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*Original Article*

# Comparison between the efficacy of self-prepared chlorhexidine varnishes and of EC 40®

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**Abstract**

It was reported that clinical application of chlorhexidine (CHX) varnish could reduce dental caries occurrence effectively. However, this form of CHX is not commercially available in Thailand. Our previous study showed that the released CHX from 20% and 40% self-prepared CHX varnishes were sufficient to inhibit the growth of *Streptococcus mutans*. This study aimed to compare the efficacy of the 20% and 40% self-prepared CHX varnishes to commercial CHX varnish EC 40®. The study included CHX release, antibacterial activity against *S. mutans* ATCC 25175, and the cytotoxic effect of CHX on fibroblasts. The results showed that the greatest amount of CHX was released by EC 40® followed by 40% and 20% self-prepared CHX, which were 4,111.29 μg, 2,408.7 μg, and 1,136.4 μg, respectively. EC 40® gave the strongest antibacterial activity; however, there was no statistical significant difference. The 20% self-prepared CHX gave the highest viability of fibroblasts. This study indicates that the self-prepared CHX should be considered to be used as antimicrobial agents for the prevention of dental caries.

**Keywords:** chlorhexidine, varnish, dental caries

## 1. Introduction

Chlorhexidine (CHX) is the most potent documented antimicrobial agent against mutans streptococci (MS) and dental caries. Different modes of administration are recommended for caries prevention (Matthijs *et al*., 2002; Ribeiro *et al*., 2007). However, it has been suggested that CHX application in varnish form results in a longer-lasting suppression of MS compared with other forms of application (Pienihakkinen *et al*., 1995; Ribeiro *et al*., 2007). Balanyk and Sandham (1989) developed the first varnish containing 10% CHX, as a way of increasing CHX and the effective release of CHX into the oral cavity. Since then, a number of studies have focused on this type of material at different concentrations (1%, 3%, 20%, 30%, 40%, and 50%) in order to assess its ability to lower the MS levels in saliva and dental biofilm. CHX varnishes with concentrations of 1% (Cervitec®),

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10% (Chlorzoin®), 20% (BioC®), and 40% (EC40®) are currently available on the market.

High and low concentrations of CHX in varnish have been reported to reduce the number of MS in plaque and saliva (Sandham *et al*., 1988; Sandham *et al*., 1991) for considerable periods of time. This long-lasting effect is probably due to the prolonged contact time between varnish and teeth. Highly concentrated varnish exhibited a pronounced CHX sustained release. Moreover, the concentration can be prepared 10-40 times higher than in other regimens (Schaeken *et al*., 1991). Some authors (Schaeken and Haan, 1989; Schaeken *et al*., 1991) examined highly concentrated CHX varnishes as supersaturated solutions of CHX-di-acetate in ethanol, stabilized by the natural resin sandarac. The optimal CHX varnish concentration suggested for suppression of MS was 40% of CHX (EC40®, Explore, Nijmegen, Netherlands). It has been documented that MS were significantly suppressed for at least 4 weeks after a single 40% CHX varnish application.

At the present, commercial CHX sandarac varnish is not available in Thailand. We have previously reported on the CHX sandarac varnish prepared at the Faculty of Dentistry, Prince of Songkla University, Thailand. It was found that the self-prepared CHX sandarac varnishes with 20% and 40% concentration could inhibit the growth of *Streptococcus mutans* in a vitro study (Musekapan *et al.,* 2005). The aim of this study was to further evaluate the properties of the self-prepared CHX sandarac varnishes at 20% and 40% compared to the commercial CHX sandarac varnish EC 40®. Three properties of CHX varnishes were investigated, which included CHX release, antibacterial activity against *S. mutans*, and the cytotoxic effect on fibroblasts.

## 2. Materials and Methods

### 2.1 Preparation of CHX sandarac varnishes

The self-prepared CHX sandarac varnishes were produced from chlorhexidine diacetate monohydrate (Fluka, Switzerland) at concentrations of 20% and 40% (w/w) in sandarac resin (Morocco). The mixtures were dissolved in 95% ethyl alcohol (w/v). The self-prepared CHX sandarac varnishes were kept in brown bottles at room temperature.

### 2.2 Release of CHX from CHX sandarac varnishes

Three sets of six glass slides, sized 22×22 mm, were filled up with 200 ml of 20% and 40% self-prepared CHX sandarac varnishes and EC 40®, and left to dry at room temperature for 20 min. Each tested glass slide was immersed in a glass container, which contained 30 ml distilled water at 37°C on a horizontal rotator. One millimeter of distilled water was sampled every 1 h during the first 6 hrs, and every 3 hrs until 12 hrs. The determination of the CHX level in each sample was performed using high performance liquid chromatography (Agilent 1100 and Walters, USA).

