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Original Research

Strong antimicrobial activity of *Hypericum perforatum L.* against oral isolates of *Lactobacillus spp.*

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Abstract: *Lactobacillus* spp. are one of the first microorganisms involved in the development of dental caries in the first years of life of the child. The purpose of this study was to investigate the antibacterial effect of alcoholic extract of *hypericin* against strains of *Lactobacillus* spp. and determine its related MIC (minimum inhibitory concentration); and cytotoxic effect against gingival fibroblasts. Antimicrobial activity and MIC were evaluated using micro broth dilution method based on CLSI (clinical and laboratory standards institute) protocols. Determination of cytotoxicity was done by using MTT assay protocol on gingival fibroblast cells at 24, 48 and 72 hours after adding different concentrations of alcoholic extract of *hypericin. Hypericin* extract had an antimicrobial effect on *lactobacillus spp.*, and its MIC was determined to be 0.625µg/ml . The IC50 value after 24, 48 and 72 hours was obtained as 0.89µg/ml, 0.7µg/ml and 0.604µg/ml, respectively. *Hypericin* extract MIC for *L.acidophilus* spp. was 0.625µg/ml and given that, MIC was less than IC50. This concentration does not have toxic effects on gingival fibroblast cells. The results of this study indicate that *hypericin* extract was able to eliminate acid producing strains in the mouth and can be evaluated as a suitable and safe substitute for mouthwashes and oral disinfectants.

Key words: *Hypericin*; *Lactobacillus*; The minimum inhibitory concentration; Gingival fibroblasts.

Introduction

The use of herbal medicines in developed countries has expanded rapidly in the second half of the twentieth century; this general acceptance of the use of herbal therapies is due to significant side effects in chemical compounds of synthetic drugs (1, 2). In the field of oral diseases, herbal treatments have also been used; these drugs have been frequently evaluated in chemotherapy-induced mucositis treatment (3).

In recent years, the use of products derived from *Hypericum perforatum L.* , known as grass tea, especially for the treatment of depression (4), has grown substantially and it has become a medicinal herb worldwide (5). *H.performatum L.* is an ever green plant that has yellow flowers and is native to Europe (6). The alcoholic extracts from this plant include a number of phenolic components, such as *hypericin* and *hyperforin*, which have antioxidant properties (7). Due to its extensive activity in the nervous system and its positive effect on the treatment of mild to moderate depression, the use of this plant has increased significantly in recent years (8-10). *Hypericin* is responsible for its antidepressant effect (11). The antibacterial effect of *hypericin* has also been proven by several studies (12, 13) and it has been used as an anti-inflammatory, antiviral and antibacterial component (14, 15). Also, the extract of this plant is a photosensitizer in PDT (Photodynamic Therapy) (16). In various studies, the effect of photochemical *hypericin* on bacterial (17) and fungal species has been investigated and its anti-Candida effects have been proven (18). *Hypericin* has also been shown to have an antibacterial effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (19).

Lactobacillus spp. are part of the normal oral microflora (20, 21) and form about 1% of the cultured oral microflora (22, 23) and are the first microorganisms that contribute to the development of dental caries in the early years of the child's life (24). The number of these microorganisms in saliva is used as a predictive test of decay risk (25) and has a positive relationship with oral dryness (26). The high levels of *Lactobacillus spp.* are associated with decay and consumption of sugars (27, 28).

Regarding the role of *Lactobacillus spp.* in decay, and according to previous studies, MIC (minimum inhibitory concentration) of *hypericin* extract has not been determined on *Lactobacillus spp.* and its cytotoxicity has not been studied on gingival fibroblasts. Therefore, the aim of this study was to investigate antibacterial effect of *hypericin* alcoholic extract against *Lactobacillus* spp. microorganisms and its MIC was determined, and simultaneously, this MIC was evaluated for cytotoxicity on gingival fibroblasts.

