



# Formulation and evaluation of antimicrobial activity of *Morus alba* sol-gel against periodontal pathogens

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## ABSTRACT

Antibiotic resistance is a major problem with inadvertent usage. Thus, there is a need to search for new antimicrobial agents of herbal origin to combat antibiotic resistance. One such plant is *Morus alba* which has a long history of medicinal use in traditional Chinese medicine. To compare the antimicrobial activity of *Morus alba* sol-gel with chlorhexidine sol-gel against ATCC standard strains of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia*. Crude extract of *Morus alba* leaves was prepared by Soxhlet method by using ethanol as a solvent. Phytochemical screening of the crude extract of *M. alba* was performed to check the various chemical constituents. *M. alba* sol-gel and chlorhexidine sol-gel were formulated using Pluronic f127 and Pluronic f108 and compared for their antimicrobial activity. The minimum inhibitory concentration of both the gels was performed using agar well diffusion technique. The minimum inhibitory concentration of *M. alba* sol-gel and chlorhexidine sol-gel against *A. actinomycetemcomitans* is 15 and 13 mm, *T. forsythia* is 7 and 17 mm, and *P. gingivalis* is 10 and 11 mm, respectively. *M. alba* possess good antibacterial activity against *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythia* and thus would be beneficial for the prevention and treatment of periodontal disease.

**Key words:** Antibacterial activity, *Morus alba*, periodontal microorganisms, thermoreversible sol-gel.

## INTRODUCTION

Chronic periodontitis is an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss, and bone loss and is characterized by periodontal pocket formation and recession of the gingiva (American Academy of Periodontology). Periodontitis has a multifactorial etiology, with the primary etiologic agents being pathogenic bacteria residing in the subgingival area. More than 700

different bacterial species colonize the oral cavity, but only a few of these are thought to be potential periodontal pathogens. (Paster *et al.*, 2000), *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* are known as the main pathogens of periodontal disease, and treatment of periodontal disease is associated with success in removing and reducing these microorganisms. (Cugini *et al.*, 2000, Slots *et al.*, 1991 & Socransky *et al.*, 1992). Treatment of periodontal pocket is based on mechanical debridement of the tooth surface followed by proper maintenance of oral hygiene. Limitations of mechanical debridement are inability to access the periodontal pockets which leads to early microbial re-colonization and recurrence of the periodontal pocket. (Caffesse *et al.*, 1986 & Greenstein *et al.*, 2000). Thus, local drug delivery systems (gels, microchip, fiber, etc.) emerged into the market. Local delivery devices are system designed to deliver agents into the periodontal pocket to retain therapeutic levels for a prolonged period of time. One such local drug delivery system which has controlled release property, less time consumption, and noninvasive is a gel. Gels are formulated using poloxamers (polyoxyethylene and polyoxypropylene units). These high-molecular-weight poloxamers have the ability to form a thermoreversible gel. (Choi *et al.*, 1998). The advantage of thermoreversible sol-gel/hydrogel system is that they are shear thinning systems which show the temperature-dependent gelation. The addition of poloxamers results in the formation of gel system. Poloxamers exhibit temperature-dependent sol to gel phase transition. The aqueous solutions of these polymers are liquid at room temperature but gel at body temperature.

Once injected, it is retained in the periodontal pocket for a long time, thus reducing the frequency of administration. (Choi *et al.*, 1998). For centuries, plants have been used throughout the world as drugs and remedies for various diseases, including infectious diseases. (Betoni *et al.*, 2006 & Lee *et al.*, 2007). These drugs serve as prototypes to develop the more effective and less toxic medicines. (Lewis *et al.*, 2006 & Sharma *et al.*, 2009). According to WHO, medicinal plants would be the best source for obtaining a large variety of drugs. (Khan *et al.*, 2009 & Santos *et al.*, 1995). Many plants have been used as remedies for diseases and offer biologically active compounds that possess antimicrobial properties. (Havyarimana *et al.*, 2012 & Kuorwel *et al.*, 2011) Of the many herbal derivatives, *Morus alba* is one such plant which has garnered great attention because of its antioxidative, antidiabetic,

antibacterial, antiviral, and anti-inflammatory properties. (Lokegaonkar *et al.*, 2011, El-Beshbishy *et al.*, 2006 & Chung *et al.*, 2003). *M. alba* also known as white mulberry is cultivated throughout the world, wherever silkworms are raised. The leaves of the white mulberry are the main food source for the silkworms. It is a popular medicinal plant that belongs to family Moraceae, and has long been used commonly in Ayurvedic and many of traditional systems of medicine. To the best of our knowledge, *M. alba* has been investigated for its use in dentistry only against pathogens causing dental caries. Hence, through this *in vitro* study, an attempt is made to explore the use of crude extract of *M. alba* in a sol-gel formulation against periodontal pathogens. Thus, the aim of the present study is to formulate and compare the thermosensitive sol-gel of *M. alba* with chlorhexidine against the periodontal microorganisms.

