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# *Ganoderma lucidum* polysaccharides associated with 5-Fluorouracil impair OSCC tumorigenesis *in vitro*

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ABSTRACT

*Backgrounds: Ganoderma lucidum* polysaccharides have been shown several anti-cancer, anti-inflammatory, and immunomodulatory properties among studies with different tumor models and its use with advanced and conventional combination therapies is a world trend. The administration of 5-Fluorouracil is already used as chemotherapy for many tumors and works on tumor remission; however, its adverse effects are still severe, impoverishing treatment and quality of life for patients. Cancer stem cells represent a subpopulation of cells with defense mechanisms against chemotherapy agents and are the main cause of relapses and metastases in cancer treatments. Also, the Epithelial-Mesenchymal Transition program increases properties related to tumor malignancy and mortality. In this scenario, the aim of the study is to evaluate the effects of *Ganoderma lucidum* polysaccharides in combination with 5-Fluorouracil on the subpopulation of cancer stem cells present in the human oral squamous carcinoma cell line SCC-9.
*Methods:* SCC-9 cells were treated *in vitro* for 72 h with different 5-Fluorouracil low doses, associated or not with *Ganoderma lucidum* polysaccharides. Cells maintained with culture media or cisplatin were used as control. All the cells were evaluated for cytotoxicity, cancer stem cells, and Epithelial-Mesenchymal Transition properties.
*Results:* The associated treatment avoided proliferation, delayed migration, slightly modified morphology of cells, increased apoptosis, decreased colony and blocked spheres formation, and downregulated cancer stem cells, Epithelial-Mesenchymal Transition, and ABC drug transporters expression. In addition, *Ganoderma lucidum* polysaccharides + 5-Fluorouracil changed the treated cells into a non-cancer stem cell phenotype, a characteristically not resistant and less proliferative population. The 5-Fluorouracil treatment alone showed remarkable modification in cellular morphology, apoptosis, and absence of holoclones; however, it upregulated the molecular expression of cancer stem cells' hallmarks.
*Conclusions:* These findings demonstrate that combining *Ganoderma lucidum* polysaccharides with a low dose of 5-Fluorouracil is effective against oral squamous cell carcinoma *in vitro* by enhancing the cells' sensitivity to drugs and reducing the characteristics associated with cancer stem cells (CSCs). This suggests the possibility of reducing conventional chemotherapy doses and improving oral squamous cell carcinoma treatment. It also highlights the potential for this combination to be used as an adjunct in Complementary Alternative Medicine (CAM).

## Introduction

*Ganoderma lucidum* (G. lucidum Leyss. ex Fr.) Karst is an edible mushroom used for over 2000 years in Asian countries, known for its several properties on human health improvement. Also known as the "plant of immortality", Reish in Japan and Ling-zhi in China, are traditionally used in Eastern countries' medicine to improve the vitality of the human body, recognized for promoting health and longevity, both fundamental aspects of health practices in Asia. It has been widely used by TCM in combination with conventional treatments against several

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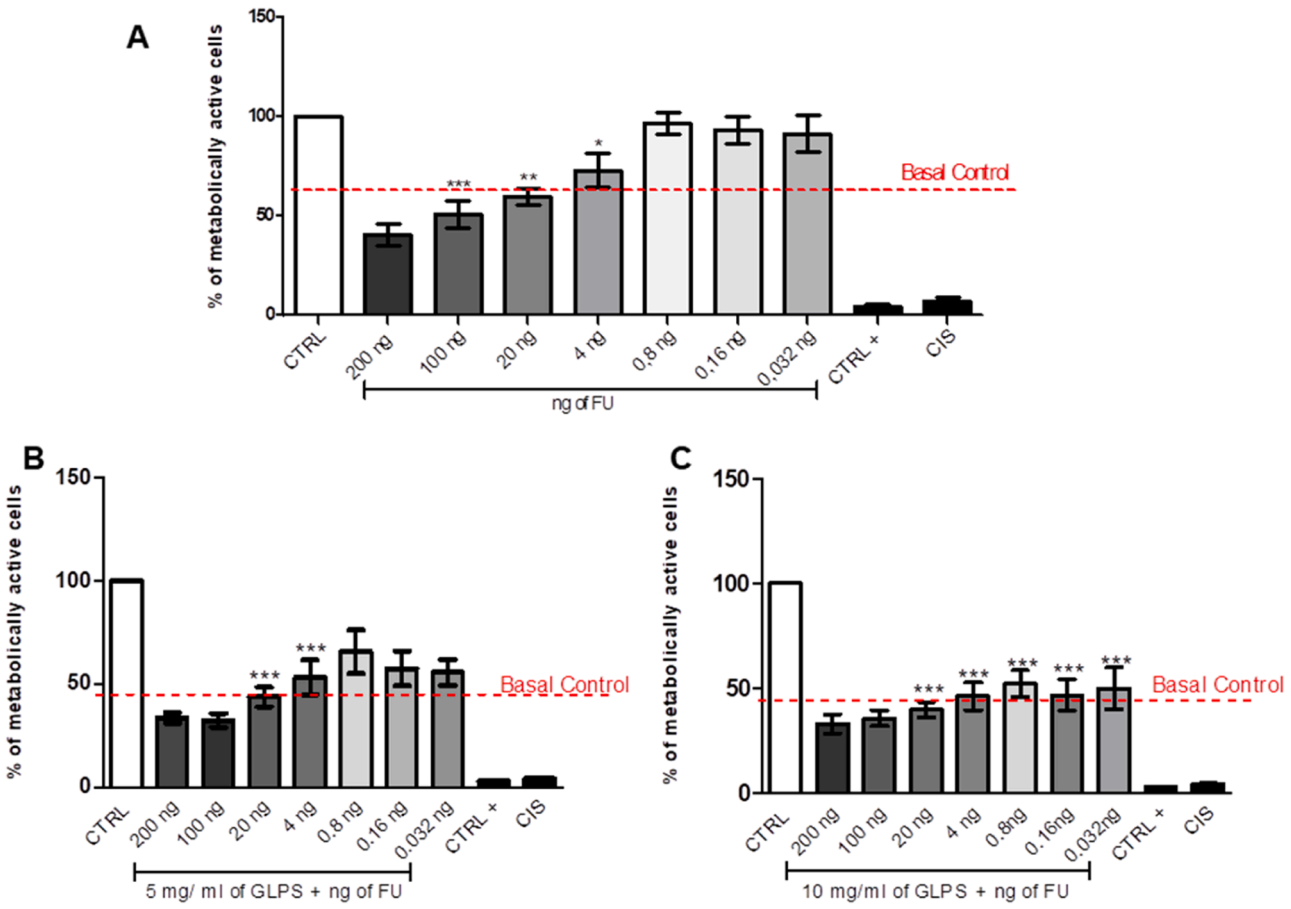


**Fig. 1.** Cytotoxicity in SCC-9 cells exposed for 72 h at different doses assessed by MTT assay. **(A)** 5-FU, **(B)** association of 5-FU doses with 5 mg/ml of GLPS and **(C)** association of 5-FU doses with 10 mg/ml of GLPS. Values are expressed as mean ± standard deviation (SD) of three independent experiments performed in triplicates. BASAL CTRL: Cells cultured for 24 h and non-treated; CTRL: Cells cultured for 96 h and non-treated; CTRL +: Positive control of death (100 % death); CIS: Cells treated with 50 μg/ml of Cisplatin. *$p < 0.05$ *versus* CTRL; ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). $n = 9$.

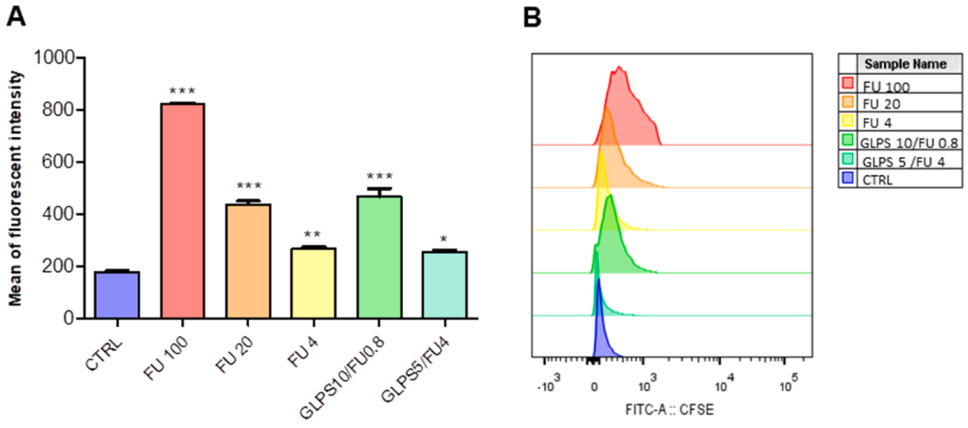


**Fig. 2.** Proliferation in SCC-9 cells exposed for 72 h at different doses assessed by CFSE assay. **(A)** Graphical representation of Mean of Fluorescent Intensity (MFI) and **(B)** Histogram showing the intensity of the CFSE stained cells treated or not (CTRL) for 72 h. **FU100, FU20** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). Values are expressed as mean ± standard deviation (SD) of one experiment performed in triplicates. *$p < 0.05$ *versus* CTRL; ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). $n = 3$.

