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Gingerly effervescent tablets: investigating the effect of cytotoxicity on gingival fibroblasts and antimicrobial properties under laboratory conditions

Zahra Aghazadeh [1], Marziyeh Aghazadeh [1], Hossein Samadi Kafil [2], Parisa Falsafi [1,*], Mahdi Rahbar [3]

[1]Dental and Periodontal Research Center, Department of Oral and Maxillofacial Medicine, Dental School, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Dental and Periodontal Research Center, Department of Operative and Esthetic Dentistry, Dental School, Tabriz University of Medical Sciences, Tabriz, Iran
*corresponding author e-mail address: ***Pf.dent@yahoo.com*** | Scopus ID 55011885000

ABSTRACT

Ginger is a medicinal plant with antioxidant, anti-bacterial, anti-inflammatory and anti-fungal properties. The shape of effervescent tablet is a new form of this product for use as a mouthwash. This study investigated the cytotoxicity and antimicrobial properties of gingerly effervescent tablets under laboratory conditions. In this study, MTT assay was done to evaluate in-vitro gingival fibroblast cytotoxicity during 24, 48 and 72 hours. In addition, the effect of antimicrobial properties on common bacterial and oral fungi was investigated. P-value was considered significantly less than 0.05. There was no significant difference between the mean of live cells in groups with 24, 48 and 72 duration (P-value = 0.071). There was no significant difference between ginger group and control group (P<0.05). Minimum inhibitory concentration (MIC) of effervescent tablet was different, ranging from 10 mg/ml for Candidas family to 20 mg/ml for Staphylococcus and 40 mg/ml for negative grams. According to the results, it can be concluded that gingerly effervescent tablets have no toxic effect after 72 hours of exposureto gingival fibroblasts. Its suitable antimicrobial properties for fungi makes it useful for the control of infection and as an ideal mouthwash.

Keywords: *Antimicrobial; Cytotoxicity Test; Fibroblasts; Ginger.*

1. INTRODUCTION

Fungal infections are one of the most commonly reported oral diseases. Denture stomatitis is a chronic inflammation of the mucous membrane that is in the area covered by a mobile denture, and is found in 11 to 67% of people having mobile dentures.

Candida albicans is the most important microorganism that plays a role in the denture pathogenesis of stomatitis [1, 2].

Today, antifungal medicines with different formulations are available for treatment, which are used topically (such as Nystatin and Clotrimazole) and systemically (Azoles and amphotericin b [3].

Long-term use of antifungal drugs can lead to side effects. In addition, the resistance to these drugs has caused restriction in the use of such antifungal compounds [4, 5]. Drug resistance is seen in immune suppressants and people with long-term use of antifungal drugs [6]. Side effects associated with common antifungal drugs include nausea, vomiting, liver dysfunction, cardiac arrhythmias, neuropathy, etc. Therefore, new researches have focused on finding effective antifungal compounds with plant origin and fewer side effects [6].

Many studies have shown that individuals engaging brushing in denture care, but this method is not suitable for the denture care. Generally, in the affected people, topical use of antifungal drugs for denture along with simultaneous consumption of these drugs in the form of intraoral is recommended [7, 8].

This researchers have investigated the inhibitory effects of ginger essential oil among the herbal essential oils on the microbes. In one study, the antimicrobial effects of this plant on Staphylococcus aureus bacteria, *Pseudomonas aeruginosa* and *Escherichia coli* were investigated. Accordingly, it was found that ginger extract has a significant inhibitory effect on these species [9].

In another study, antifungal activity of ginger essential oil against the resistance of Candida albicans to Fluconazole isolated from patients with vaginal candidiasis was investigated. In this study, it was shown that ginger essential oil has an inhibitory effect on all isolated samples of *C. albicans* [10].

According to the study conducted by Dr. Taghavi et al., the antifungal effect of ginger extract on Candida, Glabra, and Krusoei candida was evaluated. According to the obtained results, this extract has significant anti-fungal properties [11].

Dr. Islami et al. used ginger extract in the form of mouthwash in the clinical status for patients with danturastomatatyisis, it had similar therapeutic results with nystatin drops [12].

Another study on the hydro alcoholic extraction from ginger rhizome on hand and skin swelling showed that ginger has an anti-inflammatory effect on the skin and hands [13].

Recently, ginger mouthwash has been produced domestically. Based on the study conducted by Aghazadeh et al., this product was investigated in terms of anti-fungal, antimicrobial and cytotoxic properties. According to the results of this study, this mouthwash is anti-fungal without any toxic effects and has properties similar to nystatin [14].

This present study investigated the toxic effects of gingerly effervescent tablets with similar MIC in mouthwash and compare them together.

It is worth mentioning as well that the zero hypothesis of this study is that the gingerly effervescent tablets do not have cytotoxicity in gingival fibroblast cells and dose not have antimicrobial properties.

