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Effects of Fermented Wheat Germ Extract on Oral Cancer Cells: An In Vitro Study

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ABSTRACT

Oral carcinoma is one of the most aggressive cancers, and despite the advances in the therapy, its mortality is still high. An attention in cancer treatment has focused on natural compounds due to their potential beneficial effects on human health. In this study, the effects of dietary supplement Fermented Wheat Germ Extract (FWGE) on oral tongue squamous cell carcinoma (OTSCC) cells were investigated *In Vitro* using three cell lines (HSC-3, SAS, SCC-25) with variable aggressiveness. The cell viability was significantly decreased by the treatment with high concentration of FWGE in every cell line. Regarding migration and invasion, HSC-3 and SCC-25 cells were most sensitive to FWGE since their movement was significantly reduced with 5 and 10 mg/ml FWGE, while SAS was inhibited only with 10 mg/ml FWGE. Chemotherapeutic compounds (cisplatin and 5-fluorouracil) significantly reduced all OTSCC cells viability. Importantly, combination of these drugs with 10 mg/ml FWGE significantly decreased the cell viability compared to the treatment with the chemotherapeutics or FWGE alone. Based on these *In Vitro* experiments, the use of FWGE seems to improve the anticancer effects on OTSCC cells. Further *In Vivo* and clinical studies should be conducted to verify the positive effects of FWGE for OTSCC patients.

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Introduction

Oral squamous cell carcinoma (OSCC) represents one of the most aggressive types of cancers with over 350,000 new cases and 146,000 deaths in 2015 worldwide (1,2). The incidence of OSCC, and especially oral tongue carcinoma (OTSCC), is increasing and the average age of the affected patients is decreasing (3). The etiologic factors for OSCC include tobacco, alcohol, betel quid or a combined consumption of them. The prognosis et al., with several features, but in general, early diagnosis and surgical treatment of low stage tumors improve the outcome (4–8). Radiotherapy, chemotherapy and biological therapies are applied for patients with advanced or recurrent disease. However, despite the advances in the cancer therapy, the 5-years survival rate does not globally exceed 60% (9,10).

There are several challenges in current cancer treatment. Among these, the need of more effective, personalized and selective therapies (11). The toxicity of the current cancer drugs often causes series of severe side effects, which significantly worsen patients' quality of life and often leads to the interruption of the therapy (12). Efforts have been made to find compounds able to support and improve cancer therapy. A growing attention has been focused on some natural products known to be beneficial for humans.

Fermented wheat germ extract (FWGE) is produced through the fermentation of wheat germ by *Saccharomyces cerevisiae*, separation of the fermentation liquid, drying and granulation (13). Recent studies reported that two quinones, 2-methoxy benzoquinone and 2,6-dimethoxy benzoquinone, which are present in wheat germ as glucosides, are likely responsible for some of the biological properties

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of FWGE (13–15). FWGE, also known as Avemar™, has been evaluated *In Vitro* and shown to have an inhibitory action on many cancer cell lines (16). A couple of clinical studies have been conducted to test the effects of FWGE supplement on patients undergoing conventional chemotherapy for melanoma (17) and colorectal cancer (18). The results revealed significantly improved survival in the groups supplemented with FWGE compared to the controls treated with the conventional drugs only (17,18).

Although the anti-tumor activity of FWGE has been reported in some cancers, its action on OTSCC has been investigated only with one oral tongue cell line (SCC-4) *In Vitro* (19). The purpose of present study is to evaluate the effect of FWGE on three additional OTSCC cell lines with different invasive properties.

Materials and Methods

Cell Lines

The three human OTSCC cell lines HSC-3 (Japanese Collection of Research Bioresources (JCRB), JCRB0623), SCC-25 (ATCC, CRL-1628) and SAS (JCRB Cell Bank, JCRB0260) were routinely cultured in 1:1 Dulbeccós Modified Eagle Medium (DMEM):Ham´s Nutrient Mixture F-12 (Gibco, Carlsbad, CA, USA), supplemented with 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml ascorbic acid, 250 ng/ml amphotericin B and 0.4 ng/ml hydrocortisone (all from Sigma-Aldrich, St Louis, MO, USA). All cell lines were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and passaged routinely using trypsin-EDTA (Sigma- Aldrich).