## MATERIALS AND METHODS

### Chemicals

The chemicals used in the gel preparation are Pluronic f127, Pluronic f108, chlorhexidine gluconate solution, and methyl paraben. The entire chemicals were of IP grade.

### Collection of plant

The leaves of the plant *Morus alba* were collected from Rajshahi University campus, Bangladesh. It was identified and authenticated in the Department of Botany, Rajshahi University, Bangladesh.

### Preparation of plant extract

Fresh leaves of the plant were washed under running tap water and air dried for about one week and then homogenized to fine powder and stored in airtight bottle. The powder of leaves (100gm) was extracted with 100 ml ethanol using conical flask in a shaking incubator at 28°C for two days. The extract was filtered and evaporated until dryness. The extract was stored at 4°C.

### Test microorganisms

The pure culture microorganisms were collected from the Institute of Biological Science (IBSc), Department of Pharmacy, University of Rajshahi, and Env. Microbiology Lab, ICDDR, B Mahakhali, Dhaka, Bangladesh. The bacteria were used for the study of antibacterial activity as follows *A. actinomycetemcomitans* (ATCC 29523), *P. gingivalis* (ATCC 33277), and *T. forsythia* (ATCC 43037).

**Phytochemical screening**

Freshly prepared crude extract of *M. alba* leaves using ethanol as a solvent was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the crude extract was performed using the following chemicals and reagents.

**Alkaloids***Wagner's reagent*

A fraction of the extract was treated with 3–5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) and observed for the formation of reddish-brown precipitate (or coloration).

**Phenols***Ferric chloride test*

A fraction of the extract was treated with aqueous 5% ferric chloride and observed for the formation of deep blue or black color.

**Tannins***Braymer's test*

2 ml of the extract was treated with 10% alcoholic ferric chloride solution and observed for the formation of blue or greenish color solution.

**Flavonoids***Alkaline reagent test*

To the test solution (0.5–1 ml), few drops of sodium hydroxide solution (10%) were added. Formation of an intense yellow color, which turns colorless on the addition of few drops of dilute sulfuric acid, indicates the presence of flavonoids.

**Proteins and amino acids***Ninhydrin test*

About 0.5–1 ml of the sample was taken in a test tube and it was boiled with 0.2% solution of ninhydrin. Appearance of violet color confirms the presence of proteins in the sample.

**Sterols and triterpenoids***Liebermann–Burchard test*

About 5 ml of test solution was boiled with few drops of acetic anhydride, cooled, and then concentrated sulfuric acid was added along the sides of the test tube. Appearance of red color in the lower layer indicates the presence of sterols, whereas yellow color in the lower layer indicates the presence of triterpenoids.

**Carbohydrates***Molisch's test*

Few drops of Molisch's reagent were added to 2 ml of the various extracts. This was followed by a slow addition of 0.2 ml of concentrated sulfuric acid along the sides of the test tube. The mixture was then allowed to stand for 2–3 min. Appearance of a red or a dull violet color at the interphase of the two layers indicated a positive test.

**Saponins**

Froth test 1 g of the extract was dissolved in 10 ml of distilled water in a test tube and vigorously shaken for 1–2 min. Formation of honeycomb froth 1 cm in height and lasting for a minimum of 30 minutes indicates the presence of saponins.

**Cardiac glycosides***Keller-Kelliani's test*

5 ml of each extract was treated with 2 ml of glacial acetic acid in a test tube and a drop of ferric chloride solution and 1 ml of concentrated sulfuric acid were added along the sides of the test tube. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardiac glycosides. A violet ring may appear below the ring, while in the acetic acid layer, a greenish ring may form.

