

# Evaluation of Cytotoxic effect of Garlic on human gingival fibroblasts: A Preliminary Study

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## ABSTRACT

In this study we aimed to evaluate the cytotoxic effect of garlic extract on human gingival fibroblasts. Garlic extracts were prepared at 5 different concentrations as 12.5%, 6.25%, 3.12%, 1.5%, and 0.6%. Cytotoxic effects of garlic and chlorhexidine gluconat (CH) on human gingival fibroblasts were evaluated by agar diffusion method at 24 h. Garlic extracts concentrations at 6.25, 3.12, and 1.5% were not found cytotoxic on human gingival fibroblasts, whereas concentration at 12.5% was found cytotoxic, this effect was found less cytotoxic than CH. According to results of this study we could suggest that Garlic would have a promising role in the future for oral health, if appropriate solutions can be prepared.

**Key words:** Garlic; chlorhexidin gluconat; human gingival fibroblast; cytotoxicity

## ÖZET

**Horner sendromu: tüp torakostominin oldukça nadir görülen bir komplikasyonu**

Çalışmamızda insan diş eti fibroblastları üzerine sarımsak özütünün sitotoksitesi araştırıldı. %12.5, 6.25, 3.12, 1.5 ve 0.6'lık derişimlerde olmak üzere 5 farklı sarımsak çözeltisi hazırlandı. Klorheksidin glukonat ve sarımsağın insan dişeti fibroblastları üzerine olan sitotoksik etkileri 24. saatte agar difüzyon metodu ile değerlendirildi. % 6.25, 3.12 ve 1.5'lik derişimlerdeki sarımsak çözeltileri her hangi bir sitotoksik etki göstermemiştir. %12.5'luk derişimdeki sarımsak çözeltisi sitotoksik etki göstermiştir, bu etki klorheksidin glukonatin gösterdiğinden daha az bulunmuştur. Bu ön çalışmadan elde edilen verilere göre sarımsak çözeltisi ile hazırlanacak uygun solüsyonlar ağız sağlığının elde edilmesi yönünde gelecekte kullanılabilir.

**Anahtar kelimeler:** Sarımsak; klorheksidin glukonat; insan dişeti fibroblastı, sitotoksite

## Introduction

Garlic (*Allium sativum*) has been used as a medicine since ancient times and has been known to have antibacterial, antifungal and antiviral properties (1). Several centuries ago the Egyptians used garlic to treat many disease entities (2). Aristotle and Hippocrates called attention to the healing powers of garlic, and Pasteur mentioned its medicinal and antibacterial properties (3).

Garlic is composed of mainly of water (60-70g/100g fresh weight) and the most significant components, medicinally, is the organosulfur-containing compounds (3). Among other ingredients, it contains alliin/allicin and its degradation products, and sulphur-containing essential oils (4). An intact garlic bulb does not contain alliin but rather its precursor, alliin, which is hydrolyzed to alliin, pyruvate, and ammonia by the phosphopyridoxal enzyme allinase when the tissue of the bulb is disrupted (5).

These constituents of garlic, particularly alliin/allicin, have been demonstrated to possess antioxidant properties, and to inhibit the production and/or release of mediators such as adenosine, prostaglandins and thromboxanes (6). Garlic has been claimed to be beneficial in infection, tumours, diabetes, hypertension, hyperlipidemia and atherosclerosis (7). There is increasing interest in its antihypertensive and antihypercholesterolaemic activity, also (8).

Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria including species of *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus*, and *Clostridium*. Even acid fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic (9). Garlic extracts also have a strong antifungal effect, and alliin was assumed to be the main component

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responsible for the inhibition of fungal growth (10). The mode of action of allicin on the fungal cell has not yet been elucidated but it is assumed to function on thiol enzymes as in other microorganisms.

Under light of these data we aimed to evaluate garlic's cytotoxic effect on human gingival fibroblasts (HGFs). Commercially worldwide available mouth-rinse solution chlorhexidine gluconate (CH) was chosen for comparison. This is the first report that evaluated cytotoxic effect of garlic extract on HGFs.