types of cancer, improving survival and reducing the adverse effects caused by chemotherapy, directly impacting positively the quality of life of patients [1]. Among *Ganoderma lucidum* biological benefits, many studies are showing potent anti-tumor activities of its active compounds, such as polysaccharides [2–5]. *Ganoderma lucidum* polysaccharides (GLPS) have antioxidant activities and can enhance immunity, cytotoxic effects, and tumor cell apoptosis combating cancer growth when applied with advanced and conventional combination therapies [6]. When combined with chemo or radiation therapy, GLPS can improve the healthy state of cancer patients and strengthen the anticancer effect of treatment, which makes GLPS an excellent adjuvant therapeutic drug for different types of cancer [7]. In a previous *in vitro* study with Oral

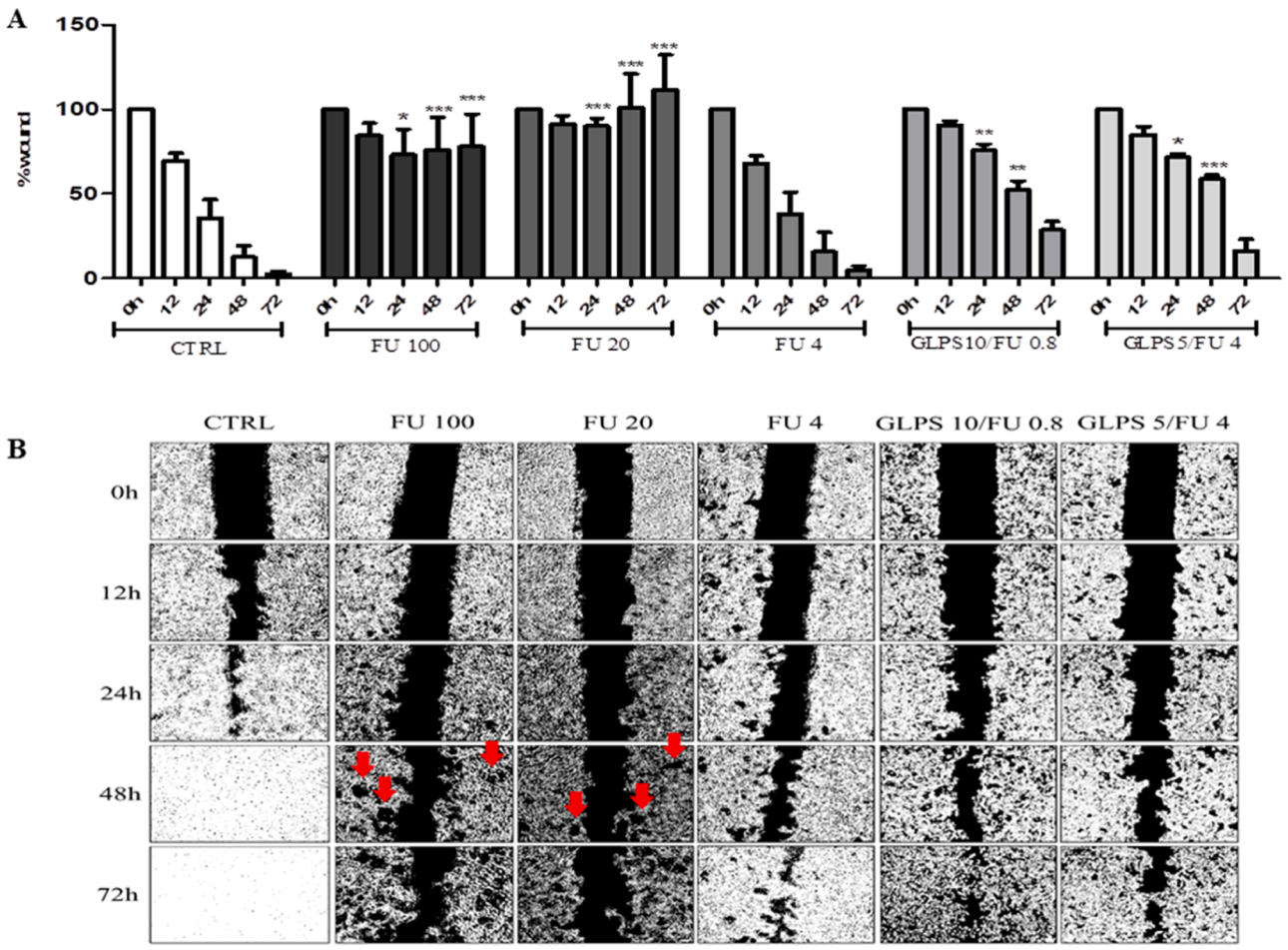


**Fig. 3.** **(A)** Graphical representation and (**B)** Microscopic images of migration assay with SCC9- treated and not treated (CTRL) cells, using wound closure analysis at 12, 24, and 72 h after scratch.,. **CTRL:** SCC-9 cells maintained with 0.5 % fetal bovine serum-supplemented culture medium. **FU100, FU20,** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). The assay was performed in triplicate in three independent experiments with distinct cell passages, and three microscopic fields were analyzed for each wound. *$p < 0.05$ *versus* CTRL; ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). $n = 9$.

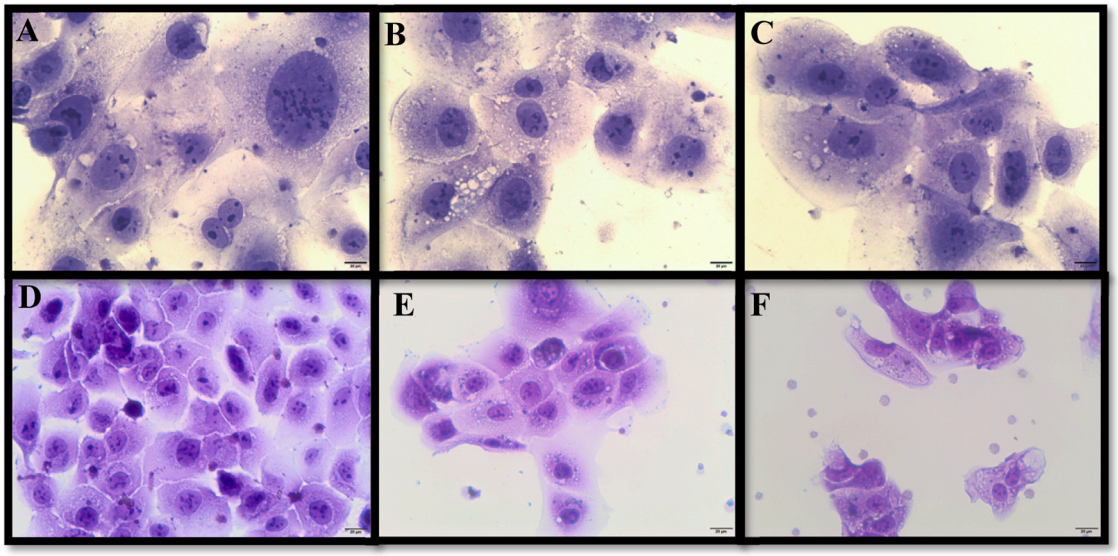


**Fig. 4.** Representative images of the morphological alterations between cells treated with (**A**) FU100, (**B**) FU20, (**C**) FU4, (**D**) CTRL, (**E**) GLPS10/FU0.8 and (**F**) GLPS5/FU4. 40x magnification in the inverted light microscope. The scale bar at the images is equivalent to 20 μm.

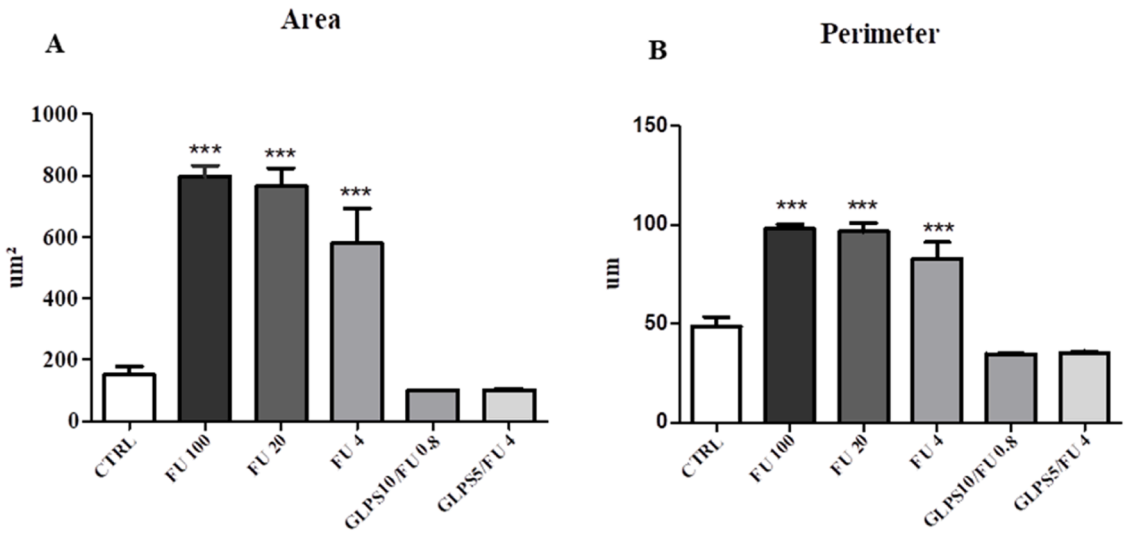


**Fig. 5.** **(A)** Values of the area (μm$^2$) and (**B**) perimeter (μm), respectively, of the nuclei of SCC-9 cells (treated or not). **FU100, FU20** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). Values are expressed as the mean and standard deviation of three independent experiments performed in duplicate. *** *versus* CTRL ($p < 0.001$). $n = 6$.

