Antimicrobial activity of noncytotoxic concentrations of *Salvia officinalis* extract against bacterial and fungal species from the oral cavity

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The use of medicinal plants can be an alternative method for the control of microorganisms responsible for human infections. This study evaluated the antimicrobial activity of *Salvia officinalis* Linnaeus (sage) extract on clinical samples isolated from the oral cavity and reference strains of *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Candida albicans, Candida tropicalis,* and *Candida glabrata*. In addition, testing assessed the cytotoxic effect of *S officinalis* on murine macrophages (RAW 264.7). Minimum inhibitory, minimum bactericidal, and minimum fungicidal concentrations of *S officinalis* extract were determined by broth microdilution method in 60 microbial samples. The cytotoxicity was checked by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The quantities of the proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) produced by RAW 264.7 were analyzed by an enzyme-linked immunosorbent assay. An *S officinalis* concentration of 50.0 mg/mL was effective against all microorganisms. Regarding cytotoxicity, the groups treated with 50.0-, 25.0-, and 12.5-mg/mL concentrations of *S officinalis* presented cell viability statistically similar to that of the control group, which was 100% viable. The production of IL-1β and TNF-α was inhibited at a 50.0-mg/mL concentration of *S officinalis*. Thus, *S officinalis* extract presented antimicrobial activity on all isolates of *Staphylococcus* spp, *S mutans*, and *Candida* spp. No cytotoxic effect was observed, as demonstrated by the survival of RAW 264.7 and inhibition of IL-1β and of TNF-α.

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Salvia officinalis Linnaeus (sage) belongs to the Lamiaceae family, from the Mediterranean region; however, it is distributed throughout the continents. It is a perennial sub-shrub species of woody stems, included in a genus with approximately 900 species. This aromatic plant can be used for culinary, medicinal, and commercial (fragrance) purposes.[1,2] In addition, some biologic effects, including antibacterial, antifungal, antileishmanial, anti-inflammatory, antitumor, antioxidant, antinociceptive, mnemonic, and antiangiogenic activities, have been attributed to *S officinalis* products such as extracts, essential oils, and bioactive molecules.[3-11]

Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, and *Candida* spp are commensal microorganisms that can cause opportunistic infections in their host. An effective method for their control is essential, either through conventional techniques, such as the use of known antimicrobials, or through alternative strategies, such as the use of plant products. Considering the increase in antimicrobial-resistant microorganisms, alternative methods involving the use of plant products may be a beneficial approach.

Therefore, this in vitro study aimed to evaluate the antimicrobial effect of *S officinalis* glycolic extract against clinical samples from the oral cavity and reference strains of *S aureus, S epidermidis, S mutans, Candida albicans, Candida tropicalis,* and *Candida glabrata.* In addition, the study analyzed the cytotoxic profile of effective concentrations of this extract on murine macrophages (RAW 264.7).

Materials and methods

The present study was approved by the Research Ethics Committee, Institute of Science and Technology, São Paulo State University (UNESP), São José dos Campos, Brazil, according to protocol 008/2010-PA/CEP.

Plant extract

Salvia officinalis glycolic extract was commercially purchased (Byofórmula) at an initial concentration of 200.0 mg/mL in propylene glycol. This extract was obtained from leaves of the plant and was chemically composed of pinene, cineole, camphor, carvacrol, thujone, ursolic acid, oleanolic acid, chlorogenic acid, caffeic acid, labiatic acid, rosmarinic acid, α- and β-amyrin, picrosalvin (carnosol), betulin, flavonoids, tannins, estrogenic substance, saponins, resins, and mucilages, according to the manufacturer.

Table. Number of clinical isolates and reference strains eliminated by each concentration of *Salvia officinalis* extract.

	Concentration (mg/mL)									
Microorganism	**50.00**	**25.00**	**12.50**	**6.25**	**3.13**	**1.56**	**0.78**	**0.39**	**0.19**	**0.09**
Staphylococcus aureus	10[a]	2	0	0	0	0	0	0	0	0
Staphylococcus epidermidis	10	8[a]	1	0	0	0	0	0	0	0
Streptococcus mutans	10[a]	0	0	0	0	0	0	0	0	0
Candida albicans	10	9[a]	1	0	0	0	0	0	0	0
Candida tropicalis	10	10[a]	0	0	0	0	0	0	0	0
Candida glabrata	10	6	3[a]	0	0	0	0	0	0	0

[a]Minimum microbicidal concentration for reference strain (ATCC).

