Biological effects of formulation containing curcuminoids and *Bidens Pilosa* L. in oral carcinoma cell line

**Abstract:** FITOPROT, which contains curcuminoids and *Bidens pilosa* L. extract, is an innovative mucoadhesive formulation indicated for the topical treatment of chemoradiotherapy-induced oral mucositis (OM) in patients with advanced and visible oral squamous cell carcinoma. The formulation is used as a mouthwash directly on tumor tissue of patients with advanced neoplasms, without triggering cancer cell proliferation or tumor invasiveness. Thus, the aim of this study was to evaluate the biological effects of FITOPROT on an oral squamous cell carcinoma cell line (SCC-4). The viability of SCC-4 cells was assessed after exposure to FITOPROT using MTT reduction assay. The effects of the mucoadhesive formulation on cell cycle progression and cell death parameters were evaluated using flow cytometry. In addition, the inflammatory profile of the tumor cells was evaluated using the cytometric bead array (CBA) assay. FITOPROT promoted a concentration-dependent decrease in cell viability and cell cycle arrest at the G2/M phase (p < 0.05). Mitochondrial membrane potential was also altered after exposure to the formulation (p < 0.05), in parallel with a reduction in VEGF and IL-8 production (p = 0.01 and p = 0.05, respectively). In summary, the results indicate that FITOPROT reduces SCC-4 cell viability, promotes cell cycle arrest, modulates mitochondrial membrane potential, and exhibits antiangiogenic and anti-inflammatory properties, thus indicating its potential for topical use in patients with OM and visible tumors in the mouth.

**Keywords:** Carcinoma, Squamous Cell; Bidens; Curcumin; Cell Survival; Cell Cycle Checkpoints.

**Introduction**

Oral squamous cell carcinoma (OSCC) is a malignant neoplasm of epithelial nature that corresponds to 90% of all cancers found in the oral cavity.¹ The majority of patients present with advanced clinical stage (III and IV) and are treated by neoadjuvant radiotherapy (RT) alone or in combination with chemotherapy (QT)², which is a therapeutic strategy associated with excellent overall survival rates.³,⁴ However, these therapeutic protocols are generally associated with the occurrence of serious side effects in the oral mucosa that are difficult to treat, such as chemoradiotherapy-induced oral mucositis (OM).³
Chemotherapy- or radiotherapy-induced OM is a debilitating inflammatory condition whose ulcerated or severe forms may result in the interruption of cancer treatment and cause negative effects on the survival and quality of life of patients with OSCC.5,6 In this context, our research group developed an innovative mucoadhesive formulation (mouthwash) based on curcuminoids and a glycerinated extract of *Bidens pilosa* L. for the topical treatment of OM, which has shown promising results *in vivo* (experimental animal models),7 *in vitro*,8 and in clinical studies (Phase I).9 A pre-clinical study (*in vivo*) was undertaken using an experimental animal model of intestinal mucositis to evaluate the safety and efficacy of this phytomedication. In that study, it was shown that FITOPROT exerted anti-ulcerative, anti-inflammatory, and antioxidant activities.7 The same research group later showed that the mucoadhesive formulation presented cytoprotective capacity in normal keratinocytes cells (*in vitro*)8 induced with 5-fluorouracil (5-FU) damage and it was clinically safe in healthy volunteers (Phase I randomized clinical trial) thus showing an important potential for the treatment of OM in patients with OSCC in advanced stage and with visible lesions in the mouth.9 Recently, a phase II clinical trial is being developed (finalizing the patient recruitment stage) to assess the safety and efficacy of FITOPROT used by cancer patients (Figure 1).

In addition to these biological properties that are important for the treatment of OM, other studies have demonstrated the antineoplastic potential of curcuminoids and *Bidens pilosa* L., and its action on the signaling pathways of cell proliferation of epithelial and hematopoietic tumors.10,11,12,13 Furthermore, *in vitro* studies have indicated that these extracts have an antiproliferative capacity through the blockade of cell cycle phases and the induction of apoptosis.10,11,12,13 In addition, curcumin also demonstrated the ability to reduce tumor size, especially those associated with other forms of cancer treatment, in experimental animal models.13

Considering previous evaluations of the safety and efficacy of FITOPROT, patients with OSCC diagnosed in advanced stages, without the...
possibility of immediate surgical resection and who are submitted to neoadjuvant RT/QRT, can use the mouthwash safely over the tumor. Thus, the in vitro evaluation of the biological effects of this phytomedication on neoplastic cells, complementing the preclinical evaluations, was necessary.²⁸⁻³⁰ The objective of this in vitro study was to evaluate the biological effects of FITOPROT, which contains curcuminoids in association with *Bidens Pilosa* L. extract, using a cell line of oral squamous cell carcinoma (SCC-4). It is believed that FITOPROT does not promote OSCC neoplastic cell proliferation and it can be used to treat OM in cancer patients.

