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# Preliminary cytotoxicity assessment of Jaftex vs. chlorhexidine mouthwashes on human gingival fibroblasts and oral squamous cell carcinoma

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## Abstract

**Background and objectives** Jaftex is a new herbal mouthwash that consists of the aqueous extract of Jaft oak, thymus, and the aqueous extract of Satureja Bachtiarica. Its cytotoxicity remains unknown.

**Methods** The sample size was determined as 180 specimens as 3 specimens for each of the 60 different combinations of 2 mouthwashes, 2 cell lines, 3 exposure times (24, 48, 72 h), and 5 mouthwash doses. Also, there were 36 positive and negative controls. Jaftex 1% and chlorhexidine 0.2% were sterilized and diluted 1:2, 1:4, 1:8, 1:16, and 1:32 of the original concentration. Each drug dilution was made available in triplicate. OCC and HuGu cells were exposed to these concentrations at 37 °C for 24 h ($n=60$), 48 h ($n=60$), and 72 h ($n=60$). The optical densities (ODs) were measured for each of the 180 experimental specimens and 36 positive and negative specimens. Also the IC50 was calculated. The results were analyzed ($\alpha=0.05$).

**Results** The viability was much higher in the case of Jaftex compared with CHX. The viability was higher in OCC compared to HuGu. All pairwise comparisons between each two dosages were significant (all $P$ values $\leq 0.013$) in a way that by reducing the dosage, the viability increased.

**Conclusions** The toxicity of Jaftex was significantly lower than that of chlorhexidine. Also, the toxicity of both mouthwashes against the HuGu cell line was higher than the OCC-18 cell line. During 24, 48, and 72 h, the mouthwash toxicity increased significantly.

**Keywords** Biocompatibility, Viability, Oral cancer cells (OCC-18), Oak jaft, Zataria multiflora (Thymus), Satureja bachtiarica

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## Introduction

Mouthwashes are widely used to maintain oral and dental hygiene. These products have synthetic and natural compounds and are effective with mechanisms such as eliminating microorganisms, inhibiting microbial growth, or inhibiting the function of microbial enzymes [1–7]. This antimicrobial function is used to control plaque, treat gingivitis, and also before oral and periodontal surgeries such as dental extraction and implant placement [1–7].

When choosing an appropriate mouthrinse, besides its antimicrobial efficacy, its biocompatibility should also be taken into account [7]. Important cells such as granulocytes, macrophages, fibroblasts, epithelial cells and osteoblasts are affected in the healing process of oral ulcers [8]. Cytotoxicity of mouthwashes in wound healing occurs when mouthwashes come into direct contact with the cells responsible for wound healing, and inhibit wound healing by exerting toxic effects on them [9].

Among all available mouthwashes, chlorhexidine is considered as the gold standard [10]. Chlorhexidine is a well-known antimicrobial substance and has a wide range of activity against aerobic and anaerobic Gram-negative and Gram-positive bacteria, yeasts, and some viruses such as HIV [7, 11]. However, its certain side effects are notable, such as changes in taste, change in the color of the teeth and oral mucosa, and dry mouth [12]. The toxic effect of chlorhexidine on human cells has been shown [13, 14]. Chlorhexidine in a commercial concentration of 0.12% or even lower concentrations, if used shortly before closing the surgical site wound, can have toxic effects on gingival fibroblast cells and adverse effects on wound healing [7, 13].

Recently, the use of herbal mouthwashes has received attention as they have natural compounds that are physiologically compatible with the body and are better in terms of toxicity than chlorhexidine [15, 16]. Jaftex is a new herbal mouthwash that consists of the aqueous extract of Jaft oak fruit as the base of the composition, the aqueous extract of Zataria Multiflora (Thymus) and the aqueous extract of Satureja Bachtiarica. The plant extract is prepared from the Medicinal Plant Growth Center of Jundishapur University of Medical Sciences, Ahvaz [1–5, 17]. As stated above, it is formed of different herbal components: Oak is one of the medicinal plants for which several therapeutic properties have been mentioned. The thin shell that covers the oak fruit is called Jaft, which has medical and industrial applications [18]. Jaft has a considerable efficacy in the treatment of bacterial and viral diseases such as oral lesions [15]. Antimicrobial properties of different species of Iranian oak have been mentioned in different studies [19–21]. Zataria Multiflora belongs to the Lamiaceae family and is found only in Iran, Pakistan and Afghanistan. Among other effects, it is an antiseptic compound [22]. Satureja Bachtiarica belongs to the Lamiaceae family, and is a native plant and one of the most important species among twelve Iranian savory species. This plant grows in the southern regions of Iran [23], and has anti-spasmodic, anti-diarrheal, antioxidant, sedative, and antimicrobial properties [24–28].

Now that the effectiveness of the Jaftex mouthwash against microorganisms has been shown in recent studies [1–5, 17], it is needed to evaluate its other aspects necessary for biomaterials, including its staining potential and biocompatibility. This is even more important since the antimicrobial effect of Jaftex is not as better than chlorhexidine [17]. Therefore, it should offer more advantages in other areas in order to be considered a viable option. These include its biocompatibility. However, its cytotoxicity has not been investigated in any study. Therefore, this research was conducted for the first time. Its goal was to compare the cytotoxicity of different doses and exposure times of Jaftex versus chlorhexidine mouthwasheses on oral cancer cell lines (oral squamous cell carcinoma, OCC-18 or IBRC C10995) versus human gingival fibroblasts (HuGu) cell lines (IBRC C10459)). These cancer cells were chosen because cytotoxicity, although an adverse effect of the mouthwash for normal tissues, might be considered useful as a local anti-cancer treatment [29]. The null hypotheses were the absence of any difference between mouthwashes, among different dosages of mouthwashes, among different exposure times, and between cell lines in terms of cell viability.

## Materials and methods

This explorative, experimental, laboratory study was performed in the Cellular and Molecular Faculty of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Due to the investigation of cytotoxicity on the cells purchased from the National Bank of Genetic and Biological Reserves of Iran and the absence of any human or animal sampling or intervention, there was no ethical concerns regarding the methodology. The protocol and ethics of this in vitro study were approved by the Medical Research Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (ethics code: IR.AJUMS.REC.1399.451). All methods were performed in accordance with the relevant guidelines and regulations (including the Declaration of Helsinki); all experimental protocols were approved by the Institutional Review Board of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

The sample size was determined as 180 specimens as 3 specimens for each of the 60 different combinations of 2 mouthwashes × 2 cell lines (OCC-18 vs. HuGu) × 3 exposure times (24 h, 48 h, 72 h) × 5 mouthwash doses. There were **also** 36 positive and negative controls (detailed below).

### Cell lines and cell culture

The tools in use were as follows: RPMI, IdeZist, Iran; 3 ml disposable pipette, SPL, Iran; Blue sampler head, JET, Iran; Crystal sampler head, JET, Iran; Yellow sampler head, JET, Iran; Falcon tube 50 cc, SPL, Iran; Falcon tube 15 cc, SPL, Iran; Oral tumor fibroblast cell line, Pasteur Institute, Iran; 96-well Plate, SPL, Iran; Filter flask, SPL, Iran; PBS, IdeZist, Iran; FBS, IdeZist, Iran; MTT, Sigma, Iran; 1.5 cc microtube, SPL, Iran; PEN/STREP Antibiotic, IdeZist, Iran; Trypsin, IdeZist, Iran; Trypan Blue, IdeZist, Iran; DMSO, IdeZist, Iran.

Two cell lines were used in this study: Oral tumor cell line (oral squamous cell carcinoma [OCC-18] or IBRC C10995) and human gingival fibroblast cell line (normal cell, HuGu or IBRC C10459) were purchased from the National Center of Genetic and Biological Resources of Iran. Both cell types had full catalogues and were already verified at that center in terms of purity and identity, using surface antigens and PCR. Then they were immersed in RPMI1640 (Roswell Park Memorial Institute) medium containing 10% heat-inactivated fetal calf serum (FCS), 25 µg/ml gentamicin, 2.5 µg/ml amphotericin B, and 0.5 mg/ml collagenase. After incubation for 18 h at 37 °C and 5% $CO_2$, the pieces were separated by gentle pipetting. Then they were centrifuged at 1500 rpm, suspended in a new complete culture medium, and finally transferred to a 25 cm cell culture flask. After expanding the culture to about 75–80% of the surface of the flask, the cells were trypsinized and transferred to larger flasks to reproduce more. A number of vials were preserved in the third or fourth cell passage and were used after thawing.

