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ANTIMICROBIAL AND ANTIPLASMID ACTIVITIES OF *MORUS ALBA L.* AGAINST POTENT ORAL PATHOGENS

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ABSTRACT

Streptococcus mutans is one of the common initiators of dental caries. Inhibition of *Streptococcus mutans* and other cariogens with phytochemicals can be well appreciated for prevention of caries progression. Development of multidrug resistance in the oral microbial population is a major challenge in caries management. The current study aims at the evaluation of antimicrobial and antiplasmid activity of *Morus alba* root extracts against common oral pathogens *Streptococcus mutans, Lactobacillus acidophilus, Candida albicans* and *Enterococcus faecalis*. The extract showed inhibitory activity against the studied bacteria under lower concentrations and against *Candida albicans* under higher concentrations. The MIC values were found to be 0.25mg/ml for *Streptococcus mutans*, 0.5mg/ml for *Lactobacillus acidophilus* and *Enterococcus faecalis* and 1mg/ml for *Candida albicans*. The extracts showed excellent antiplasmid activity against *Streptococcus mutans* and moderate activity *Lactobacillus acidophilus* and *Enterococcus faecalis*. *Morus alba* root extracts can form an excellent candidate for the management of dental caries can limits the transfer of multidrug resistance among the commensal oral microbes.

KEYWORDS: ***Morus alba, Streptococcus mutans,*** **Dental caries and multi drug resistance.**

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INTRODUCTION

Streptococcus mutans are Gram-positive bacteria present in the oral cavity which is considered as the initiator microbe for dental caries.[1] *Streptococcus mutans* induces acid production, the initial event of caries formation, recruits other microbes like *Lactobacillus acidophilus, Candida albicans* and *Enterococcus faecalis* to form a complex microbial community. The interactions and colonization of other microbes with *Streptococcus mutans* are mediated through biofilm formation.[2] The glucans and the acids produced by the *Streptococcus mutans* are beyond the buffering capacity of the salivary fluids. The associated drop in pH of the target site paves way for the elimination of non-cariogenic commensal microbes. Being acidogenic and aciduric, *Streptococcus mutans* facilitate the coaggregation and proliferation of the succeeding cariogens and leads to biofilm formation, demineralization and finally the cavitation of the tooth .[3] The elimination of pioneering biofilm initiators like *Streptococcus mutans* from the tooth surface can be a promising strategy for the prevention of dental plaque accumulation leading to cavitation. Endodontic irrigants such as chlorhexidine hypochlorite and other antimicrobial agents against the cariogenic microbes also impose serious threats like multidrug resistance which put forward the need of an efficient antimicrobial agent .[4] The non-specificity and inefficiency of current antibiotics and the plasmid mediated drug resistance are the major emerging challenges in dentistry. Hence specific antimicrobials to eliminate primary colonizers and prevention of multidrug resistance are well appreciated. Phytochemicals are gaining attention in dentistry and medicine owing to their versatile therapeutic values and minimal side effects. *Morus alba* Linn (commonly known as white mulberry or Tut) belongs to the Moraceae family is being cultivated throughout the world for silk industry .[5] Ethano-medical properties of *Morus alba* is versatile which is evident from its extensive use in traditional medicinal system.[6,7] The abundance of such medicinal properties coined the name 'Kalpavriksha' to *M. alba* in India. The plant is a repertoire for abundant active phytoconstituents like; tannins, phytosterols, sitosterols, saponins, triterpenes, flavanoids, and alkaloids. Prenylated flavonoids like moralbanone, kuwanon S, kuwanon G, kuwanon H, mulberroside C, cyclomorusin, eudraflavone B hydroperoxide, oxydihydromorusin, leachianone G and α-acetyl-amyrin from the root of *Morus alba* are also significant .[8] *Morus alba* has also been hailed for its potent antimicrobial properties. Traditionally *Morus alba* is chewed in toothache in order to avoid further cavitation of the carious tooth .[9] de Oliveira *et al*., reported that the ethanolic extract of *Morus alba* possess both antibacterial and antifungal activity.[10] Some phenolic compounds like flavonoids, stilbenes and 2-arylbenzofurans contained in *M. alba* are good antimicrobials. Antimicrobial activity of *Morus alba* is also attributed to the phyto constituents like kuwanon C, mulberrofuran G, and albanol B.[8] *Morus alba* has been reported to possess remedial effects against dental caries and impede diverse oral pathogens. But we are lacking much evidence regarding the antiplasmid activity of *Morus alba* extracts especially against cariogenic microbes. In the present study we aimed to test the major phytochemicals in the ethanolic extract of *Morus alba* whole root and to explore its antimicrobial properties against the selected oral pathogens *Streptococcus mutans, Lactobacillus acidophilus, Enterococcus faecalis* and *Candida albicans.* The evaluation of plasmid curing ability for preventing the emergence of multidrug resistant oral bacterial strains is investigated.