Microbial samples

A reference strain (ATCC [formerly American Type Culture Collection]) and 9 clinical isolates from the oral cavity of tuberculosis patients were used, totaling 60 samples. The standard strains used were *S aureus* (ATCC 6538), *S epidermidis* (ATCC 12228), *S mutans* (ATCC 35688), *C albicans* (ATCC 18804), *C tropicalis* (ATCC 13803), and *C glabrata* (ATCC 90030). The clinical isolates were previously identified and stored in the collection of microorganisms of the Laboratory of Microbiology and Immunology, Institute of Science and Technology, São Paulo State University (UNESP).

Assessment of antimicrobial activity

To determine the minimum inhibitory concentration (MIC) of the extract, the broth microdilution method was used.[12-14] Bacteria were cultured on brain-heart infusion agar (Himedia Laboratories) and yeasts on Sabouraud dextrose agar (Himedia Laboratories) for 24 hours at 37°C. The *S mutans* samples were cultured under microaerophilic conditions (95% oxygen and 5% carbon dioxide [CO_2]). Subsequently, in the case of bacteria, the inocula were standardized in sterile saline solution (0.9% sodium chloride) with turbidity adjusted to 10^6 colony-forming units per milliliter (CFU/mL) in order to reach a concentration of 5×10^5 CFU/mL when added to the microplate wells. The yeast suspension was diluted, first 50× and then 20×, to obtain a concentration ranging from 5×10^2 to 2.5×10^3 CFU/mL.

The microdilutions were carried out in TPP microplates (Sigma-Aldrich). For bacteria, 100 µL of Mueller-Hinton broth (Himedia Laboratories) was added to each well. For yeasts, 100 µL of RPMI 1640 (Himedia Laboratories) buffered with MOPS (Sigma-Aldrich) at pH 7.0 ± 0.1 was added to each well. Then 100 µL of *S officinalis* extract was added to the wells of the first column, from which the sequence of 10 serial dilutions (1:2), ranging from 50.00 to 0.09 mg/mL, was performed in the remaining wells. Finally, 100 µL of one of the standardized bacterial or fungal inocula was added to each well.

One well was designated as positive control (medium only) and another as negative control (inoculum and medium). The plates were incubated for 24 hours at 37°C (under microaerophilic conditions for *S mutans*). Subsequently, the MIC was determined based on the first well with no turbidity, adjacent to the last turbid well.

For the determination of the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the extract, 100 µL of the MIC and 100 µL of the concentrations higher than the MIC were seeded on brain-heart infusion or Sabouraud dextrose agar. After 48 hours' incubation, the MBC and MFC were determined by observing which plates had no colony growth.

Murine macrophages culture

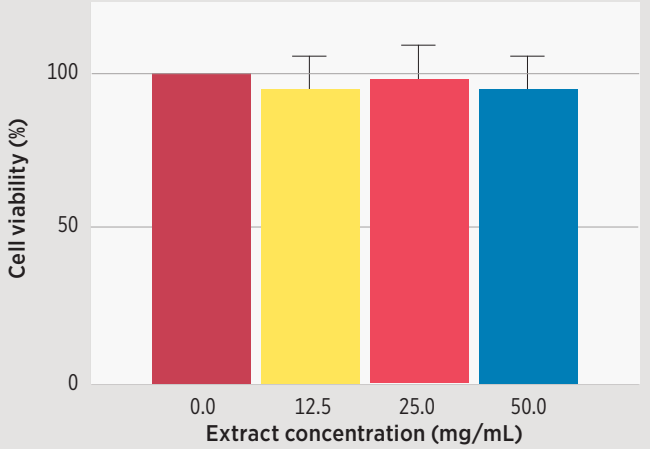
Murine macrophages (RAW 264.7), obtained from Rio de Janeiro Cell Bank, Paul Ehrlich Technical and Scientific Association, were maintained in Dulbecco's modified Eagle medium (DMEM) (LGC Biotecnologia) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Gibco). They were kept under humidified atmospheric conditions at 37°C (5% CO_2) until there was cell monolayer subconfluence. Viable cell counts were then made by the Trypan blue exclusion method (0.5%, Sigma-Aldrich).

Analysis of cytotoxicity

The cytotoxicity of *S officinalis* was analyzed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. In a 96-well microplate (Nunc, Thermo Fisher Scientific), 200 µL of DMEM with 4×10^4 viable cells was added to each well. The microplate was maintained at 37°C and 5% CO_2 for 24 hours. After that, the supernatant was discarded, and *S officinalis* extract, diluted in culture medium to the most effective concentrations (12.5, 25.0, and 50.0 mg/mL), was added. For the control group, only DMEM was used. Each experimental group consisted of 12 wells.

