

Antimicrobial activity of *Calendula officinalis*, *Camellia sinensis* and chlorhexidine against the adherence of microorganisms to sutures after extraction of unerupted third molars

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ABSTRACT

Objective: The objective of this study was to compare the antimicrobial effect of mouthwashes containing *Calendula officinalis* L., *Camellia sinensis* (L.) Kuntze and 0.12% chlorhexidine digluconate on the adherence of microorganisms to suture materials after extraction of unerupted third molars. Material and Methods: Eighteen patients with unerupted maxillary third molars indicated for extraction were selected (n=6 per mouthwash). First, the patients were subjected to extraction of the left tooth and instructed not to use any type of antiseptic solution at the site of surgery (control group). After 15 days, the right tooth was extracted and the patients were instructed to use the *Calendula officinalis*, *Camellia sinensis* or chlorhexidine mouthwash during 1 week (experimental group). For each surgery, the sutures were removed on postoperative day 7 and placed in sterile phosphate-buffered saline. Next, serial dilutions were prepared and seeded onto different culture media for the growth of the following microorganisms: blood agar for total microorganism growth; Mitis Salivarius bacitracin sucrose agar for mutans group streptococci; mannitol agar for *Staphylococcus* spp.; MacConkey agar for enterobacteria and *Pseudomonas* spp., and Sabouraud dextrose agar containing chloramphenicol for *Candida* spp. The plates were incubated during 24-48 h at 37°C for microorganism count (CFU/mL). Results: The three mouthwashes tested reduced the number of microorganisms adhered to the sutures compared to the control group. However, significant differences between the control and experimental groups were only observed for the mouthwash containing 0.12% chlorhexidine digluconate. Conclusions: *Calendula officinalis* L. and *Camellia sinensis* (L.) Kuntze presented antimicrobial activity against the adherence of microorganisms to sutures but were not as efficient as chlorhexidine digluconate.

Key words: *Calendula officinalis* L. *Camellia sinensis* (L.) Kuntze. Chlorhexidine. Antimicrobial activity.

INTRODUCTION

The sutures used in dentoalveolar surgery, such as extraction of unerupted teeth, represent a risk factor for the healing of surgical wounds because they are prone to the adherence of pathogenic bacteria. The accumulation of microorganisms on sutures may serve as a focus of odontogenic infections. These infections are caused by both aerobic and anaerobic bacteria, including species of the genera *Fusobacterium*, *Peptostreptococcus*, *Prevotella*, *Porphyromonas*, *Streptococcus*, and *Bacteroides*. Recent studies suggest that odontogenic infections and bacteremia develop at the time of suture removal and are a possible risk for bacterial endocarditis^{4,23}.

The use of oral antiseptics after surgery is an efficient method for microbial reduction and the consequent prevention of infections¹⁰. Chlorhexidine gluconate is currently the safest and most efficient antimicrobial agent used for the reduction of microorganisms in the oral cavity^{13,14,19,21,22}. However, chlorhexidine is associated with a number of adverse effects, such as the formation of stains on teeth and dentures, dysgeusia, parotid enlargement, and desquamation of the oral mucosa. These factors have encouraged the search for other antimicrobial agents¹⁴.

Within this line of research, the use of medicinal plants indicated for the treatment of infectious processes has been investigated based on the results of ethno-pharmacobotanical studies. The use of plants for the treatment of different diseases is known since ancient times. The oldest studies on medicine and medicinal plants arose in China and Egypt¹¹. Medicinal plants are valuable natural herbal products frequently used for the treatment of various diseases⁵ and represent an important source of new biologically active compounds^{11,24}. The antimicrobial compounds found in plants inhibit the growth of bacteria and fungi by mechanisms that differ from those underlying the activity of commonly used antimicrobial agents and have a significant clinical value^{7,12,30}.

Calendula officinalis L. is an annual plant of the family Asteraceae, which flourishes between May and October. Its flowers are used for medicinal preparations. This plant is native to Central Europe and the Mediterranean and grows naturally at sunny locations throughout North America and Europe²⁷. The following chemical components are found in this species: sesquiterpenes, flavonoid glycosides, triterpene saponins, triterpene alcohols, flavonoids, carotenoids, xanthophylls, phenolic acids, steroids, mucilage, tocopherol, and calenduline²⁸. The extract produced from *C. officinalis* has been widely used in Europe since the 12th century as a topical antiinflammatory agent. *In vivo* studies employing

mouthwashes containing *C. officinalis* demonstrated the efficacy of this plant in the reduction of gingival bleeding¹⁸. According to Iauk, et al.¹⁵ (2003), *C. officinalis* also presents antimicrobial activity against periodontopathogenic bacteria.