### Cytotoxicity assessment

The MTT method was used for this purpose [7]. The Jaftex 1% mouthwash (with the patent number IR139350140003008118) was obtained from the medicinal plant growth center of Jundishapur University of Medical Sciences, Ahvaz. Commercial chlorhexidine 0.2% mouthwash was obtained from a pharmacy. They were sterilized by filtration using a 0.22 micrometer filter and then stored at 4 °C.

Different concentrations of medications were prepared by serial dilution in sterile plastic centrifuge tubes. These concentrations for both mouthwashes were as follows: the initial concentration of each mouthwash (1% for Jaftex and 0.2% for chlorhexidine) was diluted to the following ones: 1:2, 1:4, 1:8, 1:16, and 1:32 of the original concentration.

In the exponential growth phase, the culture media were diluted with trypsin until a suspension of $1 \times 106$ cells per milliliter was obtained. Afterwards, 100 µl of cell suspension was added to each centrifuge tube containing 2 ml of drug solution. One of the tubes containing only the suspension in the complete culture medium was used as a control to check cell survival. Then the tubes were incubated for 1 h at 37 °C in a humid atmosphere containing 5% $CO_2$. After drug exposure, cells were washed twice with 10 ml of culture medium to remove any remaining drug and then resuspended in 2 ml of fresh complete culture medium. Then 100 µL of each suspension was added to each well of a 96-well plate. Each drug dilution was made available in triplicate.

Three wells containing only complete culture medium were used as negative controls to reduce the non-specific color. The plates were then incubated at 37 °C in a humid environment with 5% $CO_2$ for 24 h (60 specimens), 48 h (60 additional specimens), and 72 h (60 more specimens). After incubation, 20 µL of 5 mg/ml MTT solution was added to each well; and the plate was incubated for another 3–4 h. After incubation, the culture medium was removed from the wells by gentle aspiration. Then 100 µL of Dimethyl Sulfoxide was added to the wells to dissolve the formazan crystals. Finally, the plates were read on a microplate reader at a wavelength of 492 nm and a reference of 630 nm [30].

The optical densities (ODs) were measured for each of the 180 experimental specimens and 36 positive control (DMSO 10%) and negative control (untreated cells) specimens. Each of the mouthwashes (CHX or Jaftex) in each of the 3 incubation times (24, 48, 72 h) had 30 experimental specimens: 15 per OCC and 15 per HuGu. Per each of these cell lines (oral cancer cell [OCC-18] or human gum fibroblast cells [HuGu]), each mouthwash had 3 specimens per each of the 5 different concentrations (1:2, 1:4, 1:8, 1:16, and 1:32 of the initial concentration). These two-fold dilution factors are standard practice in pharmacology. At each time interval per cell line (for both mouthwashes combined), there were also 3 positive controls and 3 negative controls. OCC-18 was selected because it is the most common oral cancer. HuGu was selected to represent gingival cells.

### Outcome

#### *Cell viability*

The 3 positive controls per each time interval per cell line were averaged. The average of these 3 positive controls were used to calculate the percent of viability for that particular cell line in that particular time interval (regardless of the types of mouthwashes or their concentrations). The formula used for viability calculation was the OD of each experimental specimen × 100 / the average OD of the corresponding 3 positive controls.

#### *IC50*

The most popular and useful indicator of a drug's effectiveness is its half-maximal inhibitory concentration also known as IC50. In pharmacological research, IC50

**Table 1** Descriptive statistics and 95% CIs for OD values and viabilities (%) in the sample and different groups

| Variable | Parameter | Group | *N* | Mean | SD | 95% CI | | Min | Max |
|---|---|---|---|---|---|---|---|---|---|
| OD | – | All | 180 | 0.0969 | 0.0936 | 0.0831 | 0.1107 | 0.0010 | 0.3250 |
| | Exposure time | 24 h | 60 | 0.1149 | 0.1059 | 0.0875 | 0.1423 | 0.0030 | 0.3250 |
| | | 48 h | 60 | 0.0970 | 0.0943 | 0.0726 | 0.1213 | 0.0010 | 0.3140 |
| | | 72 h | 60 | 0.0789 | 0.0762 | 0.0592 | 0.0986 | 0.0010 | 0.2670 |
| | Cell line | HuGu | 90 | 0.0864 | 0.0827 | 0.0690 | 0.1037 | 0.0010 | 0.3050 |
| | | OCC-18 | 90 | 0.1074 | 0.1027 | 0.0859 | 0.1290 | 0.0010 | 0.3250 |
| | Mouthwash | CHX | 90 | 0.0590 | 0.0720 | 0.0439 | 0.0741 | 0.0010 | 0.2380 |
| | | Jaftex | 90 | 0.1348 | 0.0976 | 0.1144 | 0.1553 | 0.0070 | 0.3250 |
| | Dosage | 1:32 | 36 | 0.2218 | 0.0587 | 0.2019 | 0.2416 | 0.1220 | 0.3250 |
| | | 1:16 | 36 | 0.1516 | 0.0756 | 0.1260 | 0.1772 | 0.0510 | 0.3150 |
| | | 1:8 | 36 | 0.0619 | 0.0559 | 0.0430 | 0.0808 | 0.0030 | 0.1910 |
| | | 1:4 | 36 | 0.0319 | 0.0299 | 0.0218 | 0.0420 | 0.0010 | 0.0920 |
| | | 1:2 | 36 | 0.0174 | 0.0207 | 0.0104 | 0.0244 | 0.0010 | 0.0810 |
| Viability (%) | – | All | 180 | 10.2054 | 9.8897 | 8.7508 | 11.6600 | 0.1000 | 34.5000 |
| | Exposure time | 24 h | 60 | 12.2180 | 11.2644 | 9.3081 | 15.1279 | 0.3200 | 34.5000 |
| | | 48 h | 60 | 10.1917 | 9.9341 | 7.6254 | 12.7579 | 0.1000 | 33.1000 |
| | | 72 h | 60 | 8.2067 | 7.9360 | 6.1566 | 10.2568 | 0.1000 | 27.7000 |
| | Cell line | HuGu | 90 | 9.1042 | 8.7529 | 7.2710 | 10.9375 | 0.1000 | 32.5000 |
| | | OCC-18 | 90 | 11.3067 | 10.8465 | 9.0349 | 13.5784 | 0.1000 | 34.5000 |
| | Mouthwash | CHX | 90 | 6.2038 | 7.5991 | 4.6122 | 7.7954 | 0.1000 | 25.3000 |
| | | Jaftex | 90 | 14.2071 | 10.3237 | 12.0449 | 16.3694 | 0.7000 | 34.5000 |
| | Dosage | 1:32 | 36 | 23.3508 | 6.2548 | 21.2345 | 25.4671 | 12.8000 | 34.5000 |
| | | 1:16 | 36 | 15.9836 | 8.0409 | 13.2629 | 18.7043 | 5.3000 | 33.4000 |
| | | 1:8 | 36 | 6.5153 | 5.9120 | 4.5149 | 8.5156 | 0.3000 | 20.3000 |
| | | 1:4 | 36 | 3.3478 | 3.1632 | 2.2775 | 4.4180 | 0.1000 | 9.8000 |
| | | 1:2 | 36 | 1.8297 | 2.2045 | 1.0838 | 2.5756 | 0.1000 | 8.6000 |

**OD**, optical density; **MW**, mouthwash; **SD**, standard deviation; **CI**, confidence interval; **Min**, minimum; **Max**, maximum; **HuGu**, human gingival fibroblast; **CHX**, chlorhexidine; **OCC-18**, oral cancer cell (oral squamous cell carcinoma)

provides a measure of an antagonist medication's potency by indicating the amount of drug required to halt a biological process by half [31].