2. MATERIALS AND METHODS

2.1. Materials and Methods.

Gingerly effervescent tablets (Rojin Cosmetic Co, Tabriz, Iran) were used in this study. Every tablet was dissoleved in a glass of warm water (37 C and 200 ml). In a 96-well cell culture microplate, 100 µl of sabouraud dextrose (Merck KGaA, Darmstadt, Germany) (2 times for fungal species) and Muller hinton broth (two times for bacterial species)(Merck KGaA, Darmstadt, Germany) was added to each well (average value was measured to reach the desired optimal concentration) . Then, 80 µl of ginger extract was added to the first well and subsequent dilutions were prepared for subsequent wells. Subsequently, 20 µl of fungal or bacterial samples (at a concentration of 0.5 Mcfarland) were added to each well. After 18-24 h incubation at 37°C, the wells were evaluated for their turbidity.

The wells before turbidity investigation were examined in terms of minimum inhibitory concentration (MIC). This process was done for all the species. These measurements for MICs and the minimum bactericidal concentrations (MBCs) were done based on the recommendations of the Clinical and Laboratorial Standards Institution M27-A3 and CLSI M100-S22 [15-17]. Different concentrations of ginger extract (80 mg / ml - 0.625 mg / ml) were used.

MIC values were estimated using a spectroscopic method, with an absorbance measurement at 620 nm (OD620 optical density reading at 620 nm) [18]. Control tubes with Muller Hinton Agar (Merck) (without ginger extract) were used as negative control; and 70% ethanol was used as a positive control. To determine MBCs, sterile swabs were used to inoculate higher concentrations than MICs for 24-hour into the blood agar [19]. All studied species have been prepared from Iranian Type Culture Collection by the Iranian Scientific and Technology Organization (IROST). The studied species are as follows:

‹*P. aeruginosa* ATCC 27853 ‹*K. pneumoniae* ATCC 700603 ‹ S.aureus ATCC 25923 ‹*A. baumannii* ATCC 19606 ‹B. cereus ATCC 11778 ‹*C. krusei* DMS 70079 ‹*C. albicans* ATCC 0231 and two oral species of *C. albicans*.

In this study, the cytotoxicity of the gingerly effervescent tablets (Vi-one) was also investigated. For this purpose, the gingival fibroblast cell class with ID 30646 was purchased from Tehran Anistitopustor and cultured in a culture medium of FBS-10% + DMEM and antibiotics.

Based on the MTT assay protocol, in 36 wells of two-flask houses with 96-well plates, 5,000 cells were shed and allowed for 24 hours which is the required time to connect the cells. Effervescent tablets were added to the cell's environment in doses of 1, 1.2, 1.4 and 2 tablets. Each well had a control well. MTT Assay Test was done in 24, 48 and 72 hours, in all the wells. The MTT Assay method is a colorimetric method used for measuring the enzymes activity. This method causes formation of purple color in the formed sediment Formazam; and is used for evaluation of the viability percentage (cell counts and proliferation of cells).

Among the 96 wells of the plate, 36 wells were used for cultivation (three wells were considered as a case study for evaluation of the target substance; and three wells were selected as controls without the target substance. They were evaluated in three different times, and each experiment was carried out three times to reduce the probability of error).

The data obtained from the study were reported by descriptive statistics methods (abundance-percent). The Kruskal-Wallis test was used to compare the cytotoxicity in two groups due to the lack of normal distribution of data. Statistical analysis was performed using statistical software SPSS16 and P value less than 0.05 was considered meaningful.

3. RESULTS

In this study, for the investigation on the effects of antibiotics against the under study species, MIC for Nistatin against *C. albicans* and *Candida cruzei* was 32 and 16 µg/ml, respectively. MIC for Fluconazole against both *C. albicans* and *Candida kruzeye* was 16 µg/ml, indicating a poor response of these microorganisms to fluconazole and nystatin.

To remove the background of the ginger extract from the wells, various dilutions without bacteria and fungi were used; and the rates of obtained results were less than all the results; so that the final results were normalized.

MIC for *S. aureus, K. pneumoniae, B. cereus* and *A. baumannii* was 20 mg/ml and for *P. aeruginosa* and E. coli it was 40 mg/ml; however, for the isolation of fogies, it was 5 mg/ml for C. krusei and 10 mg/ml for *C. albicans*.

Table 1 describes the mean optical density (representing live cells) in different sizes of effervescent tablets and different time durations.

According to Table 1, the rate of cytotoxicity when 2 tablets of effervescent ginger were used inr 24 hours was 0.05, in 48 hours it was 0.15, and in 72 hours it was 0.32. In addition, when 1 gingerly effervescent tablets were used, in 24 hours it was 0.03, 48 hours it was 0.2, and in 24 and 72 hours it was 0.12.