Green tea is produced from the leaves of *Camellia sinensis* (L.) Kuntze (family Theaceae), and has been popularly used in Japan and China for many centuries³. Animal studies have shown that extracts of *C. sinensis* possess antimicrobial activity against *Streptococcus mutans*²². In addition, *in vitro* studies have demonstrated a strong antimicrobial action of *C. sinensis* against periodontal pathogens such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*²⁹.

Several studies have tested the use of medicinal plants in mouthwashes. Groppo, et al.¹⁴ (2002) compared the effects of 0.12% chlorhexidine with mouthwashes containing *Allium sativum* L. and *Melaleuca alternifolia* Cheel. Chlorhexidine and *Allium sativum* L. showed antimicrobial activity against *S. mutans*, but not against other oral microorganisms seeded onto blood agar. In contrast, *Melaleuca alternifolia* Cheel presented strong antimicrobial activity against all oral microorganisms studied. Lauten, et al.¹⁸ (2005) tested a mouthwash containing essential oils and extracts from four plant species [*Melaleuca alternifolia* Cheel, *Leptospermum scoparium* Forst., *Calendula officinalis* L. and *Camellia sinensis* (L.) Kuntze] in 17 patients. No differences in plaque index or gingival bleeding index were observed between the mouthwash containing medicinal plants and the placebo group (mouthwash containing 12.8% ethanol in water).

The objective of the present study was to compare the effects of mouthwashes containing *Calendula officinalis* L. (Asteraceae), *Camellia sinensis* (L.) Kuntze (Theaceae) and 0.12% chlorhexidine digluconate on the adherence of microorganisms to suture materials after extraction of unerupted third molars.

MATERIAL AND METHODS

This study was conducted according to the guidelines for research involving humans and was approved by the Ethics Committee of the São José dos Campos Dental School (FOSJC), São Paulo State University (UNESP) (protocol 062/2007 - PH/CEP).

Preparation of the mouthwashes

Three mouthwashes were prepared for this study:

Oral solution of 0.12% chlorhexidine digluconate (Becker, São José dos Campos, SP, Brazil).

A 1% *Calendula officinalis* L. tincture mixed with mouthwash containing Hamposyl L®, aspartame,

glycerine, and Nipagim® (Becker).

Mouthwash containing 25% (w/v) of a water-ethanol fluid extract (1:1) obtained from the leaves of *Camellia sinensis* (L.) Kuntze (5 g), glycerine (4 g), 70% sorbitol (3.5 g), sodium saccharin (0.5 g), sodium lauryl sulfate (0.6 g), lauryl polyglucoside (1 g), disodium phosphate (0.3 g), and distilled water (85.1g). Before preparation of the mouthwash, the alcohol of the fluid extract was completely evaporated. The mouthwash was prepared at the Laboratory of Pharmacognosy and Medicinal Plants (LAFAPLAM), Faculty of Pindamonhangaba (FAPÍ).

Patient selection

The patients were selected at the Clinic of Oral and Maxillofacial Surgery and Traumatology, FOSJC, UNESP. The patients were submitted to clinical and radiographic evaluation in order to exclude those who presented any pathological or infectious process. Eighteen patients ranging in age from 17 to 30 years with unerupted maxillary third molars indicated for extraction were selected. These patients presented good health conditions and satisfactory oral hygiene and were not using medications or any oral antiseptic solution. The 18 patients were randomly divided into 3 groups as shown in Figure 1.

Surgery for unerupted maxillary third molar extraction

A panoramic radiograph was obtained from the patients before surgery for planning of the intervention. Tooth extraction was performed according to the following standardized surgical technique: first, post-tuberosity regional anesthesia of the greater palatine nerve was performed, complemented by infiltration of the anesthetic into the bottom of the buccal sulcus at the height of the first molar and on the palatine side of the gingival mucosa close to the tooth to be extracted. Next, an incision was made in the gingival mucosa of the alveolar bone crest from the curvature of the tuberosity to the mid-portion of the distal side of the second molar, complemented by an oblique incision in the direction of the bottom of the sulcus involving the distal side of the interdental papilla of the first molar. A mucoperiosteal flap was elevated and the tooth to be extracted was localized, followed by osteotomy and tooth sectioning. Extraction was

performed with a Seldin and Potts elevator. The surgical pocket was irrigated with physiological saline. The wound was closed with interrupted 3-0 silk sutures (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil) and the flap was repositioned in its original location.