## Material and Methods

### Preparation of the methanolic extracts

The air-dried and finely ground samples were extracted by using the method as described elsewhere (11). Briefly, the sample weighing about 100 g was extracted in a Soxhlet with methanol (MeOH) at 60 °C for 6 hours. The extract was then filtered and concentrated *in vacuo* at 45 °C yielding a waxy material (10.00%, w/w). Finally, the extracts were then lyophilised and kept in the dark at +4 °C until tested (11).

### Gingival fibroblast cell culture

Cultures of fibroblasts were established from gingival biopsies obtained from healthy individual. The biopsies were stored at 4 h at 4°C in Hank's salt solution containing penicillin/streptomycin and amphotericin (all from Biochrom KG, Berlin, Germany) prior to amplification. The gingival tissue samples were cut into 1–2 mm<sup>3</sup> pieces, and then washed twice with Hank's salt solution. Thereafter, the cut biopsies were placed into tissue culture flasks (25 cm<sup>2</sup>). The explants were incubated with culture medium consisting of Dulbecco's modified Eagles medium (DMEM, Sigma Chemical Co, St. Louis, MO), 10mm HEPES, glucose (4.5 g/l), NaHCO<sub>3</sub> (3.7 g/l), penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin (2.5 mg/ml) (all from Biochrom KG, Berlin, Germany), supplemented with 10% heat inactivated fetal calf serum (FCS) (PAN Systems, Aidenbach, Germany). Cells were grown at 37°C in a humidified atmosphere of 10% CO<sub>2</sub> in air. Culture medium was renewed twice per week until cells reached confluency. For subcultivation, cells were detached from the culture flasks with 0.25% Trypsin/EDTA Solution (Sigma, UK) for 3–5 min. Cells used for the experiments proliferated in logarithmic phase between the 7th and 12th passages. Cell morphology was visualized with phase contrast microscopy (Nikon, Eclipse, TS 100).

### Agar diffusion method

The agar diffusion tests were performed according to international standards (12,13). Briefly, the cultures were harvested using 0.25% trypsin solution (Gibco, Germany). Stock cultures were seeded in 35 mm diameter cell culture petri dishes (Nunc, Wiesbaden, Germany) at a density of 1 x 10<sup>6</sup> cells/petri dish and subcultured once a week. After a confluent cell layer had formed, the medium was removed and replaced with complete medium containing 1.5% agarose (FMC BioProducts, Rockland, ME, USA). After the agarose had solidified, the cells were stained with a vital dye (neutral red; Sigma). During the following procedures the cells were protected from light to prevent cell damage elicited by photoactivation of the stain. Experimental solutions were applied by using sterile round Whatman papers in diameter 6 mm. For each garlic solution four replicate dishes and four additional dishes with positive and negative control materials were prepared. As negative control, DMEM was used, and as positive control absolute phenol was used. After an exposition period of 24 h at 37°C the cell response was evaluated by inverted microscope observation (Type IM, Zeiss, Oberkochen, Germany). Cytotoxicity was determined by the cellular uptake of neutral red, which preferentially binds to acid regions of living cells as lysosomes and proliferating DNA, staining them intensively red. The toxicity of the test substances was related to the zone of decolorization around the sample caused by unstained injured cells (zone index) and the morphological signs of cell damage within the zone (lysis index) and was estimated by the response index (zone index/lysis index) that was determined as the average response index out of the replicates (Tables I and II)

## Results

### Agar diffusion test

The garlic and CH were investigated using the agar diffusion test for 24 h. At no point in time were cytotoxic reactions detected in any of the four replicates of with mouthrinse %1.5, %3.12 and %6.25. There was no zone of decolorization around the samples. Even the cells directly under those concentrations of garlic, which could be examined by removing the materials from the agar overlay, did not show any signs of cell injury and were similar to negative controls. Therefore, with regard to Tables I and II, the response index of these concentrations

was determined to be 0/0, which means they were not cytotoxic. Concentration of garlic 12.5% and CH showed zones of decolorization of different size and degree around the samples, and cell lysis that varied. Evaluated by the scoring system of Tables I and II, the cytotoxicity was interpreted as mild for garlic sample at concentration %12.5, and moderate for CH. Phenol that was used as the positive control in the agar diffusion test caused a zone of decolorization of 6 mm around the specimen. About 60–80% of the cells in the zone were affected. Therefore, the response index was 3/4 and toxicity was interpreted as marked. The negative control DMEM did not influence the cells.

