BJOS
Brazilian Journal of Oral Sciences

Original Research

Volume 24
2025
e255546

Antifungal and wound healing potential of calendula officinalis for dental use: an *in vitro* study

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Aim: To demonstrate the antifungal and healing properties of *Calendula officinalis*. **Methods:** We included four assays: inhibition halo, germicidal test, cytotoxicity test, and scratch test. **Results:** The extract at 100% and 50% concentrations was effective, and both presentations (*Calendula officinalis* extract and *Calendula officinalis* infusion) exhibited no cytotoxic effects. Furthermore, the infusion demonstrated a high healing effect without affecting the morphology of the cell monolayer and promoted fibroblastic proliferation. The extract did not show the expected healing results. **Conclusions:** *Calendula officinalis* holds considerable potential for further research across various biological processes, highlighting its promise as a subject for continued investigation.

Keywords: Antifungal agents. Calendula. Dentistry. Phytotherapy. Wound healing.

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Editor: Dr. Altair A. Del Bel Cury

Received: January 19, 2024
Accepted: September 17, 2024

Introduction

In dentistry, some therapeutic substances have side effects. For example, chlorhexidine gluconate 0.12% (CHX) can cause staining of teeth, alteration or irritation of taste, and alteration of the oral mucosa[1]. Currently, the practice of herbal medicine has gained significant momentum in the therapeutic use of medicinal plants, either as replacements for or in combination with pharmaceutical medicines[2]. In dentistry, homeopathic remedies such as *Calendula officinalis L.* (*C. officinalis*) and its petals have been proposed due to their anti-inflammatory, antipyretic, antitumor, and healing effects[3,4].

Calendula officinalis, a well-known therapeutic herb used for millennia, belongs to the Kingdom Plantae and the Asteraceae family[5,6]. Traditionally, it has been utilized as an anti-inflammatory, immunostimulant, diaphoretic, analgesic, antiseptic, and in the treatment of open and lacerating wounds with bleeding[7].

It has been demonstrated that *C. officinalis* possesses pharmacological properties relevant to dentistry, including its antimicrobial property for controlling bacterial growth in dental biofilm[8], analgesic effect (as its use has been reported among tribal peoples who use *C. officinalis* as an analgesic)[7], anti-inflammatory action promoting hemostasis[5,9], significant benefits in controlling the progression of periodontal disease and tissue regeneration[10], healing properties[8,11,12], promotion of wound healing[13], antifungal effect against several strains of *Candida spp.*[6], and antiviral effect by suppressing herpes simplex virus replication and exhibiting anti-HIV activity[6,7].

The main purpose of this research was to determine the properties of the infusion and extract of *C. officinalis* for use in dentistry as an alternative method for mouthwash. The focus was on demonstrating the antifungal and wound healing properties of *C. officinalis* fluid extract and flower infusion. The rationale behind this study is the need to find therapeutic alternatives in dentistry that are not only effective but also reduce the side effects associated with conventional treatments.

Methods

This was an *in vitro* study included three control groups. To assess the antifungal and wound healing potential of *Calendula officinalis (C. officinalis)* for dental applications, we utilized a hydroethanolic extract of *C. officinalis*, sourced as a fluid extract from an herbalist shop in Monterrey, Mexico.

Following this, dried *C. officinalis* flowers were purchased from an herbalist shop, also located in Monterrey. For preparing the *C. officinalis* infusion, 7 grams of dried flowers were weighed, and 100 mL of sterilized water was boiled and poured over the flowers in a glass container. After steeping for 15 minutes, the infusion was filtered through a fine sieve to remove flower residues. The resulting infusion was then transferred to a sterilized glass bottle and tightly sealed for storage.

The *in vitro* study utilized drugs including Chlorhexidine Gluconate 0.12%, an orally administered antiseptic commonly used in dentistry, and Nystatin, also administered

orally and frequently used to treat oral candidiasis. These drugs were sourced from a drugstore in Monterrey, Mexico. No conflicts of interest were reported by the authors in the development of this study.

Candida albicans strains (reference ACCT 90029) were obtained from the Research and Development Center in Health Sciences at the Universidad Autónoma de Nuevo León (Monterrey, Mexico). The inoculum was prepared by adding the *Candida albicans* strain to YPD broth as a culture medium and adjusting it to a turbidity of 0.5 on the McFarland scale to ensure proper suspension. Subsequently, this inoculum was plated on Petri dishes.