The patients received 500 mg amoxicillin (Aché, São Paulo, SP, Brazil) and 50 mg sodium diclofenac (Novartis, São Paulo, SP, Brazil), and were instructed to brush their teeth using toothpaste only in the non-operated areas.

Control group

After the first surgery, i.e., extraction of the left tooth, all patients were instructed not to use any type of oral antiseptic solution.

Experimental groups

Fifteen days after the first surgery, the contralateral third molar was extracted following the same procedures as described above. However, the patients were instructed to use a mouthwash as shown in Table 1.

The mouthwash was provided in an individual package (250 mL) accompanied by a measuring cup. The patients were asked to rinse their mouth with 15 mL of the product to be tested for 30 s according to the method of Metin, et al.²⁰ (2006), twice a day (morning and night) after toothbrushing for 7 days.

Suture removal

The sutures were removed on postoperative day 7. After removal, one of the suture lines was standardized into a size of 15 mm using a sterile endodontic millimeter ruler. The suture line was transferred aseptically to a test tube containing 2 mL sterile 0.1 M phosphate-buffered saline (0.9% NaCl), pH 7.2. The samples were sent to the Laboratory of Microbiology, FOSJC, UNESP, for microbiological analysis.

Microbiological analysis

The test tube containing the suture was vortexed for 2 min (Fanem, São Paulo, SP, Brazil) in order to obtain a homogenous suspension. Decimal dilutions (10⁻¹, 10⁻² and 10⁻³) were then prepared from this suspension in sterile saline. Aliquots of 0.1 mL of the stock solution and of the dilutions were

| Number of patients | Extraction of unerupted third molar | |
|--------------------|-------------------------------------|------------------------------|
| | Left | Right |
| 6 | Control | Chlorhexidine |
| 6 | Control | <i>Calendula officinalis</i> |
| 6 | Control | <i>Camellia sinensis</i> |

Figure 1- Distribution of the patients according to experimental group

seeded in duplicate onto Petri dishes containing the following culture media: blood agar prepared with brain heart infusion agar (Difco, Detroit, MI, USA) supplemented with 5% sheep blood for total count of aerobic and facultative anaerobic microorganisms; Mitis Salivarius bacitracin sucrose (MSBS) agar prepared with Mitis Salivarius agar (Difco) supplemented with 0.2 IU/mL bacitracin and 15% sucrose for the growth of mutans group *streptococci*; mannitol agar (Difco) for the growth of *Staphylococcus* spp.; MacConkey agar (Difco) for the growth of enterobacteria and *Pseudomonas* spp., and Sabouraud dextrose agar (Difco) containing 0.1 mg/mL chloramphenicol (União Química, São Paulo, SP, Brazil) for the growth of *Candida* spp.

The blood agar, mannitol agar, MacConkey agar and Sabouraud agar plates were incubated at 37°C for 24-48 h. The MSBS agar plates were incubated at 37°C for 48-72 h in a 5% CO₂ atmosphere. After the time of incubation, plates containing 30 to 300 colonies were counted and the number of colony-forming units per milliliter (CFU/mL) obtained was log transformed (log₁₀).

Statistical analysis

The results were analyzed statistically by the paired Student's t-test using the Minitab software

(Minitab, Inc., State College, PA, USA), with the level of significance set at 5%.

RESULTS

The mean number of CFU/mL (log₁₀) of aerobic and facultative anaerobic microorganisms grown on blood agar obtained for the control and experimental groups is shown in Figure 2. The 3 mouthwashes caused microbial reduction when compared to the control group. However, this reduction was only significant for sutures from the groups using 0.12% chlorhexidine digluconate (p=0.008).

The 3 mouthwashes also reduced the growth of *Candida* spp. on Sabouraud agar (Figure 3). However, statistically significant difference in mean CFU/mL (log₁₀) was only observed between the control and chlorhexidine groups (p=0.048).

Tables 1-3 show the mean number of CFU/mL (log₁₀) of microorganisms grown on MSBS, Mannitol and MacConkey agar obtained for the control and experimental groups using mouthwash containing 25% *C. sinensis* fluid extract, 1% *C. officinalis* tincture and 0.12% chlorhexidine digluconate. No significant differences in microbial reduction were observed between the control groups and the groups using either mouthwash.

