



Original Article

Combined Antibacterial Activity of Cetylpyridinium Chloride in the Presence of Two Herbal Extracts Against *Streptococcus mutans*

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Abstract

Background: Dental caries is one of the most prevalent chronic human infections worldwide. It begins with bacterial adherence to the tooth surface and the formation of dental plaques. Among various microorganisms involved, mutans streptococci are the principal oral microorganisms involved in the initiation and development of dental caries. Cetylpyridinium chloride (CPC), a quaternary ammonium with a rapid bactericidal effect, is widely used as the active ingredient of antiseptic oral mouthrinses. Plants extracts are also extensively employed in oral hygiene products for their antimicrobial, anti-inflammatory, and astringent properties. The present study aimed to investigate the combined antibacterial activity of CPC in combination with two plant extracts that are extensively utilized in dental hygiene products.

Methods: The aerial parts of *Matricaria chamomile* and hydroalcoholic extracts of *Quercus infectoria* galls were prepared by maceration. Dried extracts were investigated for antibacterial activity against *Streptococcus mutans* by determining the minimum inhibitory concentrations (MICs). A checkerboard method was applied to investigate the combined antibacterial activity of CPC in the presence of *M. chamomile* aerial parts and *Q. infectoria* gall extracts.

Results: The results of this study indicated a synergistic effect between CPC and the hydroalcoholic extract of *Q. infectoria* galls. However, the presence of the extract of *M. chamomile* aerial parts had an antagonistic effect on the antibacterial activity of CPC against *S. mutans*.

Conclusion: Accordingly, despite several beneficial properties, plant extracts should be cautiously used in the formulation of antimicrobial products due to the probability of unwanted antagonistic interactions that destroy the product's efficacy.

Keywords: Antagonism, Antiseptic mouthwash, Cetylpyridinium, Herbal, Matricaria, Synergism



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Introduction

Dental caries is one of the most prevalent chronic human infections worldwide. It begins with bacterial adherence to the tooth surface and formation of dental plaque/biofilms. Cariogenic bacteria within these dental plaques produce acid by metabolizing carbohydrates. Persistent acidification drops the plaque pH under the critical pH (5.5), destroying enamel surfaces. The demineralization of the enamel and the degradation of the dentine organic matrix lead to the development of caries, and the inflammation of pulp that can end with tooth loss (1,2). Among various microorganisms, mutans streptococci, mainly *Streptococcus mutans*, is the principal oral microorganism involved in the initiation and

development of dental caries (3). Epidemiological studies have shown a direct correlation between the salivary level of this microorganism and the number of decayed teeth. It can easily adhere to an enamel surface and form a high-cell-density biofilm by producing irreversible interactions between bacterial cells. Its other virulence factors are acidogenicity, acid tolerance, and synthesis of water-insoluble glucan from sucrose (4).

Antibacterial mouthwash formulations containing chlorhexidine, triclosan, and cetylpyridinium chloride (CPC) are usually prescribed to overcome plaque formation and subsequent complications. CPC has been studied extensively in clinical trials and is one of only two antimicrobial mouth rinse ingredients that has received



a Category I recommendation from the advisory panel of the United States Food and Drug Administration for safety and effectiveness in reducing supragingival plaque and gingivitis (5). CPC is a quaternary ammonium compound with broad-spectrum antibacterial activity. It is a cationic surface-active agent with both hydrophilic and hydrophobic groups (6). This structure enables the compound to bind to the microbial cell surface and integrate into the cytoplasmic membrane. The disruption of membrane integrity results in the leakage of cytoplasmic components, eventually leading to cell death through interfering with cellular metabolism and the inhibition of cell growth (7). CPC has also been demonstrated to inhibit the plaque formation process through adsorption on the tooth enamel and interference with the co-aggregation of bacteria. This ability is important for the retention of CPC in the mouth and continued antimicrobial activity for a period of time after rinsing. Previous studies have reported significant reductions in salivary aerobic and anaerobic bacterial counts for up to seven hours following a single rinse with a CPC-containing product (8).