In the case of 1/2 effervescent tablet taken for 24 hours, it was 0.04, for 48 hours it was 0.3 and for 72 hours it was 0.1. When 1.4 ginger tablets were consumed in 24 hours it was 0.07, in 48 hours it was 0.18 and in 72 hours it was 0.5.

In the control group, in which the ginger tablet was not taken, the rate of cytotoxicity was 0.02 for 24 hours, 0.17 for 48 hours, and 0.3 for 72 hours, respectively.

According to Figure 1 to 3, the rate of optical density was different based on the sizes of effervescent tablets, but these changes due to the increase or decrease of the size of the tablet did not have a regular trend. That is, the use of 1.4 to 2 ginger tablets is not poisonous to gingival fibroblasts cells.

To select the appropriate test for data analysis in each of the groups, at first, the Kolmogorov-Smirnov test was performed. Regarding the fact that the test statistic rate was more than the significant rate (P> 0.05), we concluded that the data have abnormal distribution. Therefore, Kruskal-Wallis nonparametric test was used to analyze these data.

Considering the P-Value (0.071) and its larger value, it is concluded that the relationship between cytotoxicity and duration and the consumed amount is not significant. That is, the amount of time and the number of tablets from 1.4 to 2 does not have any effect on the amount of toxicity rate, and it remains non-toxic still.
The difference between the mean surviving cells of the control group and the group who have taken the effervescent tablet containing ginger is not significant. Thus, it can be said that proliferation rate and cell survival in the case study and control groups are not significantly different.

Table 1: The optical density of studied samples in different used amounts of effervescent tablet within the various durations.

The amount of effervescent tablet	Duration		
	24 hours	**48 hours**	**72 hours**
2	0.05	0.15	**0.32**
1	0.03	0.2	**0.12**
1.2	0.04	0.3	**0.1**
1.4	0.07	0.13	**0.5**
Without effervescent tablet	0.02	0.17	**0.3**

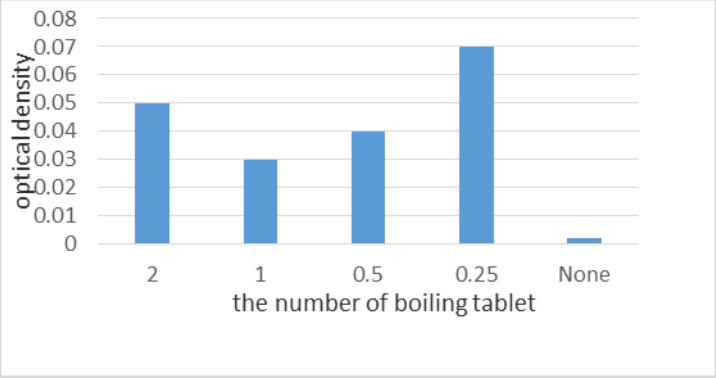

Figure 1. the rate of gingival fibroblasts living cells after 24 hours.

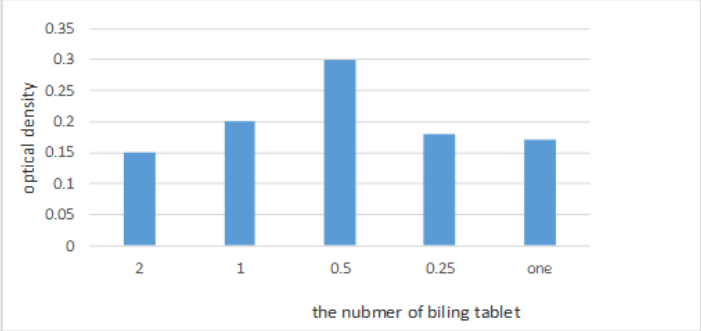

Figure 2. the rate of gingival fibroblasts living cells after 48 hours.

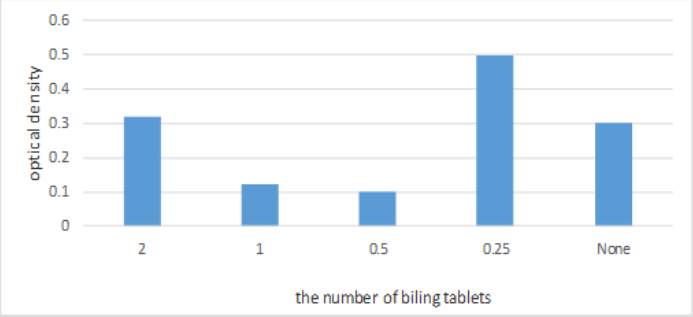

Figure 3. the rate of gingival fibroblasts living cells after 72 hours.

Discussion.