MATERIALS & METHODS

Morus alba roots for the study was collected from local areas of Thiruvananthapuram district, Kerala, India.

Preparation of Morus alba crude extract

100 g *Morus alba* roots powder was soaked in 70% ethanol for 72 h in a shaker at room temperature. After soaking the extract was filtered using a muslin cloth in a petri dish and dried using an air flow system. The dried extract was removed, weighed, dissolved in sterile distilled water and kept aseptically in air tight containers.[11]

Phytochemical analysis

The extract was diluted and the presence of major phytochemical compounds like alkaloids by Dragandroff method,[6] flavonoids by lead acetate test, phenols by ferric chloride test, steroids [12] and glycosides by ferric chloride test, saponins by frothing test and tannins by ferric chloride test were evaluated using previously reported protocols.[13]

Microbial Culture and Maintenance

The oral bacterial strains (MTCC, Chandigarh) used were *S. mutans* (MTCC 890), *L acidophilus* (MTCC 10307), *E. faecalis* (MTCC 1059) and *Candida albicans* (MTCC 227). The strains were revived by suspending them in BHI (Brain Heart Infusion) broth followed by subculturing them on specific selective media (Mitis Salivarius Agar (MSA) for *E. faecalis*, Trypticase Soy Agar (TSA) for *S. mutans*, MRS medium for *Lactobacillus acidophilus* and Potato Dextrose Agar (PDA) for *Candida albicans*).

Antimicrobial Activity

The extract was checked for its antibacterial activity against the bacteria *S. mutans, L acidophilus and E. faecalis* on Muller Hinton Agar (MHA) plates and *Candida albicans* on PDA (Potato Dextrose Agar) plates by well diffusion method. The bacterial cells were inoculated in BHI broth and *Candida albicans* in PDB (Potato Dextrose Broth) to obtain a cell density of 10^8CFU/ml (by comparing with 0.5 McFarland standard tubes). The cell suspension was swabbed on to corresponding plates. 25µl, 50µl, and 100µl of the extracts (from 100mg/ml stock in water) were added in to the separate well. Gentamycin and Clotrimazole were used as controls for the bacteria and *Candida albicans* respectively. The zone of inhibition was measured after overnight growth at 37^{o}C. Minimal inhibitory concentration (MIC) was also determined by agar well diffusion method using varying concentrations. The minimum concentration of the extract that showed the zone of inhibition is considered as MIC.[14]

Plasmid curing ability of the bacteria

The bacterial strains were grown in 25ml BHI broth with 25 µg/ml *Morus alba* extract, the cells were harvested by centrifugation and incubated at room temperature for 10 mins with 50mM Glucose, 25mM Tris-HCl, pH 8 and 10mM EDTA. The preparation of plasmid was done by the alkaline lysis method. [15, 16] The plasmid DNA so obtained was run in 1% agarose gel electrophoresis at 50 V. A control with 1 µg/ml acridine orange (standard curing agent) was also treated in a similar way for comparison.

RESULTS

Phytochemical constituents

Table 1
Phytochemical analysis of M. Alba

Parameters	Results
Alkaloids	-
Phenols	++
Glycosides	-
Terpenoids	+++
Flavanoids	++
Saponins	-
Steroids	+
Tanins	-

+++ high content,
++ moderate content,
+ presence,
– absence

The phytochemical analysis showed the presence of phenols, terpenoids, flavonoids and steroids in the ethanolic extract of the whole root of *Morus alba*. The phytochemical contents were assessed qualitatively and scored according to their amount. The one with maximum content was depicted as +++ and other were scored accordingly as ++, + and –. Alkaloids, glycosides, saponins and tannins were however found to be absent in the extract (Table 1).