Squamous Cells Carcinoma (OSCC), we showed for the first time that GLPS treatment changes tumor cell morphology and granularity, delays migration, decreases colony, impairs sphere formation, and downregulate Cancer Stem Cells (CSCs), Epithelial-Mesenchymal Transition (EMT), and ABC markers, strongly supporting the evidence that GLPS impaired SCC-9 cells tumorigenesis/tumor properties [8].

One of the most important approved drugs to treat head and neck squamous cell carcinoma (HNSCC) is 5-Fluorouracil (5-FU) [9,10]. However, the conventional administration of 5-FU at the Maximum Tolerated Dose (MTD) causes adverse effects including anemia, neutropenia, thrombocytopenia, cutaneous reactions, immunodepression-related diseases, and toxicity [10,11], thereby decreasing the patient's quality of life and reducing treatment effectiveness. Therefore, the association of 5-FU with adjuvant therapies can be a good option once the benefits of this association can effectively act against the survival and performance of CSCs, helping delay tumor progression, improving prognosis and life quality in cancer patients, as well as decreasing costs, doses and bringing data to the popular use of *Ganoderma lucidum* use during cancer treatment [12,6].

Among the malignant tumors that affect the general population, HNSCC is the sixth most frequent type of cancer in the world and reaches a mortality rate of 50 %, accounting for approximately 800,000 new cases and 400,000 deaths every year [13–15]. OSCC represents 95 % of HNSCC [16,17] and it has been a concern for public health, since even with the decrease in consumption of the potential initiators/promoters agents such as tobacco and alcohol, its incidence has been increasing, especially in young adults and women [18–20]. OSCC treatment mainly relies on surgical resection alone or in combination with chemo/-radiotherapy, but the mortality rates remain high mainly due to local recurrence and cervical lymph node metastasis [16,21]. Thus, despite advances in current therapy approaches, OSCC treatment still needs more efficient targeting of resistant tumor cells that can survive and later drive and sustain cancer regrowth and invasiveness. In the current studies on fighting cancer, much attention has been paid to a heterogeneous population of tumor cells, which exhibit high proliferation, high invasiveness, and self-renewal properties, denominated CSCs [22–25]. CSCs have been believed to underlie cancer treatment failure, recurrence, and metastasis [26–29]. In addition, CSCs are also the main cause of the inefficiency of conventional treatments to eradicate cancer [30], as they represent a subpopulation of cells with defense mechanisms against radioactivity and chemotherapy [31–35]. As a result, CSCs present increased resistance to DNA damage, increased expression of anti-apoptotic proteins [36], and elevated expression levels of ABC drug transporters that pump chemotherapeutics drugs out of the cell [33,37,38], becoming a key target to effective cancer treatment [39]. More than that, CSCs can activate the EMT program, increasing properties related to tumor malignancy and morbidity/mortality [40–42]. A hallmark of the EMT process is the downregulation of adhesion molecule (ESA), E-cadherin (responsible for keeping adhesion between cells) and/or upregulation of mesenchymal markers such as N-cadherin and vimentin, indicating a poor prognosis in carcinomas, including HNSCC [42,43].

Considering that GLPS alone can downregulate CSC, EMT, and ABC markers [8] and 5-FU is a good option already used against cancer, we believe that GLPS has the potential to enhance the sensitivity of 5-FU in tumor cells and consequently even decrease 5-FU efficient dose, possibly increasing the potential to eliminate the CSCs and modulating the EMT. Furthermore, the reduction in treatment costs would have economic benefits. Therefore, the purpose of the current study is to show how GLPS can improve a well-used chemotherapy drug, the 5-FU. For that, we compared the effectiveness of GLPS alone (earlier publication) with a low dose of 5-FU alone and combined it with GLPS on the subpopulation of CSCs from the SCC-9 OSCC cell line.

## Materials and methods

### *Cell culture*

The SCC-9 cells of oral squamous carcinoma (ATCC CRL-1629™) were cultured in DMEM/F-12 medium (Dulbecco's Modified Eagle Medium, Gibco/Nutrient Mixture, Gibco) supplemented with 10 % fetal bovine serum (Gibco), 400 ng/ml hydrocortisone (Sigma Aldrich®), and antibiotic/antimycotic solution (Gibco) and incubated in a humidified incubator at 37 °C with 5 % $CO_2$. To perform the assays, cisplatin (Blausiegel) was used as the chemotherapeutic control at a concentration of 50 μg/ml [44]. The MTT cytotoxicity assay, flow cytometry for apoptosis, epithelial phenotyping, size/granularity analysis, and real-time PCR for CSC/EMT/ABC expression were performed. The GLPS extract was obtained and used according to the protocol described in a previous study [8]. Briefly, this fraction presented a GLPS composition of ~70 % of β-glucans and 30 % of mannogalactans.

### *MTT assay*

The aim of this study was to evaluate the effects of 5-FU alone and in combination with GLPS on SCC-9 cells by using the MTT metabolic

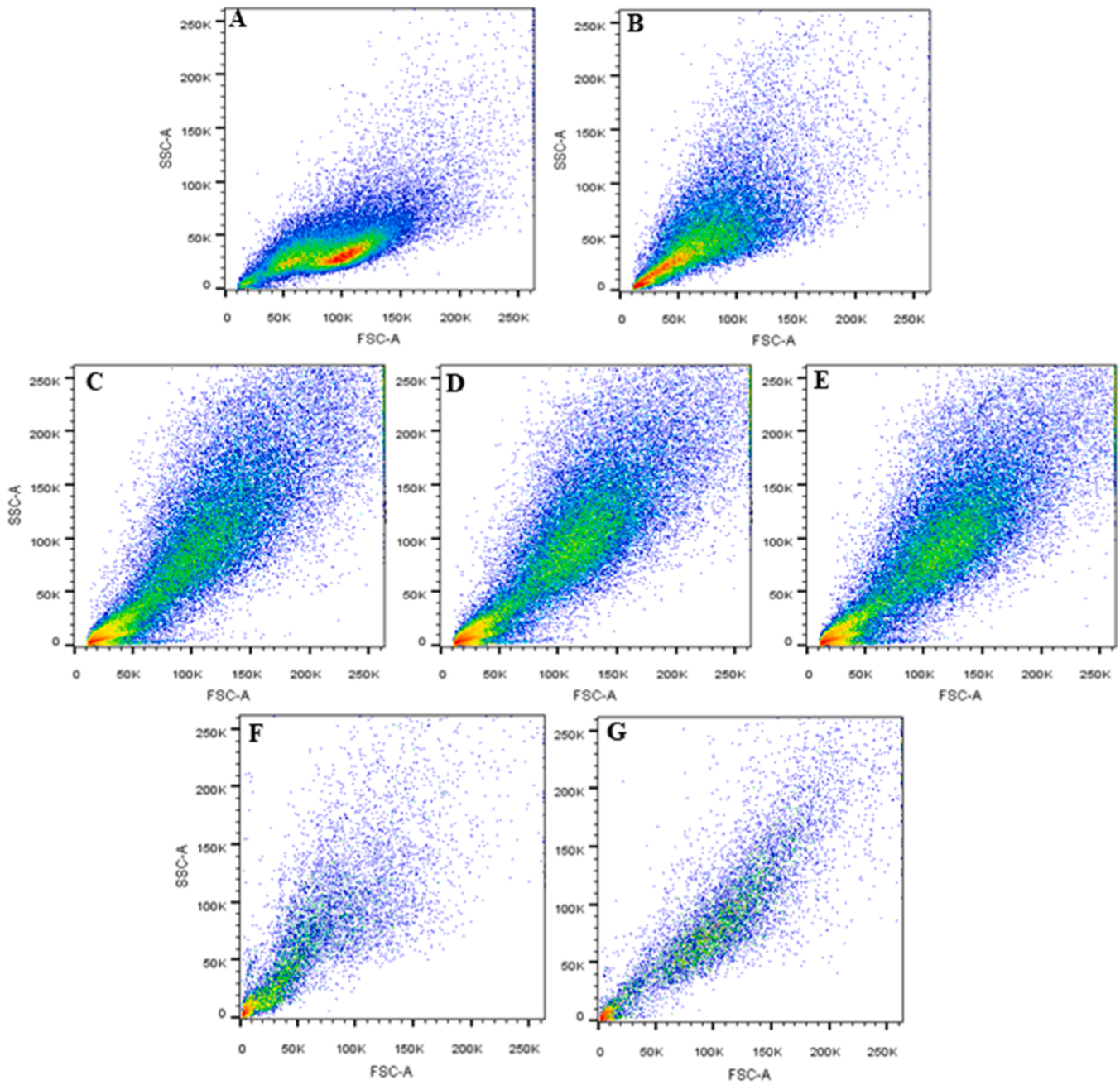


**Fig. 6.** Dot Plot type analysis of flow cytometry demonstrating the difference between size (FSC-A) and granularity (SSC-A) by treated cells compared to (**A**) CTRL (untreated SCC-9 cells). SCC-9 cells were treated with 50 μg/ml of cisplatin (**B**), FU100 (**C**), FU20 (**D**), FU4 (**E**), or (**F**) GLPS10/FU0.8 and (**G**) GLPS5/FU4, for 72 h.

activity assay. The cells were seeded at a density of 104 cells/well in 96-well plates and were treated with different concentrations of 5-FU alone (200, 100, 20, 4, 0.8, 0.16, 0.032 ng/ml), or a combination of both GLPS and 5-FU for 72 h. Cell death was assessed using distilled water only (CTRL+) or cisplatin (CIS) as a positive control. The control group was untreated (CTRL) and had 100 % viability. The assays were performed in triplicate and repeated three times. Additionally, the basal control of cells (BASAL CTRL) was determined by measuring the metabolic activity at the end of the 24-h adherence period.