Fungal infections are relatively one of the common oral diseases. Today, there are antifungal drugs with different formulations for treatment either by using topically (such as nystatin and colutriamzol) and systemically (Azoles and amphotericin B).
The long-term use of antifungal drugs leads to side effects, and the development of resistance to these drugs has limited the use of such antifungal compounds.
In this experimental study, the effect of cytotoxicity of gingerly effervescent tablets extract on gingival fibroblasts was investigated under laboratory conditions. The toxicity rate of the gingerly effervescent tablets was measured by MTT assay. For this purpose, 24 hours after the preparation of the culture medium, the gingerly effervescent tablets for 24, 48 and 72 hours were added to the culture medium. The percentage of cell survival was measured by MTT method.
In the study of Aghazadeh et al., which investigated the anti-bacterial and anti-fungal effects of Zingiber officinale, it was concluded that the relationship between ginger toxicity and the duration of time is not significant (14).
In the study conducted by Mohagheghei et al. on the cytotoxicity effect of ginger watery extract, it was concluded that by increasing duration of time, the fresh and watery extract of ginger has no cytotoxic effect on normal cells (20).
In the present study, after measuring the toxicity by MTT method, after 24, 48 and 72 hours, and then analyzing the obtained data, the results showed that the cell cytotoxicity rate of the ginger tablets is different in the passing of time, although the obtained results of analysis showed that these differences are not significant; or in the other words, duration of time has no effect on the toxicity rate of the gingerly effervescent tablets for gingival fibroblast. This result is in accordance with the obtained results of the other studies.
According to the Aghzadeh's study on the toxicity of ginger, it was conducted that the ginger has no toxicity effect (14). Based on the study conducted by Nag on investigating the antioxidant activity and toxicity of ginger, it was found that the toxicity rate significantly increases by increasing the ginger density; and in low density, it has no toxicity effect [21].
The Norfazlina study was aimed at investigating the effects of ginger and black seeds in a separate and combined form on cancer cells of patients with leukemia. From this result, it was obtained that they have toxic effect on the cancer cells [22].
In the study of Santos et al., which investigated the cytotoxicity of ginger and two other herbs, using tetrazolium (MTT) and neutral red assays, anti-proliferative activity was assessed by various mechanisms. The obtained data were analyzed; and it was concluded that ginger has no toxicity effect on the cells [23].
In the study of Sharifi far et al., aimed at investigating the cytotoxicity of essential oil and extract of ginger and cinnamon by the larvae fatality of saline shrimp test in different concentrations and solvents, it was concluded that ginger has high toxicity [24].
In the study conducted by Lee on the toxicity of ginger in an oily combination, various densities of oil containing 35.02 ± 0.30% ginger were used. The findings of his study showed that the ginger has a significant relation with toxicity; and by increasing the density of ginger, the rate of toxicity increased as well [25].
In this study, the obtained results from the data show that ginger has no toxicity effect; which is in compliance with the studies of Aghazadeh and Santos.

Regarding the conclusions of Nag's study on the low density ginger, similar results with this study were obtained; although the results are inconsistent in the high density ginger. It can be due to the difference in the formulation; because in Nag's study the essential oil with ethanol solvent was used; but in the present study, the ginger effervescent tablet has been examined.

There are some contradictions in the study conducted by Norfazlina because in the mentioned study the cancer cells have been investigated; while in the present study the gingival fibroblast was examined.

Regarding the study conducted by Sharifi Nia, the cause of contradiction is the type of investigated substance. Since the essential oil and extract of ginger have been investigated in this study, the used solvents can affect the obtained results.

In the study by Lee, ginger oil was used, which can be the reason for the inconsistency in the results of his study and this study.

In Hosseini's study, which aimed to compare the effect of ginger and nystatin mouthwashes on denture inflammation, it was concluded that ginger and nystatin have the same acceptable effect on the treatment of denture stomatitis; but ginger mouthwash has fewer side effects in comparison with nystatin [12].

Based on the present study in accordance with the study conducted by Islami, this result obtained is that the gingerly effervescent tablets have no toxic effect as complications.

This study was conducted by considering some hypothesis. Hence, in order to complete and extend the studied issue more, the following proposals in line with this study will be presented in the future. The conducted study was done within 24, 48 and 72 hours.

It is suggested that to obtain more accurate results, longer times should be considered. In addition, in this study only the MTT test was used to measure the toxicity rate.

It is proposed that other methods should be used for measuring the toxicity and the results compared in the future.

4. CONCLUSIONS

According to the obtained results, it can be said that the gingerly effervescent tablets after even 72 hours proximity to gingival fibroblast, has no toxic effect. These tabaltes had strong antimicrobial properties against Candida isolates which makes them useful for the control of infection and as an ideal mouthwash. Also with higher concentration it was able to reduce colonization of gram negative and gram positive pathogens.

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