In this study, the IC50 was measured as follows: The dose of each drug (i.e., Jaftex and chlorhexidine) was calculated by multiplying its original dose with the dilution factor. For Jaftex, the original dose was 1% or 0.01 and the dilution factors were 1:2, 1:4, 1:8, 1:16, and 1:32. Thus, the drug doses for Jaftex were 0.005, 0.0025, 0.00125, 0.000625, and 0.000313. For chlorhexidine, the original dose was 0.2% or 0.002. With the same dilution factors mentioned above, the drug doses for chlorhexidine were 0.001, 0.0005, 0.00025, 0.000125, and 0.000063.

The 3 optical densities (ODs) corresponding to each dose of Jaftex were divided by the respective control optical density (OD) in order to normalize the OD values, also called the viability values. These normalized ODs along with their respective drug dose were used for calculating the IC50.

A 4-parameter logistic regression equation including the Hill coefficient was used to calculate the IC50 from these values: At each of the 3 time intervals (24 h, 48 h, and 72 h), the dose of the drug was considered the independent variable, or X, and the 3 normalized OD values (3 viability values or 3 response values) were considered the 3 repeats of the dependent variable, or Y. The X and Y were fitted to the 4-parametric logistic regression equation involving the Hill coefficient, using the log(inhibitor) vs. normalized response within Prism 10 software (GraphPad, La Jolla, California, United States).

### Statistical analyses

Descriptive statistics and 95% confidence intervals (CI) were calculated for ODs and viabilities at each concentration of each mouthwash per each cell line at each interval. A full-factorial 4-way analysis of variance (ANOVA) was used to test the effects of each of the variables (Time, Cell lines, Mouthwashes, and Dose) and their interactions on viability. A Bonferroni post hoc test was used for pairwise comparisons following significant ANOVA results. The software in use for viability was SPSS 26 (IBM, Armonk, NY, United States). The level of significance was set at 0.05. For IC50, Prism 10 was used (GraphPad, La Jolla, California, United States).

**Table 2** Descriptive statistics for OD values and viabilities (%) in all subgroups

| | | | | | OD | | | | Vitality (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time** | **Cell** | **MW** | **Dosage** | ***N*** | **Mean** | **SD** | **Min** | **Max** | **Mean** | **SD** | **Min** | **Max** |
| 24 h | HuGu | CHX | 1:32 | 3 | 0.1697 | 0.0235 | 0.1510 | 0.1960 | 18.0800 | 2.5029 | 16.0900 | 20.8900 |
| | | | 1:16 | 3 | 0.1000 | 0.0095 | 0.0900 | 0.1090 | 10.6567 | 1.0189 | 9.5900 | 11.6200 |
| | | | 1:8 | 3 | 0.0113 | 0.0068 | 0.0060 | 0.0190 | 1.2067 | 0.7223 | 0.6400 | 2.0200 |
| | | | 1:4 | 3 | 0.0080 | 0.0036 | 0.0050 | 0.0120 | 0.8533 | 0.3855 | 0.5300 | 1.2800 |
| | | | 1:2 | 3 | 0.0073 | 0.0045 | 0.0030 | 0.0120 | 0.7833 | 0.4809 | 0.3200 | 1.2800 |
| | | | All | 15 | 0.0593 | 0.0686 | 0.0030 | 0.1960 | 6.3160 | 7.3084 | 0.3200 | 20.8900 |
| | | Jaftex | 1:32 | 3 | 0.2787 | 0.0327 | 0.2420 | 0.3050 | 29.6967 | 3.4884 | 25.7900 | 32.5000 |
| | | | 1:16 | 3 | 0.2353 | 0.0511 | 0.2010 | 0.2940 | 25.0800 | 5.4390 | 21.4200 | 31.3300 |
| | | | 1:8 | 3 | 0.1183 | 0.0205 | 0.1010 | 0.1410 | 12.6100 | 2.1913 | 10.7600 | 15.0300 |
| | | | 1:4 | 3 | 0.0590 | 0.0298 | 0.0340 | 0.0920 | 6.2867 | 3.1758 | 3.6200 | 9.8000 |
| | | | 1:2 | 3 | 0.0313 | 0.0344 | 0.0090 | 0.0710 | 3.3400 | 3.6729 | 0.9600 | 7.5700 |
| | | | All | 15 | 0.1445 | 0.1047 | 0.0090 | 0.3050 | 15.4027 | 11.1592 | 0.9600 | 32.5000 |
| | OCC-18 | CHX | 1:32 | 3 | 0.2207 | 0.0153 | 0.2090 | 0.2380 | 23.4333 | 1.6442 | 22.2000 | 25.3000 |
| | | | 1:16 | 3 | 0.1407 | 0.0146 | 0.1240 | 0.1510 | 14.9333 | 1.5144 | 13.2000 | 16.0000 |
| | | | 1:8 | 3 | 0.0120 | 0.0035 | 0.0080 | 0.0140 | 1.2667 | 0.4041 | 0.8000 | 1.5000 |
| | | | 1:4 | 3 | 0.0113 | 0.0059 | 0.0070 | 0.0180 | 1.2000 | 0.6245 | 0.7000 | 1.9000 |
| | | | 1:2 | 3 | 0.0057 | 0.0015 | 0.0040 | 0.0070 | 0.5667 | 0.1528 | 0.4000 | 0.7000 |
| | | | All | 15 | 0.0781 | 0.0910 | 0.0040 | 0.2380 | 8.2800 | 9.6704 | 0.4000 | 25.3000 |
| | | Jaftex | 1:32 | 3 | 0.3137 | 0.0103 | 0.3050 | 0.3250 | 33.3000 | 1.0817 | 32.4000 | 34.5000 |
| | | | 1:16 | 3 | 0.2987 | 0.0196 | 0.2770 | 0.3150 | 31.7000 | 2.0664 | 29.4000 | 33.4000 |
| | | | 1:8 | 3 | 0.1520 | 0.0354 | 0.1220 | 0.1910 | 16.1667 | 3.7448 | 13.0000 | 20.3000 |
| | | | 1:4 | 3 | 0.0730 | 0.0190 | 0.0540 | 0.0920 | 7.7333 | 2.0502 | 5.7000 | 9.8000 |
| | | | 1:2 | 3 | 0.0513 | 0.0263 | 0.0310 | 0.0810 | 5.4667 | 2.7791 | 3.3000 | 8.6000 |
| | | | All | 15 | 0.1777 | 0.1158 | 0.0310 | 0.3250 | 18.8733 | 12.2889 | 3.3000 | 34.5000 |

