

## *In vitro* Antibacterial Effect of Hydroalcoholic Extract of *Lawsonia inermis*, *Malva sylvestris*, and *Boswellia serrata* on *Aggregatibacter actinomycetemcomitans*

### Abstract

**Background:** Considering the increased rate of microbial resistance to antibiotics and chemical side effects of antibiotics and antiseptics used for the treatment of periodontal disease, there is a need for an alternative antimicrobial agent with fewer complications. Medicinal herbs have recently become popular as novel antimicrobial agents. This study aimed to assess the antibacterial effects of hydroalcoholic extracts of *Lawsonia inermis*, *Malva sylvestris*, and *Boswellia serrata* on *Aggregatibacter actinomycetemcomitans*. **Materials and Methods:** Hydroalcoholic extracts of the three medicinal plants were obtained by the maceration technique and *A. actinomycetemcomitans* was cultured. Antimicrobial efficacy of the three medicinal plants was compared with that of 0.2% chlorhexidine (CHX) according to the Clinical and Laboratory Standards Institute protocol using agar disc diffusion and broth microdilution techniques. All tests were repeated three times. **Results:** Hydroalcoholic extracts of all three plants had antimicrobial activity against *A. actinomycetemcomitans*. The minimum inhibitory concentration (MIC) of *L. inermis*, *M. sylvestris*, and *B. serrata* was 78.1, 156.2, and 1666 µg/mL with no significant difference between them. The MIC of CHX was 3.33 µg/mL, which was significantly higher than that of *B. serrata* extract. **Conclusion:** Given that further *in vivo* studies confirm other properties of these extracts and their safety in terms of cytotoxicity and mutagenicity, hydroalcoholic extracts of *L. inermis* and *M. sylvestris* may be used in mouthwashes or local delivery systems to affect periodontal biofilm.

**Keywords:** *Aggregatibacter actinomycetemcomitans*, *Boswellia serrata*, chlorhexidine, *Lawsonia inermis*, *Malva sylvestris*

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### Introduction

Periodontitis is an inflammatory disease of the supporting tooth structures caused by anaerobic, Gram-negative periopathogens, which is characterized by progressive destruction of periodontal ligament and alveolar bone and is associated with pocket formation, gingival recession, or both. It is among the most common oral health problems in human societies.<sup>[1]</sup> Dental plaque is an organized structure of different microorganisms and plays an important role in occurrence of periodontal disease.<sup>[2]</sup>

*Aggregatibacter actinomycetemcomitans* is among the most important periopathogens in the microbial plaque, especially in aggressive periodontitis. Its interactions with other oral pathogens affect the progression of disease and response to treatment.<sup>[3]</sup> In case of no treatment, progression of infection foci leads to

destruction of the tooth-supporting structures and bone loss, followed by eventual tooth mobility and tooth loss and its related esthetic and functional consequences, which impose high cost on patients.<sup>[1]</sup> Elimination of subgingival plaque is the mainstay of treatment for periodontal disease. In the first phase of treatment, debridement of the root surface is performed by mechanical methods such as scaling and root planing. However, mechanical debridement is difficult in deep periodontal pockets due to inadequate access, and the microbial plaque may not be completely eliminated. Such cases often require more than one session of treatment. Adjunct treatment with chemical antimicrobial agents can decrease recolonization of bacteria, enhance wound healing after treatment, and decrease the need for pocket surgery.<sup>[4-6]</sup>

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At present, chlorhexidine (CHX), as the best antiseptic agent, is used for chemical control of microbial plaque, but in case of long-term use, it can have complications such as toxicity for the connective tissue, staining of teeth and restorations, changes in sense of taste, mouth dryness, and allergic reactions. Furthermore, it has contraindications for using in children.<sup>[7-9]</sup>

In addition, extensive use of synthetic chemical agents and medications has numerous side effects and has resulted in emergence of uncommon infections and microbial resistance. Recently, the use of herbal medicines has gained increasing popularity due to their history of use in traditional medicine, containing various bioactive molecules, easy availability, and low cost.<sup>[10]</sup> Moreover, the side effects of herbal medications are often insignificant,<sup>[11]</sup> and they can be safely used. *Lawsonia inermis*, *Malva sylvestris*, and *Boswellia serrata* are among the commonly used herbal medications.