The FGH cell line was obtained from the Research and Development Center in Health Sciences at the Universidad Autónoma de Nuevo León (Monterrey, Mexico), and cultured in plates with DMEM medium supplemented with 10% fetal bovine serum (FBS) and a mixture of antibiotics and antifungals. The culture was maintained at 37°C in an atmosphere of 5% CO2. The trypan blue method was used to evaluate cell viability.

After obtaining the *C. officinalis* infusion, we performed five trials: sensidisc antimicrobial test, antimicrobial assay MIC, germicidal assay, MTT gingival fibroblast cytotoxicity assay, and wound healing assay gingival fibroblast.

Sensidisc antimicrobial test

The diffusion method on antimicrobial susceptibility test discs was employed to determine the antifungal activity of the test solutions against *Candida albicans*. An inoculum of *Candida albicans* (50 microliters) previously prepared was cultured and evenly spread on YPD agar plates. Three discs were loaded with respective mouthwash preparations of *C. officinalis* fluid extract at different concentrations: 25% (25 g/100 mL), 50% (50 mL of fluid extract in 50 mL of sterilized water), and 100% (*C. officinalis* fluid extract per se). Additionally, one disc was loaded with *C. officinalis* infusion (7 g/100 mL), one disc served as a positive control with CHX, and another disc served as a negative control with nystatin (a common drug for treating oral candidiasis). The tests were repeated three times to minimize errors. The plates were then incubated at 37°C for 24 hours. Finally, the inhibition halos were measured using a millimeter ruler. The antimicrobial susceptibility test was conducted in accordance with Standard NMX-BB-012-1974, "Multidiscs for Antimicrobial Susceptibility Testing"[14].

Antimicrobial Assay MIC

The aim of this test was to determine the minimum inhibitory concentration (MIC) of *Candida albicans* by incubating each sample for 24 hours at 37°C. A 1:100 dilution was prepared by combining 1 mL of the activated strain with 99 mL of pure YPD broth. Ten tubes were prepared with varying concentrations of *C. officinalis* extract, ranging from 15 mg/mL in tube 1 to 100 mg/mL in tube 10. The absence of growth, indicated by translucency compared to positive chlorhexidine and negative pure YPD controls, in the tube with the lowest extract concentration, was designated as the MIC. The incubation was maintained at 37°C throughout the experiment,

with absorbance readings of the culture taken every 30 minutes at 595 nm using a microplate reader.

The antimicrobial MIC broth dilution test followed the NCCLS standard: Performance Standards for Antimicrobial Susceptibility Testing, document M7 Methods for Dilution Antimicrobial Susceptibility Testing.

Germicidal Assay

For the performance of this test, the species *Candida albicans* was used as the organism of interest. The dilution procedure was followed by adding 1 mL of the inoculum of the microorganisms to the six dilutions previously prepared with a phosphate buffer solution. Each dilution was then placed in Petri dishes with gentle swirling to ensure homogeneous distribution, and the medium was allowed to solidify. After 24 hours of incubation at a constant temperature of 37°C, the colony-forming units (CFU) present in each of the Petri dishes used in the experiment were counted. The assay followed the NMX-BB-040-SCFI-1999 standard: General Methods of Analysis: "Determination of Antimicrobial Activity in Germicidal Products"[15].

MTT Gingival Fibroblast Cytotoxicity Assay

For this study, a gingival fibroblast cell suspension at a concentration of 1x10^5 cells/mL was prepared in a 96-well culture plate. After a 24-hour incubation period, the culture medium was removed, and 100 µL of *C. officinalis* infusion dilutions in DMEM were added: *C. officinalis* infusion dilutions/DMEM; infusion per se, 90g/10mL, 80g/20mL, and so on up to a concentration of 10g/90mL. A negative control (CHX) and a positive control (DMEM) were also included. After a further 24 hours of incubation, the contents of each well were removed, and cell viability was assessed by measuring the reduction of 50 µL/well of MTT solution (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium) by metabolically active cells. Cell quantification was performed using a microplate reader, and the results were recorded in the corresponding tables. For the cytotoxicity assay, the protocol by Martinez-Redríguez et al.[16] (2020) was followed.

Wound Healing Assay Gingival Fibroblast

To conduct this study, a gingival fibroblast cell suspension was prepared to achieve 90% confluence, forming a confluent monolayer. A 200-microliter (p200) pipette tip was used to create a straight line both horizontally and vertically in the cell monolayer, simulating an experimental "wound." The fibroblast cell culture was treated with the infusion at its non-toxic dose of 10 g/90 mL *C. officinalis* infusion dilution, while a positive control group was treated with DMEM culture medium. Each treatment was performed in triplicate to ensure robustness of the results. To document the wound evolution in the cells, images were captured at specific time intervals: 0 hours, 6 hours, 24 hours, 30 hours, and 48 hours, using an inverted fluorescence motorized microscope.

