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Cytotoxicity evaluation of *Curcuma zedoaria* (Christm.) Roscoe fluid extract used in oral hygiene products

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Abstract

Objective. This *in vitro* study evaluated the cytotoxic effects of the *Curcuma zedoaria* (Christm.) Roscoe (popular name: zedoary) fluid extract, as used in preparations for oral hygiene, mostly for anti-septic purposes. ***Materials and methods.*** The cell viability and cell growth were assessed by Trypan blue dye exclusion assay using the LMF cell line derived from oral mucosa. Cell viability (short-term assay) was measured 0, 6, 12 and 24 h after contact with the fluid extract. Cell growth (long-term assay) was analyzed in 1, 3, 5 and 7 days. The experimental groups were those testing the fluid extract obtained from the zedoary rhizome and the extractor liquid (ethanol 70° GL) in the concentrations of 0.01–0.0001% v/v. Fresh DMEM were used in the control cultures. ***Results.*** Short-term assay—all studied cultures maintained stable cell viability; Long-term assay—there was progressive cell growth in all studied cultures. ***Conclusion.*** According to the results, the zedoary fluid extract presents low cytotoxicity and probably can be used in the oral hygiene products.

Key Words: *cell culture, cytotoxicity, zedoary, vegetal extract*

Introduction

The use of plant extracts with known antimicrobial properties may have great significance in therapeutic treatments and may be added to oral formulations or toothpastes to create or improve the antimicrobial efficacy of these products [1,2]. *Curcuma zedoaria* (Christm.) Roscoe (commonly called zedoary) is a herbal medicine that possesses a very complex chemical composition such as essential oil, oil-resin, terpenic compounds and other constituents, with a wide spectrum of biological properties [3–9]. The main constituents normally found in the rhizome are: curcumenone (a cyclopropanesesquiterpen) and the spirolactones curcumanolide A and curcumanolide B, essential oil (containing 1–1.5% of cineol), curcumin, starch (50%), resin (3,5%), albuminoids, vitamins B1, B2, B6 and the minerals calcium, magnesium, iron, phosphorous, sodium and potassium [10,11]. Because of the presence of cineol, camphene, alpha-pinene, camphor and several aromatic compounds, this plant has been used in folk medicine, for digestive and gall bladder disorders, cough and hepatic disorders [12,13]. Additionally, its extract presents anti-inflammatory and antimicrobial activities, being broadly employed in Asian medicine [3,14]. Many studies report the use of this vegetal product for several oral therapeutic purposes, such antimicrobial agent, halitosis remedy and in periodontal illness in association to other classical approaches [7,15–17].

The chemical composition of the herbal extracts can be variable, considering the influence of cultivation conditions, soil quality, season and procedure of harvesting. Moreover, in fluid extracts, for example, the expected pharmacological actions are attributed to the interaction of the set of compounds of the extract and not due to an isolated molecule [17].

Normally, commercial products used for oral hygiene, maintenance or recovery of mouth integrity present antimicrobial action as well as the possibility of introducing any active drug that will collaborate with some specific action. Zedoary fluid extract can be

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one more option for other commercial products. For this reason, many studies have been made on zedoary considering its therapeutic potential as an antimicrobial and anti-inflammatory phytotherapeutical substance for maintenance of oral health [12–14,18].

As it may be used in products applied in the oral cavity, toxicity assays are necessary. Therefore, an *in vitro* evaluation of a zedoary cytotoxic effect on cells derived from oral mucosa is the main objective of this work.

Materials and methods

The cytotoxic effects of *Curcuma zedoaria* were tested *in vitro* using fibroblasts derived from oral mucosa [19]. The Ethical Committee of the College of Dentistry, University of Sao Paulo approved this study (number 195/02, protocol 224/02).