Drugs and Chemicals

FWGE was donated by American BioScience Inc. under form of Fermented Wheat Germ Extract – Super concentrate (FWGE-SC®), with the trademark of Metatrol®. The solution was prepared, considering the proportion indicated by the manufacturer (41 mg of FWGE-SC®= 5500 mg of FWGE). For each experiment, a fresh stock solution containing 100 mg/ml FWGE was prepared to serum free DMEM/F-12 media with 0.5% lactalbumin (Sigma-Aldrich), vortexed, centrifuged and passed through a 0.22 µm filter.

Cell Viability Assay

HSC-3 and SCC-25 cell lines were seeded into 96-well flat-bottom tissue plates with density of 5×10^3 cells per well and SAS cells with density of 2×10^3 cells. After 24 h of incubation at 37 °C and 5% CO_2, the cells were treated with different concentrations of FWGE (2, 5 and 10 mg/ml) and control cells were treated with normal media mixed with lactalbumin medium (9:1). Cell growth was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich) (20) according to the manufacturer's protocol at 24, 48 and 72 h of incubation. Absorbance was measured at the wavelength of 550 nm with Victor 3 V multilabel reader (Perkin Elmer). Each experiment was performed in triplicate and repeated three times.

3D Tumor Spheroid Invasion Assay

Spheroids were generated for three cell lines (HSC-3, SAS, SCC-25) following the protocol described by Naakka et al. (21) Briefly, the cells were seeded in a complete growth medium at a cell density of 1×10^3 cells/well into ultra-low attachment (ULA) 96-well round bottom plates (Corning) and incubated at 37 °C for 4 day. After visual confirmation, spheroids were embedded in 50 µl gel containing 0.5 mg/ml Myogel (22), 0.5 mg/ml of Fibrinogen (Merck), 0.3 U/ml Thrombin (Sigma-Aldrich) and 33.3 µg/ml of Aprotinin (Sigma-Aldrich). The plate was then transferred to the incubator at 37 °C for 30 min in order to allow gel to solidify. 100 µl of complete culture medium containing different concentrations (2, 5 and 10 mg/ml) of FWGE were gently added on top of gel. Control wells were added with normal medium combined with lactalbumin (9:1). Spheroids were imaged at 0 h and, after 2, 3, 4, 7, 9 and 11 day of incubation, using a Nikon Eclipse TS100 inverted light microscope, with 4x objective magnification, connected to a Canon PowerShot S50 camera. Fiji software (23) was used to measure the area covered by spheroids. The change in spheroid area at each time point compared with the area at 0 h was calculated. Each experiment was performed in triplicate and repeated three times.

Transwell Invasion Assays

Transwell migration and invasion assays were performed in 6.5 mm inserts with an 8 µm pore size (Corning). For invasion assays, membranes were coated with 50 µl of MyoGel (22,24) (2.4 mg/ml), an extracellular matrix developed in laboratory of University of Oulu solidified with 0.8 mg/ml type I collagen from rat tail (Corning) in serum free medium. The HSC-3 and SAS cells were seeded into the upper chamber with a density of 70,000 cells, diluted in

100 µl medium containing 0.5% of lactalbumin instead of FBS. Then, 100 µl of medium containing indicated amounts of FWGE suspended in lactalbumin medium was added on top of the cells. As a chemoattractant, 500 µl of medium supplemented with 10% FBS was used in the lower chamber.

The Transwell plates were incubated at 37 °C for 48 h for migration assay and for 72 h for invasion assay. Then, the cells were fixed in 4% neutral buffered formaldehyde for 1 h and washed once with PBS. Cells were stained with 1% Toluidine Blue in 1% Borax for 10 min at room temperature and washed several times with deionized water. Then, non-invaded cells on the upper surface of the filter were carefully removed with a wet cotton swab. Toluidine blue stain was eluted with 1% SDS and absorbance was measured at 650 nm using a Victor2 Microplate Reader (Perkin Elmer Wallac) (22). All the experiments have been performed in triplicate and repeated from three to five times. The average of the results is reported.