After 24 hours' exposure to *S officinalis*, the supernatant was discarded, and the wells were washed with sterile phosphate-buffered saline (PBS) (Cultilab). Then, MTT solution (Sigma-Aldrich), at a concentration of 0.5 mg/mL PBS, was added to the microplate, which was incubated for 1 hour under light protection. The MTT solution was removed, and 100 µL of dimethyl sulfoxide (Sigma-Aldrich) was added to each well. The microplate was incubated for 10 minutes, protected from light, and then homogenized in a shaker for an additional 10 minutes.

Chart 1. Cell viability after exposure to *Salvia officinalis* extract.

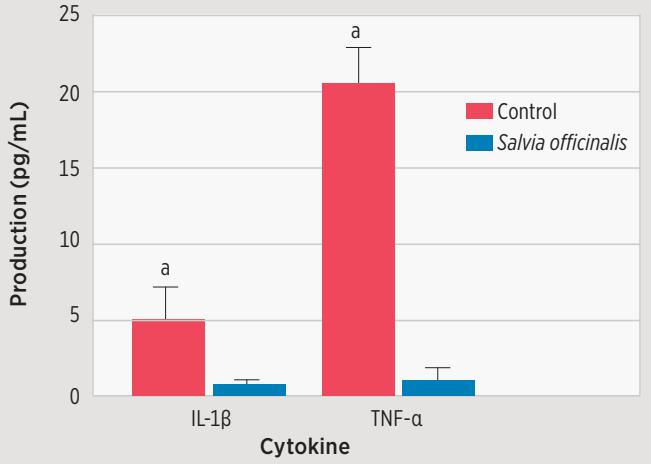


Cell viability presented by RAW 264.7 cell culture either not exposed or exposed to concentrations of *Salvia officinalis* extract for 24 hours.

Error bars represent the standard deviation.

No statistically significant difference was observed among the experimental groups ($P > 0.05$; analysis of variance and Tukey test).

Chart 2. Production of proinflammatory cytokines in cell culture after exposure to *Salvia officinalis* extract.



Abbreviations: IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α.

Production of proinflammatory cytokines by RAW 264.7 cell culture either not exposed (control; 0.0 mg/mL) or exposed to a concentration of 50.0 mg/mL of *Salvia officinalis* extract for 24 hours.

Error bars represent the standard deviation.

[a]A statistically significant difference was observed in comparison with the control group ($P < 0.05$; analysis of variance and Tukey test).

The optical density of each well in the microplate was read by a spectrophotometer (BioTek Instruments) at a 570-nm wavelength. The optical density was converted to the percentage of cellular viability, and the values of treated groups were compared to the values of the control group.[15]

Quantification of proinflammatory cytokines

To verify the stimulus given to macrophages after exposure to the extract, RAW 264.7 cells were grown in wells of a 24-well plate at a concentration of 10^6 viable cells/mL DMEM for 24 hours at 37°C and 5% CO_2. The plate was divided, and the first group (n = 12) was treated for 24 hours with the most effective concentration found in the evaluation of the microorganisms. The second group (n = 12) underwent no treatment except replacement of the culture medium.

After incubation, the supernatant from each sample was collected and kept at –20°C for further quantification of cytokines. Release of the proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) by macrophages was quantified with an enzyme-linked immunosorbent assay. Commercial kits (R&D Systems, No. DY401 for IL-1β and No. DY410 for TNF-α) were used as suggested by the manufacturer. The absorbance of the wells was measured in a microplate spectrophotometer at 450 nm, and the values obtained were converted into picograms per milliliter, according to the standard curve for IL-1β or TNF-α, with the aid of the GraphPad Prism 5.0 program (GraphPad Software).

Statistical analysis

The means and standard deviations obtained from the cytotoxicity assay and quantification of proinflammatory cytokines were subjected to analysis of variance and Tukey testing. Values were considered statistically significant at $P \leq 0.05$.

Results

All 60 microbial strains (100.0%) were eliminated after treatment with *S officinalis* extract at 50.0 mg/mL (Table). Concentrations of 25.0 mg/mL and 12.5 mg/mL were partially effective, causing elimination of some microorganisms (58.3% and 8.3% of strains, respectively). Concentrations below 12.5 mg/mL had no effect on the isolates.

