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Original Article

Evaluation of cytotoxic effects of Anbarnesa on fibroblast L929: Can it be used as a mouthwash?

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ABSTRACT

Aims: In Iranian traditional medicine Anbarnesa (derived from smoke from burning female donkey's stool) has been used to treat ulcers and inflammatory conditions like stomatitis and ear infections (otitis). We assess the properties of Anbarnesa as an alternative mouthwash.

Materials and Methods: In this experimental study, Anbarnesa smoke was analyzed using aGC-mass device. The smoke collected was dissolved at different densities in propylene glycol and incubated in Dulbecco's modified Eagle's medium in direct contact with fibroblast cells. Assessment of cytotoxicity was done at 1, 24 and 72 h. Cell viability was measured by methyl thiazolyl tetrazolium test, and ELISA Reader machine was used to read the results. Data were analyzed using one-way ANOVA test.

Results: The findings of this study showed Anbarnesa was nontoxic in 1/64, 1/128 and 1/256 dilutions. In 1/32 dilution, toxicity was seen after 72 h. In dilutions, 1/8 and 1/16 toxicity were seen in the 1st h.

Conclusion: According to the initial results of Anbarnesa may be used as an alternative mouthwash with fewer side-effects for plaque control and prevention of periodontal disease.

KEY WORDS: Cytotoxicity, fibroblast, traditional medicine

INTRODUCTION

In order to prevent periodontal disease, plaque control is important.[1] Today, mechanical and chemical methods for plaque control are in vogue.[1] Antibacterial agents such as mouthwash in addition to mechanical methods, are effective in the treatment of periodontal diseases.[2]

An ideal mouthwash is the one that has no allergic reactions,[3] no teeth or oral mucosa staining,[4] possesses anti-plaque and antibacterial properties,[5] has minimal cytotoxic effects on cells,[6-8] is sustainable and has a good taste.[9]

Such a mouth rinse with all the above-mentioned properties is not present yet. Hence, various types of mouthwash have been introduced of which, due to its beneficial effect, chlorhexidine (CHx) has been recommended;[4,10] but it has many side-effects, prime among them is cytotoxicity.[8]

Traditional medicine and herbal remedies have been used in various fields of medicine.[11] In Iranian traditional medicine Anbarnesa smoke (derived from burning dried dung of the female donkey), is used for the treatment of inflammatory oral lesions such as aphthous ulcer and other inflammatory lesions such as otitis.[12] Composition of animal manure is organic matter basically organic carbon, water-soluble organic carbon, organic N, carbohydrates, humic acid-like carbon and fulvic acid-like carbon.[13] Since ancient times, humans have used smoke of medicinal plants to cure illness. In a study shows that in 50 countries, usage of medicine smoke is common, and one type is medicinal smoke of Anbarnesa.[14]

Fibroblast cells are dominant cells in periodontal ligament (PDL), and are important in maintaining healthy gingiva and surrounding tissues.[1,15] The biological effects of the mouth rinses have not been sufficiently studied; the present work was done to determine the cytotoxic effect of Anbarnesa sebum 1 on cultured mice fibroblasts in category L929.

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MATERIALS AND METHODS

In this experimental *in vitro* study, 198 samples with positive and negative controls of mice fibroblast cells obtained from the Pasteur Institute cell bank (Tehran, Iran) were studied. According to the standard ISO 10993:5, 6 replicates were used. The original concentrations, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 dilution were prepared and studied. Assessments were done after 1 h, 24 and 72 h after contact with the cells.