Treatment with Chemotherapeutic Drugs Alone and in Combination with FWGE

Cisplatin (Cis) was dissolved in culture medium, while 0,1% DMSO was used to dissolve 5-fluorouracil (5-FU). Stock solutions were then diluted in culture medium to final concentration of 0.5 µg/ml Cis or 5-FU. Combined chemotherapeutic treatment was carried out by mixing Cis and 5-FU at 0.1 µg/ml and 0.25 µg/ml final concentration, respectively. The concentration of chemotherapeutics was chosen based on previous experiments testing a range of concentrations from 0,1 µg/mL to 20 µg/mL, for both single drugs and combinations of them (data not shown). FWGE was used with concentration of 5 and 10 mg/ml alone or in combination with Cis, 5-FU or both. For F-FU tests, control cells were treated with 0.1% DMSO.

HSC-3 and SAS cells were seeded in 96-well plates, at a density of 2×10^3 cells/well. Cells were allowed to attach overnight and then incubated with compounds, or with DMSO only, for 24, 48 and 72 h, after which cell viability was evaluated by MTT assay as described above. All experiments were performed in triplicate and repeated three times.

Statistical Analysis

Analysis of data were performed using IBM SPSS Statistics Version 25 (SPSS, Inc.) and GraphPad Prism Version 6.01 (GraphPad Software, Inc.). Data distribution was evaluated using Kolmogorov-Smirnov and Shapiro-Wilk tests. ANOVA and Kruskal-Wallis tests were applied for multiple comparisons, while Dunn and Tukey tests were used for post-hoc analysis.

Results

FWGE Reduces OTSCC Cell Viability in Dose-Dependent Manner

To assess the effects of FWGE on cell viability of OTSCC cell lines, cells were treated with different concentrations of the compound and analyzed by MTT assay was performed at time points 24, 48 and 72 h. The concentrations of FWGE were decided based on the previous studies (3,19,25,26). An initial experiment of cell viability was carried out by testing three concentrations (0.1, 1 and 10 mg/ml). Since doses 0.1 and 1 mg/ml had no effect on OTSCC cell viability, two additional concentrations (2 and 5 mg/mg) were explored. Based on the response data, the concentrations of 2, 5 and 10 mg/ml were chosen for further experiments. The highly invasive HSC-3, and less invasive SCC-25 cells treated with 5 and 10 mg/ml FWGE showed a significant ($p < 0.001$) reduction in their viability compared to untreated cells (Figure 1(a, c), Supplementary Table 1). Concerning SAS, a significant ($p < 0.01$) cytotoxicity was detected only upon treatment with the highest concentration (10 mg/ml) (Figure 1(b), Supplementary Table 1),

Cell Movement is Differentially Affected by FWGE in OTSCC Cell Lines

3D Tumor Spheroid Invasion Assay

The invasion of spheroids formed by the OTSCC cell lines was tested in human leiomyoma-based extracellular matrix, Myogel (21). The invasion area of spheroids was compared between untreated cells, and cells treated with 2, 5 and 10 mg/ml of FWGE at different time points. On day 2 and four HSC-3 cells treated with 5 and 10 mg/ml FWGE invaded significantly less than controls (Figures 2(a) and 3(a)). On day 7, only 10 mg/ml FWGE was significantly effective (Figures 2(a) and 3(a), Supplementary Table 2). SCC-25 cells, treated with 5 and 10 mg/ml FWGE invaded significantly less on day 7, 9 and 11 (Figures 2(b) and 3(b), Supplementary Table 2), whereas with SAS cells, only the highest concentration of FWGE (10 mg/ml) had a significant reduction on spheroid invasion on day 2, 3 and 4 (Figures 2(c) and 3(c), Supplementary Table 2).