**Methodology**

**Cell culture**

Oral squamous carcinoma cells (SCC-4), acquired from the American Type Culture Collection (Rockville, MD, USA), were cultivated in a mixture of Dulbecco’s modified Eagle's medium (DMEM):Ham’s F12 medium (1:1) (Sigma-Aldrich, St. Louis, USA), supplemented with 10% fetal bovine serum (Invitrogen/Life Technologies, Carlsbad, USA), l-glutamine (2 mM), sodium pyruvate (0.5 mM), hydrocortisone (500 ng/mL) (Sigma-Aldrich, St. Louis, USA), penicillin (100 IU/mL), and streptomycin (100 µg/mL) (Invitrogen/Life Technologies, Carlsbad, USA). During cultivation, the cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C. Cell viability > 90% was considered as adequate to carry out the proposed assays.

**Cytotoxicity assay and exposure of SCC-4 cells to FITOPROT**

Cytotoxicity assay was performed using the tetrazolium salt reduction assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)].¹⁴ The SCC-4 cells were cultivated in 96-well plates overnight (1.5 × 10⁴ cells/well). Then, they were treated with decreasing concentrations of FITOPROT (2%–0.01%), which were experimentally determined by Santos Filho et al.,⁶ for 24 h and incubated with culture medium containing MTT (0.5 mg/mL). After 3 h of incubation, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) (100 µL/well), and the optical density was measured using a microplate reader (Thermo Fisher Scientific, Waltham, USA) at 560nm. This assay was performed in sextuplicate. The relative cell viability was determined in comparison to the negative control (unexposed cells) for establishment of non-cytotoxic concentrations that were employed in further mechanistic analyses.

**Cell cycle progression analysis**

To evaluate the influence of the mucoadhesive formulation on cell cycle phases, the SCC-4 cells were seeded in 6-well plates (3.0 × 10⁵ cells/well) and treated with FITOPROT for 24 h using the inhibitory concentration of 20% of cell growth (IC₂₀), which was previously determined in the cytotoxicity assay. After this period, the cells were harvested by trypsinization and fixed with 1.0 mL of 70% ice-cold ethanol. After fixation, the cells were incubated for 1 h with a solution of RNAs (200 µg/mL) and propidium iodide (50 µg/mL) (both from Sigma-Aldrich, St. Louis, USA) and then washed with phosphate-buffered saline (PBS) for further assessment in a flow cytometer (BD FACSCANTO II, BD Bioscience, USA). This assay was performed in sextuplicate.

**Mitochondrial membrane potential (ΔΨₘ) assessment**

Rhodamine 123 staining was used for the evaluation of the mitochondrial membrane potential (ΔΨₘ). The SCC-4 cells (3.0 × 10⁵ cells/well) were seeded in 6-well culture plates and pretreated with IC₂₀ of FITOPROT for 24 h. After exposure, the cells were washed using PBS and then incubated with 200 µL of rhodamine 123 (1 µg/mL) (Sigma-Aldrich, St. Louis, USA) at 37°C for 1 h, followed by another wash using PBS. The cells were then suspended in 200 µL of PBS and again incubated at 37°C for 20 min before being analyzed in a flow cytometer (BD FACSCANTO II, BD Bioscience, USA). This assay was performed in sextuplicate.

**Evaluation of caspase activity in SCC-4 cells after exposure to FITOPROT**

The expression of caspases 3/7, 8, and 9 was analyzed in sextuplicate using the CaspaTag™ in situ...
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The SCC-4 cells were pretreated with FITOPROT (IC$_{20}$) for 24 h and then washed with 2.0 mL of PBS, centrifuged at 1500 rpm for 5 min, and incubated at 37°C with 10 μL of the corresponding reagent for each caspase for 1 h. After incubation, the cells were washed twice with 2.0 mL of PBS, resuspended in 200 μL of PBS, and then analyzed by flow cytometry.