Chemical composition of C. officinalis

C. officinalis, commonly known as marigold, contains a diverse array of chemical compounds that contribute to its therapeutic properties. The primary constituents present in the plant are listed in Table 1.

Table 1. Phytochemical analysis results of *C. officinalis* flowers and leaves, (+) Present, (-) Absent

Metabolite/Test	Chemical Test	Result
Alkaloids	Dragendorff	-
Carbohydrates	Molisch	+
Coumarins	Baljet Test	+
Double Bonds	Beayer Test	+
Flavonoids	Shinoda	+
Phenolic Hydroxyl Groups	Ferric Chloride	+
Sterols and Triterpenes	Liebermann-Burchard	-
Xanthophylls	Xanthophylls test	-

Statistical analysis

Each study was accompanied by a specific statistical analysis. In the antimicrobial susceptibility test, inhibition halo values were compared using a single halo measurement taken 24 hours post disk placement, adhering to Standard NMX-BB-012-1974, "Multidiscs for Antimicrobial Susceptibility Testing"[14]. The antimicrobial MIC broth dilution test was conducted following the NCCLS standard: Performance Standards for Antimicrobial Susceptibility Testing, document M7 Methods for Dilution Antimicrobial Susceptibility Testing.

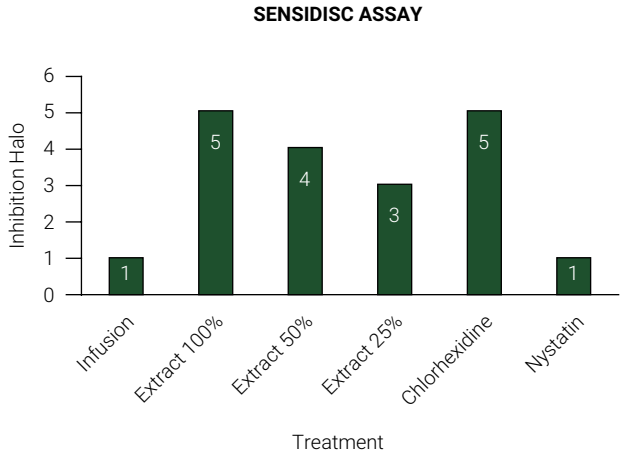
In the germicidal assay, the initial plate readings were averaged to calculate viable cell counts, and the percentage reduction was determined following the NMX-BB-040-SCFI-1999: General Methods of Analysis: "Determination of Antimicrobial Activity in Germicidal Products"[15]. For the cytotoxicity assay, culture plates were read on a microplate reader at 570 nm, and the cytotoxicity was calculated using the formula from Martinez-Rodríguez et al.[16] (2020). The wound healing assay data were processed using SPSS v23, with normality tests, Kruskal-Wallis (K-W), and Mann-Whitney U tests conducted to identify significant differences between groups, setting the significance level at $p \leq 0.05$.

Results

Sensidisc antimicrobial test

The antimicrobial susceptibility test discs applied to *Candida albicans* indicate that a 5 mm inhibition halo is observed when using 100% *C. officinalis* extract. When using 50% *C. officinalis* extract, the inhibition halo measures 4 mm. For 25% *C. officinalis* extract, the inhibition halo is the same as that of *C. officinalis*. Additionally, the *C. officinalis* flower infu-

sion results in a 1 mm inhibition halo. On the other hand, the positive control (CHX) shows a 5 mm inhibition halo, while the negative control (Nystatin) has a 1 mm inhibition halo.



Source: Created by the Author
Figure 1. Bar chart. Halo of inhibition. The results of the susceptibility test are observed.

Antimicrobial assay MIC

The test tubes were incubated for a duration of 24 hours. Upon completion of this incubation period, turbidity was observed in tube 1, containing a concentration of 15 mg/mL, indicating the presence of microbial growth. Conversely, from tube 2 through tube 10, the contents remained translucent, suggesting that no appreciable microbial growth had occurred. These results indicate that the minimum inhibitory concentration (MIC) is located within tube 2, which contained a concentration of 20 mg/mL. The minimum inhibitory concentration (MIC) of the positive control, chlorhexidine (CHX), was observed in tube 2, which contained a concentration of 10 mg/mL.

Table 2. MIC Antimicrobial Assay. The results of various concentrations of *C. officinalis* applied to *Candida albicans* are shown.