**Table 2** (continued)

| | | | | | OD | | | | Vitality (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time** | **Cell** | **MW** | **Dosage** | ***N*** | **Mean** | **SD** | **Min** | **Max** | **Mean** | **SD** | **Min** | **Max** |
| 48 h | HuGu | CHX | 1:32 | 3 | 0.1553 | 0.0300 | 0.1290 | 0.1880 | 16.2667 | 3.1533 | 13.5000 | 19.7000 |
| | | | 1:16 | 3 | 0.0940 | 0.0214 | 0.0700 | 0.1110 | 9.8333 | 2.2502 | 7.3000 | 11.6000 |
| | | | 1:8 | 3 | 0.0097 | 0.0021 | 0.0080 | 0.0120 | 1.0000 | 0.2646 | 0.8000 | 1.3000 |
| | | | 1:4 | 3 | 0.0057 | 0.0021 | 0.0040 | 0.0080 | 0.5667 | 0.2082 | 0.4000 | 0.8000 |
| | | | 1:2 | 3 | 0.0050 | 0.0040 | 0.0010 | 0.0090 | 0.5000 | 0.4000 | 0.1000 | 0.9000 |
| | | | All | 15 | 0.0539 | 0.0646 | 0.0010 | 0.1880 | 5.6333 | 6.7779 | 0.1000 | 19.7000 |
| | | Jaftex | 1:32 | 3 | 0.2347 | 0.0258 | 0.2070 | 0.2580 | 24.6333 | 2.7301 | 21.7000 | 27.1000 |
| | | | 1:16 | 3 | 0.1700 | 0.0289 | 0.1490 | 0.2030 | 17.8333 | 3.0436 | 15.6000 | 21.3000 |
| | | | 1:8 | 3 | 0.1043 | 0.0121 | 0.0930 | 0.1170 | 10.9667 | 1.2583 | 9.8000 | 12.3000 |
| | | | 1:4 | 3 | 0.0483 | 0.0310 | 0.0210 | 0.0820 | 5.0667 | 3.2517 | 2.2000 | 8.6000 |
| | | | 1:2 | 3 | 0.0130 | 0.0040 | 0.0090 | 0.0170 | 1.3667 | 0.4509 | 0.9000 | 1.8000 |
| | | | All | 15 | 0.1141 | 0.0854 | 0.0090 | 0.2580 | 11.9733 | 8.9668 | 0.9000 | 27.1000 |
| | OCC-18 | CHX | 1:32 | 3 | 0.2047 | 0.0096 | 0.1960 | 0.2150 | 21.6000 | 1.0149 | 20.7000 | 22.7000 |
| | | | 1:16 | 3 | 0.0880 | 0.0184 | 0.0670 | 0.1010 | 9.2667 | 1.8930 | 7.1000 | 10.6000 |
| | | | 1:8 | 3 | 0.0090 | 0.0072 | 0.0030 | 0.0170 | 0.9333 | 0.7767 | 0.3000 | 1.8000 |
| | | | 1:4 | 3 | 0.0057 | 0.0031 | 0.0030 | 0.0090 | 0.5667 | 0.3055 | 0.3000 | 0.9000 |
| | | | 1:2 | 3 | 0.0050 | 0.0026 | 0.0030 | 0.0080 | 0.5000 | 0.2646 | 0.3000 | 0.8000 |
| | | | All | 15 | 0.0625 | 0.0810 | 0.0030 | 0.2150 | 6.5733 | 8.5534 | 0.3000 | 22.7000 |
| | | Jaftex | 1:32 | 3 | 0.3083 | 0.0051 | 0.3040 | 0.3140 | 32.4667 | 0.5686 | 32.0000 | 33.1000 |
| | | | 1:16 | 3 | 0.2513 | 0.0361 | 0.2140 | 0.2860 | 26.5000 | 3.7590 | 22.6000 | 30.1000 |
| | | | 1:8 | 3 | 0.1177 | 0.0090 | 0.1090 | 0.1270 | 12.4000 | 0.9539 | 11.5000 | 13.4000 |
| | | | 1:4 | 3 | 0.0650 | 0.0201 | 0.0460 | 0.0860 | 6.8333 | 2.1595 | 4.8000 | 9.1000 |
| | | | 1:2 | 3 | 0.0447 | 0.0206 | 0.0290 | 0.0680 | 4.7333 | 2.1733 | 3.1000 | 7.2000 |
| | | | All | 15 | 0.1574 | 0.1095 | 0.0290 | 0.3140 | 16.5867 | 11.5274 | 3.1000 | 33.1000 |

**Table 2** (continued)

| | | | | | OD | | | | Vitality (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time** | **Cell** | **MW** | **Dosage** | ***N*** | **Mean** | **SD** | **Min** | **Max** | **Mean** | **SD** | **Min** | **Max** |
| 72 h | HuGu | CHX | 1:32 | 3 | 0.1343 | 0.0137 | 0.1220 | 0.1490 | 14.0667 | 1.4189 | 12.8000 | 15.6000 |
| | | | 1:16 | 3 | 0.0723 | 0.0191 | 0.0510 | 0.0880 | 7.5667 | 2.0257 | 5.3000 | 9.2000 |
| | | | 1:8 | 3 | 0.0093 | 0.0015 | 0.0080 | 0.0110 | 0.9667 | 0.2082 | 0.8000 | 1.2000 |
| | | | 1:4 | 3 | 0.0057 | 0.0012 | 0.0050 | 0.0070 | 0.5667 | 0.1155 | 0.5000 | 0.7000 |
| | | | 1:2 | 3 | 0.0040 | 0.0030 | 0.0010 | 0.0070 | 0.4000 | 0.3000 | 0.1000 | 0.7000 |
| | | | All | 15 | 0.0451 | 0.0540 | 0.0010 | 0.1490 | 4.7133 | 5.6626 | 0.1000 | 15.6000 |
| | | Jaftex | 1:32 | 3 | 0.1977 | 0.0095 | 0.1880 | 0.2070 | 20.6667 | 0.9504 | 19.7000 | 21.6000 |
| | | | 1:16 | 3 | 0.1470 | 0.0270 | 0.1290 | 0.1780 | 15.3667 | 2.8113 | 13.5000 | 18.6000 |
| | | | 1:8 | 3 | 0.1043 | 0.0121 | 0.0930 | 0.1170 | 10.9000 | 1.2530 | 9.7000 | 12.2000 |
| | | | 1:4 | 3 | 0.0487 | 0.0166 | 0.0310 | 0.0640 | 5.0667 | 1.7616 | 3.2000 | 6.7000 |
| | | | 1:2 | 3 | 0.0090 | 0.0020 | 0.0070 | 0.0110 | 0.9333 | 0.2517 | 0.7000 | 1.2000 |
| | | | All | 15 | 0.1013 | 0.0710 | 0.0070 | 0.2070 | 10.5867 | 7.4245 | 0.7000 | 21.6000 |
| | OCC-18 | CHX | 1:32 | 3 | 0.1880 | 0.0190 | 0.1690 | 0.2070 | 19.5000 | 2.0000 | 17.5000 | 21.5000 |
| | | | 1:16 | 3 | 0.0750 | 0.0235 | 0.0510 | 0.0980 | 7.8000 | 2.4515 | 5.3000 | 10.2000 |
| | | | 1:8 | 3 | 0.0077 | 0.0042 | 0.0030 | 0.0110 | 0.7667 | 0.4163 | 0.3000 | 1.1000 |
| | | | 1:4 | 3 | 0.0023 | 0.0015 | 0.0010 | 0.0040 | 0.2333 | 0.1528 | 0.1000 | 0.4000 |
| | | | 1:2 | 3 | 0.0023 | 0.0012 | 0.0010 | 0.0030 | 0.2333 | 0.1155 | 0.1000 | 0.3000 |
| | | | All | 15 | 0.0551 | 0.0754 | 0.0010 | 0.2070 | 5.7067 | 7.8254 | 0.1000 | 21.5000 |
| | | Jaftex | 1:32 | 3 | 0.2553 | 0.0132 | 0.2410 | 0.2670 | 26.5000 | 1.3748 | 25.0000 | 27.7000 |
| | | | 1:16 | 3 | 0.1470 | 0.0132 | 0.1320 | 0.1570 | 15.2667 | 1.3796 | 13.7000 | 16.3000 |
| | | | 1:8 | 3 | 0.0870 | 0.0053 | 0.0810 | 0.0910 | 9.0000 | 0.5292 | 8.4000 | 9.4000 |
| | | | 1:4 | 3 | 0.0503 | 0.0140 | 0.0370 | 0.0650 | 5.2000 | 1.4526 | 3.8000 | 6.7000 |
| | | | 1:2 | 3 | 0.0300 | 0.0137 | 0.0180 | 0.0450 | 3.1333 | 1.4295 | 1.9000 | 4.7000 |
| | | | All | 15 | 0.1139 | 0.0846 | 0.0180 | 0.2670 | 11.8200 | 8.7843 | 1.9000 | 27.7000 |

**OD**, optical density; **MW**, mouthwash; **SD**, standard deviation; **Min**, minimum; **Max**, maximum; **HuGu**, human gingival fibroblast; **CHX**, chlorhexidine; **OCC-18**, oral cancer cell (oral squamous cell carcinoma)

