Research Article

In-Vitro Antimicrobial Activity of Selected Medicinal Plant Extracts against Dental Caries Causing Pathogens

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ABSTRACT

The antimicrobial efficacy of the following extracts: methanolic, ethanolic, acetone, petroleum ether, and aqueous extracts of *Azadirachta indica* leaves (Neem), *Eugenia caryophyllata* flower buds (cloves) and *Ficus Bengalensis* bark (Banyan) were tested against standard cultures of dental pathogens, Bacteria: *Enterococcus faecalis* (*E. faecalis*), *Staphylococcus aureus (S. aureus)* and Fungi: *Candida albicans* (*C. albicans*) and the results were compared with clinical isolates using the agar well diffusion method and Minimum inhibitory concentration. The findings indicate that clove extracts, neem extracts (except aqueous extract) had a significant inhibition and *Ficus* extracts does not show significant activity against dental pathogens. Among the clove extracts, petroleum ether extracts demonstrated strong inhibition of 40 ± 0.27 mm and Minimum Inhibitory Concentration (MIC) is less than < 0.5 mg/ml. In future, this extract may prove to be a be a significant source for a novel, potent herbal remedy for dental infections.

Keywords: Antimicrobial activity, agar well diffusion method, Dental caries, Clove, neem.

INTRODUCTION

Since the beginning of civilization, medicinal plants have always been a component of human culture. They are the foundation of many indigenous traditional medical systems in India[1]. The importance of medicinal plants as a possible source of bioactive chemicals has been recognized by pharmacological investigations. In affluent nations, traditional medicine comprising chemicals derived from medicinal plants is utilized by approximately 80 % of the population[1]. Since ancient times, people have utilized plants to treat infectious diseases, and they are thought to be a significant source of novel antimicrobial compounds[2]. Numerous studies have been conducted to investigate the antibacterial properties of extracts from herbal plants, such as roots, stems, leaves, or flowers.[3.4]

According to World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs[5]. In order to promote the use of plant materials as potential sources of antimicrobial agents, their biological activity and composition must be carefully assessed before usage [6]. The development of innovative antibacterial medications has been accelerated by the rise of germs resistant to antibiotics. The increase in the rate of multiple drug-resistant microorganisms is attributable to the rash use or mismanagement of antibiotics that has resulted in many antibiotics losing their effectiveness against certain microorganisms [7]. The hunt for novel antimicrobial drugs has become necessary due to the adverse effects of synthetic chemicals and the emergence of bacterial resistance to already prescribed antibiotics[8]. The antibiotics, chemical compounds that restrict growth and the microbe's susceptibility to these substances are all quantitatively determined using microbiological assays. Dental problems remain one of the most widespread diseases of the mankind [8]. Dental cavities are sometimes epidemic-level conditions in developing nations, particularly among the impoverished. Since the 19th century, when sucrose became a daily used sweetener by many people worldwide, the increasing prevalence of dental caries had also been noticed [8].

Dental caries is an infectious microbial disease that results in localized dissolution and destruction of the calcified tissues of the teeth [9]. Dental caries is also cause bad breath and foul tastes. An edentulous mouth may result from an infection that, in severely advanced cases, spreads from the tooth to the surrounding soft tissues. Dental caries is one of the public health concerns for several reasons [9]. Teeth affected with dental caries are sources of infection that can cause an inflammation of dental pulp, periodontium and gums. If left untreated, this disease gradually leads to teeth loss, which causes chewing difficulties and aesthetic problems [9].

In India, neem plant is referred to as village plant pharmacy because of its ability to cure many diseases ranging from bad teeth, gum problems, ulcers, dysentery, bed bugs and malaria [10]. Cloves are dried aromatic unopened floral buds and the clove oil is widely used as a perfume and food flavouring [11, 12], as a medicine for the treatment of asthma, rheumatoid arthritis, acne, warts, scars and some allergic disorders [13], as a general antiseptic in medical dental practices [14]. Clove bud oil, has been used by dentists for a long time as a dressing in dentistry for minor wounds, as an analgesic in painful and infective diseases of the oral

cavity, commonly used as general hygiene. *Ficus bengalensis* is a large evergreen tree found throughout India. The medicinal ingredients from this plant are said to be quite beneficial in treating a number of conditions, including dysentery, diarrhoea, diabetes, leucorrhoea, menorrhagia, nervous disorders, tonic and astringent. Its phytochemical and pharmacognostic properties includes antioxidant, anticancer, analgesic, anti-inflammatory, antipyretic, analgesic, anti-rheumatic, anti-ulcerogenic activities [15].