*Cell proliferation assay*

To assess cell proliferation, a total of $10^6$ cells were treated with a 3 μM solution of carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma-Aldrich, Missouri, United States) for 15 min at room temperature while shielded from light. The labeled cells were then seeded and exposed to GLPS and/or 5-FU for 72 h before being analyzed for changes in fluorescence intensity using flow cytometry. Reductions in CFSE fluorescence levels were used to indicate cellular proliferation. The assay was performed in duplicate one time.

*Migration assay*

To perform the scratch wound healing formation migration assay, SCC-9 cells were seeded in 12-well plates at a density of $3 \times 10^5$ cells/well and allowed to adhere for 12 h, following the protocol of Camargo et al. [8]. In addition, metastatic SCC-9 lineage (LN) was included as a control for migration. Cells were photographed within the first 12 h and then every 24 h under an inverted microscope to monitor migration along the wound until 72 h of the experiment. The wound area was

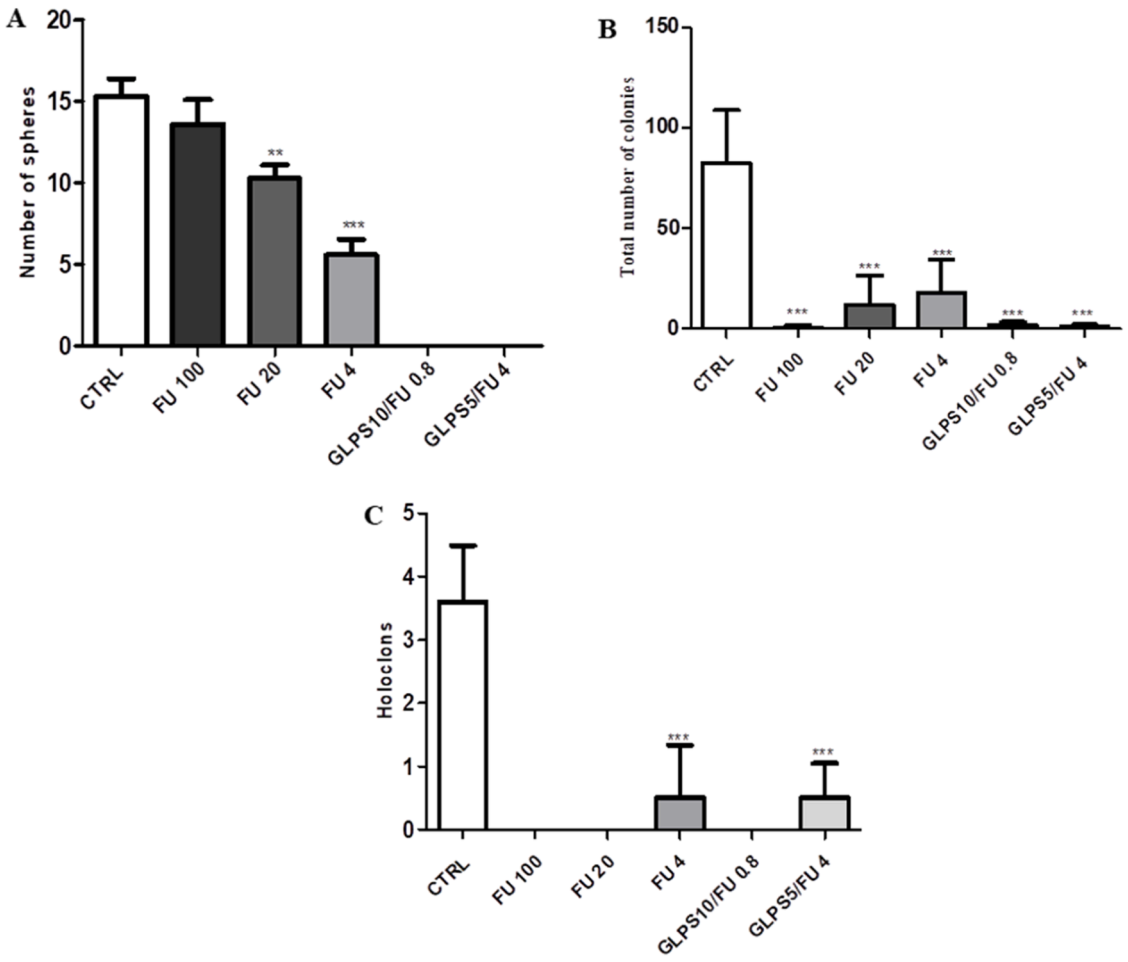


**Fig. 7. (A)** Quantification of sphere formation assay by SCC-9 cells treated cells. Note the absence of sphere formation by SCC-9 cells at GLPS / FU post-treatment. (**B**) The number of colonies formed by SCC-9 cells, after treatment. (**C**) The number of holoclones (characteristic of CSC). **CTRL**: untreated tumor cells, **FU100, FU20,** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). Values expressed as the mean and standard deviation of the duplicate of three independent experiments. . ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). $n = 6$.

measured using Image J software (Zeiss), and the percentage of wound closure was calculated. The assay was conducted in triplicate and repeated in three independent experiments using distinct cell passages, and three microscopic fields were analyzed for each wound.

*Morphological analysis*

To analyze the morphological changes in SCC-9 cells upon treatment, the cells were initially seeded in T75 cell culture flasks and then detached and reseeded at a density of $10^3$ cells per well in 8 chamber Culture Slides (Falcon). The cells were allowed to adhere for 24 h in complete DMEM/F-12 culture medium before being treated with various concentrations of 5-FU alone (200, 100, 20, 4, 0.8, 0.16, 0.032 ng/ml), GLPS alone at 5 mg/ml (GLPS 5) and 10 mg/ml (GLPS 10), or a combination of both. After treatment, the cells were fixed with 4 % paraformaldehyde for 15 min and stained with 1.5 % crystal violet solution in absolute ethanol diluted with distilled water (1:40 ratio) for 5 min. The stained slides were then dried at room temperature, mounted using Permount® (Fisher Chemical®) and a glass cover, and photographed under an inverted light microscope (Olympus CKX41) using the Olympus CellSens Standard program until a total of 600 cells were in each group. The area and perimeter of nuclei were measured using Image J software (Image J 1.52a. Wayne Rasband, National Institutes of Health, United States of America). The assays were performed in duplicate in three independent experiments with distinct cell passages.

*Colony and spheroid culture formation*

After each treatment, SCC-9 cells were detached and seeded in 6-well plates (Costar) at a low density of 250 cells/well and maintained for 10 days in moist chamber culture to allow for adherence and formation of colonies. The number of colonies with >50 cells was counted under an inverted microscope, and holoclones corresponded to the CSC subpopulation [8,45]. Three independent experiments were performed in duplicate and analyzed by three different researchers.

To assess the growth in suspension as tumorspheres, the cell concentration was adjusted to 1000 cells/ml culture medium, and cells were held in suspension by the use of 24 well ultra-low adhesion plates (Corning®) at 37 °C under 5 % $CO_2$ in a humid chamber. After being monitored for 21 days, tumorspheres with a diameter of about 50 µm were quantified by inverted microscopy (Olympus CKX41) to determine their clonal capacity [8,46,47]. The assays were performed in duplicate in three independent experiments with distinct cell passages and analyzed by three different investigators.