Henna with the scientific name *L. inermis* is a shrub or tree from the family of Lythraceae. It often grows in tropical areas in Africa and Asia. In Iran, it is often found in Balouchestan.<sup>[12-15]</sup> Chemical composition of henna includes lawsone, mucilage, mannite, gallic acid, and tannic acid.<sup>[16]</sup> The main molecule produced by this plant is lawsone, which is primarily concentrated in the leaves and comprises 0.5%–1.5% of the henna.<sup>[17,18]</sup> This molecule has high capacity for bonding to proteins and confers staining properties for coloring of skin, hair, nails, leather, silk, and wool.<sup>[19]</sup> *L. inermis* has antihyperglycemic,<sup>[20]</sup> antiulcer,<sup>[21]</sup> antihemorrhagic, and hypotensive<sup>[22]</sup> properties as well. Roots, leaves, flowers, and seeds of this plant are used for treatment of different diseases in traditional medicine. Leaves are effective for the treatment of diarrhea, bloody diarrhea, and leprosy; flowers are effective for the treatment of anemia, insomnia, burning sensation, cardiopathy, and fever; and the seeds are effective for the treatment of intermittent fever. Furthermore, in traditional medicine, it is used for the treatment of edema, bronchitis, menstrual disorders, rheumatism, hemorrhoids, jaundice, enlarged spleen, and cutaneous diseases.<sup>[23]</sup> Anti-inflammatory properties,<sup>[24]</sup> anticancer properties, and antioxidant properties<sup>[25]</sup> are among the most important characteristics of *L. inermis*. The antibacterial effects of *L. inermis* on Gram-positive bacteria such as *Staphylococcus aureus* and Gram-negative bacteria such as *Escherichia coli* have been documented.<sup>[10,12,26]</sup> In a previous study, subcutaneous injection of ethanolic extract of *L. inermis* in rats showed no toxicity in 300 mg/kg concentration.<sup>[27]</sup>

*M. sylvestris* is a plant from the family of Malvaceae. It commonly grows in Europe, North Africa, and South-West Asia. In Iran, it grows around Tehran, Gilan, and central and southern parts of the country such as Qeshm and Bandar Abbas.<sup>[28-30]</sup> This plant has dark green leaves with five lobes and dentate margins and red to blue-purple

flowers arranged along the axis of the leaves.<sup>[15]</sup> Photochemical constituents of *M. sylvestris* include several classes of terpenoids such as monoterpenoids, diterpenoids, and aromatic compounds. It also contains mucilage, volatile essences and small amounts of tannin, malvin, and malvidin.<sup>[29-32]</sup> In Iran, *M. sylvestris* is used as a vegetable and medicinal plant. Flowers of *M. sylvestris* are used for the treatment of wounds, eczema, cutaneous infections, bronchitis, and gastrointestinal problems. It also has strong anti-inflammatory, antioxidant, anticancer, anticough, and laxative properties. According to Wang (2005), anthocyanin in the composition of *B. serrata* decreases the plasma cholesterol and total glyceride levels. They also showed that *B. serrata* extract protected the mice against gastric ulcers caused by ethanol, and this property can be related to the high mucilage content.<sup>[33,34]</sup> Antibacterial activity of this extract against *S. aureus*, *E. coli*, and *Enterococcus faecalis* has been confirmed.<sup>[33]</sup>