**Table 3** The results of the **full-factorial 4-way** ANOVA

| Predictor | F | *P* |
|---|---|---|
| Corrected Model | 76.042 | < 0.0000001 |
| Intercept | 4932.670 | < 0.0000001 |
| Exposure time | 63.508 | < 0.0000001 |
| Cell line | 57.434 | < 0.0000001 |
| Mouthwash | 758.403 | < 0.0000001 |
| Dose | 797.995 | < 0.0000001 |
| Time × Cell line | 3.515 | 0.0328605 |
| Time × Mouthwash | 14.689 | 0.0000020 |
| Time × Dose | 9.355 | < 0.0000001 |
| Cell line × Mouthwash | 9.662 | 0.0023497 |
| Cell line × Dose | 11.195 | 0.0000001 |
| Mouthwash × Dose | 36.111 | < 0.0000001 |
| Time × Cell line × Mouthwash | 2.974 | 0.0548625 |
| Time × Cell line × Dose | 1.385 | 0.2097291 |
| Time × Mouthwash × Dose | 1.441 | 0.1865998 |
| Cell line × Mouthwash × Dose | 1.119 | 0.3506774 |
| Time × Cell line × Mouthwash × Dose | 1.058 | 0.3974151 |

## Results

There was no missing data. Descriptive statistics and 95% CIs are presented in Tables 1 and 2. The 4-way ANOVA (R-squared = 0.974, Adjusted R-squared = 0.961) showed that the effects of all variables and most interactions were significant (Table 3): The viability was much higher in the case of Jaftex compared with CHX (Fig. 1). The viability was higher in OSCC compared to HuGu (Figs. 1 and 2). According to the Bonferroni test, all pairwise comparisons between each two dosages were significant (all *P* values ≤ 0.013) in a way that by reducing the dosage, the viability increased (Figs. 2 and 3). Also, there were significant pairwise differences between 3 exposure times (all *P* values < 0.000001, Bonferroni) in a way that the highest viability was in the 24th hour, which was significantly higher than viability in both 48th and 72nd hours; the viability in the 48th hours was significantly greater than that in the 72nd hour (Fig. 3).

The IC50 values were calculated for both drugs against both cell types, at each of the 3 intervals (Fig. 4; Table 4). Both mouthwashes had some degree of toxic effect against both HuGu and OCC-18 cell lines at 24, 48 and 72 h. The time variable itself affected the IC50 value.

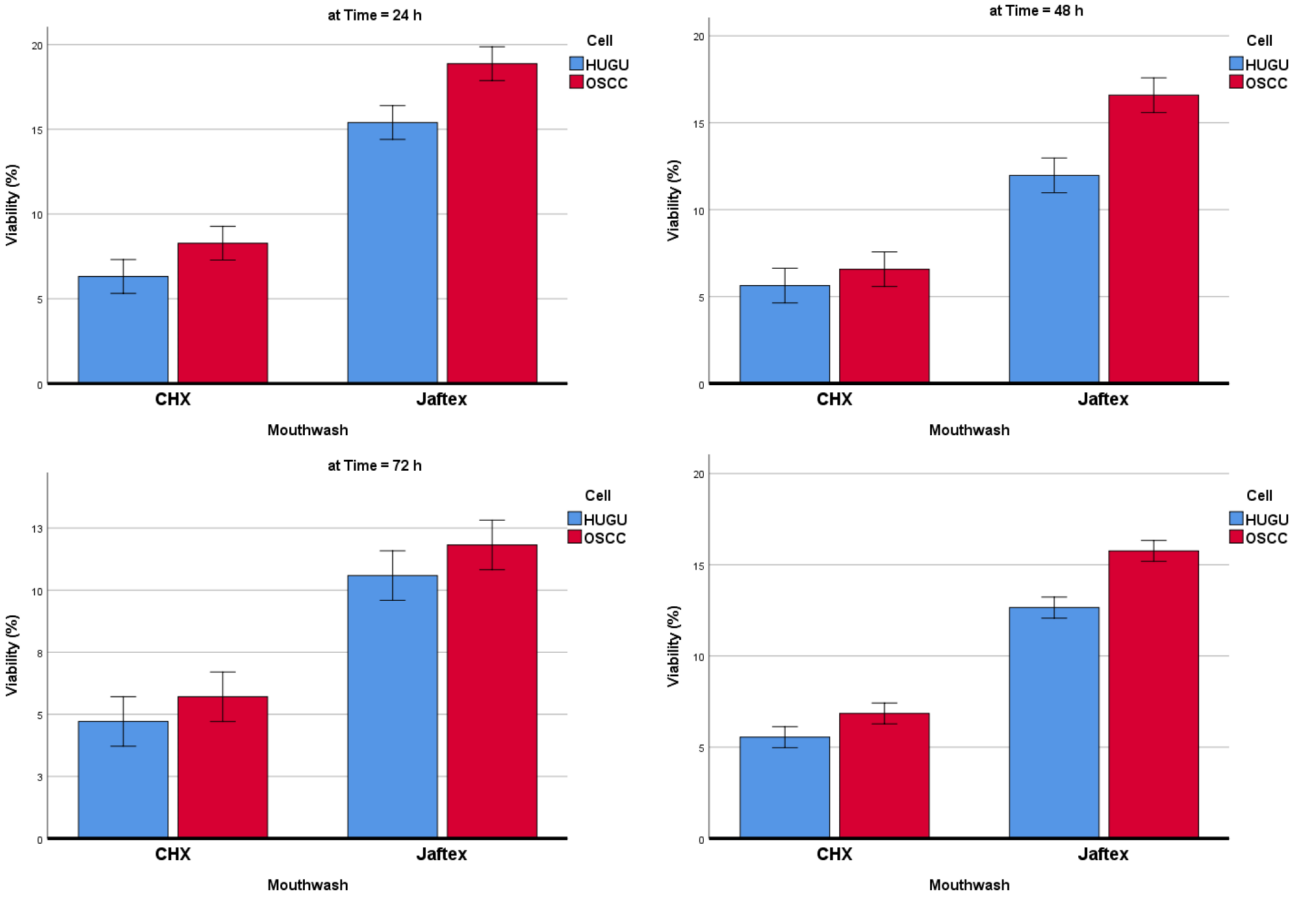


**Fig. 1** Estimated marginal means (and 95% CIs) for viability percentages observed in various subgroups at each of the intervals and in all intervals combined. The bottom-right panel shows results about the comparison of the viabilities of Jaftex versus CHX on both cell lines, while the other three panels show viability of cell lines under the influence of Jaftex and CHX at different time intervals

