF (table 3). Four of these belonged to genus *Bacillus*, one to *Lysinibacillus* which was originally included under *Bacillus* genus and one to *Brevundimonas*.

Antibacterial activity of herbal extracts against oral isolates

All the oral isolates were found to be sensitive to Guava leaves, Jamun bark and Betel leaves extract. Jamun bark extract and Betel leaves extract were found to be more effective with greater zone of inhibition against the isolates. Guava leaves extract showed maximum effect against colony 7 and 8. Colony 5, 6, 7 showed resistance against Heeng extract, but colony 1, 2 and 8 showed maximum zone of inhibition. These results are depicted in table 4.

Table 1: Colony Characteristics of the oral isolates

Colony character	Colony 1	Colony 4	Colony 5	Colony 6	Colony 7	Colony 8
Size	3mm	3mm	2mm	Pinpoint	2mm	3mm
Shape	circular	circular	Oval	circular	circular	circular
Colour	cream	Cream	Cream	cream	orange	yellow
Margin	entire	Entire	Entire	entire	entire	entire
Elevation	convex	Flat	Flat	convex	convex	flat
Opacity	opaque	opaque	opaque	opaque	opaque	opaque
Consistency	smooth	smooth	smooth	smooth	smooth	smooth
Gram characters	Gram positive short rods	Gram positive thin rods	Gram positive Rods	Gram positive rods	Gram negative rods	Gram positive rods

Table 2: Biochemical Characteristics of the oral isolates

Biochemical	Colony 1	Colony 4	Colony 5	Colony 6	Colony 7	Colony 8
Glucose	+ ve	+ve	-ve	-ve	+ve	-ve
Mannitol	-ve	-ve	-ve	-ve	-ve	-ve
Galactose	+ve	-ve	-ve	-ve	+ve	-ve
Maltose	+ve	-ve	-ve	-ve	+ve	-ve
Sucrose	+ve	+ve	-ve	-ve	+ve	-ve
Fructose	+ve	+ve	-ve	-ve	-ve	-ve

Table 3: Oral isolates identified by MALDI TOF

Colony No.	Bactria Identified by MALDI-TOF
C-1	*Bacillus licheniformis Gibson 46* (MCC 2047T)
C-4	*Bacillus sp.* DMVJ16CS (MCC 2505)
C-5	*Lysinibacillus fusiformis* DSM 2898T DSM
C-6	*Bacillus sp.* DMVM_13SAS (MCC 2472)
C-7	*Brevundimonas vesicularis* DSM 7226T HAM
C-8	*Bacillus sp.* DMVJ1CS (MCC 2448)

Table 4: Antibacterial activity of herbal extracts against oral isolates

Oral isolates	Zone of inhibition (mm)			
	Guava leaves	Jamun bark	Heeng powder	Betel leaves
Colony 1	11mm	15mm	19mm	12mm
Colony 4	13mm	18mm	18mm	15mm
Colony 5	11mm	12mm	-	13mm
Colony 6	14mm	11mm	-	10mm
Colony 7	18mm	12mm	-	12mm
Colony 8	18mm	14mm	19mm	15mm

DISCUSSION

Dental caries is one of the most common chronic infectious diseases in the world. The tendency to opt for therapeutic agents from natural sources is greatly increasing in the medical field and is also true for the dental problem management as dental caries is an ancient disease of mankind. To identify therapeutic agents from natural sources for the management of dental disease, many studies are being conducted. Most of the agents being evaluated are plant extracts and are aimed at the management of periodontal disease and dental caries. With this aim to determine the antibacterial efficacy of four herbal extracts on oral isolates, we isolated six bacterial species from the dental caries of four patients. The isolates were further identified by MALDI-TOF. The well reported pathogens of dental caries like *Streptococcus mutans* and *Streptococcus mitis* were not isolated in the current study as the swabs were taken superficially, but these isolated bacteria can have synergistic effect with the oral *streptococci* to aggravate the disease condition.