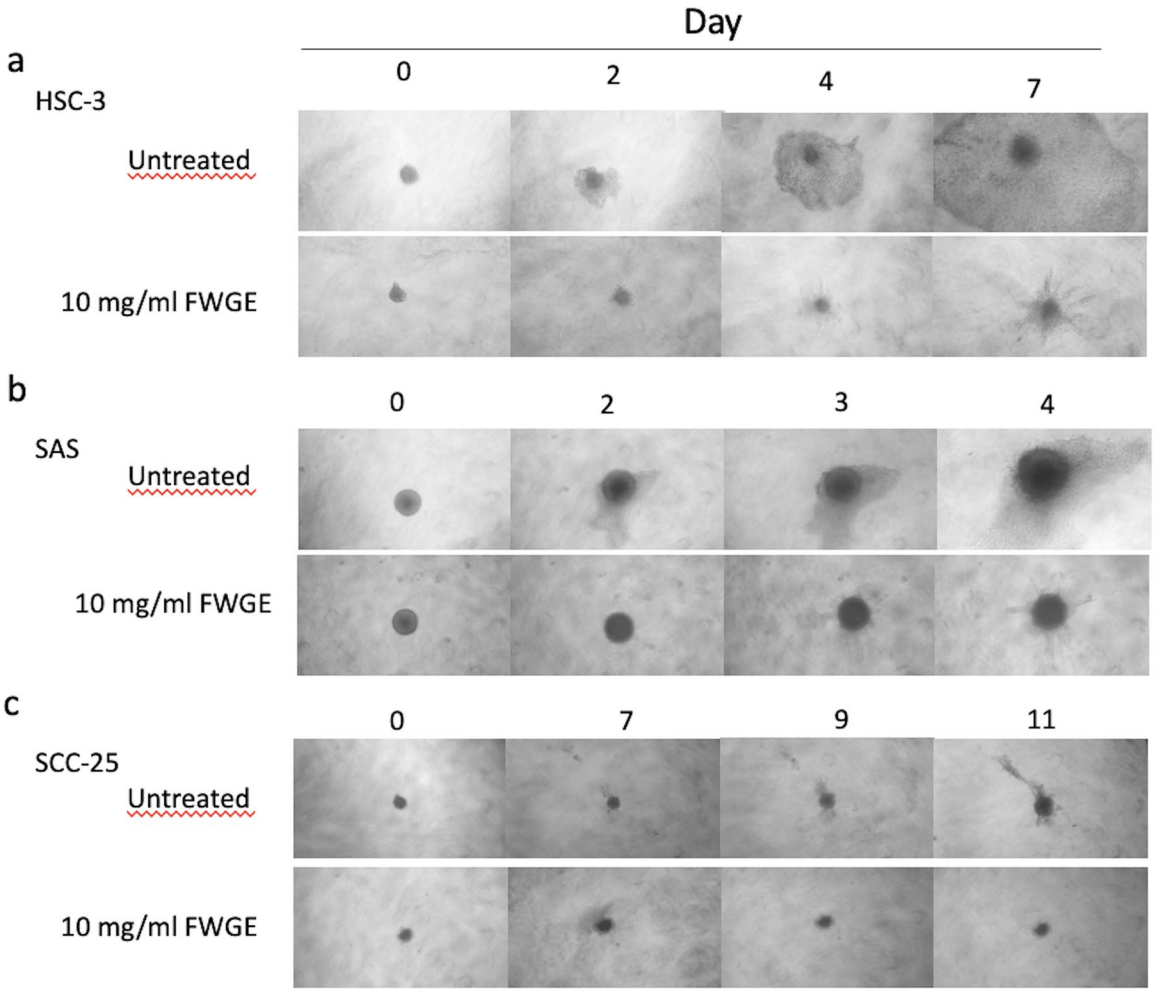

Figure 1. Effect of FWGE treatment (2, 5 and 10 mg/ml) on HSC-3 (a), SAS (b), SCC-25 (c) cell viability assessed by MTT assay at 24, 48 and 72 h,. The optical density (OD) was measured at 570 nm. Columns and bars represent mean and standard deviation (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