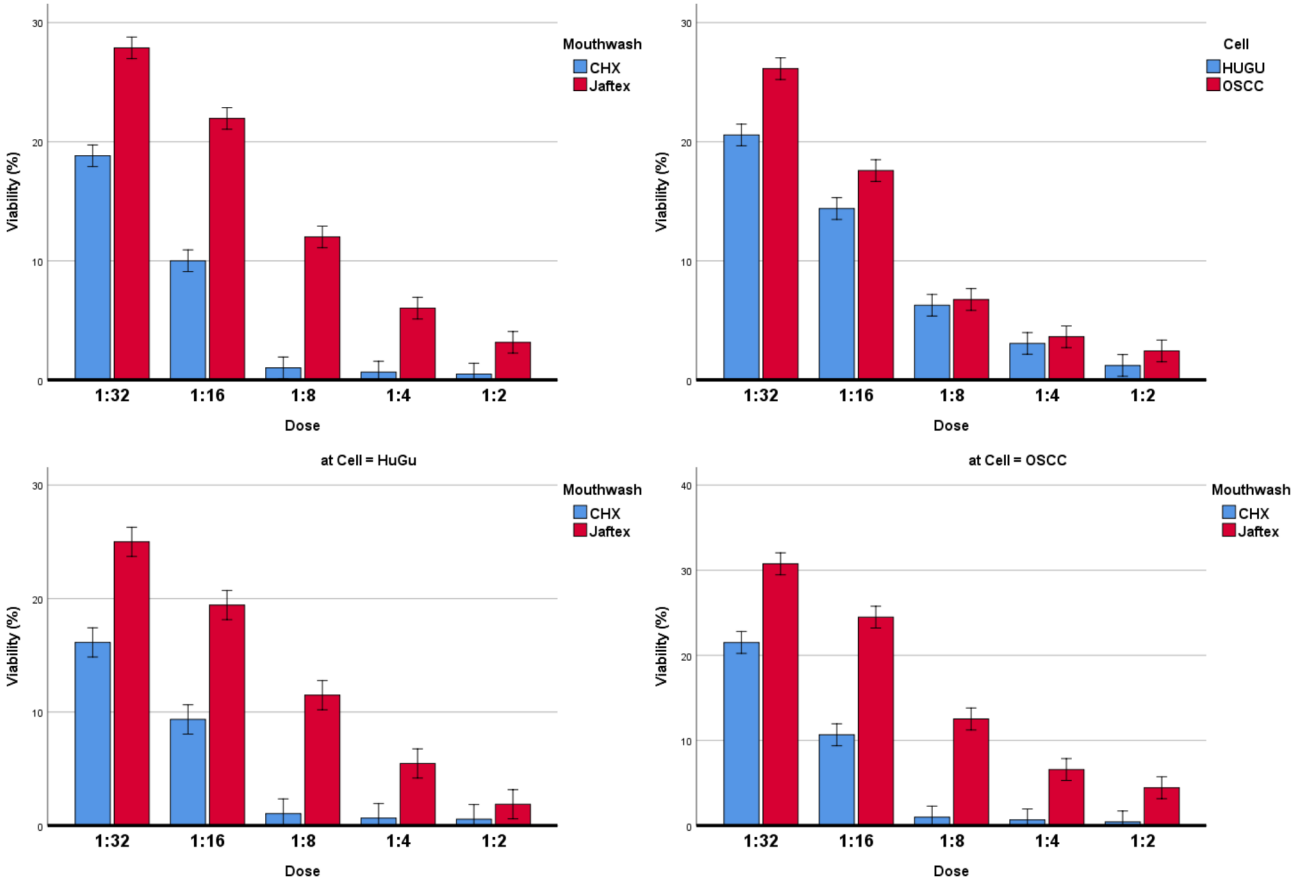


**Fig. 2** Estimated marginal means (and 95% CIs) for viability percentages observed in various subgroups and at different doses of mouthwashes. The bottom two panels show the viabilities of Jaftex versus CHX on each of cell lines separately, at different dosages. The top left panel show the viabilities of Jaftex versus CHX on both cell lines combined, at different dosages. The top right panel show the viabilities of both mouthwashes combined on each of the cell lines separately, at different dosages

## Discussion

The purpose of this study was to investigate the toxicity of Jaftex and chlorhexidine mouthwashes on gingival fibroblast cell lines (HuGu) and oral cancer cell lines (OCC-18). MTT assay showed cytotoxicity for Jaftex in all tested concentrations on OCC-18 and HuGu cell lines. Cell lines obtained from tumors provide the possibility of examining cancer cells in a simple and controlled environment [32]. Jantermi et al., who investigated the cytotoxic effect of Zataria Multiflora in cervical cancer cell lines (HeLa), stated that the IC50 after 24 and 48 h was 35.59 and It was 10.48 µg/ml [33]. Plants like Zataria Multiflora are rich in phenolic compounds such as carvacrol and thymol, which showed antioxidant and antitumor activity on various cell lines such as Hep-2 and human laryngeal carcinoma [34, 35]. A study investigated the effect of Satureja Khuzestanica cytotoxicity on human colon adenocarcinoma (SW480), breast adenocarcinoma (MCF7), and choriocarcinoma (JET3) cell lines; they reported IC50 as 62.5, 125 and 125 µg/ml respectively [30]. Another research concluded that the essential oils of certain plants can be used in the treatment of breast and ovarian cancer and infectious diseases. Antimicrobial effects were also significant [36]. Jantermi et al. investigated the cytotoxic effect of Zataria Multiflora in normal fibroblast cell lines and expressed IC50 after 24, 48 and 72 h as 6.04, 25.11 and 66.71 µg/ml respectively. Considering that the IC50 of the extract in cell lines was less than 100 µg/ml, Zataria Multiflora was classified as a potentially toxic substance [33]. In the study by Babadi et al. [37], the MIC of Jaftex against different bacteria (Streptococcus mutans, Streptococcus sanguinis, Streptococcus salivarius and Lactobacillus casei) in the range of 0.0625 to 0.5 µg/ml and MBC was between 0.125 and 0.5 µg/ml [37]. These values are much lower than the values in which 50% of normal fibroblast cell line growth inhibition occurs. Therefore, it can be said that it is better to use Jaftex in the initial concentration available in the market (1%) for antioxidant activity and to fight cancer cells, and in lower concentrations for antimicrobial activity. Numerous variables, including illnesses, food, and topical or systemic drugs, are known to disrupt homeostasis and cause cell apoptosis [38, 39].

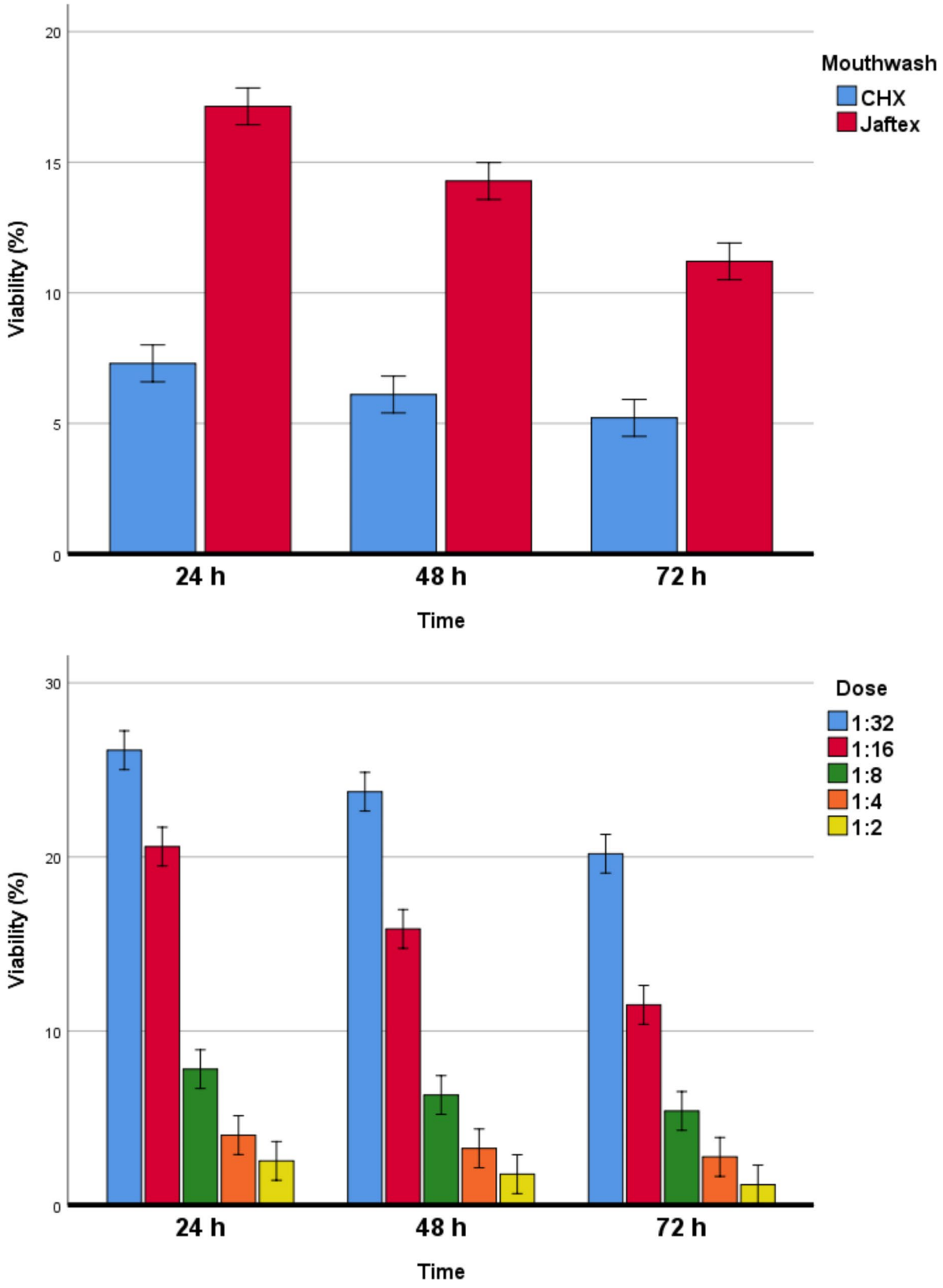


**Fig. 3** Estimated marginal means (and 95% CIs) for viability percentages observed at different intervals as well as different doses of mouthwashes. The top panel shows the effect of each of the mouthwashes on cell viability of both cell lines combined, at 3 intervals of 24 h, 48 h, and 72 h. The bottom panel shows the effect of the duration of exposure of both cell lines combined to different mouthwash dosages at 3 intervals of 24 h, 48 h, and 72 h