*B. serrata* belongs to the family of Burseraceae, which is ethnic to Saudi Arabia, Yemen, and Somalia and is also cultivated in India. This plant has secretory canals. A groove is created in the body of the shrub in order for its white juice, which is resinous, to leak out. History of the use of *B. serrata* resin dates back to 3000 years ago. After exposure to air, it becomes solid and can be found in amorphous pieces, white to yellow-brown in color.<sup>[28,35]</sup> Boswellic acid is the most important constituent of *B. serrata* resin. Evidence shows that Acetyl-11-Keto- $\beta$ -boswellic acid (AKBA) is the main constituent of resin, which is a pentacyclic terpenoid. It has a selective nonoxidative inhibitory pattern for the 5-lipoxygenase pathway, which is the main pathway for the production of leukotrienes from the arachidonic acid in cellular immunity. AKBA has significant activity against a number of inflammatory diseases such as cancer, arthritis, chronic colitis, ulcerative colitis, Crown's disease, and bronchial asthma.<sup>[36-41]</sup> New pharmacological studies have shown that in addition to anti-inflammatory effects, it regulates the immune system, inhibits the synthesis of leukotrienes, has anti-oxidant properties, and protects the liver.<sup>[42,43]</sup> It also lowers the blood sugar<sup>[44]</sup> and triglycerides<sup>[45]</sup> and positively affects the memory.<sup>[42]</sup> It has been used for the prevention<sup>[46]</sup> and treatment of Alzheimer's disease.<sup>[47]</sup> Antimicrobial activity of *B. serrata* against *S. aureus*, *E. coli*,<sup>[39]</sup> and *Streptococcus mutans* and its antifungal activity against *Candida albicans*<sup>[48]</sup> have been confirmed. Several studies have assessed the effect of medicinal plant extracts on oral pathogens and have shown the positive efficacy of extracts of plants such as *B. serrata* (AKBA) on *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella intermedia*,<sup>[49]</sup> *M. sylvestris* on *A. actinomycetemcomitans*,<sup>[50]</sup> and *L. inermis* on aerobic bacteria such as *S. aureus*. However, it has been shown that hexane and dichloromethane in the

extract of *M. sylvestris* and KBA in *B. serrata* extract<sup>[49]</sup> have no antibacterial activity. There are also controversies regarding the effect of *L. inermis* on Gram-positive and Gram-negative<sup>[27]</sup> bacteria and its minimum inhibitory concentration (MIC).

Considering the possible effect of geographical location and type of solvent of extract on its antimicrobial activity and since the effects of hydroalcoholic extracts of these herbs on *A. actinomycetemcomitans* have not been previously evaluated, this study aimed to assess the effect of hydroalcoholic extracts of *B. serrata*, *M. sylvestris*, and *L. inermis* on *A. actinomycetemcomitans*.

## Materials and Methods

### Extraction and preparation of herbal extracts

*B. serrata* was purchased from the Shirazi Bazar in Tehran, *M. sylvestris* dried flowers were obtained from the Medicinal Plant Research Center, and *L. inermis* leaves were obtained from the farms of Dezful in spring of 2016. Hydroalcoholic extracts of the plants were obtained using the maceration technique (soaking and shaking in solvent). Plants were first milled by electric mortar and then 1000 g of each plant was weighed by a digital scale. Each sample was separately placed in a 2000 mL Erlenmeyer flask, and 2000 cc of solvent (ethanol and water with 50/50 ratio) was added to it (5:1 powder/solvent ratio). Erlenmeyer flasks were capped with aluminum foils, and they were stored at room temperature for 72 h. The solutions were filtered using a Buchner funnel. After filtering, the extracts were concentrated by a rotary device and this was repeated three times for each extract. Dried concentration was weighed, labeled, and refrigerated at 4°C. For microbial assessment, 20 mg of each extract was weighed by a digital scale and dissolved in 2cc of 2% dimethyl sulfoxide using a shaker.

### Preparation of bacteria

*A. actinomycetemcomitans* standard anaerobic Gram-negative bacteria (JP2 Nov99) were used in this study. Standard strains of *A. actinomycetemcomitans* were obtained in vials from the cellular and molecular laboratory of dental school, and the vials were broken under aseptic conditions. Their contents were poured into tubes containing tryptic soy agar. The tubes were then placed in an anaerobic jar and incubated at 37°C for 24 h (type A GasPak, Merck). The bacteria were then passaged on chocolate agar medium and incubated again under the same conditions mentioned earlier.

### Assessment of antimicrobial activity

#### *Disc diffusion and well-plate methods*

Plates containing tryptic soy broth were prepared by dissolving 40 g of medium in 100 mL of distilled water and addition of 10% sheep blood. To assess the antimicrobial

activity of extracts, according to the CLSI protocol, four concentrations of 12.5, 25, 50, and 100 µL were prepared.

Four plates were swab cultured with 0.5 McFarland standard concentrations of bacteria next to the flame.