Enterococcus faecalis is a nonspore-forming, facultative anaerobic Gram-positive coccus. It is responsible for up to 90% of human *enterococcal* infections in humans [16], associated with different forms of periradicular diseases and asymptomatic chronic periradicular lesions. *E. faecalis* is resistant to calcium hydroxide because of this. it causes root canal treatment failures [17]. *S. aureus* is a presumed pathogen for many oral diseases, such as oral mucositis, periodontitis, peri-implantitis, endodontic infections and even dental caries [18-20]. *S. aureus* is a Gram-positive, non-spore forming, non-motile, grape like clusters and the most important coagulase positive pathogen from *staphylococci* due to mixture of invasiveness, toxic mediated virulence, antibiotic resistance and some strains have developed drug-resistant varieties [21, 22]. A common colonizer of mucosal surfaces in the gastrointestinal, reproductive, and oral cavities is the fungus *Candida albicans*, which may be a component of the commensal microbiota in the general population. Often seen as dental plaque in children with severe early childhood tooth decay, causes widespread and excruciating tooth decay with systemic repercussions that impact millions of toddlers globally [23] with the background of literatures, the current study aims to find the antimicrobial efficacy of neem leaves, clove flower bud and banyan bark against dental pathogens.

MATERIALS AND METHODS

Plant material and extraction

Fresh leaves of *Azadirachta indica*, flower buds of *Eugenia caryophyllata* and bark of *Ficus Bengalensis* were collected from local herbal market. They were shade dried, powdered and the bioactive metabolites of the collected plant materials were extracted using five different solvents ethanol, ethanol, petroleum ether, acetone and aqueous in the ratio (1:10 w/v).

Pathogens and Culture Media

The pathogenic cultures used for the antimicrobial activity were obtained from microbial type culture collection and gene bank (IMTECH, Chandigarh, India). They were *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25723), *C. albicans* (MTCC 3017) and their clinical isolates. Prior to any antimicrobial test, they were subcultured on freshly prepared agar plates and kept on an agar slant at 4°C. Mueller-Hinton Agar (MHA) and Sabouraud's Dextrose agar (SDA) were used for the antibacterial and antifungal activity respectively. Mueller-Hinton Broth (MHB) was used for the Minimum inhibitory concentration (MIC) determination.

Antimicrobial screening

Agar well diffusion method was used to screen the antimicrobial activities of different solvents plate were inoculated by dipping sterile cotton swabs into the standard inoculum suspension (1 x 10^6 cfu/mL) and a lawn culture was made by spreading onto the surface of the MHA and SDA plate and the plates were allowed to dry for 10 min. Wells of 6mm diameter were punched out on the inoculated plates using a sterile cork borer. Subsequently, 20 µl of the different herbal extracts were added to the respectively labelled wells. For a duration of 24 hours, the plates were incubated at 37°C. The diameter of the zone of inhibition around the wells was measured in millimetres. The detection of antimicrobial activity was done by measuring the zone of inhibition including the diameter of the well after the period of incubation. DMSO at a concentration of 10% was employed as a negative control[24].

Determination of Minimum inhibitory concentration

The minimum inhibitory concentration of the herbal extracts was determined by broth dilution assay in sterile disposable 96 well microtitre plates (Zellkulter, Germany) as per CLSI (Clinical Laboratory Standards Institute). 100 µl of filter sterilized RPMI 1640 was dispersed into all the 12 wells of each row. 100 µl of the stock solution (1mg/µl) of the extract was added to the well A1 and mixed well. Doubling dilution of the herbal extracts was done from well A1 through A11 to achieve concentrations ranging from 500 – 0.5 mg/ml. A12 well was used as culture control (no herbal extract). 100 µl of the culture suspension (1.5 x 10^8 cfu/ml) was added to all the wells. After 24 hours at 37°C, the plate was incubated. The MIC was identified as the extract's lowest concentration at which bacterial growth is totally inhibited. 5 µl of the suspension from each well was spot inoculated on respectively labelled SDA plates and incubated at 37°C for 24 hours. After incubation, the plates were observed for the presence of creamy white buttery colonies. Minimum fungicidal concentration (MFC) was calculated as the lowest concentration of extract that resulted in 100 % growth reduction compared to the culture control.

Statistical analysis

All the test was conducted in triplicate experiments and the data are represented as mean ± standard deviation (SD). The results were analysed statistically using Microsoft excel.

RESULTS AND DISCUSSION

Agar well diffusion assay was carried out using aqueous, ethanol, methanol, petroleum ether and acetone extracts of neem, ficus and clove were tested against standard dental isolates *E. faecalis*, *S. aureus, C. albicans* and compared with its clinical isolates. **Table 1; Plate 1A; 1B; 1C** reveals that the antibacterial activity of clove extracts

inhibited all the isolates except aqueous extract of *E. faecalis* and its clinical isolate. The petroleum ether and acetone extracts of clove showed promising activity against *C. albicans and S. aureus* with the zone of inhibition ranging 15-40 ± 0.80 mm.