Apart from the above-mentioned antimicrobial agents, phytochemicals with antimicrobial and antiplaque properties are extensively used in oral hygiene products for the prevention and treatment of dental caries, as well as for antioxidant, analgesic, and elimination of bad breath effects (9,10). The incorporation of herbal products is usually associated with higher consumer acceptance due to the decreased risk of adverse effects and the promotion of antibiotic resistance (11). However, when using herbal products in combination with another antimicrobial agent, the type of interactions they have against the intended microorganisms should undergo careful investigation. The interactions observed in the case of using two or more antimicrobial agents are additive, antagonistic, or synergistic effects, predominantly based on the antibacterial mechanisms of action and the target sites of the agents. While antagonism can ruin antimicrobial therapy, the synergistic effect reduces the risk of drug resistance and side effects for the patients (12). The present study sought to evaluate the combined antibacterial activity of CPC in the presence of 2 herbal products, extracts from *Matricaria chamomile* aerial parts or *Quercus infectoria* galls, against *S. mutans*. Both plants are famous for their antibacterial and antiplaque ingredients and are extensively employed in commercial oral hygiene products and mouthrinses.

Materials and Methods

Materials and Strains

The standard strain of *S. mutans* (ATCC 35668) was obtained from the Persian Type Culture Collection of the Biotechnology Department at Iranian Research Organization for Science and Technology, Tehran, Iran. Muller-Hinton Broth (MHB) and resazurin sodium were purchased from Merck (Dartmouth, Germany) and Sigma-Aldrich (St Louis, MO, USA), respectively.

Preparation of the Extracts

The *Q. infectoria* galls and aerial parts of *M. chamomile* were randomly collected from the wild populations of Lorestan and Hamedan Heights, respectively. The plants were dried in shade and then crushed and extracted by the hydroalcoholic solvent (80:20 v/v, ethanol/water). The extraction was performed in closed flasks on a shaker at room temperature for 24 hours. The mixtures were then filtered, and the solvent was removed by a rotary evaporator and then dried in a water bath at 50°C.

Minimum Inhibitory Concentration Determination

The minimum inhibitory concentration (MIC) of CPC and herbal extracts against *S. mutans* were separately determined using the micro-dilution method according to the Clinical and Laboratory Standards Institute Guideline (13). CPC was dissolved in sterile deionized water to obtain 100 µg/mL stock solution, which was further diluted to 0.1, 0.2, 0.5, 0.75, 1.5, 3.125, 6.25, 12.5, 25, and 50 µg/mL. The extracts were dissolved in sterile deionized water and serially diluted to obtain 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 mg/mL concentrations. The microbial inoculum was prepared by dispersing a proper amount of the colonies formed on the 24-hour culture of *S. mutans* in the 0.9% sodium chloride solution to obtain the turbidity equivalent to half McFarland standard. The MHB culture medium was inoculated by adding 0.1% v/v of the inoculum to obtain the final microbial concentration of 10⁵ CFU/mL. In the 8-well columns of a microtiter plate, 100 µL of various concentrations of the extract were mixed with 100 µL of the inoculated MHB (n=4). Inoculated and un-inoculated MHB were used as positive and negative controls, respectively. After incubating the microtiter plates at 37°C for 24 hours, the growth of the microorganism was investigated using the resazurin colorimetric test. An adequate amount of resazurin sodium salt was dissolved in distilled water to obtain a 0.01% w/v solution, which was then dispensed in the wells of the microtiter plates to the final concentration of 0.001% w/v. The plates were incubated at 37°C for 30 minutes and then assessed visually. Any color changes from purple to pink or colorless were recorded as positive microbial growth. The lowest concentration of the extract that inhibited the growth under the explained condition was considered MIC. This test was performed in triplicate, and data repeated in at least two experiments were reported as MICs.

Checkerboard Assay

The combined antimicrobial effect of CPC in the presence of the herbal extracts against *S. mutans* was separately determined using the checkerboard method (14). The inoculated MHB culture medium was dispensed in the wells of a microtiter plate (200 µL for a and b and 100 µL in other wells (Figure 1). The serial dilutions of CPC and the extract in sterile deionized water were poured in the wells of the microtiter plate in the order shown in

Figure 1 to obtain a range of concentrations of $1/8 \times \text{MIC}$ to $8 \times \text{MIC}$. After incubating at 37°C for 24 hours, the plates were visually investigated for microbial growth using a resazurin colorimetric assay. The well of the microtiter plate containing the lowest concentrations of both CPC and the extract that inhibited the growth of microorganism were determined. The fractional inhibitory concentration index (FICI) was calculated to evaluate the combined antimicrobial effect of CPC and the extract against *S. mutans* (Equation 1). FIC for CPC or the extracts was calculated by dividing its MIC in combination (A, B) by the MIC when acting alone ($\text{MIC}_A, \text{MIC}_B$) (15).

$$\text{FICI} = \text{FIC}_A + \text{FIC}_B = A/\text{MIC}_A + B/\text{MIC}_B \quad (1)$$

Results

Determination of Minimum Inhibitory Concentration

Table 1 provides the obtained MIC amounts for CPC, *M. chamomile*, and *Q. infectoria* gall extracts. Resazurin colorimetry was used for determining the wells of microtiter plates with microbial growth. Resazurin is an oxidation-reduction indicator utilized for the determination of cell growth. Its blue non-fluorescent color changes to a fluorescent pink color after being oxidized by the enzymes of viable cells. The resazurin colorimetric method is especially useful when studying colorful and opaque substances that interfere with determining the signs of microbial growth. This method has been previously shown to be efficient and accurate for the evaluation of antibacterial herbal products (16).