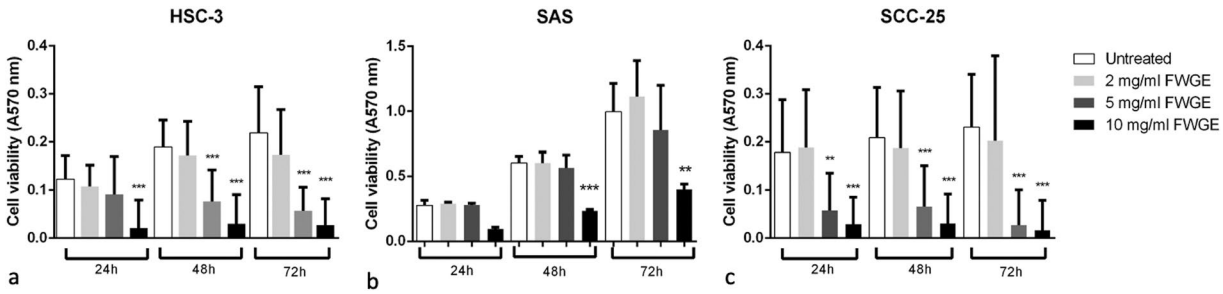

Figure 2. Control and 10 mg/ml FWGE treated spheroids of HSC-3 (a), SAS (b) and SCC-25 (c) cells imaged at 0 h and after indicated days, using a Nikon Eclipse TS100 inverted light microscope, with 4x objective magnification, connected to a Canon PowerShot S50 camera.

Transwell Migration and Invasion Assay

Transwell assay was used to evaluate whether treatment with FWGE could affect OTSCC cells vertical migration through a semipermeable membrane and invasion through Myogel. Here we compared only two cell lines, HSC-3, and SAS, to see if those differences seen in viability and spheroid assay are seen also in vertical migration and invasion.

HSC-3 cells treated with 5 and 10 mg/ml FWGE significantly ($p < 0.01$) reduced their migration and invasion(Figure 4(a)), but in case of SAS cells, only the highest concentration of FWGE (10 mg/ml)

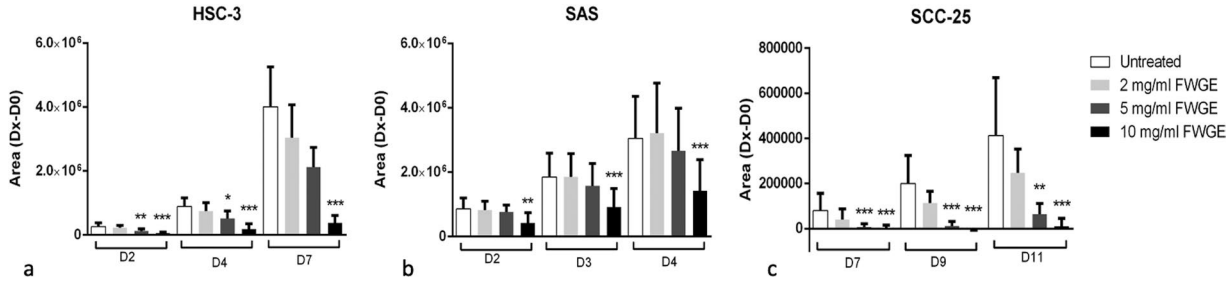

Figure 3. Spheroid invasion assay of HSC-3 (a), SAS (b) and SCC-25 (c) cells. The columns represent the difference in the spheroid area between a specific timepoint (day 2, 3, 4, 7, 9 or 11) and the beginning of the analysis (day 0). Columns and bars represent mean and standard deviation (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

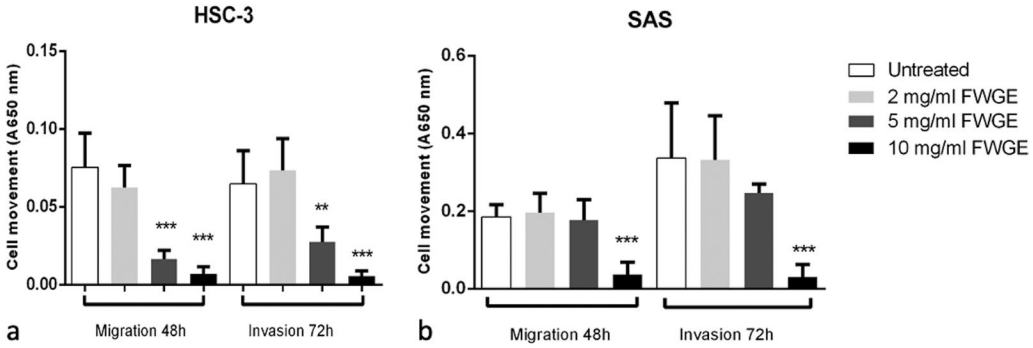

Figure 4. Transwell migration and invasion of 2, 5 and 10 mg/ml FWGE treated of HSC-3 (a) and SAS (b) cells. The migrated (48 h)/invaded (72 h) cells were stained with Toluidine Blue, the stain was dissolved in 1% SDS solution and the absorbance at 650 nm was measured. Columns and bars represent mean and standard deviation (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