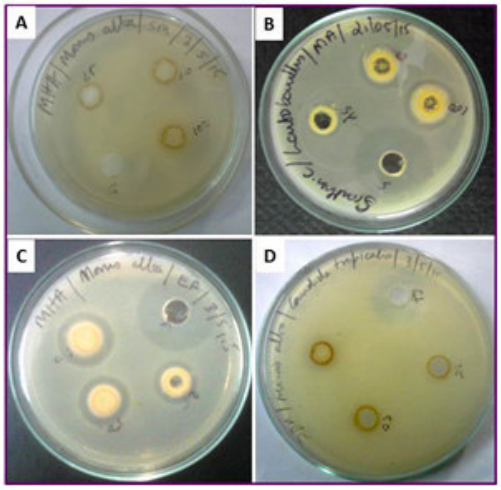
Antimicrobial Activity

The antimicrobial activity of the ethanolic extract of *Morus alba* whole root against the studied oral pathogens *Streptococcus mutans, Enterococcus faecalis, Lactobacillus acidoplhilus* and *Candida albicans* were found to be appreciable (Figure 1) (Table 2). The extract inhibited *S. mutans* (MTCC 890), *Lactobacillus acidophilus* (MTCC 10307*), Enterococcus faecalis* (MTCC 1059)and *Candida albicans* (MTCC 227) in a concentration-dependent manner. The MIC values of the bacteria were found to be 0.25 mg/ml for *Streptococcus mutans* and *Enterococcus faecalis* and 0.5 mg/ml for *Lactobacillus acidophilus.*

Table 2
Antimicrobial effects of M. Alba

S. mutans **(MTCC 890)**	
Concentration (mg)	**Zone (mm)**
2.5	19
5	22
10	28
Gentamycin (10 µg)	38
L. acidophilus **(MTCC 10307)**	
Concentration (mg)	**Zone (mm)**
2.5	12
5	14
10	17
Gentamycin (10 µg)	28
E. faecalis **(MTCC 1059)**	
Concentration (mg)	**Zone (mm)**
2.5	16
5	17
10	20
Gentamycin (10 µg)	26
C. albicans **(MTCC 227)**	
Concentration (mg)	**Zone (mm)**
2.5	0
5	0
10	15
Clotrimazole (10 µg)	25

Figure 1
Antimicrobial activities of M. alba. (A) S. mutans, (B) L. acidophilus, (C) E. faecalis and (D) C. albicans.



Plasmid curing ability of M. alba against the cariogenic bacteria

The antiplasmid activity of *Morus alba* root extracts with respect to oral pathogens has not been investigated so far. We made an attempt to validate the efficiency of *Morus alba* root extracts to inhibit the plasmid DNA of the oral pathogenic bacteria using acridine orange as a control (Figure 2). The results showed appreciable activity for *Streptococcus mutans* when compared to the standard curing agent acridine orange. But minimal curing activity was found for *Lactobacillus acidophilus* and *Enterococcus faecalis.*

Figure 2A
Antiplasmid activity of M. alba - agarose gel electrophoresis.