Table 1- Mean number and standard deviation of colony-forming units (CFU)/mL (log₁₀) of microorganisms isolated from suture materials and grown on Mitis Salivarius bacitracin sucrose agar obtained for the control groups and groups using *Camellia sinensis*, *Calendula officinalis* and chlorhexidine mouthwash

| Groups | Mouthwash | Control | p |
|------------------------------|-----------|-----------|-------|
| <i>Camellia sinensis</i> | 1.49±1.57 | 2.19±1.36 | 0.098 |
| <i>Calendula officinalis</i> | 1.83±0.74 | 2.60±0.41 | 0.054 |
| Chlorhexidine | 2.10±1.17 | 3.68±0.63 | 0.081 |

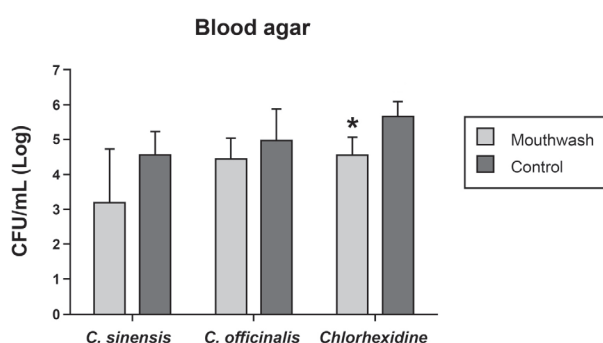


Figure 2- Mean number and standard deviation of colony-forming units (CFU)/mL (log₁₀) of microorganisms isolated from suture materials and grown on blood agar obtained for the control groups and groups using *Camellia sinensis*, *Calendula officinalis* and chlorhexidine mouthwash *Statistically significant difference between the mouthwash and control groups of chlorhexidine (Student's t-test)

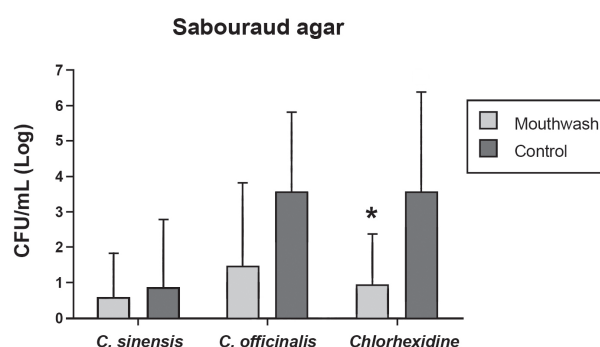


Figure 3- Mean number and standard deviation of colony-forming units (CFU)/mL (log₁₀) of microorganisms isolated from suture materials and grown on Sabouraud agar obtained for the control groups and groups using *Camellia sinensis*, *Calendula officinalis* and chlorhexidine mouthwash *Statistically significant difference between the mouthwash and control groups of chlorhexidine (Student's t-test)

Table 2- Mean number and standard deviation of colony-forming units (CFU)/mL (\log_{10}) of microorganisms isolated from suture materials and grown on Mannitol agar obtained for the control groups and groups using *Camellia sinensis*, *Calendula officinalis* and chlorhexidine mouthwash

| Groups | Mouthwash | Control | p |
|------------------------------|-----------|-----------|-------|
| <i>Camellia sinensis</i> | 0.08±0.95 | 1.77±1.52 | 0.174 |
| <i>Calendula officinalis</i> | 0.17±0.41 | 0.23±0.57 | 0.363 |
| Chlorhexidine | 0.28±0.69 | 1.05±0.83 | 0.081 |

Table 3- Mean number and standard deviation of colony-forming units (CFU)/mL (\log_{10}) of microorganisms isolated from suture materials and grown on MacConkey agar obtained for the control groups and groups using *Camellia sinensis*, *Calendula officinalis* and chlorhexidine mouthwash

| Groups | Mouthwash | Control | p |
|------------------------------|-----------|-----------|-------|
| <i>Camellia sinensis</i> | 0.96±1.53 | 1.50±1.68 | 0.343 |
| <i>Calendula officinalis</i> | 0.12±0.29 | 0.22±0.53 | 0.363 |
| Chlorhexidine | 0.12±0.29 | 0.48±0.76 | 0.177 |

DISCUSSION

Microorganisms of the oral microbiota can adhere to suture materials, a fact favoring their passage into the surgical wound and causing odontogenic infections and bacteremia. This situation is favored or prevented, depending on the adsorption properties of the suture material and oral hygiene care during the postoperative period²³.