**Fixed oil and fats***Filter paper test*

A small quantity of extract was pressed between the filter paper. Appearance of oil stain on the paper indicated the presence of fixed oils. The chemical constituents were identified by the characteristic color changes and precipitation reactions using standard protocol. Trease et al., 1989 & Harborne et al., 1998

**Sol-gel preparation**

The chemicals used for gel preparation were of IP grade. Both the gels were prepared using the same chemicals except for the active ingredients. Initially, 18 g of Pluronic F 127 (18%, w/v) was slowly dissolved in 100 ml of distilled water (maintaining ice bath at 4°C) with continuous stirring using a magnetic stirrer. It is followed by the addition of 6 g of Pluronic F 108 (6%, w/v) to the above solution and stirred at a low speed (300–400 rpm) in order to avoid frothing. The above solution was stored in a container, sealed with aluminum foil, and refrigerated overnight at 4°C until clear solution was obtained. The above steps are common for both the gels. For *M. alba* gel, 16 g of *M. alba*

extract (16%, w/v), and for chlorhexidine gel 1 ml of chlorhexidine gluconate (1%, w/v) were added slowly to the above solutions with continuous stirring using a magnetic stirrer followed by the addition of a preservative, 100 mg of (0.1% w/v) of methyl paraben. The final volume of the gel was 100 ml. The chlorhexidine and *M. alba* sol-gel were stored in a refrigerator at 4°C until further investigations are carried out. (Schmoika *et al.*, 1972 & Joshi *et al.*, 2013)

### Antimicrobial activity

The antimicrobial assay was determined by the agar well diffusion method. Antibacterial activity of the gels was determined on brucella agar medium. In a conical flask, 4.3 g of brucella agar base was suspended in 100 ml of distilled water. The conical flask was heated to boil to dissolve the medium completely. Later it was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. It was cooled to 50°C (for *T. forsythia*, 100 µl of 0.001% of *n*-acetyl muramic acid was added) and then aseptically added 5% (v/v) sterile defibrinated sheep blood. It was mixed well before pouring into sterile Petri plates. The medium was poured into sterile disposable Petri plates. After the medium was solidified, plates were kept in a refrigerator until further use. The Petri plates were removed from the refrigerator and dried in an incubator at 37°C. *A. actinomycetemcomitans*, *P.*

*gingivalis*, and *T. forsythia* were grown in thioglycollate broth mixed with horse serum, incubated for 48 h under anaerobic conditions. With the help of a micropipette, 50 µl of bacteria as per 0.5 McFarland standards were taken, spread on the agar plate, and lawn cultured with the help of a sterile swab. Two wells were made in the nutrient agar plate using a sterile cork borer measuring 4 mm depth and 6 mm diameter. In one well, 70 µl of chlorhexidine gel and in another well 70 µl of *M. alba* gel were filled with the help of micropipette. The plates were then incubated for 48 h in an anaerobic jar within 20–30 min of agar well diffusion. After incubation, the plates were observed for the zone of inhibition around the well and the diameters of the inhibition zones were measured in mm using a Hi antibiotic zone scale. (Isenberg *et al.*, 1972) The assay was carried out in triplicates and the result thus obtained is taken as the mean of the three readings.

### RESULTS

Table 1 shows the qualitative phytochemical investigation of *M. alba* that showed the presence of alkaloids, carbohydrates, glycosides, saponins, sterols, triterpenes, fats and oils, phenols, tannins, flavonoids, proteins, and amino acids in ethanolic extract.

**Table 1: Phytochemical analysis of crude extract of *M. alba* leaves using ethanol solvent**

Chemical constituent	Ethanol
Alkalioids	+
Carbohydrates	+
Proteins and amino acid	+
Glycosides	+
Saponins	+
Tanins	+
Flavonoids	+
Fixed oils and fat	+
Sterols	+
Triterpenes	+
Phenols	+

**Table 2: Antibacterial activity of *Morus alba* and chlorhexidine gel against periodontal microorganisms (zone of inhibition) by agar well diffusion method**

Microorganisms	<i>M.alba</i> gel(mm)	Chlorhexidine gel (mm)
A. <i>actinomycetemcomitans</i>	15	13
B. <i>P. gingivalis</i>	10	11
C. <i>T. forsythia</i>	07	17

## DISCUSSION

In the present *in vitro* study, thermosensitive/thermoreversible gels were formulated and antimicrobial activities of gels have been assessed for periodontal pathogens using agar well diffusion method. Gels are the polymeric network which increases the contact time of the drug at the administration sites, thereby increasing the absorption of the drug due to its mucoadhesive property. However, due to its semisolid nature, there is difficulty in application. To ease the application procedure, the sol-gel has been formulated in such a way that it is liquid during application, but turns out into a gel when it comes into contact with the oral mucosa.(Choi *et al.*, 1998) . The ideal gel formulation for the treatment of periodontitis should exhibit a high value of mucoadhesion and retention within the pocket for the desired period of time, show controlled release of the drug, and be easily delivered into the periodontal pocket preferably using a syringe.(Jones *et al.*,2000).