*Annexin V apoptosis detection and CSC phenotyping*

To quantify apoptotic cell death, as well as the phenotype of cells post-treatment, $10^5$ SCC-9 cells (treated and non-treated), as well as CIS-treated SCC-9 cells (CIS), were analyzed by flow cytometry as previously described in Camargo et al. [8]. Annexin $V^-$ / DRAQ7$^-$ were considered

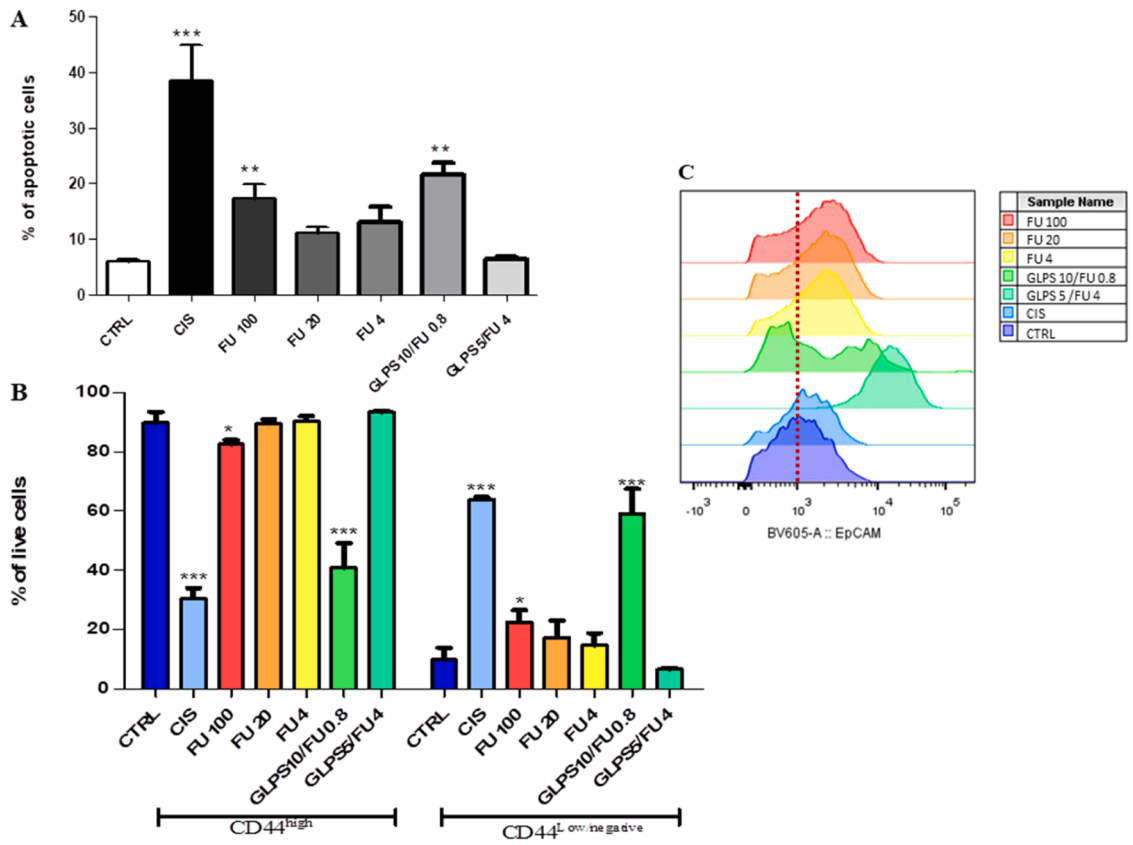


**Fig. 8.** **(A)** Percentage of apoptotic cells after 72 h of treatment. (**B**) Percentage of live SCC-9/CD44$^{+}$ cells (Annexin-/DRAQ7-) assessed by flow cytometry after 72 h of treatment. (**C**) Histogram showing the intensity of the epithelial marker ESA (EpCAM) in live SCC-9/ CD44$^{+}$ cells treated or not (CTRL) for 72 h. **CTRL**: Untreated SCC-9 cells. **CIS**: Cisplatin-treated SCC-9 cells (50 μg/ml), **FU100, FU20** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). . *$p < 0.05$ *versus* CTRL; ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). $n = 6$.

live cells and the CD44$^{+}$ cells were then divided into CD44$^{high}$ (CSC) and CD44$^{low}$ (non-CSC) phenotypes. The CD44$^{high}$ cells were analyzed for ESA$^{high}$ (epithelial) and ESA$^{low}$ (mesenchymal) phenotype expression [48]. The Anexin$^{+}$/Draq$^{-}$ cells were considered apoptotic cells. The assay was performed in triplicate in three independent experiments with distinct cell passages and 10 thousand events were considered.

*CSC/EMT/ABC transcripts*

To verify the expression of CSC and EMT-related genes as well as the ABC drug transporters, Real-Time PCR (qPCR) was performed. Briefly, the total RNA of treated cells and control groups was extracted with the MagMAX ™ mirVana ™ Total RNA Isolation Kit (Applied Biosystems ™ # A27828) according to the manufacturer's instructions and with an automated pipettor (MicroLab Nimbus, Hamilton). To obtain the cDNA was used the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems ™ # 4,374,967), according to the manufacturer's guidelines, uses random primers to initialize the cDNA synthesis. Amplification assays and analysis of the results were conducted as detailed in Camargo et al. [8]. Three independent experiments with distinct cell passages were separately pooled in triplicates for qPCR assays.

*Statistical analysis*

Data were presented as the means ± standard deviation (SD) of the minimum of three independent experiments. One-way analysis of variance (ANOVA) and Tukey-Kramer test or Two-way ANOVA and Bonferroni were used to assess significant differences between groups. *P*-value (P) $< 0.05$ was considered to be statistically significant. GraphPad Prism 6 (GraphPad Software), and Statistica (StatSoft) software were used.

## Results

*5-FU alone and associated with GLPS are cytotoxic in a dose-dependent manner*

SCC-9 cells were treated with GLPS and 5-FU combination at various concentrations for 72 h and cytotoxicity was analyzed by MTT. Among all tested doses, only **FU100, FU20** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS5/FU4** (GLPS 5 mg/ml *plus* 4 ng of 5-FU) and **GLPS10/FU0.8** (GLPS 10 mg/ml *plus* 0.8 ng of 5-FU) significantly reduced cellular metabolic activity compared with CTRL, without promoting noticeable cytotoxicity since the percentage of cells was close to those of the BASAL CTRL. Doses less than 0.8 of 5-FU were not chosen for the association since 0.8 is already low enough for the same biological effects as other low doses, which could easily lead to technical errors. The chosen of GLPS doses of 10 mg/ml and 5 mg/ml were previously chosen as described by Camargo et al. [8]. Briefly, the GLPS doses significantly reduced cellular metabolic activity compared with CTRL, without promoting noticeable cytotoxicity since the percentage of cells was close to those of the BASAL CTRL (GLPS 10 and GLPS 5) [8].

5-FU alone exerted a dual effect on treated cells depending on its concentration; while high doses of 100, 20, and 4 ng/ml significantly reduced cellular metabolic activity compared to CTRL ($P < 0.001$, 0.01, and 0.05, respectively), doses lower than 4 ng/mL had no significant on treated cells, which showed metabolic activity similar of CTRL (Fig. 1**A**).

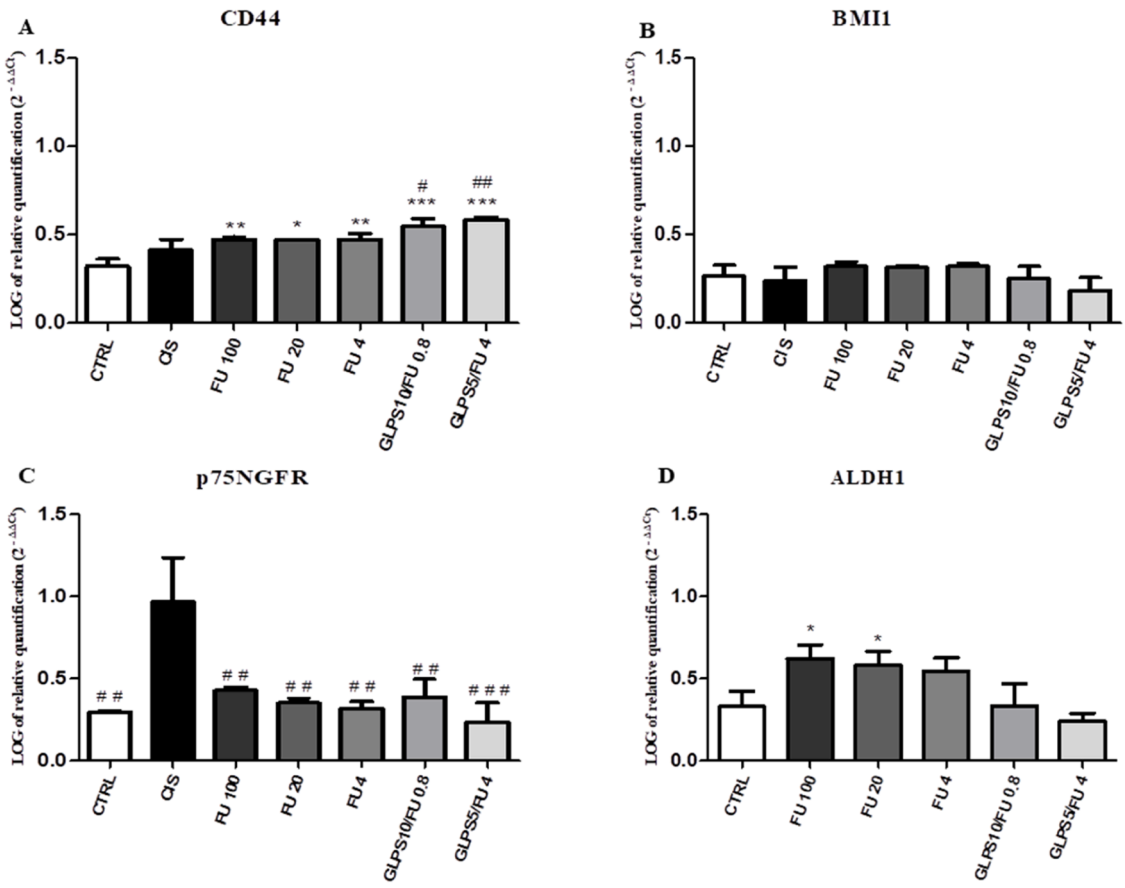


**Fig. 9.** Expression of CSC markers in SCC-9 cells cultured in clonal density under treatment and controls. **CTRL:** untreated SCC-9 cells; **CIS:** SCC-9 cells treated with 50ug/ml of cisplatin (conventional MTD of chemotherapy); **FU100, FU20** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). *$p < 0.05$ *versus* CTRL; ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). # $p < 0.05$ *versus* CIS; ## *versus* CIS ($P < 0.01$) and ### *versus* CIS ($P < 0.001$). Values are expressed as mean and standard deviation, from one PCR assay with a triplicate of independent experiments in triplicate. ALDH1 target was not detectable in CIS-treated cells using the PCR technique.