## Discussion

Many hundreds of plants worldwide are used in traditional medicine as treatments for bacterial

infections. Some of these have also been subjected to in vitro screening but the efficacy of such herbal medicines has seldom been rigorously tested in controlled clinical trials. Conventional drugs usually provide effective antibiotic therapy for bacterial infections but there is an increasing problem of antibiotic resistance and a continuing need for new solutions. Although natural products are not necessarily safer than synthetic antibiotics, some patients prefer to use herbal medicines, and garlic is one them.

Allicin, main active component of garlic, reacts very rapidly with free thiol groups, via thiol-disuophide exchange and, therefore, it is thought that its main mechanism of antimicrobial action is through interaction with thiol-containing enzymes, including cysteine proteases and alcohol dehydrogenases (10,14). Because these enzymes tend to be essential for bacterial nutrition and metabolism it has been suggested that development of resistance to allicin arises 1000-fold less easily than it does to certain antibiotics (15). In addition, certain oral streptococci and lactobacilli have been shown to be sensitive to garlic extract and a mouth wash containing garlic extract was more effective at reducing the total salivary bacterial count and the mutans streptococcal count (16,17).

Fungal infections have become an important aspect of modern infectious disease practice. The prominence of fungi as pathogens may be due to the longer survival of immunocompromised patients, the recent development and usage of broader-spectrum antibiotics, or the wider use of immunosuppressive and cancer chemotherapeutic agents. Oral candidosis is a frequent problem in patients with HIV infection and fluconazole-resistant *Candida* species present particular difficulties for elective treatment. Garlic is reported to have antifungal properties and another garlic component, allicin, has been used for prevention of thrush in neonates (10,18).

The development of new therapies for treatment of oral cavity diseases is of great importance since the systemic and local administration of antimicrobials brings about several problems. Some of these problems are: selection of multiresistant microorganisms, interbacterial transfer of resistance determinants and unpleasant side effects. A relatively large number of chemical agents, which are mostly synthetic compounds, have been use for many

**Table I. Toxicity evaluation by the size of the zone of decolorization and the degree of cell lysis using the Agar Diffusion Test**

<i>Index</i>	<i>Description of Zone</i>
<b>Zone</b>	
0	No detectable zone around or under specimen
1	Zone limited to area under specimen
2	Zone extends less than 0.5 cm beyond specimen
3	Zone extends 0.5-1.0cm beyond specimen
4	Zone extends greater than 1.0cm beyond specimen but does not involve entire dish
5	Zone involves entire dish
<b>Lysis</b>	
0	No observable Cytotoxicity
1	Less than 20% of zone affected
2	20% to 39% of zone affected
3	40% to 59% of zone affected
4	60% to 80% of zone affected
5	Greater than 80% of zone affected

**Table II. Scoring System for estimating Cytotoxicity in the Agar Diffusion Test**

<i>Response Index</i>	<i>Interpretation of Cytotoxicity</i>
0/0- 0.5/0.5	None
1/1- 1.5/1.5	Mild
2/2- 3/3	Moderate
>4/4	Marked

Diameter of test specimen = 5 mm; response index = zone index/lysis index.

purposes, control of dental plaque, elimination of oral pathogens, against malodor, etc.

All the researches mentioned have shown clearly the potential antibacterial and antifungal activities of garlic. It is better the use of standardized preparations of garlic. The results of the present study suggest that garlic containing mouthrinse is not cytotoxic on HGFs. In addition, some studies related to dental caries and periodontal diseases were needed to determine effects of garlic on oral diseases.