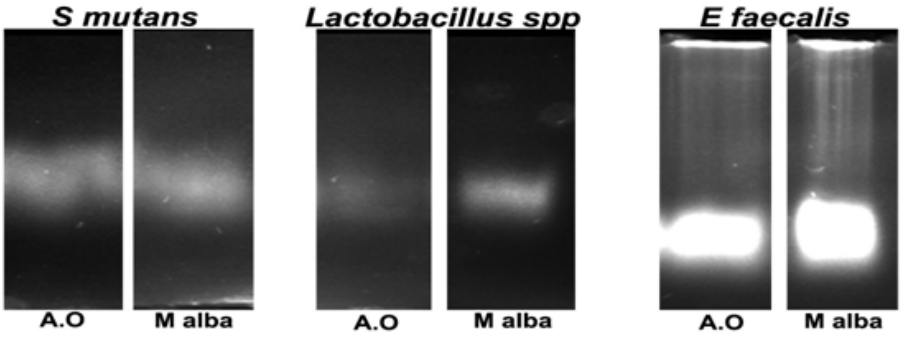
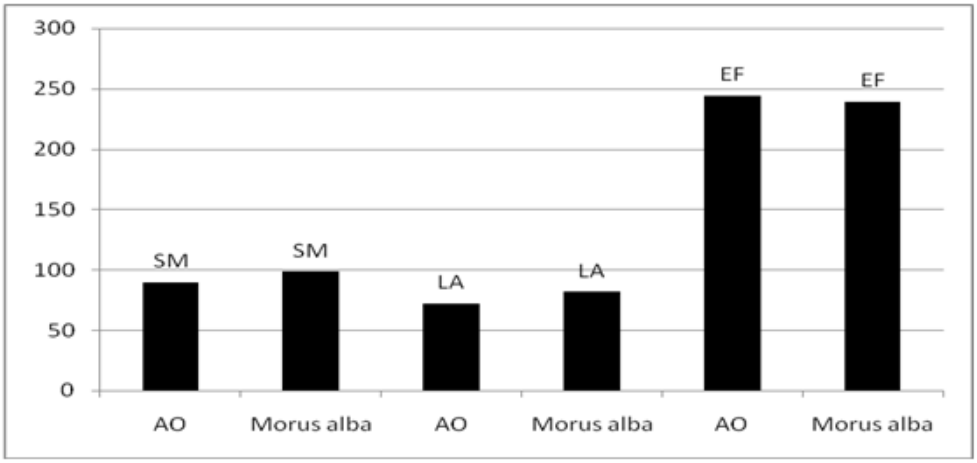


Figure 2B
Plasmid curing effects of Morus alba extracts in comparison with Acridine orange (AO) on Oral bacteria. Along X axis – Effect of Morus alba extracts and acridine orange on studied pathogens Streptococcus mutans (SM), Lactobacillus spp (LA) and E fecalis (EF): Along Y axis – Relative difference in band intensity of plasmids in arbitrary units as determined using ImageJ analysis software.