The same pattern was observed in comparison to BASAL CTRL, however without statistical significance. While doses higher than 20 ng/mL decreased cellular metabolic activity, doses lower than 4 ng/mL caused an increase in the percentage of metabolically active cells.

Association of 5-FU with GLPS 5 mg/mL decreased at 20 and 4 ng/mL (Fig. 1**B**); GLPS 10 mg/ml *plus* 20, 4, 0.8, 0.16, 0.032 ng of 5-FU (Fig. 1**C**) did not allow for an increase in metabolic activity when compared to CTRL (number of cells in culture for an amount of 96 h, and without treatment). At the same time, these concentrations did not result in reduced metabolic activity obtained in the first 24 h of culture (adherence period), as verified when compared with BASAL CTRL (number of cells after 24 h adherence period and immediately before the treatments). No significant effect on cellular metabolic activity was observed upon treatment of 5-FU alone or in association with GLPS 5 or 10 when compared to BASAL CTRL. However, although not statistically significant, higher concentrations of 5-FU.

On the other hand, in the presence of higher concentrations of 5-FU (associated or not with GLPS), the SCC-9 cells showed reduced metabolic activity concerning BASAL CTRL, which can be interpreted as a potential for excessive cytotoxicity, as it results in several cells below baseline for the other analyzes.

Treatment with CIS resulted in the lowest percentages of metabolically active cells, similar to CTRL+. In contrast, the lower concentrations of 5-FU alone or associated with 10 mg/ml GLPS allowed the metabolic activity of cells in a similar way to CTRL.

*5-FU alone and associated with GLPS inhibited the proliferation of SCC-9 cells*

To analyze the effect of selected working doses on tumoral cell proliferation, the SCC-9 cells were CFSE tested. CFSE is a cell-permeant dye and is equally partitioned to daughter cells during cell division, allowing the indirect assessment of proliferation; then loss of CFSE is indicative of proliferation. Fig. 2**A-B** shows less proliferation in a dose-dependent manner of selected MTT doses against CTRL, a condition that allowed cells to spontaneously proliferate.

SCC-9 cells treated with 5-FU alone or in association with GLPS were less proliferative than non-treated cells but at different extents. FU 100 alone exhibited the lowest (4 times lower) proliferative potential when compared to CTRL ($P < 0.001$), followed by FU 0.8/GLPS 10 (2.5 times lower; $P < 0.001$), FU 20 (2 times lower; $P < 0.001$), FU 4 (1.5 times lower; $P < 0.01$). FU4/GLPS5 – treated cells exhibited the closest proliferative potential to CTRL.

*5-FU alone and associated with GLPS-impaired SCC-9 cell migration*

The rate of cell migration in the presence of all the treatments was lower than CTRL, in which the wound was closed at 48 h. The most effective treatments to impair migration were FU 100 and FU 20, which could be significantly noticed from the 24 h and kept the wound open until the end of 72 h (Fig. 3). Also, these doses induced the formation of empty spaces at the end of the 48 h period, suggesting cell death (Fig. 3**B** – **arrows**). At a lower extent, FU 0.8/GLPS10 and FU 4/GLPS5 also

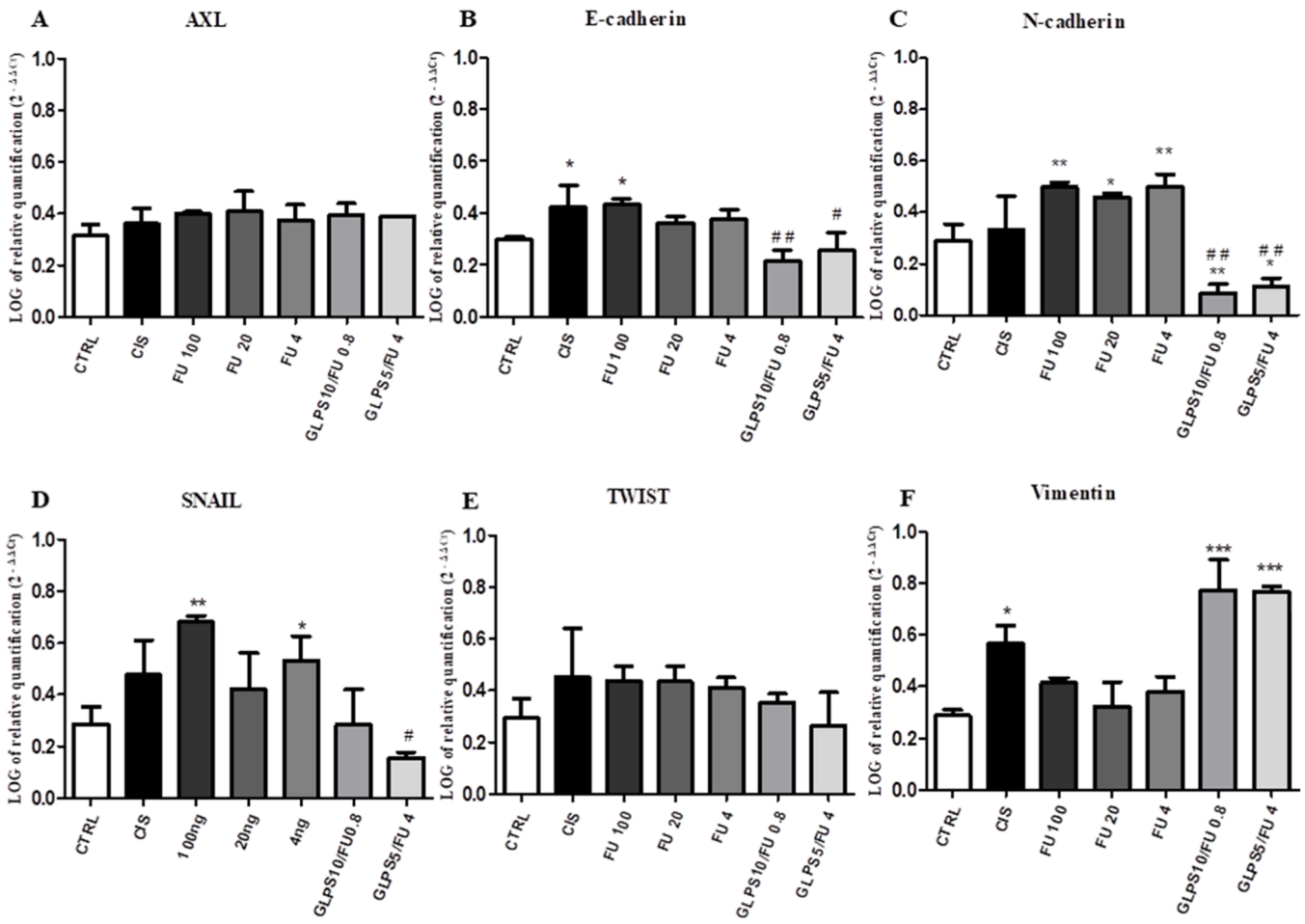


**Fig. 10.** Expression of EMT markers in SCC-9 cells cultured in clonal density with treatment and controls. **CTRL**: untreated SCC-9 cells; **CIS:** SCC-9 cells treated with 50 μg/ml of cisplatin (conventional MTD chemotherapy); **FU100, FU20** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). *$p < 0.05$ *versus* CTRL; ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). #$p < 0.05$ *versus* CIS; ## *versus* CIS ($P < 0.01$). Values are expressed as mean and standard deviation, from one PCR assay with a triplicate of independent experiments. $n = 3$.

inhibited cell migration from the 24 h period, but the wound was much narrower at the end of the 72 h in comparison to treatment with 5-FU alone (Fig. 3).

*Only 5-FU alone changed the morphology of treated cells*

To evaluate the morphological changes in treated SCC-9 cells, an optical microscope analysis was performed, comparing treatments and CTRL (Fig. 4**D**). Larger and more separated cells were observed after treatment with 5-FU (Fig. 4**A-C**). The association of 5-FU and GLPS resulted in intra-cytoplasmic vacuolization (Fig. 4**E** and **F**).

Regarding the area and perimeter measurements, the nuclei size of 5-FU-treated cells was significantly increased in comparison to CTRL, which showed an average area (μm$^2$) of 301.1 ± 68.8 and perimeter (μm) of 60.8 ± 6.4. The larger nuclei area (2.5 times) and perimeter (1.6 times) were from FU100-treated cells (797.1 ± 79.8 μm$^2$ and 98.0 ± 4.7 μm, respectively, followed by **FU20 (**764.3 ± 135.3 μm$^2$ and 96.5 ± 9.5 μm, respectively) and **FU4** (579.1 ± 253.1 μm$^2$and 82.6 ± 19.3 μm, respectively) (Fig. 5**A and B**). No nuclear morphological changes were detected after treatment with 5-FU associated with GLPS.