Materials and Methods

MIC determination

A standard species of *Lactobacillus acidophilus* (ATCC Medium 416) (from Iranian Institute of Science

and Technology) and oral isolates of *Lactobacillus* spp. isolates were prepared. The hydroalcoholic extract of the *H.perforatum L.* was prepared from Poursina company (Tehran, Iran), which contained 0.1mg/ml of *hypericin*. The filtration solution was sterilized and stored in darkness. All bacteria were cultured in MRS-Agar (Man Rogosa Sharpe Agar) (Merck Schuchardt, Germany) media for 24 hours before being tested for live and fresh isolates. The MIC test was performed using a 96-well sterile plates, by microdilution method based on CLSI (clinical and laboratory standards institute) protocol (29). In a 96-well plate, dilution series of the under study substance (*hypericin* alcoholic extract) was prepared at a concentration of 16 µg/ml to 0.019 µg/ml. Beside the flame of light and under the hood, to prevent bacterial contamination, a fresh bacterial culture (18-24 hours) were prepared in normal saline and the bacterial density was adjusted to 0.5 McFarland (30). This suspension was diluted with MRS agar at 1: 100 ratio and then 100 µl of it was added to each well; thus, in each well, there was an estimated 10^6 cfu/ml bacteria. Anaerobic condition was provided by using GasPak C anaerobic system (Becton Dickinson, UK) in anaerobe jar (28). After incubation for 24 hours under anaerobic conditions at 37 °C, the tray-reading stand was made for this purpose, the plate underneath the light was observed. The presence of turbidity, indicating bacterial growth, was noted on a special table. According to the definition of the concentration, the last (most dilute) well without any curvature was created equal to the MIC. Extract control house, culture medium and bacteria were also separately included.

For testing of MBC (minimum bactericidal concentration), wells without a specific turbidity were grown on MRS-agar medium and after 24 hours incubation under anaerobic conditions at 37 °C, the minimum concentration of extract which the bacteria has no growth in it, was reported as lethal concentration of MBC (Fig. 1) (30).

This experiments were repeated three times and the data obtained from the study were analyzed by descriptive statistics methods.

Evaluation of cytotoxicity

The gingival fibroblast cell line was prepared by the HGF1-PI1 identification code from the Tehran Pasteur Institute and cultured in a flask in DMEM medium (dulbecco's modified eagle's medium) with FBS (fetal bovine serum) 20% and penicillin-streptomycin, then incubated at 37°C and Co2 at 5% pressure and it was multiplied.

Every 24 hours, a complete culture medium (penicillin-streptomycin + FBS 20% + DMEM) was added to the flask containing cells and cells were counted by a Neubauer chamber under an optic microscope.

MTT assay

In 96-well plates, one well was used for MIC concentrations that were pre-assigned and 4 other wells at a concentration of 2, 4, 1/2 and 1/4 MIC, and a positive control well (no *hypericin* extract) were determined. Initially the gingival fibroblast cells replicated in the flask were trypsinized and removed from the floor, and in the target wells, 5000 cells per 200 microliter were placed in a complete environment. The cells were doubled for 24 hours. After this time, *hypericin* extract was added to the wells at concentrations of MIC and 2, 4, 1/2 and ¼ of MIC. After 24 hours, 30 µl of MTT (MTT powder with concentration of 5mg/ml + PBS sterile) was added to each well. After incubation for 4 hours at 37 °C, 180 µl of supernatant was removed and 150 µl of DMSO (dimethyl sulfoxide) was added and placed on a shaker for 20 minutes (DMSO, soluble MTT powder and readable to the ELISA device). Then, the optical density (OD) was evaluated by an ELISA reader (bioteck, United states) device at 570^{nm} and 490^{nm} wavelengths (29).

The same steps were repeated for 48 hours and 72 hours after adding *hypericin* extract. These tests were repeated four times and the data obtained from the study were analyzed by descriptive statistics.

Statistical analysis

Chi-square test (or Fisher exact test) was performed for data analysis. P values below 0.05 were considered significant. Statistical analysis was done by Spss. 21 software.