Curcuma zedoaria extract

Fluid extract was obtained from the zedoary powder rhizome. The plant was identified at the Botanic Institute (SP, Brazil) and stored as reference under the registry number 338498. The characterization of pulverized rhizomes was: 7%, >0.420 mm; 7%, 0.250–0.420 mm; 24%, 0.177–0.250 mm; 37%, 0.149–0.177 mm; and 25%, <0.149 mm. To obtain the fluid extract, the fractionated percolation method was performed, employing ethanol 70° GL as extractor liquid [4], a process in which the material is extracted till exhaustion. The extract was phytochemically characterized as reported in previous published work [20]. To characterize the fluid extract obtained, a thin-layer chromatography (TLC) was performed, using as parallel standards the ethanolic solutions of camphor (10% w/v), curcumin (50% w/v) and essential oil (25% v/v). All compounds were present in the extract.

Cell culture

The LMF cell line derived from oral mucosa were grown in Dubelcco's Modified Eagle medium (DMEM, Sigma Chemical™ Co., St. Louis, MO) supplemented by 10% fetal bovine serum (Cultilab™, Campinas, Sao Paulo, Brazil) and 1% of antibiotic/antimycotic solution (Sigma Chemical™ Co.) in a 5% CO_2 atmosphere with 95% relative humidity. Cell viability was assessed by the Trypan blue dye exclusion assay.

Experimental groups

The experimental groups were as follows: GI–control, cultures grown in fresh DMEM; GII–control, DMEM with the addition of extractor liquid (ethanol at 70° GL) at final concentrations in culture medium of 0.01%, 0.001% and 0.0001% v/v, to verify the influence of the extractor liquid; GIII–DMEM with *C. zedoaria* fluid extract, at final concentrations in culture medium of 0.01%, 0.001% and 0.0001% v/v.

The responses from a short-term assay and a long-term assay were analyzed. These assays measure the cell viability and the retention of the self-renewal capacity of the cells, respectively.

Short-term assay (cell viability)

Cells (2×10^4 cells/plate) were plated on 60 mm Petri dishes. Three days later, when the cell reached confluence, the culture medium was exchanged by the experimental culture media (GI, GII and GIII). The evaluations were performed immediately and 6, 12 and 24 h later, when the cells were counted in a hemocytometer using the Trypan blue exclusion assay.

Long-term assay (cell survival)

Cells (1×10^3 cells/plate) were plated on 60 mm diameter culture dishes. Three hours after plating the cell culture media were exchanged by the experimental media (GI, GII and GIII). Then, 1, 3, 5 and 7 days later three dishes of each group were submitted to the Trypan blue exclusion assay. The data was used for obtaining the cell growth curves.

Trypan blue dye exclusion assay

The Trypan blue dye exclusion assay was carried out as described previously [21]. Briefly, cell counts were determined by counting the viable cells in a hemocytometer. Only the dead cells were stained by the dye and were excluded of the counts. For each time period, three dishes of each group were counted. The number of viable cells harvested from each Petri dish was obtained by the following mathematical equation:

$$\mathrm{UC} \times \mathrm{D} \times 10^4 \ \#\mathrm{SQ} \qquad (1)$$

where UC = unstained cell count (viable cells), D = the dilution of the cell suspension and #SQ = number of squares of the hemocytometer counted.

The viability percentage of the cell populations of each Petri dish was obtained by the following mathematical equation:

$$\mathrm{UC}/\mathrm{TC} \times 100 \qquad (2)$$

where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells).

Statistical analysis

Each data point corresponded to the mean ± SD (standard deviation) of either cell count or percentage of cell viability from three dishes. The data were compared by analysis of variance (ANOVA)

Table I. Short-term assay—percentage of cell viability: 0–24 h.

	Mean cell viability ± SD (%)		
Experimental groups	0.01%	0.001%	0.0001%
I	94.64 ± 3.40	99.44 ± 0.56	97.59 ± 1.81
II	98.89 ± 1.11	98.02 ± 1.98	97.59 ± 1.77
III	97.05 ± 1.77	96.33 ± 2.27	97.62 ± 1.86

complemented by the Tukey's test. The significance level employed was 5% ($p \leq 0.05$).

Results

Tables I and II show the mean percentages of cell viability in the short- and long-term assays, respectively. Overall the cell viabilities ranged from 90–100% in all experimental groups, regardless of the concentration of the fluid extract obtained from the zedoary rhizome or the extractor liquid (ethanol 70° GL) tested. There were no statistical differences in cell viability amongst the experimental groups in both assays.