Tube No.	Extract concentration mg/mL	Result
N° 1	15 mg/mL	Presence of bacterial growth
N° 2	20 mg/mL	Bacterial growth absence
N° 3	30 mg/mL	Bacterial growth absence
N° 4	40 mg/mL	Bacterial growth absence
N° 5	50 mg/mL	Bacterial growth absence
N° 6	60 mg/mL	Bacterial growth absence
N° 7	70 mg/mL	Bacterial growth absence
N° 8	80 mg/mL	Bacterial growth absence
N° 9	90 mg/mL	Bacterial growth absence
N° 10	100 mg/mL	Bacterial growth absence

Germicidal Assay

Table 3 demonstrates effective germicidal activity, indicating the sensitivity of *Candida albicans* to a 50% extract of *C. officinalis*, along with the cell count.

Table 3. Germicidal Test. The percentage of the germicidal effect of *C. officinalis* applied to *Candida albicans* is shown in this table.

Test Microorganism	Treatment application	Initial C.V	C.V Final	Percentage reduction
ACCT 90029: Candida albicans	24hrs	105 x 108 CFU/mL	<10 CFU/mL	99.999%

C.V. Viable UFC Colony Forming Units.

MTT fibroblast cytotoxicity assay

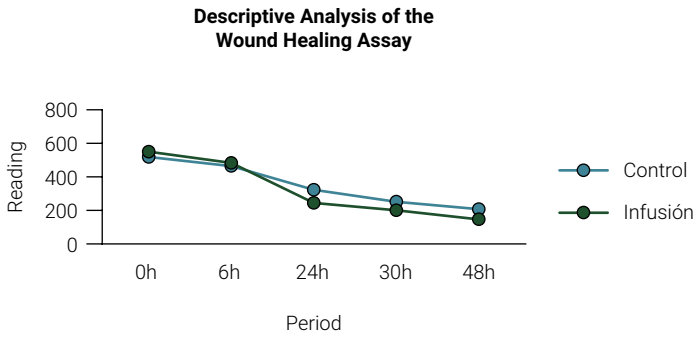
Cell cytotoxicity test showed no negative effects on viable cells during a 24-hour incubation period with the *C. officinalis* extract, unlike the positive control group. However, the negative control group exhibited a high level of cytotoxicity after 24 hours. Therefore, the *C. officinalis* extract had a cytotoxicity percentage of 62%.

Regarding the *C. officinalis* infusion, the 90/10 dilution (DMEM/infusion) was determined to be a non-toxic dose as it resulted in an increase in cell number after 24 hours of incubation compared to the positive control. Conversely, the negative control group showed significant cytotoxicity during the same period. By using the formula, the cytotoxicity percentage for the *C. officinalis* infusion was calculated to be -23%.

Scratch assay gingival fibroblast

The data show that in the control group, the initial value (0 hours) is 514.760 µm and remains constant until 30 hours. At 48 hours, the value decreases to 205.01 µm, indicating a significant decrease from the initial value ($p < 0.015$).

In the experimental group, the initial value (0 hours) is 545.945 µm and shows an increase ($p > 0.085$) compared to the reference value. At 6 hours, the value is 478.885 µm, which represents a decrease compared to 0 hours but is still higher than the reference value, indicating that the healing effect of the *C. officinalis* infusion is most effective at 6 hours. Subsequently, at 24 hours, the value decreases significantly ($p < 0.015$) compared to 0 hours, and this trend continues in the following hours (30 and 48 hours).



Source: Created by the Author.
Figure 2. The graph shows significant differences between the treated groups and the control, determined by the descriptive analysis, at a level of P < 0.05. Dots represent the mean ± S.E.M. of the groups, demonstrating the greatest healing effect at 6 hours of application.

Discussion

The aim of this project was to explore and scientifically validate the use of *C. officinalis*, renowned for its multiple therapeutic benefits, in dental practice. The main results obtained indicated that the antimicrobial susceptibility test discs applied to *Candida albicans* showed a greater halo of inhibition compared to the control group. In addition, it was found that the infusion and extract of *C. officinalis* did not present cytotoxicity in gingival fibroblast cells after 24 hours of application. Finally, a significant effect on cell migration was observed 6 hours after the treatment application.

The antimicrobial susceptibility test by Vinola et al.[3] (2021) showed that *C. officinalis* has antimicrobial and antifungal activity against *E. faecalis* and *C. albicans*[3]; however, chlorhexidine is more potent. Bermúdez Hoyos et al.[17] (2016) determined that the cream with the addition of 40% garlic extract and 20% *Calendula* showed the best results, with a reduction of 99% for *P. aeruginosa*, 98.8% for S. aureus, and 99.9% for *T. rubrum*. Cardona Rivero et al.[18] (2019) determined the minimum inhibitory concentration (MIC) of the 70% ethanolic extract of *C. officinalis* against *Escherichia coli* using the tube dilution method, presenting an MIC of 25.0 mg/mL.