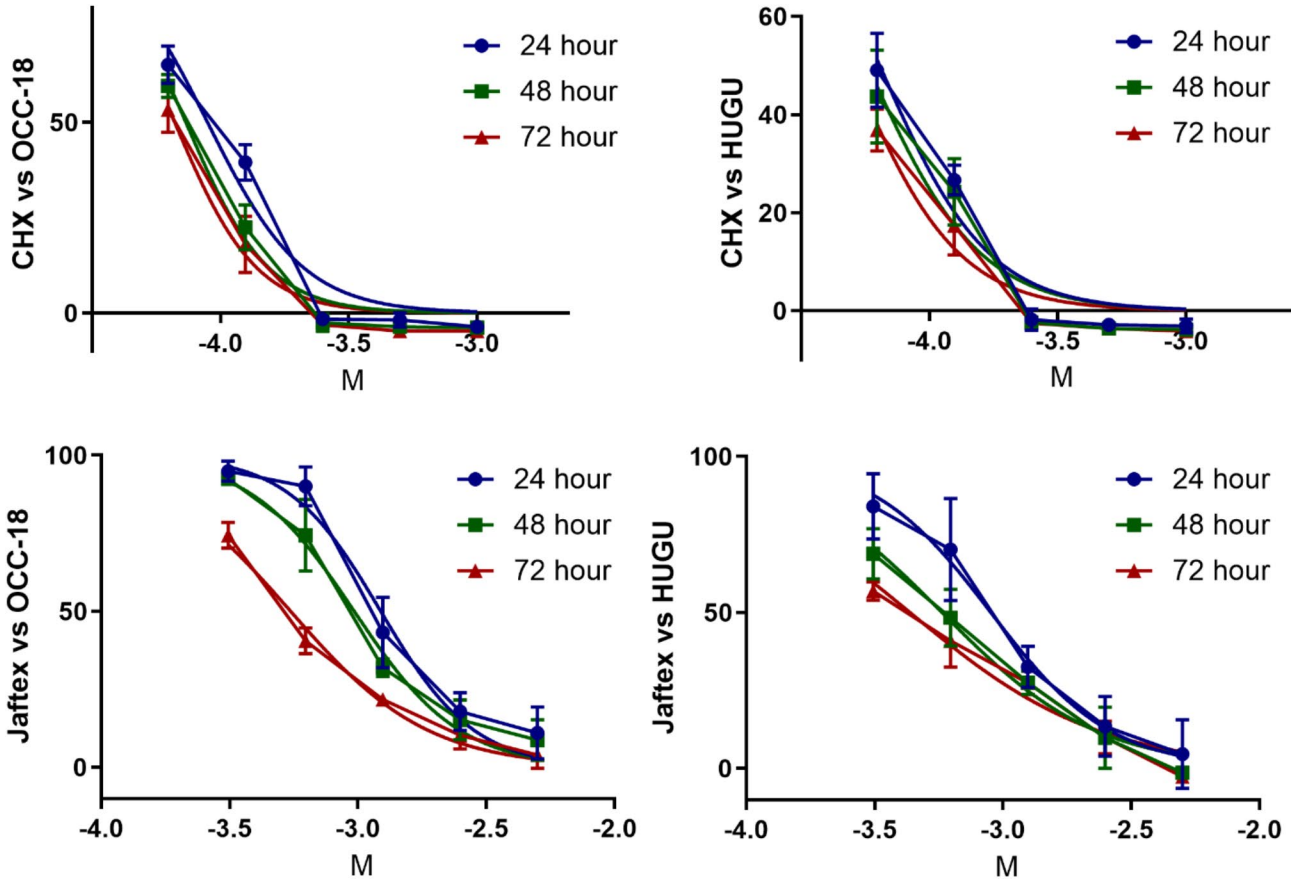


**Fig. 4** Curves representing normalize of transform of drug doses versus cell responses, for both drugs and both cell types, in all 3 intervals. HuGu, human gingival fibroblast; CHX, chlorhexidine; OCC-18, oral cancer cell line (oral squamous cell carcinoma). The IC50 values were a part of the formulas of the 4-parameter logistic regressions depicted above, and is thus calculated by fitting the data to the curve

Oral antiseptics are topical medications that are administered for difficult-to-reach regions after routine brushing or during the recovery period after surgery. Chlorhexidine is the most often utilized oral rinse in this regard. Many of the mechanisms that can contribute to their antimicrobial effects might as well negatively affect the human cells. For example, depending on the concentration, it has either a bactericide or a bacteriostatic effect [39, 40]. However, longer durations and higher concentrations of chlorhexidine may cause elevated intracellular calcium, oxidative stress, and disruptions in mitochondrial function [39, 41]. It should be noted that the studies on other herbal mouthwashes had other methodological differences such as mechanisms of action, target cells, or test settings.

Chlorhexidine is a common mouthwash that has been accepted as the gold standard in dentistry. Chlorhexidine in low concentrations causes apoptosis and in high concentrations causes cell necrosis, and properties such as inhibition of DNA synthesis and cell death have been mentioned [7, 42, 43]. In the present study, the cytotoxicity of chlorhexidine was significantly higher than that of Jaftex. The biological implications of this finding may be that Jaftex is safer to use for long-term use in normal tissues, while chlorhexidine is more potent as a potential antitumor mouthwash. Rajabalian et al. [7] in their study using the MTT method stated that in the medicinal concentrations available on the market of both chlorhexidine and Persica mouthwash for macrophages, epithelial cells, gingival fibroblasts, and Osteoblasts are toxic. At concentrations higher than 0.1%, Persica showed a considerable toxicity in all cell lines. Chlorhexidine with a concentration of 0.001% showed toxic effects only on human gingival fibroblasts. They also stated that the toxic effects of chlorhexidine and Persica are significantly reduced in the presence of FCS (fetal calf serum) [7]. In another study, Muller et al. investigated the toxic and antimicrobial effects of 12 different mouthwashes on gingival fibroblast cells, L929 (mouse aneuploid fibrosarcoma cells) and HSC-2 cells (mouse human oral epithelial cancer) using the MTT method; they showed that chlorhexidine 0.05%, ethanol, pegylated hydrogenated castor oil and sodium dodecyl sulfate mouthwashes have moderate toxic effects. Other mouthwashes including 0.2%

**Table 4** The IC50 values obtained in 4 sets of 2 drugs versus 2 cell types, using the 4-parameter logistic regression employing the Hill coefficient (log(inhibitor) vs. normalized response)