Table 1: Agar well diffusion assay of clove extracts

Microbial Strain	Diameter (mm) of the zone of inhibition of microbial strains against Clove extracts (20 µl / well)				
	Aqueous	Methanol	Ethanol	Petroleum ether	Acetone
E. faecalis ATCC 29212	-	24±0.56	22±0.80	13±0.23	12±0.36
E. faecalis Clinical isolate	-	24±0.06	24±0.56	13±0.40	12±0.58
S. aureus ATCC 25923	12±0.47	21±0.10	18±0.34	15±0.34	22±0.51
S. aureus Clinical isolate	13±0.11	22±0.12	21±0.19	17±0.39	23±0.20
C. albicans MTCC 3017	13±0.14	36±0.21	36±0.35	40±0.27	40±0.34
C. albicans Clinical isolate	12±0.22	32±0.43	32±0.45	32±0.22	36±0.45

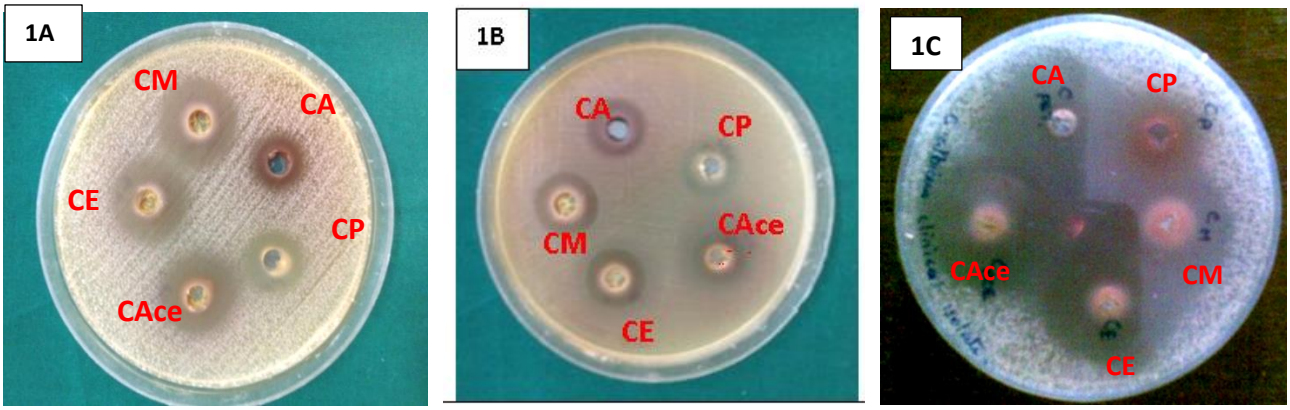

Plate 1A. shows the antimicrobial activity of clove-agar diffusion assay against *S. aureus*
Plate 1B. shows the antimicrobial activity of clove-agar diffusion assay against *E. Faecalis*
Plate 1C. shows the antimicrobial activity of clove-agar diffusion assay against *C. albicans*
CE - Clove Ethanol; CA - Clove Aqueous; CM - Clove Methanol; CP - Clove Petroleum Ether; CACe-Clove Acetone

Table 2: Agar well diffusion assay of neem extracts

Microbial Strain	Diameter (mm) of the zone of inhibition of microbial strains against neem extracts (20 µl / well)				
	Aqueous	Methanol	Ethanol	Petroleum ether	Acetone
E. faecalis ATCC 29212	-	13±0.23	15±0.19	13±0.23	11±0.23
E. faecalis Clinical isolate	-	11±0.43	14±0.33	11±0.41	-
S. aureus ATCC 25923	-	15±0.25	20±0.56	17±0.38	15±0.67
S. aureus Clinical isolate	-	18±0.18	21±0.43	20±0.34	18±0.45
C. albicans MTCC 3017	-	-	-	-	-
C. albicans Clinical isolate	-	-	-	-	-

Table 3: MIC of clove, neem extracts against dental caries pathogens

Microbial strains / Extracts	*E. faecalis* ATCC 29212	*E. faecalis* clinical isolate	*S. aureus* ATCC 25923	*S. aureus* clinical isolate	*C. albicans* MTCC 3017	*C. albicans* Clinical isolate
MIC of Clove extracts (mg/ml)						
Methanol	15.6	15.6	3.9	1	2	3.9
Ethanol	15.6	7.8	3.9	<0.5	1	<0.5
Petroleum Ether	15.6	15.6	2	<0.5	1	2
Acetone	15.6	7.8	1	<0.5	<0.5	1
MIC of neem extracts (mg/ml)						
Methanol	500	500	250	62.5	31.25	125
Ethanol	>500	>500	7.8	2	15.6	3.9
Petroleum Ether	500	500	62.5	15.6	500	>500
Acetone	>500	>500	31.25	15.6	>500	>500