DISCUSSION

Plant extracts have been used in dentistry for centuries. The phytochemicals inhibit the growth of oral pathogens, limits biofilms, dental plaque, reduces bacterial adhesion to the surfaces and subsides the symptoms of oral diseases. Also the increase in drug resistant strains to mostly used antibiotics has stimulated the search for new antimicrobial agents, from natural origin. The secondary metabolites present in plants like alkaloids, flavonoids, tannins, phenols, saponins and several other aromatic compounds contribute to the antimicrobial property.Salem *et al*., has reported the presence of flavonoids, alkaloids, steroids, saponins and tannins in the methanolic extract of sapwood, heartwood and bark of *Morus alba.*[17]In addition Oliveira *et al*.,[10] revealed the presence of coumarins, flavonoids, tannins, and triterpenes which plays a major role in antimicrobial activity of *Morus alba* leaves. According to Islam *et al*.,[9] the flavonoid, kuwanon-G, isolated from the root bark of *M*. *alba* is effective against *S*. *mutans*. The antimicrobial activity along with the antioxidant activity contributed by the presence of flavonoids and other phytochemicals can be attributed to prevention and the further progression of the carious lesion**.**Islam *et al*.,[9] reported anticariogenic potential of the ethanolic extract of *M. alba* leaves can be attributed to their ability to inhibit biofilm formation by *S*. *mutans*. Reports showed that Kuwanon G from *Morus alba* has potent antibacterial activity against the oral pathogens like *Streptococcus mutans, Streptococcus sobrinus, Streptococcus sanguis* and *Porphyromonas gingivalis*. The ethanolic extract from *Morus alba* leaf showed high antifungal activity against *Candida albicans, Candida krusei* and *Candida tropicalis.* [10] Gunjal *et al*.,[18] has reported the efficient antibacterial effect of the *Morus alba* formulation against the major periodontal pathogens like *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia.* [18] Saleem *et al*.,[17] demonstrated that the ethyl acetate fractions of *M. alba* bark bears the highest antibacterial activity with an lowest MIC of 16µg/ml against *Bacillus* species and 32 µg /ml for *Staphylococcus aureus.*[17] A recent report suggests that the antimicrobial activity of the crude extract might be due to the presence of lipophilic compounds that bind to microbial membrane.[19] The antimicrobial activity against the chosen organisms can be signified to the abundance of phytochemicals. One of the important challenges of antibiotics in dentistry is their side effects and the emerging multiple drug resistance in a multi population of oral microbiome. The plasmid mediated gene transfer plays a significant role in the emergence of drug resistant strains among oral microbial community. The agents that inhibit the plasmid DNA replication and plasmid mediated gene expression can be well appreciated for dental therapeutics. Still there is a gap in literatures regarding the antiplasmid activity of phytochemicals against the oral pathogens despite the use of various herbal products in dentistry. Also the literatures regarding the activities of ethanolic extract of the whole root of *Morus alba* against the oral pathogens are lacking. The oral cavity may serve as a reservoir for extra oral pathogens too. Reports revealed the presence of methicillin resistant strains of *Staphylococcus aureus*, colonizing in the oral cavity as well. Wang *et al*.,[20] demonstrated the drug resistant gene transfer specifically between *Streptococcus gordonii and T. denticola*. Moreover the resistance to erythromycin have been reported in *P. gingivalis* and *Tannerella forsythia,* the two major periodontal pathogens.[21] Also the plasmid mediated resistance towards chlorhexidine (CHX), the conventional irrigant against oral bacteria hurdles the efficiency of CHX in caries management.[22] More evaluations are needed for the plasmid inhibitory effects of *M. alba* root extracts in these organisms. The plasmid curing activity of ethanolic extract of *Morus alba* root potentiates the prevention of *Streptococcus mutans* mediated drug resistance in oral cavity. Being the initiator of the dental caries formation, the inhibition of plasmids of *Streptococcus mutans* are advantageous in arresting the drug resistance and can be extrapolated for the caries management.The ethanolic whole root extracts of *Morus alba* showed appreciable antimicrobial activities against the oral pathogens *Streptococcus mutans*, *Lactobacillus acidophilus, Candida albicans* and *Enterococcus faecalis.* It also exhibited a potent antiplasmid activity against *Streptococcus mutans* comparable to the standard curing agent acridine orange. This will aid in the destruction of free plasmids and also hinders the natural gene transfer process among the drug resistant oral *Streptococcus mutans* and other colonizing communities in the oral cavity thereby preventing the development of multidrug resistant strains. Being the initiator of dental caries, the inhibition of *Streptococcus mutans* is advantageous for preventing the coaggregation by secondary and tertiary colonizers which reduces the dental plaque formation. More over the antimicrobial activity against other microbes especially *Lactobacillus acidophilus, Candida albicans* and *Enterococcus faecalis* is an added benefit for *Morus alba* in caries management. Hence the incorporation of the active components of *Morus alba* root extract in the dentifrices would adjunct the process of oral hygiene and health.

CONCLUSION

The ethanolic whole root extract of *M. alba* can be a potent candidate for the management of dental caries owing to its phytochemical content and antimicrobial activity against the cariogenic microbes. Plasmid curing activity of the extract can be appreciated for the prevention of plasmid mediated gene transfer in multidrug resistance development in oral bacteria.The ethanolic whole root extracts of *Morus alba* showed appreciable antimicrobial activities against the oral pathogens *Streptococcus mutans*, *Lactobacillus acidophilus, Candida albicans* and *Enterococcus faecalis.* It also exhibited a potent antiplasmid activity against *Streptococcus mutans* comparable to the standard curing agent acridine orange. This will aid in the destruction of free plasmids and also hinders the natural gene transfer process among the drug resistant oral *Streptococcus mutans* and other colonizing communities in the oral cavity thereby preventing the development of multidrug resistant strains. Being the initiator of dental caries, the inhibition of *Streptococcus mutans* is advantageous for preventing the coaggregation by secondary and tertiary colonizers which reduces the dental plaque formation. More over

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