For the well-plate test, seven wells were created in each plate with 8-mm diameter using a sterile Pasteur pipette. Four wells were assigned to different concentrations of extracts, one well was for 0.2% CHX, one well was for doxycycline (Padtan Teb), and the last well was for the dimethyl sulfoxide negative control group. For the disc diffusion method, 6-mm blank paper discs (Bahar Afshan, Iran) were soaked in different concentrations of extracts and the positive and negative control groups, and after drying, they were placed in plates. After preparation, all plates were placed in anaerobic jar (type A GasPak, Merck) and incubated at 37°C for 48 h. Next, the plates were removed from the jar and the diameter of growth inhibition zone (if present) was measured by a ruler with 0.01-mm accuracy. The results were reported as means. All tests were repeated in triplicate.

#### *Broth microdilution method*

For this test, a row with eight wells in a 96-well microplate was assigned to diluted concentrations of each extract. For each extract, first 100 µL of Mueller-Hinton broth was added to each well, and then, 100 µL of the extract was added to the first well. Using a sampler, dilution was continued to the 8<sup>th</sup> well. Next, 10 µL of 1/20<sup>th</sup> suspension with 0.5 McFarland standard concentration was added to each well. CHX and doxycycline were used as positive controls. After placement in an anaerobic jar and incubation at 37°C for 24 h, contents of all wells were cultured on blood agar to determine MIC and minimum bactericidal concentration (MBC). The lowest concentration of extracts inhibiting bacterial growth was recorded as MIC and the lowest concentration of extracts causing death of bacteria was recorded as MBC. All tests were repeated in triplicate.

## Results

Based on the results of disc diffusion method, growth inhibition zone was only noted around hydroalcoholic extract of *B. serrata*. In the well-plate method, none of the extracts showed antimicrobial activity against *A. actinomycetemcomitans* except for the control group [Table 1]. Hydroalcoholic extract of *B. serrata* with 100 µL concentration showed the highest antimicrobial activity against *A. actinomycetemcomitans* but had no significant difference with CHX and doxycycline ( $P > 0.05$ ) [Figure 1].

Table 2 shows the MIC and MBC of hydroalcoholic extracts of *L. inermis*, *M. sylvestris*, and *B. serrata* against *A. actinomycetemcomitans*. In broth microdilution method, all extracts showed antimicrobial activity against *A. actinomycetemcomitans*. The highest antimicrobial

**Table 1: Antimicrobial activity of chlorhexidine, doxycycline, and hydroalcoholic extracts of the leaves of *Lawsonia inermis*, flowers of *Malva sylvestris*, and resin of *Boswellia serrata* by disc diffusion and agar well-plate methods**

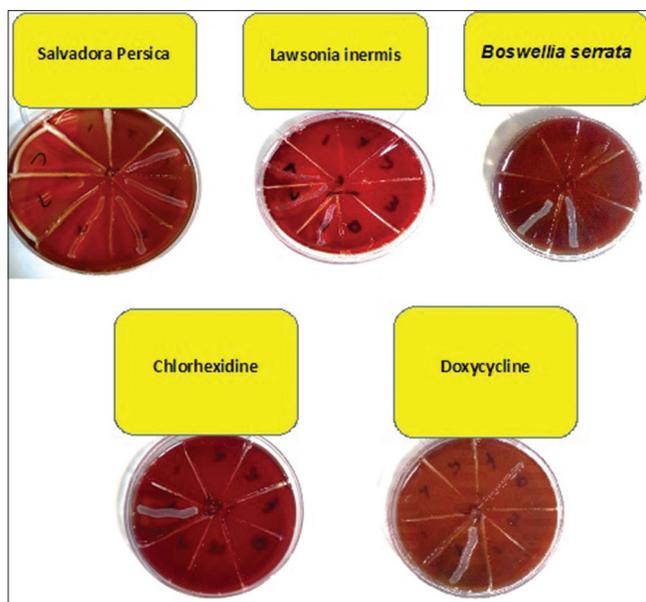
Tested materials	Microorganism/inhibition zone (mm), mean±SD (n=3) <i>Aggregatibacter actinomycetemcomitans</i> (JP2 NOV99)	
	Disc diffusion method	Agar well-diffusion method
MSH	-	-
LIH	-	-
BSH (100 µl)	10.33±1.15	-
BSH (50 µl)	9.66±0.57	-
BSH (25 µl)	2.66±4.61	-
BSH (12.5 µl)	-	-
CHX gluconate (0.2%)	12.66±0.57	13±0.81
Doxycycline (30 µg)	11±1	11.33±1.24