significantly ($p < 0.01$) inhibited their migration and invasion through Transwell (Figure 4(b), Supplementary Table 3).

Combination of Chemotherapeutic Drugs with FWGE Significantly Enhanced the anticancer Effects

To first test the effect of DMSO on cell viability, HSC-3 and SAS cells were grown in culture medium containing DMSO concentrations ranging from 0.05 to 1.25% and cell viability was tested with MTT assay. DMSO concentration 0.1% showed no significant effect on cell viability ($p > 0.05$) when compared with normal culture medium (data not shown), thereby this concentration was used as a solvent for 5-fluorouracil in further experiments.

The comparison between chemotherapeutic drugs revealed that the treatment with Cisplatin (0.5 µg/mL) inhibited HSC-3 cell viability more than 5-fluoruracil (0.5 µg/mL) alone, or by combined drugs (cis 0.1 µg/ml + 5-FU 0.25 µg/mL) ($p < 0.05$). When cisplatin, 5-fluorouracil or both were used in combination with FWGE at high concentration (10 mg/ml), cell viability of HSC-3 and SAS cells was further reduced compared to cells treated only with chemotherapeutic drugs (Figure 5(a and b), Supplementary Table 4).

Discussion

Several natural compounds have been analyzed to explore their potential anticancer properties for disease prevention (27) or importantly, also to improve the current cancer treatment and reduce the side effects of common anticancer therapies (28,29). The extract of wheat germ fermented by *Saccharomyces cerevisiae* contains a high amount of benzoquinone that exerts an antiproliferative and cytotoxic activity against several tumor cells (3,16). In this study, the effects of FWGE on highly aggressive and metastatic HSC-3, invasive SAS, as well as on less invasive SCC-25 OTSCC cell lines (30–34) were investigated to see if FWGE affects their viability, migration and invasion. Finally, the combination of FWGE with commonly used chemotherapeutic drugs, cisplatin and 5-fluorouracil, on cancer cell viability was analyzed.

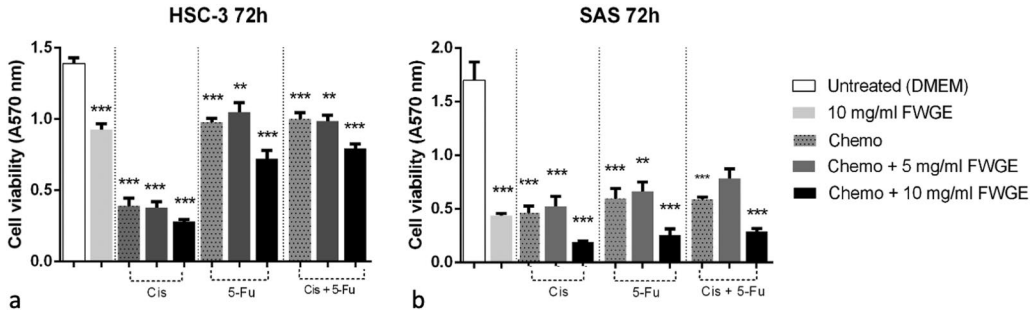

Figure 5. Effect of chemotherapeutic drugs and FWGE, used alone or in combination, on HSC-3 (a) and SAS (b) cell viability assessed by MTT assay at 72 h, after treatment. The optical density (OD) was measured at 570 nm. Columns and bars represent mean and standard deviation (SD). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with. Cis: Cisplatin; 5-Fu: 5-Fluorouracil; FWGE: Fermented Wheat Germ Extract.