**Expression analysis of proteins involved in proliferative (Ki-67 and cyclin D1), cell death pathways (cytochrome c), and angiogenesis (VEGF)**

The SCC-4 cells (2.5 × 10$^5$ cells/mL) were seeded in 75-cm$^2$ flasks and incubated overnight for adhesion. Cells were then treated with IC$_{20}$ of FITOPROT for 24 h. Then, the untreated and treated cells were washed twice with PBS–BSA (0.1%, w/v) and centrifuged at 1,500 rpm for 5 min at 25°C. Next, the cells were suspended with BD Cytofix/Cytoperm™ solution and incubated at 4°C for 20 min. This was followed by washing twice with PBS-Tween 20 (0.05%), after which the cells were centrifuged (1,500 rpm, 25°C, for 5 min) and incubated with specific monoclonal antibodies [FITC-conjugated anti-Ki-67 (MIB-1, DAKO), FITC-conjugated anti-cyclin D1 (G124-326, BD Biosciences), FITC-conjugated anti-cytochrome c (sc-13561), or FITC-conjugated anti-VEGF (Alexa Fluor 488 Conjugated - IC2931G, RD Systems)] in the dark for 30 min at room temperature. After incubation, the cells were again washed twice with PBS-Tween 20, centrifuged at 1,500 rpm for 5 min at 25°C, and suspended in 200 μL of PBS for flow cytometry analysis. This assay was performed in triplicate.

**Measurement of inflammatory cytokines**

The SCC-4 cells (7.5 × 10$^4$ cells/well) were seeded in 24-well culture plates and then treated with IC$_{20}$ of FITOPROT for 24 h. After treatment, the cells were washed twice with PBS (1 mL/well) and cell lysates were obtained treating with 250 μL/well of PBS solution containing 0.5% (v/v) Triton X-100 and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA). The cell lysates were stored at -80°C until analysis. The concentrations of the cytokines interleukin (IL)-6, IL-1β, IL-8, IL-12p70, IL-10, and TNF-α were evaluated by cytometric bead array (CBA) assay, using the human inflammatory cytokine kit (BD Bioscience, cat. no. 551811, San Diego, USA). Fluorescence was measured using flow cytometry [FACSCantoII flow cytometer with a 407-nm laser capable of detecting and distinguishing fluorescence emission between 576 and 670 nm (BD Bioscience)], where 2000 events were obtained using BD FACSDiva™ software and analyzed using the FCAP Array™ software. The Bradford assay using bovine serum albumin (Fermentas Life Sciences, Vilnius, Lithuania) was carried out to measure the total protein concentration (PerlongDNM-9602 Microplate Reader, Buena Park, USA) using a standard curve. The cytokine concentration values, corrected by the total protein value in the cell lysates, were expressed in picogram per milligram of protein.

**Statistical analysis**

Data obtained from the cytotoxicity tests, the cell cycle progression assays, and the expression analysis of Ki-67, cyclin D1, cytochrome c, caspases, and VEGF and inflammatory cytokines (IL-6, IL-1β, IL-8, IL-12p70, IL-10, and TNF-α) were expressed as median and minimum and maximum values. The Shapiro-Wilk test was used to evaluate data normality. Percentage viability relative to that of control (cells not exposed to compounds) and the IC$_{50}$ and IC$_{20}$ values were determined using nonlinear regression analysis. ANOVA and Student’s $t$-tests were used to evaluate cell viability, cell cycle phases, and expression of Ki-67, cyclin D1, cytochrome c caspase activity and VEGF by flow cytometry in the groups. The Mann-Whitney test was used to compare the treated and control groups in terms of the concentration of inflammatory cytokines.

A statistical significance level of 5% (p≤0.05) was used for all statistical tests, and data were analyzed using the Statistical Package for Social Sciences program (SPSS for Windows, version 23.0, SPSS Inc., Chicago, USA) and the GraphPad Prism 5.01 software (GraphPad Inc., San Diego, USA).
Results

Viability of SCC-4 cells exposed to FITOPROT
The MTT assay results indicated that treatment with FITOPROT significantly reduced the viability of treated SCC-4 cells (IC$_{50} = 0.1\%$) compared to that of control cells (p = 0.01) (Figure 2).

Effects of FITOPROT on cell cycle progression and proliferation
Cell cycle progression assay was carried out using flow cytometry to identify the cell cycle phases (sub-G1, G0/G1, S, and G2/M) in each individual cell. The results revealed a smaller number of FITOPROT-treated SCC-4 cells in the G0/G1 phase (preparation for cell division) compared to that of control (p = 0.006). The number of cells in the synthesis phase (S phase) was similar in both groups (p > 0.05). In addition to a reduced number of cells in the G0/G1 phase and non-return to the interphase, there was a significant increase in the treated SCC-4 cells that remained in the G2/M phase compared to that in the control group (p = 0.01) (Figure 3). Furthermore, the expression of Ki-67 and cyclin D1 cell proliferation proteins by FITOPROT-treated SCC-4 cells was similar to that in the control group (p > 0.05), indicating an absence of proliferation stimuli by the mucoadhesive formulation.