Figure 1 illustrates the cell growth of LMF cells of all experimental groups. There was significant growth from the beginning to the end of the experimental time in all experimental groups. There were no statistical differences amongst the groups, except between groups I and II and groups I and III ($p = 0.01$).

Discussion

Pharmacological research on vegetal products to be used in medicine and dentistry is a constant due to the significant therapeutic contribution that many plants may offer. Zedoary is a plant originating in Asia, and its rhizome, after being dried and pulverized, is commercially available to be administered orally in the form of powder, capsule or fluid extract [5,14]. With a hypothesis that this plant could be useful as a therapeutic alternative in dentistry, applied to oral tissues, the aim of this study was to test the effect of the fluid extract on the viability and growth of human oral fibroblasts in culture [22].

One of the zedoary applications is in halitosis control, mainly due to its antimicrobial activity and essential oil flavouring. However, a study conducted with a halimeter to detect volatile sulfur compounds

Table II. Long-term assay—percentage of cell viability: 1–7 days.

	Mean cell viability ± SD (%)		
Experimental groups	0.01%	0.001%	0.0001%
I	95.99 ± 3.09	97.66 ± 1.92	98.33 ± 1.66
II	97.88 ± 2.12	97.03 ± 2.52	93.47 ± 4.31
III	94.09 ± 3.80	96.79 ± 2.11	95.18 ± 3.11

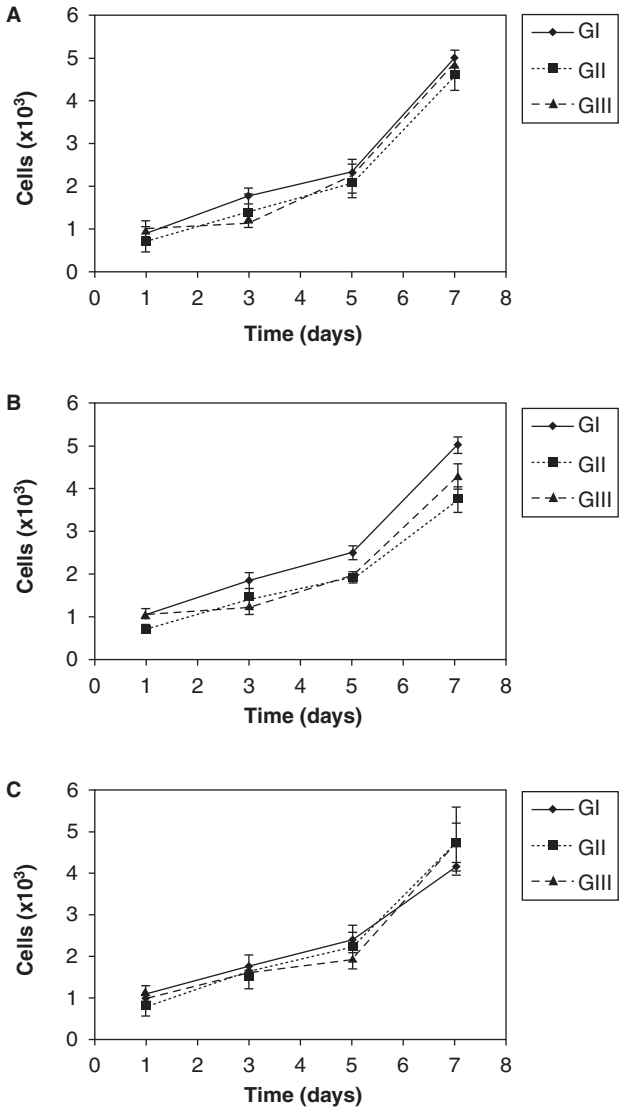

Figure 1. Cell growth curves of the LMF cell line grown in different concentrations of the *C. zedoaria* fluid extract, as follows: 0.01% (A), 0.001% (B), 0.0001% (C). GI, control; GII, extractor liquid; GIII, *C. zedoaria* fluid extract.