When the cytotoxicity of the plant extract was examined, no statistically significant difference was observed between the control group and treated groups ($P > 0.05$) (Chart 1). The macrophage cultures showed mean (SD) viabilities of 95% (9.0%), 98% (10.5%), and 95% (9.7%) after 24 hours of exposure to 12.5, 25.0, and 50.0 mg/mL of *S officinalis*, respectively.

With regard to the production of proinflammatory cytokines by the culture of RAW 264.7 exposed to 50 mg/mL of *S officinalis* extract, significant inhibition of IL-1β synthesis was observed (Chart 2). The mean (SD) IL-1β production was 5.07 (2.51) pg/mL in the untreated group. In the group that had contact with the extract, the mean (SD) production was 0.74 (0.50) pg/mL, a reduction of approximately 85.4% ($P < 0.05$). In addition, TNF-α production significantly decreased. Mean (SD) production in the control group was 20.61 (3.08) pg/mL, whereas in the treated group it only reached 1.01 (0.92) pg/mL, a reduction of approximately 95.1% ($P < 0.05$).

Discussion

In the present study, *S officinalis* extract showed effective antimicrobial action on 60 samples, including clinical isolates and reference strains of 6 different species capable of causing opportunistic infections (*S aureus, S epidermidis, S mutans,*

C albicans, C tropicalis, and *C glabrata*). The antimicrobial effect and other biologic actions presented by *S officinalis* have been attributed to the presence of numerous bioactive compounds in this plant. Among the most important molecules identified are α-thujone (34.7%), camphor (23.5%), 1,8-cineole (11.5%), and carvacrol (7.4%).[16]

The *S officinalis* extract at a concentration of 50.0 mg/mL provided 100.0% of elimination of the microorganisms evaluated in the present study. In addition, reductions in the CFU/mL of the microorganisms were detected at lower concentrations of the extract; concentrations of 25.0 and 12.5 mg/mL resulted in reductions of 58.3% (35 isolates) and 8.3% (5 isolates), respectively.

The isolates of *S aureus* showed sensitivity to the concentration of 25.0 mg/mL, with elimination of 2 isolates, and 50.0 mg/mL, with elimination of all 10 strains, including the reference strain. In another study, antimicrobial action of this plant extract was also reported against *S aureus*.[3] In that study, its bacteriostatic effect was verified with an MIC of 5.8 μL/mL, which significantly decreased the CFU/mL from 10^6 to 4.5×10^3 after 8 hours' exposure to the extract and to 10^3 after 24 hours. On the other hand, the control group exhibited significant growth of the bacterium, from 10^6 to 4.5×10^8 after 8 hours' incubation and to 10^9 after 24 hours.[3] Like the extracts, *S officinalis* essential oil has showed an inhibitory capacity on *S aureus*, as demonstrated in a disc-diffusion test in agar.[16] Application of the essential oil resulted in the formation of a zone of inhibition that was approximately 14.1 mm in diameter.[16]

In the present study, *S epidermidis* strains were shown to be the most sensitive to *S officinalis* extract, since, at a concentration of 12.5 mg/mL, a clinical isolate was eliminated and, at 25.0 mg/mL, 8 strains were eliminated, including *S epidermidis* ATCC 12228. The highest concentration (50.0 mg/mL) killed all the strains analyzed. The essential oil of *Salvia* spp provided a positive effect when used as an adjuvant to tetracycline in the in vitro control of *S epidermidis*, showing a significant reduction of CFU/mL from 7 to 3 log after 6 hours' exposure, to 2 log after 10 hours, to 1.5 log after 24 hours.[17] Additionally, the union of these products inhibited expression of a tetracycline resistance gene in *S epidermidis* isolates resistant to this antimicrobial agent.[17]

Among the bacteria evaluated in the present study, *S mutans* was the most resistant to *S officinalis* extract, even though the 10 isolates analyzed were eliminated when exposed to the highest concentration (50.0 mg/mL) of the extract.