Determination of Fractional Inhibitory Concentration Index

Table 2 summarizes MIC, FIC, and FICI amounts when CPC antibacterial activity was investigated in the presence of *M. chamomile* or *Q. infectoria* gall extracts, separately. As shown in Figure 1, in the checkerboard assay, the antibacterial activity of two agents is investigated in

Table 1. MIC of CPC, *Matricaria chamomile*, and *Quercus infectoria* gall Extracts Against *Streptococcus mutans*^a

Microorganism	<i>M. chamomile</i>	<i>Q. infectoria</i>	CPC
<i>S. mutans</i>	5 mg/mL	0.312 mg/mL	0.5 µg/mL

Note. MIC: Minimum inhibitory concentration; CPC: Cetylpyridinium chloride.

^aExperiments were performed in triplicate, and results repeated at least in two separate tests were reported as MICs.

Table 2. Combined MICs and Calculated Amounts of FIC and FICI Against *Streptococcus mutans*^a

Herbal Extract	Extract MIC in Combination	FIC	CPC MIC in Combination	FIC	FICI
<i>Quercus infectoria</i>	0.156	0.5	0.125	0.25	0.75
<i>Matricaria chamomile</i>	5	1	1	2	3

Note. MIC: Minimum inhibitory concentration; FIC: Fractional inhibitory concentration; FICI: Fractional inhibitory concentration index; CPC: Cetylpyridinium chloride.

^aExperiments were conducted in triplicate, and results repeated at least in two separate tests were reported as combined MICs.

double serial dilutions. This technique determines the MIC of the agents alone and in combination at different concentrations. FIC and FICI values represent the ratio of changes in the MIC of each agent and both of them, respectively.

Discussion

The present study was designed to investigate the antibacterial activity of *Q. infectoria* galls and *M. chamomile* herbal extracts against *S. mutans* as the main cariogenic microorganism. It further evaluated the type of interaction and combined antibacterial activity when using these herbal extracts in combination with CPC. Although both extracts were effective against *S. mutans*, the extract of *Q. infectoria* galls was dramatically more potent in inhibiting the growth of this microorganism. *Q. infectoria* is a small oak belonging to the Fagaceae family native to the Middle East and southern Europe. Spherical 1-2.5 cm in diameter galls are formed from chemical reactions after the deposition of wasps' larvae into their young branches (17). *Q. infectoria* galls are mainly composed of tannins (50-70%), flavonoids, gallic acid, alkaloids, sterols, polyphenols, volatile oils, saponins, glycosides, reducing sugars, organic acids, anthraquinones, proteins, and amino acids (17-19). Some studies indicated various pharmacologic effects of *Q. infectoria* galls (i.e., anti-inflammatory, anti-tumor, antioxidative and anti-radiation, cardiovascular protective, hepatoprotective, antidiabetic, and anti-bacterial activity effects). There are several reports on the anti-microbial activity of the extract of *Q. infectoria* galls against bacteria, yeast, and even multi-drug resistant species (20-22). Potent antibacterial activity is attributed to the presence of chemical components such as carbohydrates, lipids, mucilage, saponins, and tannins, including tannic acid with antimicrobial activity.

Matricaria chamomile L. or German chamomile is an annual plant of the composite family Asteraceae. The plant is used to treat sore stomachs and irritable bowel syndrome and is used as a gentle sleep aid. It is also employed for laxative, anti-inflammatory, and bactericidal properties, and as a mouthwash against oral mucositis

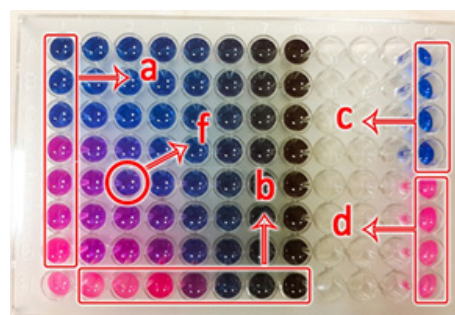


Figure 1. Order of Adding Different Concentrations of CPC and the Extract for Checkerboard Assay: Column a: CPC, Column b: *Q. infectoria* Galls or Aerial Parts of *M. chamomile* Extracts at $1/8$ to 8 fold MIC, Column c: Negative Control, Column d: Positive Control, and Well F: FIC. Note. CPC: Cetylpyridinium chloride; MIC: Minimum inhibitory concentration; *Matricaria chamomile*; *Q. infectoria*; *Quercus infectoria*

(23,24). The antibacterial properties of *M. chamomile* are related to its phenolic, flavonoid, and flavonol compounds such as caffeic acid, eugenol, rosmarinic acid, and tannic acid (25).