(VSC), one of the main gases responsible for halitosis, demonstrated that infusions from *C. zeadoaria* and *Camellia sinesis* were not effective in neutralizing mouth odor [18]. However, it must be stated that the chemical composition of the extracts can differ a lot when different methodologies of extraction or extractor liquids are employed, since the dielectric constant of the solvents leads to different interactions with the phytochemical compounds, affecting the final composition of the extract. In previous work published by Wilson et al. [9], no activity was observed in a *C. zedoaria* extract against *Staphylococcus aureus*. However, a study from our group determinates the activity against *S. aureus* in a concentration of 20 mg/mL [4].

Bacterial plaque in the oral cavity is one precondition of periodontal illness. Although physical removal is indispensable, a supplementary

mouthwash can be important in some cases [12]. Many products are marketed for this purpose and medicinal plants have been additional resources to specific oral disorder treatment, as alternatives to conventional medicines. Thus, the evaluation of zedoary toxicity upon oral tissues is important to allow its potential use in compositions designated for supplementary oral hygiene, since it possesses antimicrobial, anti-inflammatory and analgesic activities [3,8,13,23].

The antimicrobial activities of the zedoary fluid extract (in 1000 mg/mL, 500 mg/mL, 250 mg/mL and 100 mg/mL concentrations) and products commercially available (without dilution) for oral hygiene have been evaluated. There was no difference in antimicrobial action between the tested products. A linear regression method to evaluate the microbial reduction obtained as a function of time resulted into an effectiveness of 99.999% reduction of the targeted microbial population (*Streptococcus mutans*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Candida albicans*) within 60 s [24]. Other studies in the literature are consistent with this plant microbial activity [9,25].

The minimum inhibitory concentrations (MIC) against fungi (*Aspergillus niger*, *Candida albicans* and *Trichophyton mentagrophytes*) were evaluated employing the fluid extract concentrations of 100 mg/mL, 50 mg/mL, 20 mg/mL and 10 mg/mL, obtaining MIC values of 20 mg/mL for the three strains evaluated [4]. As *C. albicans* are involved in several oral ulcerative processes as opportunistic infectious agent, the extract could be useful in mouthrinses or other preparations to treat this condition.

Measurements of cytotoxicity and viability *in vitro* are specifically designed to assay viability or survival as the major parameter of response to a substance under study measured by retention of the self-renewal capacity of cells [26].

In this study, the assays were divided into two parts: short-term and long-term assay. The short-term assay aims to evaluate the immediate cytotoxicity, expressed by cell viability after contact with the extract, while the long-term assay is used to evaluate cell growth and cell viability during the presence of the extract. All concentrations of the extract tested led to responses similar to those of control groups. These findings suggest that the fluid extract of *C. zedoaria* has a discrete toxic effect to the cells, once significant differences were found between concentrations. However, this effect showed no consequences to the self-renewal capacity of the cells, as observed in cell growth assays. The differences observed between groups I and II and between groups I and III on the first day could be attributed to the effect of the extractor liquid (ethanol) in the cells, since group I is the only group with an absence of this compound.

The differences between concentrations showed that the lower concentration (0.0001% v/v) is less cytotoxic than higher concentration (0.01% v/v). This result was expected, but while there were no differences between groups in cell growth assay, it can be concluded that all concentrations can be used safely in oral pharmaceutical formulations concerning immediate exposure to the extract. Moreover, the extract was shown to be safe in long-term exposure to the cells, once the cell growth was similar in all experimental groups.

A previous study from Wilson et al. [9] evaluated the antimicrobial activity of several dry extracts from zedoary against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Proteus mirabilis* and *Klebsiella pneumoniae*. The ethanolic extract showed antimicrobial activity in the range between 0.08–0.15 mg/mL. The higher concentration tested in this work comprises with the concentrations evaluated by Wilson et al. [9] (0.01% = 0.1 mg/mL) and showed no significant cytotoxic activity.

Conclusion

Measurements of cytotoxicity and viability are specifically designed to assay viability or survival as the major parameter of response to a substance under study when possible, thereby establishing a more cost-effective and more reproducible substitute for a test otherwise performed in animals.

Both short-term assay response and long-term assay survival indicate retention of the self-renewal capacity of the cells and, so, low cytotoxicity of *C. zedoaria* fluid extract. Therefore, the results can be a preliminary indication of biocompatibility to use the extract in oral formulations that aim to complement oral hygiene.

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