Results

The results were similar through all three repetitions, in that the growth of *Lactobacillus* spp. were stopped at a concentration of 0.625µg/ml. As a result, *hypericin* extract MIC for *Lactobacillus* spp. was 0.625µg/ml (Figure 1).

Hypericin extracts cell cytotoxicity was measured based on the percentage of viability of the cells compared to the control group by MTT assay at different exposure times (24, 48 and 72 hours). Based on an average of 4 replicates, IC50 (the amount of drug that causes death of 50% of the cells) values at 24, 48 and 72 hours after exposure were 0.89µg/ml, 0.7µg/ml and 0.604µg/ml, respectively. In addition, changes in cytotoxicity were

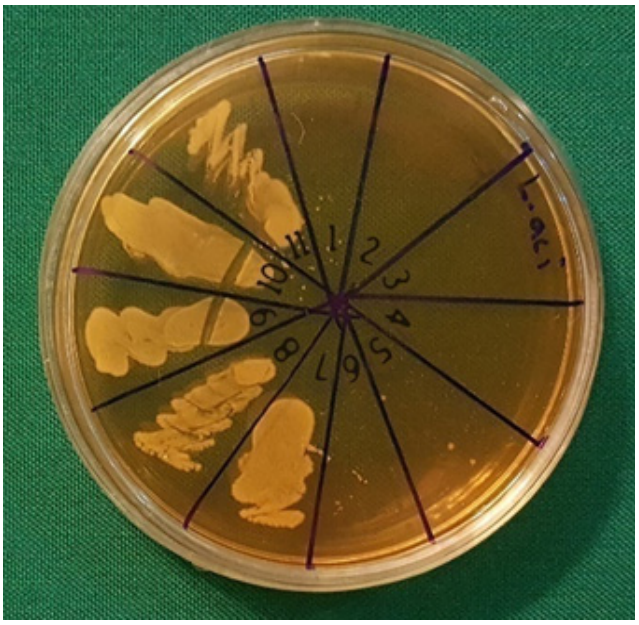

Figure 1. Determination of MBC of *L. acidophilus* isolates through theirs cultures on a MRS-agar medium. Different concentrations of *hypericin* in µg/ml: 1:16, 2:15, 3:5, 4:0.625, 5:0.3125, 6:0.156, 7:0.078, 8:0.039, 9:0.019, 10:0.009, 11:0.004

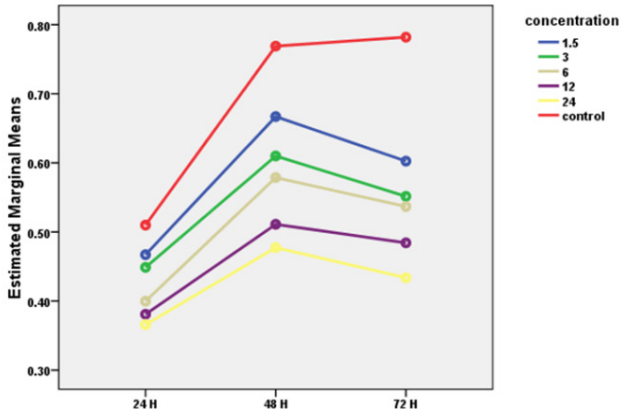

Figure 2. Levels of *hypericin* extract and cell cytotoxicity at different periods.

significant at different concentrations over time (P-value <0.001) (Figure 2).

Discussion

Regarding the role of *Lactobacillus* spp. in dental caries and the efficacy of pharmaceutical compounds of *hypericin extract* against *Streptococcus mutans* and fungal infections, the antibacterial effect of this compound on *Lactobacillus* spp. microorganisms were investigated and its MICs were determined. At the same time, this MIC with regarding to the cytotoxicity of gingival fibroblasts was investigated by MTT assay.