| Drug v cell | Parameters | | 24 h | 48 h | 72 h |
|---|---|---|---|---|---|
| Jaftex vs. OCC-18 | Best-fit values | LogIC50 | -2.91 | -3.01 | -3.26 |
| | | HillSlope | -2.39 | -2.12 | -1.63 |
| | | IC50 | 0.00122800 | 0.00096830 | 0.00054730 |
| | Std. Error | LogIC50 | 0.02899000 | 0.02549000 | 0.01895000 |
| | | HillSlope | 0.34350000 | 0.23650000 | 0.11950000 |
| | 95% CI | LogIC50 | -2.973 to -2.848 | -3.069 to -2.959 | -3.303 to -3.221 |
| | | HillSlope | -3.133 to -1.649 | -2.626 to -1.604 | -1.884 to -1.367 |
| | | IC50 | 0.001063 to 0.001419 | 0.0008530 to 0.001099 | 0.0004981 to 0.0006015 |
| | Goodness of Fit | R squared | 0.94770000 | 0.95980000 | 0.97390000 |
| | | Sum of Squares | 1003.00 | 679.50 | 255.70 |
| | | Sy.x | 8.79 | 7.23 | 4.44 |
| Jaftex vs. HUGU | Best-fit values | LogIC50 | -3.047 | -3.237 | -3.362 |
| | | HillSlope | -1.855 | -1.435 | -1.174 |
| | | IC50 | 0.0008975 | 0.0005793 | 0.0004346 |
| | Std. Error | LogIC50 | 0.03891 | 0.03289 | 0.03951 |
| | | HillSlope | 0.2866 | 0.1651 | 0.1384 |
| | 95% CI | LogIC50 | -3.131 to -2.963 | -3.308 to -3.166 | -3.447 to -3.277 |
| | | HillSlope | -2.474 to -1.236 | -1.792 to -1.079 | -1.473 to -0.8746 |
| | | IC50 | 0.0007396 to 0.001089 | 0.0004918 to 0.0006822 | 0.0003571 to 0.0005290 |
| | Goodness of Fit | R squared | 0.9128 | 0.9354 | 0.922 |
| | | Sum of Squares | 1379 | 659.6 | 545.6 |
| | | Sy.x | 10.3 | 7.123 | 6.478 |
| CHX vs. OCC-18 | Best-fit values | LogIC50 | -4.05 | -4.13 | -4.18 |
| | | HillSlope | -2.32 | -2.69 | -2.72 |
| | | IC50 | 0.00008916 | 0.00007341 | 0.00006640 |
| | Std. Error | LogIC50 | 0.02649000 | 0.01705000 | 0.02028000 |
| | | HillSlope | 0.32780000 | 0.30000000 | 0.39680000 |
| | 95% CI | LogIC50 | -4.107 to -3.993 | -4.171 to -4.097 | -4.222 to -4.134 |
| | | HillSlope | -3.031 to -1.614 | -3.341 to -2.044 | -3.578 to -1.864 |
| | | IC50 | 7.815e-005 to 0.0001017 | 6.744e-005 to 7.990e-005 | 6.003e-005 to 7.345e-005 |
| | Goodness of Fit | R squared | 0.93720000 | 0.96250000 | 0.94190000 |
| | | Sum of Squares | 744.90 | 347.10 | 450.90 |
| | | Sy.x | 7.57 | 5.17 | 5.89 |
| CHX vs. HUGU | Best-fit values | LogIC50 | -4.19 | -4.24 | -4.31 |
| | | HillSlope | -2.06 | -1.99 | -2.08 |
| | | IC50 | 0.00006410 | 0.00005745 | 0.00004948 |
| | Std. Error | LogIC50 | 0.03021000 | 0.04132000 | 0.04007000 |
| | | HillSlope | 0.32280000 | 0.40000000 | 0.38960000 |
| | 95% CI | LogIC50 | -4.258 to -4.128 | -4.330 to -4.151 | -4.392 to -4.219 |
| | | HillSlope | -2.754 to -1.360 | -2.853 to -1.125 | -2.920 to -1.237 |
| | | IC50 | 5.516e-005 to 7.450e-005 | 4.678e-005 to 7.056e-005 | 4.054e-005 to 6.039e-005 |
| | Goodness of Fit | R squared | 0.91900000 | 0.87690000 | 0.90150000 |
| | | Sum of Squares | 549.70 | 719.00 | 398.90 |
| | | Sy.x | 6.50 | 7.44 | 5.54 |

**HuGu**, human gingival fibroblast; **CHX**, chlorhexidine; **OCC-18**, oral cancer cell line (oral squamous cell carcinoma). **Std Err**, standard error; **CI**, confidence interval

chlorhexidine and cocamidopropyl betaine have strong antimicrobial and toxic effects. However, cetylpyridinium chloride has a strong toxic effect but a moderate antibacterial effect [44]. The studies that investigated the cytotoxicity of chlorhexidine in medicinal concentrations available on the market by the MTT method, mentioned moderate to strong toxicity for it. However, in a study conducted by Banerjee et al. using the method of measuring micro nucleotides before and after the effect of chlorhexidine on buccal mucosa cells, they stated that in the concentrations of drugs available on the market during 9 month, chlorhexidine did not show any toxic effect

on buccal mucosa cells [8]. Due to the contradiction in the results for different methods of determining cytotoxicity such as MTT and the evaluation of micro nucleotides, it is suggested that in future studies the cytotoxicity of Jaftex mouthwash should be investigated using the micro-nucleotide method.

This preliminary study was limited by some factors. Its design was in vitro, which is not applicable to clinical settings. There are many variables at play in the oral cavity, which are all absent in laboratory settings. Nevertheless, this is at the same time necessary for the purpose of our study to first rule out such confounding variables and have complete control over the settings in a laboratory study, in order to be able to calculate the IC50 value. This is standard practice in pharmacology, and might not be practically done using clinical in vivo studies. Moreover, the sample should have been larger, comprising more cell lines and more oral rinses. Of course, cell type availability and the limited budget and time projected for this preliminary study disallowed us from expanding the research in this regard; this was a limitation of many previous studies as well. In light of our budget limitations, we selected HuGu (human gingival fibroblasts) as an important and common gingival cell. We also selected OSCC cells as the most common form of oral cancer. It should be noted that the oral mucosa comprises numerous cells with different sensitivities to various chemicals; and the same goes for cancers as well. Furthermore, the short duration of incubation and the format of the duration of examination disallows any generalizations to long-term situations. In clinical settings, no mouthwash is used for 72 h or even for 24 h continuously. On the other hand, in clinical settings, a mouthwash may be used intermittently for a very short time each day, for years. In addition, the limited availability of the plants in use would make it difficult for many countries to produce similar mouthwashes. However, it should be noted that this patented product is supposed to be produced, if proven effective and safe, by manufacturers with access to these plants. It can become available at many other areas through exportation. Besides, it should be taken into account that chlorhexidine is a potent antimicrobial agent that has been used for many years as the gold standard for effective antimicrobial control; therefore, the reason for comparing Jaftex with chlorhexidine was not to suggest that Jaftex is necessarily as potent and effective; the reason for this comparison was chlorhexidine being the gold standard of antimicrobial mouthwashes. Many questions should be answered regarding mechanisms of actions, efficacy, and safety of mouthwashes before being able to recommend them for clinical use. It should be known how Jaftex works, how and why it has cytotoxic effects, and whether it is safe and at which doses. For this purposes, future in vitro and animal studies need to assess many aspects including the possibility and safety of this mouthwash, and if deemed effective and safe, then clinical trials should examine its safety and effectiveness in humans.

## Conclusions

Within the limitations of this preliminary short-term in vitro study, it might be concluded that the toxicity of Jaftex was significantly lower than that of chlorhexidine. Also, the toxicity of both mouthwashes against the HuGu cell line was higher than the OCC-18 cell line. Increasing the exposure time of both mouthwashes increased their toxicity. Due to its cytotoxicity, it might be possible that this herbal mouthwash be used in high concentrations for antitumor treatments and in low concentrations for antimicrobial treatments. Of course, before any suggestions for human use, many questions around this new mouthwash should be answered (such as the ones counted above) via future in vitro and animal studies followed by randomized clinical trials, if deemed safe and effective.

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**Author contributions**
Hamed Saberikia: data curation, interpreting the findings, writing the thesis, critical review and final approval of the manuscript; Mohammad Rashno: study conception and design, data curation, interpreting the findings, supervision of the project, critical review and final approval of the manuscript; Fatemeh Babadi: study conception and design, data curation, interpreting the findings, supervision of the project, critical review and final approval of the manuscript; Vahid Rakhshan: Statistical analyses, data validation, calculation of IC50 values, interpreting the findings, drafting and revising the article, creating the tables and figures, and final approval of the paper. All authors read the final draft and agreed to submit the manuscript to this journal.

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**Data availability**
The data are available from the corresponding author upon request.

## Declarations

**Ethics approval and consent to participate**
The protocol and ethics of this in vitro study were approved by the Medical Research Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (ethics code: IR.AJUMS.REC.1399.451). All methods were performed in accordance with the relevant guidelines and regulations (including the Declaration of Helsinki); all experimental protocols were approved by the Institutional Review Board of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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