MSH: Hydroalcoholic extract of *Malva sylvestris*, LIH: Hydroalcoholic extract of *Lawsonia inermis*, BSH: Hydroalcoholic extract of *Boswellia serrata*, -: No inhibition zone, SD: Standard deviation, CHX: Chlorhexidine

**Table 2: Antimicrobial activity of chlorhexidine, doxycycline, and hydroalcoholic extracts of the leaves of *Lawsonia inermis*, flowers of *Malva sylvestris*, and resin of *Boswellia serrata* by broth microdilution method**

Microorganism	MIC (mg/ml)					MBC (mg/ml)				
	MSH	LIH	BSH	CHX	Doxycycline (30 µg)	MSH	LIH	BSH	CHX	Doxycycline (30 µg)
<i>Aggregatibacter actinomycetemcomitans</i> (JP2 NOV99)	1.666	0.156	0.078	0.003	0.009	3.33	0.312	0.156	0.007	0.039

MIC: Minimum growth inhibitory concentration, MBC: Minimum bactericidal concentration, MSH: Hydroalcoholic extract of *Malva sylvestris*, LIH: Hydroalcoholic extract of *Lawsonia inermis*, BSH: Hydroalcoholic extract of *Boswellia serrata*, CHX: Chlorhexidine gluconate (0.2%)



**Figure 1: Minimum inhibitory concentration and minimum bactericidal concentration of three herbal essences, chlorhexidine, and doxycycline on *Aggregatibacter actinomycetemcomitans* in broth microdilution assay following the culture on blood agar well plate**

activity belonged to the hydroalcoholic extract of *B. serrata* (MIC of 78 µg/mL) and the lowest antimicrobial activity belonged to the hydroalcoholic extract of *M. sylvestris* (MIC of 1666 µg/mL), which had a significant difference with CHX and doxycycline ( $P < 0.05$ ).

## Discussion

This study showed the strong antibacterial effect of hydroalcoholic extract of *B. serrata* on *A. actinomycetemcomitans* using broth microdilution method. In this study, hydroalcoholic extracts of *L. inermis* and *M. sylvestris* showed weaker antimicrobial activity than that of *B. serrata*, respectively, and MIC of *M. sylvestris* was significantly lower than that of CHX. The positive antibacterial effect of hydroalcoholic extract of *B. serrata* on *A. actinomycetemcomitans* in broth microdilution method and also the direct correlation with concentration of *B. serrata* extract and diameter of growth inhibition zone in disc diffusion method in our study were in agreement with the results of Kafshdar Goharian<sup>[51]</sup> who assessed the antibacterial effect of organic extract of *B. serrata* on *A. actinomycetemcomitans*. However, the MIC reported in their study was 780 µg/mL, which was higher than the MIC in our study (78.1 µg/mL).

It means that the hydroalcoholic extract of *B. serrata* has a stronger antimicrobial activity than its organic extract, which may be due to the type of solvent, season of collection of plant samples, and tested concentrations in broth microdilution method. No other study was found on the effect of *B. serrata* extract on *A. actinomycetemcomitans*. However, some studies have assessed the effect of *B. serrata* on other anaerobic Gram-negative periopathogens of the oral cavity. For

instance, Raja *et al.*<sup>[49]</sup> purified *B. serrata* and assessed the effect of its constituents on *P. gingivalis*, *P. intermedia*, and *F. nucleatum*. The MIC of AKBA in *B. serrata* for the aforementioned bacteria was 32, 4, and 128 µg/mL, respectively. They reported AKBA to be the most effective antimicrobial constituent of *B. serrata*. Weckesser *et al.*,<sup>[52]</sup> in Germany, reported that alcoholic extract of *B. serrata* had no effect on *F. nucleatum* and *P. intermedia*, and the MIC of extract for *P. gingivalis* was 1 µg/mL. Difference in the results of the two studies may to some extent indicate the positive effect of purification of extract for increasing its antimicrobial activity. In the current study, hydroalcoholic extract of *B. serrata* had growth inhibition property in disc diffusion test while agar well-diffusion method did not show such property. Valgas *et al.*<sup>[53]</sup> showed that the well-plate method was superior to the disc diffusion because the free hydroxyl groups in the discs may prevent the diffusion of cationic molecules of the extract. However, this result was not in accordance with ours, which may be due to the better superficial diffusion of extract from the disc into the agar compared to the well-plate method.