Hydrolysable tannins such as tannic acid present in both *Q. infectoria* galls and *M. chamomile* extracts are considered the main antibacterial components. They effectively permeate through the cell wall peptidoglycan of Gram-positive bacteria and disrupt the cell membrane. In addition, they can inhibit the adherence of bacteria to their host cells by having structural similarity to the bacterial-binding receptors (26). More potent antibacterial activity of the extract of *Q. infectoria* galls is attributed to the higher content of tannins. The galls of *Q. infectoria* contain about 50-70% of tannins in composition that yields >2000 mg/mL tannins concentration in the hydroalcoholic extract (The effect of extraction temperatures on tannin content). Tannins concentration is less than 200 mg/mL in the hydroalcoholic extract of *M. chamomile* (27).

FICI values obtained from the checkerboard assay are usually interpreted as the synergistic effect for $FICI < 0.5$, partial synergism for $0.5 \leq FICI < 1$, an additive effect for $FICI = 1$, and an antagonistic effect for $FICI > 1$. According to the European Committee on Antimicrobial Susceptibility Testing, when the combined activity is not greater than the sum of the activity of individual components, an additive interaction is present. Exceeding the combined activity from the sum of the individual activities is classified as synergistic interaction, and antagonistic interaction is whereby the combined activity of the components is lower than the activity of the most potent one (28). The estimated FICI values in our study demonstrated a partial synergistic interaction against *S. mutans* for the combination of CPC and *Q. infectoria* gall extract, while adding the *M. chamomile* extract resulted in an antagonistic effect on the CPC antibacterial activity.

Partial synergism is typically observed when the components are acting through similar mechanisms of action or on similar microbial targets (29). Membrane disruption is assumed the main antimicrobial mechanism of tannic acid as the main component of both CPC and the *Q. infectoria* gall extract (30). The same results have been reported by previous studies investigating the combined activity of antimicrobial agents with a similar mechanism of action. Noel et al reported additive interaction for every combination of cationic membrane-active disinfectants against a range of microorganisms. They proposed that the broadly overlapping mechanisms of antimicrobial activity provide no opportunity for a potent synergistic interaction greater than the sum of the activity of the components (29). The antagonistic effect of the *M. chamomile* extract on CPC antibacterial activity probably resulted from the interaction of some components of the extract with CPC that destroy its chemical structure or inhibit its antibacterial activity through chemical binding or complexation. Several previous studies have

reported the same results when evaluating the combined activity of herbal products together or in the presence of pharmaceutical products. For instance, Onaku et al reported the antagonistic effect of the *Carica papaya* extract on the antimalarial activity of artesunic acid, the main compound in the treatment of malaria (31).

Conclusion

In recent years, the biomedical application of natural products has gained attention, and the importance of multi-target combination therapies has come to the forefront. However, the prediction of the combination effect within complex mixtures such as herbal extracts remains a challenging task. The present study aimed to examine the combined antibacterial activity of CPC in the presence of two herbal extracts that are widely used in oral hygiene products. Although both *Q. infectoria* galls and *M. chamomile* extracts showed antibacterial activities against *S. mutans* probably due to their tannin ingredients, totally different interactions were observed from their combinations with CPC. The partial agnostic interaction of the extract of *Q. infectoria* galls and CPC is the result of their same antibacterial mechanism for disrupting the bacterial cell membrane. The antagonistic effect of the *M. chamomile* extract may be due to the physical or chemical incompatibility of CPC with some ingredients of the extract rather than a pharmacologic interaction. It can be concluded that the complex nature of herbal products may exert different interactions when used in combination therapy products, and precise investigations are needed to prevent unwanted interactions, leading to the decreased therapeutic effect or induction of adverse effects.

Authors' Contribution

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Project administration: Shabnam Pourmoslemi.

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Supervision: Shabnam Pourmoslemi.

Validation: Shabnam Pourmoslemi.

Visualization: Shabnam Pourmoslemi.

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Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

Not applicable.

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