One of the isolate was *Brevundimonas vesicularis*, which is considered of minor clinical importance and is an opportunistic pathogen, that affect patients that are suffering with underlying medical conditions and diseases.[15]Over the past 20 years, *Brevundimonas vesicularis* has rarely been reported as a pathogen causing human infection. There are few reports of its isolation as a contaminant of water in dental unit reservoirs.[16,17]

Lysinibacillus fusiformis was also one of the identified oral isolates. *L. fusiformis* was originally known as *Bacillus fusiformis* prior to 2007; at which point it was reclassified to the genus *Lysinibacillus*, along with its close relative *Bacillus sphaericus*.[18]

Some researchers believed that *L. fusiformis* infections could only occur as a symbiotic relationship with certain *spirochaete* species.[19] Vincent gingivitis[20], also called Vincent infection or trench mouth is an acute and painful infection of the tooth margins and gums that is caused by the symbiotic microorganisms *Bacillus fusiformis* and *Borrelia vincentii.*

Bacillus licheniformis another oral isolate, is an aerobic, Gram-positive, spore-forming rod, and is ubiquitous in the environment. *B. licheniformis* is increasingly recognized as a human pathogen and causes serious infections, mainly in immunocompromised patients.[21] It was isolated in cases with bacteremia, peritonitis, food poisoning and eye infections.[22]It was also isolated as ß-lactamase-producing bacteria, in subgingival plaque from patients with refractory periodontitis in Norway.[23]

In the present study cultural method employed was agar well diffusion method to determine the antimicrobial activity of different herbal extracts.

The paste of tender leaves of guava has been traditionally used to maintain oral hygiene. Guava has shown antibacterial activity against both Gram-positive and Gram-negative bacteria.[24]In addition; extracts rich in guava flavonoids have demonstrated their potential for preventing dental caries due to the growth inhibition of the oral flora.[25]Our results go very well in accordance with all the studies.

Traditional Indian medicines like Ayurveda and Unani prescribe jamun for different health problems including diabetes, dental issues, digestive disorders, liver trouble and skin ailments. The leaves have antibacterial properties and used for strengthening teeth and gums.[26]Essential oils extracted from the Jamun leaves have been reported to exert antibacterial properties against *Bacillus sphaericus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Samonella typhimurium.*[27] In this study we observed similar activity of jamun tree bark, which was effective against both Gram positive and Gram negative isolates but maximum activity was seen against Gram positive ones.

Betel leaves extract increases the salivation which increases the amount of peroxidase, lysozyme and antibodies to combat against bacterial growth in the oral cavity. The fresh betel leaves possess antimicrobial, act against ringworm, antifungal, so it act as antiseptic and antihelminthic effects.[28] The leaf has a significant antimicrobial activity against broad spectrum micro-organisms[29] against both Gram positive and negative bacteria, which is also reflected from our results. Betel leaf extract was seen to be effective against all the six oral isolates.

Antibacterial activity of methanolic extract of Asafoetida was carried out against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli* and was shown to be significantly effective.[30]Present study also showed antibacterial activity of Asafoetida against the Bacillus species but a different study[31]revealed no activity of any extract of Asafoetida against *Bacillus subtilis*.

Overuse of antibiotics has become the major factor for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms. The worldwide emergence of *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus* and many other ß-lactamase producers has become a major therapeutic problem. Thus, in light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. For this reason, we opted to determine the effect of herbal products on the organisms isolated from dental caries, looking for new leads to develop better drugs.

CONCLUSION

Thirteen organisms were isolated from four dental caries samples. The organisms were identified by comparing their morphological and biochemical characters in Bergey's manual and the confirmative identification was done by MALDI-TOF. Jamun tree bark, guava leaves and betel leaves showed inhibition against all the isolated colonies, but three colonies were resistant to heeng extract. The antibacterial activities of these plants can be further enhanced if the active components are purified and proper dosage for administration is determined.

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