Flow cytometric analysis of morphological changes concerning size and internal complexity (forward scattered x side scattered) of treated cells confirmed that 5-FU alone indeed increased both the size and granularity of SCC-9 cells (Fig. 6**C-E)**. Changes were slight in GLPS5/FU4 treated cells (Fig. 6**G**).

*Cells treated with 5-FU associated with GLPS cannot form spheres and its colony formation was quite reduced*

When stem cell properties were evaluated, we observed that the formation of spheres was aborted under 5-FU/GLPS association and decreased in a dose-dependent manner under 5-FU treatment alone, in comparison to CTRL (Fig. 7**A**). The number of colonies was significantly reduced compared to CTRL after all treatments ($P < 0.001$), mainly in the associations of 5-FU with GLPS and FU100 alone (Fig. 7**B**). Holoclone formation (CSC colony formation) was aborted after treatment with both high doses of 5-FU (FU100 and FU 20), and a low dose of 5-FU associated with GLPS 10 (FU0.8/GLPS10). Other treatments decreased holoclone formation (Fig. 7**C**).

*Treatments changed the phenotype of cells and type of death, highlighting GLPS10/FU0.8 and FU100*

To further assess the effects of treatments on cell death and CSC phenotype, SCC-9 cells were multiple stained for Annexin V, DRAQ7, and CD44, and then submitted to flow cytometry, when the whole population was gated into CD44$^{high}$ (CSC) and CD44$^{low}$ (non-CSC) subpopulations. When compared to CTRL, treatments with CIS($P < 0.001$), FU0.8/GLPS10 ($P < 0.01$), and FU100 ($P < 0.01$) resulted in a significant increase in the percentage of apoptotic cells (Fig. 8**A**), and consequently in a direct negative impact on cell viability under these

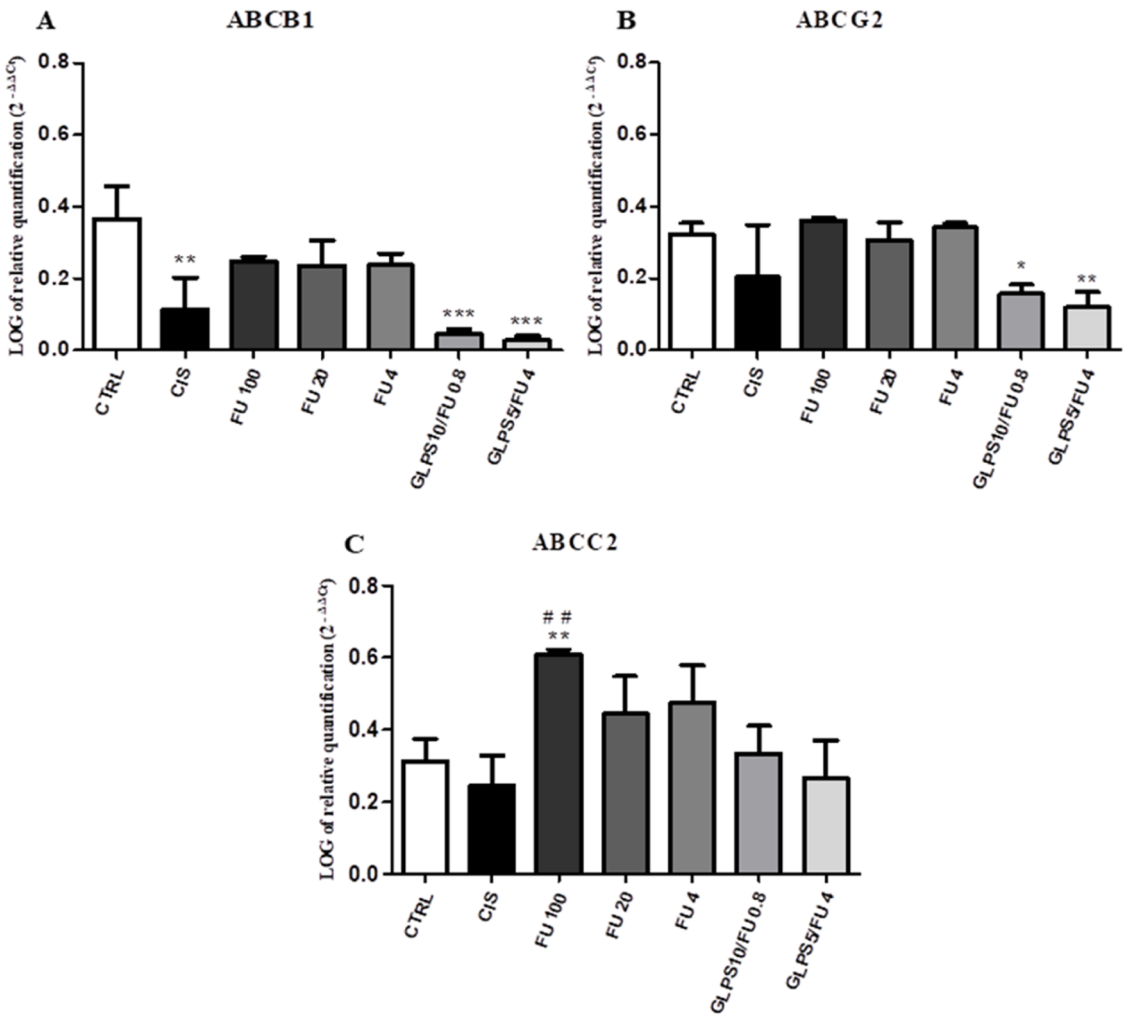


**Fig. 11.** Expression of ABC markers in SCC-9 cells cultured in clonal density with treatment and controls. **CTRL**: untreated SCC-9 cells; **CIS:** SCC-9 cells treated with 50 μg/ml of cisplatin (conventional MTD chemotherapy); **FU100, FU20** and **FU4** (5-FU doses of 100 ng/ml, 20 ng/ml, and 4 ng/ml, respectively); **GLPS10/FU0.8** (GLPS 10 mg/ml plus 0.8 ng of 5-FU) and **GLPS5/FU4** (GLPS 5 mg/ml plus 4 ng of 5-FU). *$p < 0.05$ *versus* CTRL; ** *versus* CTRL ($P < 0.01$) and *** *versus* CTRL ($P < 0.001$). *## versus* CIS ($P < 0.01$). Values are expressed as mean and standard deviation, from one PCR assay with a triplicate of independent experiments. $n = 3$.

treatments. $CD44^{high}$ cells were mainly affected by the treatment with CIS ($P < 0.001$), FU0.8/GLPS10 ($P < 0.001$), and FU100 ($P < 0.05$), showing a reduction of 66, 54, and 9 % in viability (Fig. 8**B and C**). This reduction reflected an increase of $CD44^{low}$ sub-population on CIS, FU0.8/GLPS10, and FU100, respectively. Specifically, the efficiency of affecting the CSC subpopulation was similar between FU0.8/GLPS10 association and CIS. This result about the change in CD44 molecules indicates a trend towards a non-CSC profile among treated cells. The dot plots of viability and apoptosis can be seen in Supplementary Fig. 1.

Interestingly, when the remaining CSC subpopulation ($CD44^{high}$) was analyzed, the cells showed an epithelial profile ($CD44^{high}/ESA^{high}$) (Fig. 8**C**, peak on the right of the dotted line), regardless of treatment.

*5-FU associated with GLPS downregulate CSC, EMT and ABC expression*

All treatments regulated to some extent the expression of CSC markers, except *BMI1* (Fig. 9). Compared to CTRL, 5-FU alone upregulated *CD44* and *ALDH1*, while the association with GLPS only increased *CD44* transcript (Fig. 9**A** and **D**).

When compared to CIS, only *p75NGFR* and *CD44* were regulated variably. While all treatments downregulated *p75NGFR*, only the associations with GLPS upregulated *CD44* (Fig. 9**A** and **C**). Interestingly, CIS treatment did not interfere significantly with the expression of the CSC markers compared to CTRL.

Treatments in general also regulated variably the expression of EMT markers, except *AXL* and *TWIST* (Fig. 10). Compared to CTRL, 5-FU alone significantly upregulated E-cadherin, N-cadherin, and SNAIL, while the association with GLPS downregulated N-cadherin and upregulated Vimentin (Fig. 10**B-D** and **F**).

CIS significantly upregulated E-cadherin and Vimentin compared to CTRL (Fig. 10**B and F**). When compared to CIS, treatment with 5-FU alone resulted in a similar pattern of expression of the EMT markers, so only the associations with GLPS modulated gene expression, by downregulating both cadherins and *SNAIL* transcripts (Fig. 10**B-D**). Indeed, the immunofluorescence can show the upregulation of E-cadherin proteins caused by 5-FU on SCC-9 cells (Supplementary Fig. 2).

*ABCB1* and *ABCG2* transcripts showed a remarkable downregulation after treatment with FU0.8/GLPS10/ and FU4/GLPS5/ (Fig. 11**A** and **B**), while CIS treatment significantly downregulated only *ABCB1* (Fig. 11**A**). Differently, 5-FU exerted no significant impact on the expression levels of *ABCB1* and *ABCG2,* but FU100 significantly upregulated ABCC2 compared to CTRL and CIS (Fig. 11**C**).