### 2.3 Antibacterial activity of CHX sandarac varnishes against *Streptococcus mutans*

An agar diffusion test was used to assess the antibacterial activity of CHX sandarac varnishes against *S. mutans*. Twenty-five milliliters of brain heart agar was seeded with 500 µl of overnight culture of *S. mutans* ATCC 25175 at 50°C, and was poured into a plate where 0.6 mm diameter well-cups were placed. After the media was set, the well-cups were removed. Then 100 µl of the tested CHX solutions, 20%, 40% self-prepared CHX varnishes, and EC 40®, were filled into the wells. The diameter of the inhibition zone was measured after 24 hrs incubation at 37°C. The experiments were performed in triplicate.

### 2.4 Cytotoxic effect of CHX sandarac varnishes on mouse fibroblasts

Mouse fibroblasts, Balb/c 3T3 ATCC CCL 163 (provided by Dr. Carl T. Hanks, University of Michigan, School of Dentistry, Ann Arbor, Michigan) were used to test the cytotoxicity of CHX. Balb/c 3T3 mouse fibroblasts were cultured in DMEM (Gibco, USA) supplemented with 10% calf serum, 2 mM L-glutamine, 125 IU/ml penicillin, 125 µg/ml streptomycin, and 50 µg/ml gentamycin, at pH 7.2, and at 37°C in a 5% $CO_2$ incubator.

Cells were seeded in 24 well-plates (TPP, Trasadingen, Switzerland) at a density of $5\times10^4$ cells per well, complete with medium. The filter membrane (SUPOR-200, PALL Gelman Sciences, Michigan) with a diameter of 5 mm, each was soaked with various CHX sandarac varnishes; 20%, 40% and EC 40®. Then, each filter membrane was immersed in each cell culture well. After 24 hrs exposure, cell viability was evaluated by the MTT (3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide) assay, which is based on the ability of the mitochondrial enzyme succinate dehydrogenase to convert the yellow water-soluble tetrazolium salt (MTT) into formazan crystals in metabolically active cells. The water-insoluble, dark blue product was stored in the cytoplasm of cells, would be dissolved in DMSO afterwards to generate a blue color. The color intensity was directly proportional to the amount of viable cells. Optical density was read at 570 nm (microplate reader, Biorad, USA). The experiments were performed in triplicate.

### 2.5 Statistical Analysis

Statistical analysis was performed using SPSS version 10.0 (SPSS, Chicago, IL). The results were analyzed by one-way analysis of variance and the Tukey test. Statistical significance was considered for values of $p<0.01$ (99% confidence interval).

## 3. Results

The profile of CHX released from various CHX

sandarac varnishes during 12 hrs were demonstrated in Figure 1. CHX shows a burst-release (high value during a short period of time) at the beginning of the release time followed by a slow-release. The data show that the 20% and 40% self-prepared CHX sandarac varnishes have the highest release rate at the 3rd h of the experiments with concentrations of CHX of 8.13±2.05 μg/ml and 29.46±4.1 μg/ml, respectively. The release rate then dropped rapidly and was stable at the 4th h. The EC 40® has the highest rate of release at the 6th h of the experiments with a concentration of CHX of 20.84±2.70 μg/ml. By the 12th h of the experiments, the release rates of CHX were found to be 8.11±0.80 μg/ml, 3.34±0.20 μg/ml, and 2.56±0.02 μg/ml, for EC 40®, 40%, and 20% of self-prepared CHX varnishes, respectively. By 12 h, EC 40® had the highest accumulated CHX of 4,111.29 μg, while 40% and 20% of self-prepared CHX sandarac varnishes showed lower accumulated CHX with 2,408.7 μg and 1,136.4 μg, respectively (Figure 2).

Antibacterial activity of CHX sandarac varnishes against *S. mutans* ATCC 25175 showed that EC 40® gave the strongest activity with an inhibition zone of 24.5±2.1 mm. The 40% and 20% of self-prepared CHX sandarac varnishes exhibited inhibition zones of 21.5±2.1 mm and 21.3±1.8 mm, respectively (Figure 3). However, there were no statistical differences among those inhibition zones ($p>0.05$).

The effects of various concentrations of CHX sandarac varnishes on the viability of mouse fibroblasts were determined by MTT assay. The percentage of viability of mouse fibroblasts was 88.56±11.22, 55.31±20.67 and 47.34 ±6.75, for 20%, 40% self-prepared CHX varnishes, and EC 40®, respectively. There is a statistical difference of the percentage of viability between the 20% self-prepared CHX varnish and the 40% self-prepared CHX varnish and EC 40® ($p<0.001$), but there is no statistical difference between the 40% self-prepared CHX varnish and EC 40® ($p>0.05$).

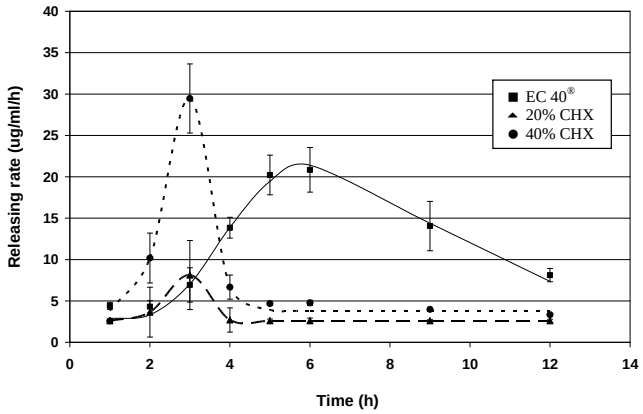


Figure 1. Release pattern of CHX from different CHX varnishes within 12 hrs.