Effects of FITOPROT on mitochondrial membrane potential (ΔΨm), caspase activity, and cytochrome c release
Accumulation of rhodamine 123 in the mitochondrial matrix was significantly higher in the SCC-4 group treated with FITOPROT (IC$_{20}$) than in the control group (p = 0.006) (Figure 4), demonstrating a possible mitochondrial membrane hyperpolarization. On the other hand, the expression of cytochrome c and caspases 3/7, 8, and 9 by SCC-4 cells treated with

Figure 2. Reduction in viability (%) of FITOPROT-treated SCC-4 cells (IC$_{20} = 0.0625\%$ and IC$_{50} = 0.1\%$).

Figure 3. Distribution of SCC-4 cell density (%) in each phase of the interphase (sub-G1, G0/G1, S, and G2/M). *Statistically significant difference between groups (ANOVA test).

Figure 4. Mean Rhodamine 123 fluorescence in FITOPROT-treated SCC-4 cells (IC$_{20}$) and control. *Statistically significant difference between groups (Student’s t-test).
FITOPROT (IC_{50}) was similar to that in the control group (p > 0.05).

**VEGF, TNF-α, IL-6, IL-1β, IL-8, IL-12p70, and IL-10 production**

Regarding the antiangiogenic property of FITOPROT, there was a significant decrease in VEGF production in the group of SCC-4 cells treated with the formulation (IC_{50}) when compared to that in the untreated SCC-4 cells (p = 0.01) (Figure 5).

Evaluation of the cell lysates revealed a lower IL-8 cytokine production by the FITOPROT-treated SCC-4 cells than that in the control group (p = 0.05). In addition, FITOPROT did not induce increased production of the cytokines TNF-α, IL-6, IL-1β, IL-12p70, and IL-10 by the SCC-4 cells, with the concentrations being similar to those in the control group (p > 0.05) (Table).  

![Figure 5. Mean VEGF production by FITOPROT-treated SCC-4 cells (IC_{50}) and control group. *Statistically significant difference between groups (Student’s t-test).](image)

**Table.** Effects of FITOPROT (0.0625%) on the levels (pg/mg) of inflammatory cytokines produced by SCC4 cells.

| Cytokine level (pg/mg) | Control Median (Min-Max) | FITOPROT-treated SCC4 cells Median (Min-Max) | p-value |
|------------------------|--------------------------|--------------------------------------------|---------|
| TNF-α                  | 11.94 (9.44–13.31)       | 19.94 (11.88–24.26)                       | 0.27    |
| IL-6                   | 218.56 (28.43–243.87)    | 32.26 (10.61–695.02)                      | 0.12    |
| IL-1β                  | 49.82 (17.43–75.49)      | 15.34 (9.64–19.83)                        | 0.12    |
| IL-8                   | 874.53 (725.04–1835.67)  | 11.95 (6.49–708.77)                       | 0.05*   |
| IL12p70                | 8.36 (8.06–12.63)        | 18.17 (11.88–20.53)                       | 0.82    |
| IL-10                  | 2.24 (0–3.31)            | 10.59 (0–11.88)                           | 0.13    |

*Statistically significant difference between groups (Mann-Whitney test).

**Discussion**

Our *in vitro* study findings showed that FITOPROT does not induce tumor proliferation, as observed by the compromising cell viability, and it reduced the number of cells preparing to divide (G0/G1 phase) and prevented their return to the interphase by maintaining them in the G2/M phase checkpoint. Consistent with our findings, Ip et al.\(^1^5\) observed that curcumin inhibits the growth of the SCC-4 cell line and induces cell death in a dose-dependent manner, promoting the arrest of G2/M phase. In addition, Mazzarino et al.\(^1^6\) demonstrated that *in vitro* exposure to curcumin loaded with mucoadhesive nanoparticles for 24 h caused a significant reduction in the viability of SCC-9 human oral cancer cells. It has also been reported that curcumin acts synergistically with epigallocatechin-3-gallate, a blocker of the G2 phase, and thereby inhibits the growth of tumor cells.\(^1^7\)

A recently published systematic review highlighted that curcumin exhibited antiproliferative capacity in head and neck tumor cells in all the *in vitro* studies evaluated. Furthermore, an increase in pro-apoptotic and a reduction in anti-apoptotic proteins were observed in some studies.\(^1^3\) Curcumin also exhibited the ability to reduce tumor size in experimental animal models, especially when associated with other forms of cancer treatment. This reduction in tumor size was accompanied by an increase in pro-apoptotic proteins (caspases 3, 8, and 9, cytochrome c, and Bax) and a reduction in anti-apoptotic proteins (BCL-2, PCNA, XIAP, and C-FLIP) and cell cycle protein (cyclin D1).\(^1^3\)
FITOPROT), Wu et al. demonstrated that this extract reduces cell viability and exhibits antiproliferative and proapoptotic activities in colorectal cancer cells.