In literature there are studies evaluated cytotoxic effects of clinical materials on HGFs. Tsourounakis et al. (19) evaluated mouthwash for its ability to affect fibroblast survival and migration, as well as long term effects on cell viability. The results of their *in vitro* study indicated that dilute essential oils displayed no detectable detrimental effects on human gingival and periodontal ligament fibroblasts, while dilute CH reduced both cell migration and long term survival. Trubiani et al. (20) analyzed the cell response to acrylic based resins Ivoclar, Tokuso and Coldpack in basal conditions, unpolished, and after the polishing procedure performed to reduce the surface roughness. Their *in vitro* results using HGFs showed a decrease of cell growth at 24 h of incubation, in samples seeded on resins in basal conditions and after the polished procedure. After 24 h of culture in presence of polished and unpolished resins a spontaneous release was present of pro-inflammatory cytokines such as Interleukin-6 (IL-6) and -8 (IL-8), which was higher in unpolished resins, indicating that the polished procedure, minimizing the cytotoxicity process, could contribute to reduce the gingival inflammation processes. Yang (21) evaluated the potential toxicological implication of BAPP (three-dimensional structure of the dental resin composites is 2,2-bis[4-(acryloxypropoxy)phenyl]propane) on HGFs. With increasing concentrations of BAPP, the mode of cell death shifted from apoptosis to necrosis. Investigator stated that BAPP-induced cytotoxicity and genotoxicity on HGFs were mediated by DNA damage and the activation of caspases 3, 8, and 9. Yu et al. (22) evaluated the cytotoxic effects of nickel ion on HGFs and explore the mechanism, and they observed the effects of the ion salt solution on the proliferation of cultured cells *in vitro*. They concluded that the inhibition effect of Ni<sup>3+</sup> on the proliferation of HGFs was partly due to its potential of inducing apoptosis, and the mechanism of apoptosis is via

the caspase-3 activation. The further studies will be fulfilled by animal experiment or evidence-based medicine method. Asgari et al. (23) assessed the adhesion of HGFs to mineral trioxide aggregate (MTA) and calcium-enriched mixture (CEM) cement using a scanning electron microscope. HGFs displayed a favorable biologic response in contact with MTA and CEM. Nowakowska et al. (24) evaluated the cytotoxic effects of the vasoconstrictor experimental gingival retraction agents (VEGRAs) in a dynamic setting. The cytotoxic effect, measured by cell viability lower than the 50% threshold, was not observed at any time period, even 24 h after application of 0.05% HCl-tetrahydrozolinebased self-manufactured retraction gels. Willershausen et al. (25) demonstrated the possible effect of different endodontic calcium hydroxide and chlorhexidine-based gutta-percha points, on two different human cell culture systems. They stated that chlorhexidine containing gutta-percha points showed the highest effect on cell growth inhibition. Wilken et al. (26) studied *in vitro* cytotoxic effect of 0.2% chlorhexidine gluconate in water, 0.15% benzydamine-HCl in 8.5% ethanol and 1% povidone iodine in 10% ethanol on HGFs. Results indicated that cells were immediately fixated by 10% chlorhexidine gluconate in water, 20% povidone iodine and 70% benzydamine-HCl. Mariotti et al. (27) examined the effects of chlorhexidine on HGFs proliferation as well as collagen and non-collagen protein production in cell culture. They stated that chlorhexidine will induce a dose dependent reduction in cellular proliferation and that concentrations of chlorhexidine that have little effect on cellular proliferation can significantly reduce both collagen and noncollagen protein production of HGFs *in vitro*. Hence, the introduction of commercially available concentrations (0.12%) or diluted commercial concentrations (as low as 0.00009%) of chlorhexidine to surgical sites for short periods of time prior to wound closure can conceivably have serious toxic effects on gingival fibroblasts and may negatively affect wound healing. Pucher et al. (28) utilized human fibroblasts derived from skin and oral tissues to test the effects of chlorhexidine on viability, growth, collagen gel contractions, and total protein synthesis. They stated that chlorhexidine is highly cytotoxic to cells *in vitro*, but various cell functions

such as proliferation, collagen gel contraction, and protein synthesis are affected to different degrees by the drug.

Garlic could have a promising role in the future medicine, if appropriate solutions can be prepared. These results give hope to us that garlic a natural product can be used for oral rehabilitation of patients for various purposes.

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