Salvia officinalis extract, besides eliminating bacteria, also demonstrated a fungicidal effect on *Candida* spp. Most isolates of *C albicans* were sensitive to the 25.0-mg/mL concentration, with 9 isolates eliminated, including the reference strain. At 50.0 mg/mL of *S officinalis*, all the strains were eliminated, whereas only 1 strain was sensitive to the 12.5-mg/mL concentration. The antifungal action of *S officinalis* essential oil was previously demonstrated on a reference strain (*C albicans* 90028) and 2 clinical isolates via the formation of zones of inhibition of 31, 24, and 24 mm, respectively.[4] These zones were significantly greater than those found in the group treated with 0.2% chlorhexidine, which were 23, 21, and 21 mm, respectively. In addition, *S officinalis* essential oil was able to inhibit the adhesion of *C albicans* in polymethyl methacrylate, reaching levels of 89% (reference strain ATCC 90028), 96% (clinical strain 1), and 95% (clinical strain 2) adhesion reduction with application of 1× MIC.[4] In the control group treated with 0.2% chlorhexidine, the corresponding values were 98%, 98%, and 96%, respectively. Thus, in the case of the clinical samples, *S officinalis* demonstrated an antifungal effect similar to that obtained with chorhexidine.[4]

In the present study, all *C tropicalis* strains analyzed were eliminated by the concentrations of 25.0 and 50.0 mg/mL of *S officinalis* extract. *Candida glabrata* seemed to be the most sensitive fungal species to *S officinalis* extract, with 3 samples eliminated, including the reference strain *C glabrata* ATCC 90030, at a concentration of 12.5 mg/mL, and 6 samples eliminated at 25.0 mg/mL. At 50.0 mg/mL, all isolates were eliminated.

During MTT assessment of the cytotoxic activity of the *S officinalis* extract, not even the highest concentration used against the microorganisms resulted in significant reduction of macrophage viability after 24 hours of exposure. The values found were statistically similar to that of the control group, which was 100% ($P > 0.05$). However, in a study by Abu-Darwish et al, which evaluated the cytotoxic effect of *S officinalis* essential oil at a concentration of 1.25 μL/mL, there was a significant decrease in cell viability—to a mean of 10.69% (SD 1.44%)—after 24 hours' exposure.[18] At lower concentrations of 0.64, 0.32, and 0.16 μL/mL, the percentages of cell viability were similar to those of the control group.[18]

In addition, *S officinalis* extract has been reported to be effective in controlling the growth of pancreatic tumor cells (RINm5F).[19] At a concentration of 150 μg/mL, *S officinalis* achieved reductions of approximately 50% in the viability of these cells in comparison with a control group.[19] This plant product also showed dose-dependent 50% inhibitory concentrations (IC50) for other tumor cell lines, including those associated with non-Hodgkin B-cell lymphoma (167 μg/mL), human leukemic monocyte lymphoma (205 μg/mL), human acute myelocytic leukemia (179 μg/mL), human breast carcinoma (142 μg/mL), human prostate cancer (76 μg/mL), and mouse fibrosarcoma (40 μg/mL).[7] In contrast, a normal cell line, nontumoral human umbilical vein endothelial cells, showed an IC50 value of greater than 600 μg/mL.[7]

In the present study, the production of proinflammatory cytokines (IL-1β and TNF-α) was also quantified to determine whether *S officinalis* extract provided any stimulus for their synthesis by macrophages. However, the plant extract resulted in significant inhibition of both cytokines. The IL-1β level in the treated group was reduced by approximately 85.4% compared to the control group. The inhibition of TNF-α was even greater: approximately 95.1% less in the treated group than in the control group. Extracts of *S officinalis* in its aqueous fraction (AF), containing volatile components and water, and dry fraction (DF), containing nonvolatile constituents of the infusion, also modulated the production of other cytokines, such as IL-6 and IL-8, in human gingival cells.[6] Incubation of the fibroblasts and fractional extracts resulted in significant reductions of IL-6 production: approximately 50% (AF) and 75% (DF). Levels of IL-8 decreased by 90% (AF) and 100% (DF). In addition, it was shown that *S officinalis* infusion promoted complete inhibition of these cytokines.[6]

Conclusion

Salvia officinalis has been shown to be a plant with ample antimicrobial capacity, acting significantly on clinical samples and reference strains of microbial species responsible for the diseases of oral health. In the present study, *S officinalis* extract showed antimicrobial activity against all microorganisms evaluated and presented no cytotoxic effect on murine macrophages (RAW 264.7). It also contributed to the control of proinflammatory cytokine production.

The potential of *S officinalis* as a therapeutic agent is remarkable, since it has been shown to control the development of opportunistic pathogens without damaging other important elements such as cells. With the effective action of *S officinalis* proven in vitro, the next step is to design more complex research approaches, such as in vivo and clinical tests, to examine the role of *S officinalis* in formulations of commercial products for medical and dental practice.

Author information

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