Regarding cell death induction mechanisms, FITOPROT promoted a hyperpolarization of the internal mitochondrial membrane, which was demonstrated by rhodamine 123 staining by the treated tumor cells. We hypothesized that this biological event induced by curcuminoids and *Bidens pilosa* L. may precede the release of apoptosis signaling proteins and DNA fragmentation. The interaction of chemical substances with the mitochondria can impede the exchange of ATP between the internal microenvironment of this organelle and the cellular cytosol, thus resulting in hyperpolarization. This process precedes mitochondrial rupture and the release of apoptosis-promoting factors responsible for DNA fragmentation and phosphatidylserine externalization. Although alteration in some apoptotic signals in FITOPROT-treated SCC-4 cells was not observed (caspase activation and cytochrome c release), probably due to treatment time, mitochondrial alterations showed impairment in cellular homeostasis, which can trigger different cell death pathways. Similar to our findings, Alcazar et al. demonstrated that treating T lymphocytes with the chemical camptothecin for 3 h promoted the hyperpolarization of the mitochondrial membrane followed by the release of cytochrome c in the cellular cytosol. Recently, Ma et al. showed that treating T-cell lymphomas with celecoxib promoted an increased expression of Bax and rhodamine 123 accumulation by the tumor cells, as well as an induction of tumor apoptosis.

In addition to these biological effects, FITOPROT also demonstrated the ability to reduce VEGF expression by SCC-4 cells, showing an antiangiogenic potential that could compromise OSCC proliferation and invasion. Tissue expression of VEGF has been recently indicated as a marker of metastasis and tumor recurrence in patients with OSCC, and, therefore, regulating the expression of this inflammatory mediator may be an important option for the control of tumor progression. Furthermore, Pan et al. demonstrated the ability of curcumin to reduce VEGF expression in vitro in a hepatocellular carcinoma cell line associated with tumor size reduction in vivo of the same tumor. In addition, Lou et al. observed that curcumin was able to suppress VEGF in primary endothelial cells of a human infant hemangioma cell line. Consistent with these published data, Huang et al. indicated that curcumin can be used as a novel therapeutic target for angiogenesis by inhibiting NF-κB/VEGF signaling in mesenchymal stem cells derived from human gastric carcinoma. Regarding the other inflammatory mediators, we observed maintenance of TNF-α, IL-1β, IL-6, IL-12p70, and IL-10 levels and a reduction in IL-8 concentration in the FITOPROT-treated SCC-4 cells. Taken together, these results indicate that the mucoadhesive formulation contributes to the control of the inflammatory response in the OSCC tumor microenvironment. Jin et al. demonstrated that the mRNA levels of IL-8, JAK, and STAT3 (jointly responsible for angiogenesis) in the colorectal carcinoma cell lineage were reduced after treatment with curcumin combined with epigallocatechin-3-gallate. Contributing to the above-described anti-inflammatory properties of curcumin, Fei et al. demonstrated that treatment with *Bidens pilosa* L. promotes a reduction in the levels of IL-8 and TNF-α in vascular endothelial cell supernatants obtained from patients with Henoch-Schonlein purpura.

In conjunction, these results demonstrated that FITOPROT does not promote cancer cell proliferation but may impair some tumor-derived cell functions instead. However, evaluation of a larger number of oral carcinoma cell lines may increase the robustness of such outcomes and allow the assessment of such effects in other cancer phenotypes. As observed by our group in a previous study, the formulation does not promote such alterations in normal keratinocytes and, additionally, acts protecting these cells from chemically-induced damage, restoring cell redox basal status, mitochondrial function, and proliferative activity.

**Conclusions**

This in vitro study demonstrated that FITOPROT has antiproliferative, antiangiogenic, and anti-inflammatory properties upon SCC-4 cells. However,
further investigation of the specific mechanisms of action that favor these antitumor properties is necessary. Furthermore, the findings demonstrated that FITOPROT did not exert negative effects in evaluated cancer cells, such as increase in cell proliferation and tumor invasiveness. Therefore, we suggest that this formulation can be potentially used in the form of a mouthwash by patients with chemoradiotherapy-induced OM and visible tumors in the mouth.

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