A study by Aneja and Joshi, 2010 reported that the antibacterial activity was observed maximum in methanolic extract of clove against *S. aureus,* showed 21.32 mm and anticandidal activity was observed in acetone, methanol and ethanol extracts between 20-24 mm[25,] which is similar to our present results. Similarly, the antimicrobial activity of clove oil and clove extract on the indigenous oral microbiota that cause dental caries. Both the clove oil and its extract were effective against gram positive, gram negative bacteria and fungi[26]. **Table 2** indicates that the antimicrobial activity of neem extracts inhibited all the dental pathogens except aqueous extract and extracts of *Ficus* bark was ineffective against all dental pathogens. The alcoholic extract of *F. bengalensis* bark was found to be effective against *Actinomyces viscoscus* [27], in present study it was confirmed that *F. bengalensis* bark extracts were ineffective in all the dental pathogens.

It was reported that neem leaves are analysed using different solvents like methanol, chloroform, petroleum ether, ethanol extracts by disc diffusion and cup plate method. The comparative studies indicated that the chloroform extract showed better antimicrobial activity against *S. aureus* with a zone of 18mm. Neem leaf extracts showed considerable antimicrobial activity against four target pathogens and in agar well diffusion method it showed maximum antimicrobial activity against *Enterococcus* and *S. aureus* [28]. However, in the present study the antibacterial activity was observed maximum in ethanolic extracts of neem against *S. aureus* oral isolate with a zone of 21 ± 0.43 mm and petroleum ether extract against *S. aureus* oral isolate with a zone of inhibition 20 ± 0.34 mm and moderate activity was found against *E. faecalis* in all the solvents extracts.

MIC was determined by dilution broth method. In current research in comparison to neem and clove extracts, the maximum MIC < 0.5 µg/µl was observed in ethanolic extracts of cloves against *S. aureus* oral isolate and *C. albicans* oral isolate, petroleum ether extract against *S. aureus* oral isolate and acetone extracts against *C. albicans* whereas, neem extract doesn't show activity upto > 2µg/µl in any of the extracts **(Table 3)** and the maximum MIC 2 µg/µl was observed in neem ethanol extracts of *C. albicans* oral isolate. It was reported that neem alcohol extracts were found effective against *C. albicans*, *Streptococcus mutans* and *E. faecalis* with the MIC value of 1.88%, 7.5% and 3.75 % and 7.5% in aqueous extracts [29].

In the previous literature the acetone, ethanol and methanol extracts of *Barleria prionitis* showed MIC of 100 µg/µl in *S. mutans* and *C. albicans* and also reported that the antibacterial activity of *S. aureus* in methanol extract was found to be comparatively more resistant than *S. mutans* and it survived up to 12.5 mg/ml thus having the MIC of 25 mg/ml [30]. The antifungal activity of *Candida* was less resistant than *S. cerevisiae* and it survived upto 25 µg/µl, thus having the MIC of 50 mg/ml in acetone, methanol and acetone extracts [31]. In the present study the methanol extracts of neem showed MIC 31.25 µg/µl for *C. albicans* clinical isolate and methanol extracts of clove shown MIC 2 µg/µl against *C. albicans* and *C. albicans* oral isolate. The neem leaf extracts have a significant antimicrobial effect against *E. faecalis* (from root samples) and *C. albicans* [32] but, the present study the lower MIC 500 µg/µl was observed in neem methanol extract of *E. faecalis* and *E. faecalis* oral isolate; petroleum ether against *E. faecalis*, *E. faecalis* oral isolate and *C. albicans* oral isolate.

CONCLUSION

Dental caries is one of the most prevalent chronic diseases of people worldwide; individuals are susceptible to these diseases throughout their lifetime. These dental infections are caused by *Staphylococcus sp.*, facultative anaerobes like *E. faecalis* and opportunistic pathogens like *C. albicans* causes gingivitis and cavitation. Previously several studies found that the neem, cloves and *Ficus* is commonly used as traditional medicinal plants due to the antimicrobial potential that cures many diseases including dental caries causing organisms. In the present study it was understood that petroleum ether and acetone extracts of cloves was highly resistance to dental pathogens followed by methanol and ethanol extracts against *C. albicans* whereas, in neem it is found to be highly resistant in ethanol extracts against *S. aureus* clinical isolate. Therefore, comparing the three plant of interest, clove and neem are highly resistant to dental isolate and they can be by mixed in different combinations for the next level of studies and in future it can be recommended to tooth paste industries for better benefits to the peoples.

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