Based on the viability assay, all three OTSCC cell lines revealed to be sensitive, albeit to varying degrees, to the treatment with FWGE. Although the concentrations 5 and 10 mg/ml FWGE reduced significantly cell viability in HSC-3 and SCC-25 cells, surprisingly, in case of SAS, no effect was seen with 5 mg/ml, but the highest concentration (10 mg/ml) reduced also their viability. Yang et al. have evaluated the action of Avemar® on OTSCC (SCC-4) cell line. They had similar results, even though the inhibitory effect was reached with lower concentration of FWGE (0.1–1.6 mg/ml) (19). Such result may be due to the different product used in the experiment, but also be related to the different reactivity of OTSCC cell lines to this compound. This is supported by our results with three OTSCC cell lines with different aggressiveness.

Spheroid assay was also conducted with all three cell lines, and here the invasive capacity of all the cell lines was inhibited by the highest concentration (10 mg/ml) of FWGE, and no effect was observed using the lowest concentration (2 mg/ml). Again, in SAS the concentration of 5 mg/ml did not produce any significant effect, but the invasive capacity of SCC-25 was strongly inhibited by this concentration. In addition, HSC-3 were sensitive to the 5 mg/ml treatment fot the first 4 day, but no longer on day 7. In case of Transwell vertical migration, the same difference between the two cell lines was present: HSC-3 cells with the concentrations of 5 and 10 mg/ml FWGE migrated significantly less than controls, whereas with SAS cells only 10 mg/ml FWGE significantly inhibited invasion. For the Transwell invasion, we used the Myogel (22,24) matrix as in the spheroid invasion assay, since we have demonstrated that Myogel contains human tumor derived compounds, such as MMP-2, tenascin-C and collagen types XII and XIV, unlike mouse tumor derived commercial matrix, Matrigel®. Furthermore, myoma tissue shows similar characteristics with the metastatic niche (35), and it has shown to be suitable also for preclinical drug testing (36). Also in Myogel invasion, FWGE treated HSC-3 and SAS cells behaved similar as in spheroid and migration assays. This confirms the different aggressiveness of these cells, observed already in previous studies (37,38), and demonstrates that oral tongue carcinoma cells display variable phenotypes and respond differently on drugs and natural compounds, like FWGE. Our results are in line with some previously reported data, showing that Avemar® inhibits OTSCC cell migration and invasion (19).

Cisplatin and 5-fluorouracil are chemotherapeutics used for the treatment of advanced oral cancer (39,40). Despite their wide use, the problem is that their therapeutic action is accompanied by multiple side effects and cancers frequently show resistance to the treatment (41,42). Moreover, additional compounds have been proposed to enhance chemosensitivity of OSCC toward cisplatin and 5-fluorouracil (43,44). Our results demonstrated that FWGE is able to enhance the anti-tumor effects of these chemotherapeutics in OTSCC cells. Indeed, combined treatment with chemotherapeutics and FWGE at high concentration (10 mg/ml) exerted significantly higher inhibitory effects on cancer cell viability, compared to treatment with chemotherapy or FWGE alone. However, the combinations of chemotherapy drugs with low concentration FWGE (5 mg/ml) had no effect compared with the chemotherapy alone or even slightly promoted cell viability, although not significantly. A similar effect has been noticed by Mueller et al. (3) in the treatment of human colon cancer cell lines HT29 and HCT-8: if the FWGE preceded 5-FU treatment, an antagonism trend between compounds was observed. Regarding the other combinations with higher FWGE concentrations, a

synergistic effect was observed in terms of cell viability inhibition, similarly to results reported in other studies (3).

Taken together, albeit with different efficacy depending on the OTSCC cell line, FWGE showed anticancer effects and, especially in high concentrations, its association with chemotherapeutics strengthens their inhibitory power. Further studies, including *In Vivo* experiments, are necessary to identify the optimal combinations of chemotherapy with natural compounds to provide optimal drug efficacy with lower toxicity on the patients.

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Disclosure of Interest

All authors declare that there are no competing interests, and no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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Authors' Contributions

KZ, MR, DS conceived and designed the experiments, performed the experiments, analyzed the data and wrote the paper. TS, LLM, ME conceived and designed the experiments, contributed reagents and wrote the paper. AS, GT performed the experiments, analyzed data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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