The results of MIC testing of hydroalcoholic extract of *L. inermis* on *A. actinomycetemcomitans* in our study were in agreement with that of Akter *et al.*<sup>[26]</sup> who reported the antimicrobial activity of *L. inermis* on Gram-negative bacteria. However, our findings were in contrast to those of Papageorgiou *et al.*<sup>[54]</sup> who showed that photochemical constituents of *L. inermis* had antimicrobial activity only against Gram-positive bacteria. Our results regarding the effect of *L. inermis* on Gram-negative bacteria were also in line with those of Bhuvanewari *et al.*, Habbal *et al.*, and Hussain *et al.*<sup>[14,55,56]</sup>

Antimicrobial effect of *M. sylvestris* has only been evaluated against a few microorganisms. According to Benso *et al.*,<sup>[50]</sup> methanolic and chloroformic extracts of *M. sylvestris* have antimicrobial activity against *A. actinomycetemcomitans* and other anaerobic periopathogens, namely, *F. nucleatum*, *P. gingivalis*, and *P. intermedia*. Lower MIC reported in their study compared to ours may be due to different methodology, part of plant used, and type of extract. No other study was found on the effect of *M. sylvestris* extract on *A. actinomycetemcomitans*. However, Razavi *et al.*<sup>[33]</sup> showed that methanolic extracts of the flower and leaves of *M. sylvestris* had high antimicrobial activity against *S. aureus*, *Streptococcus agalactiae*, and *E. faecalis*. Lower MIC reported in their study compared to ours may be due to the difference in type of extract, parts of the plant used, and more importantly, type of bacteria.

In the current study, *M. sylvestris* and *L. inermis* extracts showed antimicrobial activity only in broth microdilution method, and agar disc diffusion test did not show any growth inhibition zone. The reason may be

that we did not isolate the constituents of the extracts. Thus, in comparison with antibiotics, the presence of agar in the medium may inhibit the diffusion of extract and thus, no or weak antimicrobial activity was observed in agar disc diffusion test. However, in broth microdilution method, the inhibitory effect of agar is not present, and the extracts are in contact with bacteria in a liquid environment; thus, they can easily show their bactericidal or bacteriostatic properties. Lin<sup>[57]</sup> compared the antimicrobial properties of chitosan using different methods and showed that broth microdilution yielded better results than the disc diffusion method. They concluded that broth microdilution is a suitable and fast screening method compared to other techniques, and their results were somehow in line with ours. Moreover, considering the presence of saliva and gingival crevicular fluid in the oral environment, broth microdilution can better simulate the oral cavity than agar disc diffusion test and can yield more reliable results.

In general, difference in the results of studies comparing medicinal herbs may be due to the use of different parts of the plant, geographical location, weather conditions, time of harvesting, and method of obtaining the extract and its purification. Significant difference in antimicrobial efficacy of plant extracts and CHX may be due to the cationic nature of CHX, enabling it to adhere to all surfaces with a negative charge, and release at a specific time point. Thus, direct comparison of CHX with medicinal plant extracts may not be logical.

Previous studies have provided evidence regarding leukotoxicity of JP2 genotype of *A. actinomycetemcomitans* and its association with local aggressive periodontitis. Considering the positive antibacterial effects of hydroalcoholic extracts of *L. inermis* and *B. serrata* on *A. actinomycetemcomitans*, further studies are required to isolate the effective constituents of these extracts for the treatment of periodontal disease. Due to the unavailability of other anaerobic periopathogens such as *P. gingivalis*, *F. nucleatum*, and *P. intermedia* and lack of experience for culturing these strains, they were not evaluated in this study, and this was a limitation of our study. Further, *in vitro* studies are required to assess the efficacy of effective constituents of extracts on artificial dental biofilm. Furthermore, cytotoxicity and mutagenicity tests need to be performed. Eventually, mouth rinses or liquids for topical application must be prepared using these extracts and tested *in vivo*.

## Conclusion

Hydroalcoholic extracts of *B. serrata* and *L. inermis* may be suitable alternatives for chemical antiseptics for the treatment of periodontal disease. Future studies can further elucidate the efficacy of these extracts as mouthwash or local delivery systems for the elimination of periodontal biofilm.

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## Conflicts of interest

There are no conflicts of interest.

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