This result is similar to that obtained in this study, as the extract of *C. officinalis* showed an MIC of 20 mg/mL. Verma et al.[6] (2018) concluded that *C. officinalis* has antifungal activity against *Candida albicans* as well as against other fungal pathogens. Regarding our cytotoxicity study, it was found that both presentations of *C. officinalis* had a lower cytotoxic effect compared to Acuña Piña and Sánchez Flórez[19] (2023). Cell viability for both oregano oil, *calendula* oil, and turmeric oil did not present a statistically significant difference, indicating that the implementation of these oils does not affect fibroblast cell activity.

On the other hand, Fronza et al.[20] (2009) obtained similar results to our *in vitro* study on wound healing assay, demonstrating that both extracts of *C. officinalis* stimulated the proliferation and migration of fibroblasts at low concentrations; for example, 10 µg/mL enhanced cell numbers by 64.35% and 70.53%, respectively. Similarly,

Madrid Ahumada et al.[21] (2010) demonstrated that the major proliferative effect was achieved by the ethanolic extract of *C. officinalis* in concentrations of 750 and 500 µg/mL at 12 hours.

C. officinalis contains several chemical compounds that act on *Candida albicans*. These compounds affect motor and cytoskeletal proteins, as well as the cytoskeleton, lipid transport, and synthesis, thereby compromising the function and integrity of the cell membrane.

The primary strengths of this study are found in the economic and accessible nature of *C. officinalis*, making it readily available to the general population. Unlike many commonly used dental medications, *C. officinalis* is characterized by the absence of adverse side effects, providing a safer alternative for patients. An innovative approach to dental practice is introduced through the incorporation of sustainable, natural products. The potential for *C. officinalis* to replace chemical-based treatments not only allows for the reduction of healthcare costs but also aligns with the growing demand for eco-friendly and health-conscious medical practices, thereby significantly contributing to the advancement of dental medicine.

A notable limitation of our study is the use of a single type of fibroblast cell, which may affect the generalizability of our findings to other oral structures. Additionally, financial constraints limited the scope of our research. Therefore, further studies are essential to evaluate the therapeutic applications of *C. officinalis* across a broader range of oral tissues, including oral mucosal and labial cells. We acknowledge these limitations and strongly encourage future research to address these gaps.

The primary application of *C. officinalis* in dental practice is its incorporation into daily oral care routines as a natural alternative to conventional products. Its proven antimicrobial and antifungal properties, especially against *Candida albicans*, make it effective for maintaining oral hygiene and preventing infections. Additionally, its non-cytotoxic nature ensures safety for oral tissues, while its promotion of cell migration aids in wound healing and tissue regeneration. Integrating *C. officinalis* into mouthwashes, toothpaste, and topical gels can enhance patient health and well-being by reducing dependence on synthetic chemicals.

In conclusion, significant antifungal activity was demonstrated by the extract of *C. officinalis*. The minimum inhibitory concentration (MIC) of the *C. officinalis* extract was determined to be 20 mg/mL. Both forms of presentation exhibited no harmful effects on cells. The infusion of the flower extract revealed a noticeable healing effect, particularly six hours after application, without compromising the structure of the cell layer and promoting the growth of fibroblasts. These results suggest that *C. officinalis* holds substantial potential for further research in various biological processes, highlighting its promise as a subject for continued investigation.

Acknowledgements

The authors are grateful for the support provided by the Oral Microbiology Laboratory and the Undergraduate Research Office of the Faculty of Dentistry at the Universidad Autónoma de Nuevo León.

Conflict of Interest

The authors have no conflict of interest to disclose.

Data availability

Datasets related to this article will be available to the corresponding author upon request.

Author Contribution

Nadxiely Ixchel Ricardez-Garcia: Conceptualization, Methodology, Research, Writing- original draft, Acquisition of funds. **Myriam Angélica de la Garza-Ramos:** Supervision, Validation, Resources, Visualization, Project Management, Formal Analysis, Conceptualization. **Arturo A. Cienfuegos-Sarmiento:** Resources, Methodology, Conceptualization. **Guillermo Cano-Verdugo:** Writing- revision and editing, Visualization, Formal analysis. We declare that all authors actively participated in two distinct criteria related to authorship.

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