The results of the present study demonstrated the adherence of a large number of microorganisms to the suture material after third molar extraction. According to Banche, et al.² (2001), contamination of suture material in the oral cavity mainly originates from the saliva which contains approximately 7.5×10^8 microorganisms/mL.

The largest mean number of CFU/mL (\log_{10}) of microorganisms adhered to the suture material was observed when the isolates were grown on blood agar, followed by MSBS, whereas smaller numbers of microorganisms grew on mannitol and MacConkey agar, which are selective for *Staphylococcus* and enterobacteria, respectively. These results agree with literature data reporting that *staphylococci* and enteric *bacilli* usually are not found in the oral cavity^{8,31}; if present, they occur in smaller numbers and are part of the transitory microbiota.

The smallest number of microorganisms adhered to the suture material was observed when the isolates were grown on Sabouraud agar, which is selective for the growth of *Candida* spp. According to Appleton¹ (2000), yeast of the genus *Candida* colonize the mucosal membranes of approximately 75% of the healthy population and are usually present in small numbers.

Thus, the use of an antiseptic for the control of microorganism adherence to suture material is an

extremely important procedure, reducing the risks of transitory bacteremia, especially in patients with a serious predisposition to bacterial endocarditis and those with immunological dysfunction⁶. Several plant-derived mouthwashes are used for oral hygiene, but studies investigating the action of these phytotherapeutic agents on microbial adherence to suture materials are scarce.

In the present study, the 3 mouthwashes tested showed a tendency for microbial reduction, but significant differences were only observed for the mouthwash containing 0.12% chlorhexidine digluconate when the microorganisms were grown on blood and Sabouraud agar. These findings agree with the literature on the action of chlorhexidine on aerobic and anaerobic Gram-positive and Gram-negative bacteria and yeast. Chlorhexidine has high affinity for the cell wall of microorganisms and induces cell surface alterations that lead to the loss of osmotic balance and cytoplasmic precipitation⁹.

In the present study, although the antimicrobial activity of chlorhexidine was more efficient than that of the *C. sinensis* and *C. officinalis* mouthwashes, these plants also showed a tendency for reduction of the number of microorganisms. The antimicrobial activity of *C. sinensis* has been attributed to its main component, catechin, which acts directly on the bacterial plasma membrane, causing irreversible damage and bacterial death¹⁶. In addition to its antimicrobial activity, *C. officinalis* also possesses high topical antiinflammatory activity due to the presence of saponins and flavonoids in its phytochemical composition¹⁰. Zitterl-Eglseer, et al.³³ (1997) observed that *C. officinalis* extracts exerted antiinflammatory activity similar to that of prostaglandin inhibitors.

Regarding the effects of mouthwashes on healing process, chlorhexidine, in a concentration- and time-

dependent manner, affects negatively fibroblasts and keratinocyte cell proliferation *in vitro* and can impair wound healing^{17,32}. Kozlovsky, et al.¹⁷ (2007) demonstrated that application of 0.1% and 0.2% chlorhexidine solution on an excisional wound in rats did not have a negative effect on the rate of wound closure. On the other hand, *C. sinensis* and *C. officinalis* showed potent wound healing activity. Qin, et al.²⁶ (2010) verified that chitosan green tea polyphenol complex has enhanced the healing of incision wounds in rats by increasing the breaking strength of the wounds. Preethi and Kuttan²⁵ (2009) evaluated the effects of *C. officinalis* flower extract on excision wounds made in rats and observed that the percentage of wound closure was 90.0% in the extract-treated group, whereas the control group showed only 51.1% on eighth day of wounding. The days needed for reepithelization were 17.7 for the control animals and 13.0 for the extract-treated group. These data suggest the use of mouthwashes containing *C. sinensis* and *C. officinalis* during the postoperative period after unerupted third molar extraction.

CONCLUSION

It may be concluded that antiseptics with mouthwashes containing *Calendula officinalis* L. and *Camellia sinensis* (L.) Kuntze showed a tendency for reducing the number of microorganisms adhered to suture materials after extraction of unerupted third molars. However, the antimicrobial activity of the plant extracts was not as efficient as that of chlorhexidine.

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