The results showed that *hypericin* extract had antibacterial properties against *Lactobacillus* spp. and IC50 was greater than the MIC, which indicates the absence of toxicity of *hypericin* extract in this MIC.As the distance of MIC and IC50 increases, the drug becomes safer. In the present study, the MIC against *Lactobacilli* spp. after 24h were MIC=0/625µg/ml and IC50 was =0.89µg/ml a. Regarding the difference between these two digits, it should be noted that, firstly, the MIC dose was less than IC50. This result indicates the possibility of using the drug in a therapeutic dose without producing toxic effects (in the *in vitro* condition). Secondly, regarding difference between these two values (MIC and IC50), *hypericin* extract exposed directly to gingival fibroblasts *in vitro*, while in the clinic and in the oral environment after oral administration of the drug to reach fibroblast cells and its affect, *hypericin* extract should be transported across epithelium barrier which bioavailability and tissue toxicity will change.

Several previous studies investigated oral application of *hypericin* extract. It is also currently being prepared and used by the pharmaceutical companies for Migraine and nervous problems and reported to have no harmful effect (11). Of course, the aim of this study was to evaluate the cytotoxicity of this compound on gingival fibroblasts in direct exposure to oral topical use, which was performed in laboratory conditions and was different from the clinical setting and the rate of local/mouthwash is different from the oral dose (systemic). Obviously, the study of changing laboratory values in the form of oral mucosal or oral use requires the development of an *in vivo* study.

Given the importance of developing drug resistance to antibacterial drugs, in recent years, numerous studies have been conducted on the antimicrobial effects of different herbs (29, 31). These studies have shown that some herbs have the same effects as chemical drugs or far more (32). In 1999, Shempp et al. showed that *hypericin* has an antimicrobial effect against *Staphylococcus aureus* with MIC =1µg/ml (33). Paula pero et al. (2013), in their study on bone marrow cells of six rats with a weight of 100 g each, measured the cytotoxicity of the *hypericin* by chromosomal aberration at three concentrations of 0/3, 3, and 30mg/ml with peritoneal injection and concluded that *hypericin* does not have toxic effects on these cells (34).

Kashef et al. (2012) determined the effect of *hypericin* (concentrations of 0.1, 0.3, 0.6 and 1 µg/ml) with duration of laser radiation (3, 5 and 10 minutes) on reducing the activity of microorganisms. They concluded that *hypericin* with energy of 48 J/cm^2 reduced the growth of microorganisms of *Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli* and has bactericidal properties. However, *Pseudomonas aeruginosa* had a relative resistance to other microorganisms. Also, based on the MTT protocol, cell cytotoxicity was evaluated on abdominal skin fibroblasts cells and showed that *hypericin* does not have toxic effects on skin fibroblasts (19). Zenuz et al., in 2016 (35), investigated the effects of photodynamic therapy in the presence of methylene blue and *hypericin* on two microorganisms of *enterococcus faecalis* and *Pseudomonas aeruginosa* and concluded that the use of *hypericin* at a concentration of 100 µg/ml with and without laser therapy has the bactericidal properties against two bacteria: *Enterococcus faecalis* and *Pseudomonas aeruginosa*. By decreasing the concentration of *hypericin* to less than 100 µg/ml, its antibacterial properties decreased, microorganisms grown in culture medium.

Silva et al., (2015), tested the cytotoxicity of *hypercin* on liver cells using the MTT assay and showed that *hypericin* had a toxic effect on the cells in the concentration of 10µM and this effect is dependent on concentration and on exposure time (36). Hwang et al. (2001), based on the MTT protocol, examined *hypericin* cell cytotoxicity at various concentrations of 1-10 µM on human ovarian cells, and reported the IC50 after 24 hours and 72 hours, respectively, as 5µM and 7/5µM (37).

The results of this study showed that in the *in vitro* condition, the minimum inhibitory concentration of *hypericin* for *Lactobacillus* spp. is 0.625µg/ml and does not have toxic effects on gingival fibroblast cells. Therefore, according to the high potency of antimicrobial activity against *Lactobacillus* spp. (which plays an important role in the onset of caries in children), it can be considered as a natural product with this concentration as mouthwash, especially in children or in oral health care products such as gels and dressings.

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Conflict of Interest

None to declare.

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