In the present study, the antibacterial activity of *M. alba* gel was found to be 15 mm, 10 mm, and 7 mm against standard strains of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia*, respectively. Chlorhexidine gel was used as a positive control which is accepted as gold standard in dentistry. It has a broad spectrum of antimicrobial activity against a wide variety of bacteria (both Gram-positive and Gram-negative) and fungi. Its mechanism of action being decreasing the pellicle formation, alteration of bacterial adhesion to the tooth surface, and alteration of bacterial cell wall, ultimately leading to cell death.(Armitage *et al.*, 2000). The most widely used concentration of chlorhexidine gel formulations available in the market is 1%. Hence, in the present study, the same concentration was used in the formulation of the gel.

Antimicrobial activity revealed that a zone of inhibition of both the gels against *A. actinomycetemcomitans* and *P. gingivalis* was almost similar. Whereas the diameter of the inhibition zone was greater for chlorhexidine gel than *M. alba* gel against *T. forsythia*. Even though the *M. alba* gel demonstrates less antimicrobial activity against *T. forsythia* compared to chlorhexidine gel, it has an added advantage over adverse effects.(Flotra *et al.*, 1971).

Antimicrobial activity of mucoadhesive gel prepared from the extract of *Quercus brantii* and *Coriandrum sativum* showed  $17.23 \pm 0.2$  mm zone of inhibition against *P. gingivalis*, while *M. alba* sol-gel showed 16 mm.(Aslani *et al.*, 2013). Literature reveals few studies

of *in vitro* antibacterial activity of gel, as most of the studies of the gels have been *in vivo*. The antimicrobial activity of *M. alba* sol-gel could not be compared with other sol-gel as most of the gels have not been assessed for their antimicrobial activity against periodontal pathogens.

In the present study, leaves of the *M. alba* plant have been assessed for its antibacterial activity and thus found to be effective against periodontal microorganisms. In another study, root bark of *M. alba* was found to possess antibacterial activity against periodontal pathogens like *Actinobacillus actinomycetemcomitans* and *P. gingivalis* with minimum inhibitory concentration of 1000 µg/ml and 8 µg/ml, respectively.(Park *et al.*,2003). Kuwanon C, Mulberrofuran G, and Albanol B present in the *M. alba* leaves showed strong antibacterial activity with minimum inhibitory concentrations ranging from 5 to 30 mg/ml.(Sohn *et al.*,2004).

Therefore, all the parts of the *M. alba* plant were found to possess antibacterial activity. Phytochemical analysis of *M. alba* extract revealed the presence of all major bioactive compounds like tannins, flavonoids, saponins, and alkaloids. These bioactive compounds are known to act by a different mechanism and exert antimicrobial action. Tannins, belonging to polyphenol family, have been reported to possess the antibacterial activity against periodontal pathogens.(Petti *et al.*, 2009). Tannins precipitate microbial proteins and prevent the development of microorganisms.(Petti *et al.*, 2009). Polyphenols increase the antioxidant ability of oral fluids and prevent periodontal disease.(Evans *et al.*, 2002) Therefore, tannins present in *M. alba* extract might be responsible for exerting antimicrobial activity against periodontal pathogens. This promising gel might lead to a new avenue in the prevention and treatment of periodontitis.

Although the antibacterial activity of sol-gel was assessed against periodontal pathogens, the concentration of each phytoconstituent and the chemical constituent that might be responsible for the activity was not determined. Further, *in vivo* studies should be conducted to know the effectiveness of *M. alba* sol-gel.

## CONCLUSION

Periodontal disease is a multifactorial disease, caused by the interaction of host, agent, and environmental factors. It is important to remember that *in vitro* tests do not reflect the real condition found in periodontal pockets.

They do not consider the above factors into consideration. From the present study, it can be concluded that *M. alba* possess antibacterial activity against periodontal pathogens, but chlorhexidine gluconate was found to be more effective. As the antibacterial activity is assessed against single microorganism, the exact interaction cannot be assessed by in vitro studies. Further in vivo studies should be conducted to prove its antibacterial activity against periodontal microorganisms without causing major local or systemic adverse effects. Indeed, further work should be done to elucidate main chemical compounds and their exact mechanism of action responsible for the antibacterial activity.

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### Competing interests

Authors have declared that no competing interests exist.

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