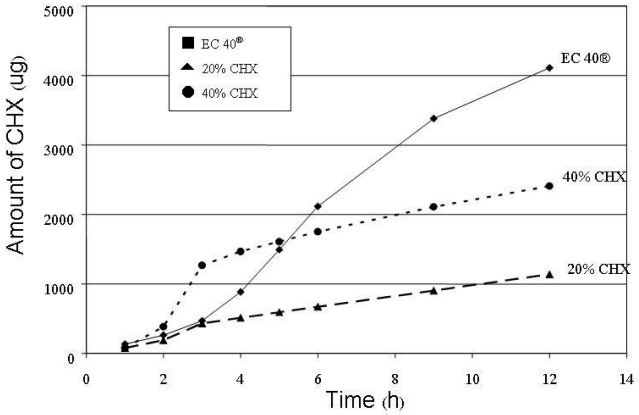


Figure 2. Accumulated amount of CHX of different CHX varnishes within 12 hrs.

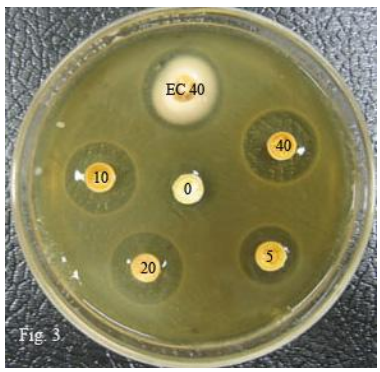


Figure 3. Antibacterial activity of different concentrations (%) of CHX varnish.

## 4. Discussion

CHX-containing varnishes have been introduced in recent years, which have brought a new concept into preventive dentistry (Balanyk and Sandham, 1985; Sandham *et al.*, 1988; Schaeken *et al.*, 1989; Huizinga *et al.*, 1990). It has been proven that CHX-containing varnishes are an excellent alternative way for the prevention of dental caries. As the varnish is a new and potentially high effective vehicle, it was chosen for this study (Ribeiro *et al.*, 2007).

Our previous study investigated the efficacy of different concentrations (5%, 10%, 20% and 40%) of self-prepared CHX sandarac varnishes against *S. mutans* ATCC 25175 (Musekapan *et al.*, 2005). It was found that the amount of CHX released from 20% and 40% self-prepared CHX varnishes exceeded the minimum inhibitory concentration (1.50±0 μg/ml) and the minimum bactericidal concentration (3.0±0 μg/ml) of *S. mutans*. The present study was undertaken to further evaluate the relative efficacy of 20% and 40% self-prepared CHX varnishes compared to EC 40® in terms of properties, including the ability of CHX release, antibacterial activity against *S. mutans*, and the cytotoxic effect on mouse fibroblasts.

The present study showed that by 12 hrs, EC 40® gave the highest amount of CHX followed by 40% and 20% self-

prepared CHX varnishes. Such amounts were sufficient to inhibit the growth of *S. mutans*. In agreement with the previous studies, the high release rates of CHX depend on the high prepared CHX varnishes concentration (Ribeiro *et al*., 2007). Many studies showed that a treatment with highly concentrated CHX varnishes can effectively suppress MS for a prolonged period of time (Schaeken *et al.*, 1991; Ie and Schaeken, 1993; Attin *et al.*, 2003). Such prolonged decrease in MS levels appears to depend upon the CHX concentration. Studies evaluating concentrations between 10% and 50% show that the 40% CHX varnish is the most effective one against MS (Schaeken *et al.*, 1989; Schaeken and Haan, 1989; Schaeken *et al.*, 1991; Sandham *et al.*, 1992). The application of varnishes at concentrations >40% leaves an unpleasant taste in the mouth for several hours following treatment (Schaeken and Haan, 1989; Attin *et al*., 2003). This can be mitigated by reducing the concentration of CHX or by decreasing the time of contact with the application surface. For this reason, most studies using 40% CHX varnish included in their methodology a less frequent application, a longer period of time between application intervals, and a removal of the varnish within 8-15 min. However, the cytotoxic effect of CHX varnish on the fibroblasts has not been mentioned so far.

Considering the cytotoxicity of the contacted cells, the study of the cytotoxic effect of different CHX varnishes in this work showed that the 20% self-prepared CHX varnish gave the highest cell viability (88.56±11.22 %). This was significantly higher compared to the 40% self-prepared CHX varnish (55.31±20.67 %) and the EC 40® (47.34±6.75 %). Thus, the results of this study suggested that the 20% self-prepared CHX varnish may have the most appropriate concentration for a CHX varnish. If the 40% CHX varnish is to be used, one should be aware of the time of contact with the application surface.

In conclusion, this study provides evidence that the 40% and 20% self-prepared CHX varnishes are effective in the growth inhibition of *S. mutans* ATCC 25175. It should be considered to be used for prevention of dental caries in high risk subjects.

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