## Discussion

Complementary therapies (CT) have been gaining ground in the world West encouraged by the search for new alternatives in the treatment of diseases such as cancer [49]. The use of TCM is the main

component of CT, once it decreases the side effects caused by chemotherapy or radiotherapy, the resistance to the drugs as well as helps immunomodulation impairing allergies and infections [49,50]. The combinations of treatments have been widely studied in the therapeutic protocol, aiming to reach the best of each substance involved [6].

Well known for TCM as well as CT, the use of *Ganoderma lucidum*, also known as Lingzhi or Reshi, has been extensively described as a species capable of curing various diseases and recognized for promoting health, longevity, and the treatment of various human diseases, including cancer. Its use with different drugs is strengthening the effect of conventional therapies and consequently improving the healthy state of patients [1,6,7]. However, the use of GLPS with chemotherapeutic drugs has been poorly studied in HNSCC [51,52] despite the use of TCM has been studied for OSCC on CT [53].

For this reason, and considering that *in vitro* GLPS treatment alone resulted in CSC elimination, inhibition of cell migration, and upregulation of the sensibility of OSCC to drugs [8], we set out to study the combination of GLPS with 5-FU. This chemotherapeutic drug is already well accepted and widely used for HNSCC; however, although effective, 5-FU causes several adverse effects, reducing the efficiency of treatment, survival, and quality of life of patients [9,10,54]. Thus, believing that the association of GLPS with 5-FU could improve the current chemotherapy, we designed this present study comparing the *in vitro* treatments of the SCC-9 cell line with distinct doses of 5-FU alone and associated with GLPS.

Treatment with GLPS/FU association for 72 h interfered with important functional activities, as well as in molecular and protein aspects of SCC-9 tumor cells, which are related to cancer progression and the stem-tumor profile. Compared to CTRL, both GLPS/FU associations resulted in increased apoptosis, inhibition of proliferation and migration. Even though these results are expected for treatment with 5-FU, the association with GLPS allowed a low-dose drug response. According to Zeng & Xiao [52], the GLPS itself is already responsible for the reduction of tumor cell proliferation and migration associated with suppression of miR-188/BCL9/β-Catenin pathway on OSCC cell line but can be used to improve the drug response on chemotherapy. Also observed a decrease in CSC phenotype properties such as colonies and spheres formation, and $CD44^{high}$ sub-population, as well as a downregulation of the EMT marker N-cadherin and ABCB1/ABCG2 drug transporters transcripts, recognized as chief culprits in the development of MDR. Interestingly, these effects were mainly observed when the lowest dose of 5-FU was associated with the highest dose of GLPS (GLPS10/FU0.8), which may reflect an important clinical implication. The downregulation of drug transporters' expression indicates a cellular sensibility to drugs and a decrease in CSC characteristics [33,55,56], which may be associated with a better response to chemotherapy and lower potential for invasiveness, recurrence and metastasis [26,28,32,39]. Studies with GLPS in combination with different chemotherapeutic drugs show an increase in the sensitivity of cancer cells against chemotherapy, by enhancing oxidative stress, DNA damage, apoptosis and due to the inhibition of ABCB1 expression, as we showed [5].

Accordingly, the effectiveness of this association could be validated when the trials demonstrated that the remaining $CD44^{high}$ cells from the treatment showed epithelial phenotype ($CD44^{high}/ESA^{high}$). This phenotype is more sensitive to treatments, being correlated with decreased tumor resistance in primary tumors [23–25]. More than that, compared to CTRL, the treatment with GLPS/FU association resulted in the absence of sphere formation, and downregulation of colony formation. Since the ability to form spheres and colonies *in vitro* represents the functionality of the CSC subpopulation [57–59], our results also suggest that GLPS plus 5-FU association can impair CSC properties.

Another finding was the increase in $CD44^{low}$ population, a non-CSC phenotype; decreasing tumor heterogeneity in a characteristically non-therapy-resistant and less proliferative tumor [40,60]. These findings follow Rodrigues et al. [61], which shows that the $CD44^{low}$ population expresses molecular changes that may function as a form of "differentiation therapy" in OSCC that can potentiate the effects of chemotherapeutic agents. This can be reinforced by the induction of apoptosis and the absence of holoclones observed after GLPS10/FU0.8 treatment. Holoclones represent colonies of tumor stem cells, with an epithelial phenotype [62] and are maintained by continuous proliferation [45]. The association of 5-FU and GLPS also resulted in intra-cytoplasmic vacuolization, suggesting autophagy and reinforcing spontaneous cell death, as suggested by Feng et al. [63] and reinforced by Chiu 2015 when using a Ganoderma protein [64].

However, the benefits against tumor cells were also observed in this study with 5-FU alone, but after the highest doses of treatment. Besides the association suggests spontaneous cell death, 5-FU alone dramatically changed the morphology of cells when compared to GLPS/FU, suggesting senescent cells [65]. This can happen in response to DNA-damaging drugs, such as 5-FU [66–68]. Low doses of anti-tumor drugs can activate signaling pathways, such as p53, that determine the evolution of the disease [69]. Low doses can also activate apoptotic pathways and when the tumor suppressor genes (*e.g.* p53) are activated and/or oncogenes inactivated, in a malignant tumor, cells can become senescent [70,71]. In malignant tumors, senescence is a mechanism of tumor suppression in which cells are unable to proliferate and can be eliminated by the immune system, resulting in tumor regression [66,72]. Furthermore, the treatment with 5-FU alone resulted in only subtle interference with sphere formation, and tumor cells showed upregulation of both cadherins, SNAIL, and ABCC2 expression. This leads us to understand that the association may be more effective in the treatment of tongue cancer than 5-FU alone, as it can broadly modify tumor cells phenotypically and molecularly, contributing to tumor regression and sensitivity. Accordingly, natural products have been effective in sensitizing different cancer cells to therapy drugs *in vitro* and *in vivo* [73,74]. Suárez-Arroyo et al. [75], demonstrated that *Ganoderma lucidum* extract (GLE) potentiates the effects of carboplatin in breast cancer through the modulation of the DNA Damage Response and cancer cell stemness, while the treatment combination significantly reduced tumor volume by 33 % and tumor weight by ~40 %. More than that a combination of GLE and 5-FU caused sensitization of cancer cells to 5-FU, resulting in improved anticancer effect *in vitro*, reduced tumor volume and increased survival in mice, in comparison with 5-FU alone [76].

The principal aim of chemotherapy is to induce apoptosis in cancer cells, however, it results in several side effects, such as gene mutation, cellular toxicity, and drug resistance [1]. The GLPS mechanism such as metabolizing enzymes induction, inhibition of urokinase plasminogen activator (uPA) expression and urokinase plasminogen activator receptor (uPAR) when used in association to 5-FU can reduce the adverse effects by different pathways activations and/or suppression, improving chemotherapy and reducing its negative effects [6]. Also, Yang et al. [77] showed that GLPS can enhance the antitumor effects of 5-Fluorouracil by upregulation of the DAP10/PI3K/ERK signaling pathway.

Besides 5-FU alone, Cisplatin (CIS) was used as tumor cell death control by the action of a chemotherapeutic [44]. At the dose of 50 mg/ml on SCC-9 cells, CIS induced the death of 97 % of cells measured by MTT and showed the highest indices of apoptosis by flow cytometry. The remaining 3 % of live cells showed $CD44^{low}$ phenotype but with CSC and EMT molecular expressions similar to CTRL or higher. Only the expression of ABCB1 was decreased compared to CTRL. These results of CIS treatment suggest that even with high indices of apoptosis, the remaining cells show results associated with cancer progression and metastasis and chemotherapeutic resistance [42,43].

Taken together, the results reinforce the efficiency of the GLPS natural compound used in OSCC treatment *in vitro*, including tongue cancer. The highlighting results also encompass the occurrence of apoptosis, the absence of sphere formation and the decrease in CSC, EMT and ABC expression at associated-treated cells. The associated treatment also altered CSC biological properties, and improved the sensitivity of cells, leading them to death. More than that, these results associated with previous studies, suggest that the use of GLPS with 5-FU is a promising

association, reducing the use of 5-FU doses and improving the therapy results against CSCs on OSCC, besides possibly reducing adverse reactions and improving the quality of life for cancer patients during chemotherapy.

## Limitations

This study has some limitations that should be acknowledged to provide a comprehensive understanding of its scope and potential implications. These limitations highlight weaknesses in the design, analysis, and interpretation of the experiment, while also recognizing the strengths that contribute to the study's overall value:

1 Generalizability:

One of the primary limitations of this study is the relatively generalizability of the findings, once it is an *in vitro* culture to broaden it to a population may be limited. However, this information can be very useful for the prospect of new therapies and pharmacological associations.

2 Writing:

Another difficulty encountered was the writing of the manuscript. Since data regarding the GL study alone at OSCC had already been published by the group, great care was taken in compiling and analyzing the results used in combination with 5-FU. To clarify both the potential of GL as an antitumor, as well as the potential of its use in combination with 5-FU.

3 Internal Validity:

The careful control of variables within the experiment enhances the internal validity of the results, allowing for a clearer understanding of the causal relationships studied.

4 Detailed Data Collection:

The meticulous approach to data collection enabled a rich dataset, providing valuable insights into the research question.

In conclusion, while this study is not without limitations, the strengths of the research design and execution contribute to its significance and potential impact. Addressing these limitations and building upon the strengths can guide future research endeavors in refining and expanding upon the current findings.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.prmcm.2023.100310.

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