First, Anbarnesa smoke was analyzed using aGC-mass device and substances such as hexane, citric acid and dimethylamine were reported. After ensuring the effectiveness of materials, Anbarnesa was burned inside a closed container with its walls coated with propylene glycol. After cooling the environment, substances in the smoke adhered to the walls. This was repeated several times to accumulate enough smoke residue on the walls. Then 10 mL propylene glycol solution was added to the container and mixed well with the material on the walls. Next, the contents were transferred to a falcon tube to separate impurities, and the suspended samples were centrifuged for 30 min. Using a Pasteur pipette, the supernatant fluid was transferred to another tube. This solution was named Anbarnesa sbmu 1. The concentration of the liquid was measured using liquid chromatography and diluted to concentrations of 0.2% and minimum inhibitory concentration values were evaluated against different bacterial species and compared with control samples of propylene glycol. Growth inhibitions on species of *Streptococcus mutans*, *Streptococcus salivarious*, *Streptococcus sanguinis*, and *Streptococcus pyogenes* after the exposure to this solution were assessed for antibacterial properties.

Complete Dulbecco's modified Eagle's medium (DMEM) with embryonic bovine serum was used for the culture.

After distribution of cells in wells of three plates, the plates were incubated for 24 h. After this, the culture medium was removed from wells and then 200 µl of sample (prepared by dilution) was placed in each of the wells. In each plate, 6 wells of negative controls and 6 wells of positive controls were assigned. The sample plates were incubated at temperatures 37°Cat 98% humidity and 5% CO_2.

The methyl thiazolyl tetrazolium (MTT) test was used to assess cytotoxicity based on color change and the production of formazan. For the MTT test, tetrazolium bromide salt (Sigma-Aldrich, USA) at a ratio of 5 mg/mL in phosphate buffer solution was mixed with the DMEM at ratio of 1/10. ELISA reader machine (Anthoos, 2020, Australia) was used to read the optical density (OD). Data of OD were divided to OD of negative controls to assess cell viability. The data were statistically analyzed using computer SPSS 17 software (IBM Chicago, IL) and one-way ANOVA test.

RESULTS

Mean and standard deviation of OD sat different concentrations at 1 h are presented in Table 1. For measuring the cell viability, OD at different concentrations are divided to ODs of negative controls shown in Diagram 1.

Cell viability over 70% meant that the concentration was not cytotoxic to fibroblast cells. Cell survival between 50% and 70% meant that the concentration had a cytotoxic effect on half of the fibroblast cells. And cell survival below 50% meant that the concentration is cytotoxic to fibroblast cells.

Means and standard deviations of OD sat different dilutions at 24 and 72 h are presented in Tables 2 and 3, respectively. Furthermore, cell viability at 24 and 72 h is shown in Diagrams 2 and 3 respectively.

There was no toxicity at dilutions of 1/32, 1/64, 1/128 and 1/256. The ANOVA test was used to compare toxicity

Table 1: Mean and SD results of various concentrations in 1 h

	Negative control	Positive control	Orginal	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
N											
Valid	6	3	3	6	6	6	6	6	6	6	6
Missing	0	3	3	0	0	0	0	0	0	0	0
Mean	0.08783	0.000533	0.07100	0.3733	0.03133	0.06117	0.22617	0.10033	0.07600	0.07550	0.06983
SD	0.017406	0.002517	0.014000	0.023235	0.013140	0.028868	0.320373	0.018949	0.020209	0.014923	0.015065
Minimum	0.057	0.003	0.057	0.002	0.016	0.026	0.089	0.078	0.043	0.049	0.049
Maximum	0.104	0.008	0.085	0.064	0.054	0.103	0.880	0.123	0.093	0.091	0.090

SD: Standard deviation

Table 2: Mean and SD results of various concentrations in 24 h

	Negative control	Positive control	Orginal	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
N											
Valid	6	3	3	6	6	6	6	6	6	6	6
Missing	0	3	3	0	0	0	0	0	0	0	0
Mean	0.17533	0.00300	0.03200	0.04517	0.01633	0.01500	0.01833	0.08737	0.12967	0.11833	0.13550
SD	0.021860	0.000000	0.008888	0.034214	0.013140	0.005574	0.014081	0.011201	0.011518	0.011201	0.014516
Minimum	0.150	0.003	0.057	0.025	0.011	0.003	0.069	0.078	0.117	0.106	0.117
Maximum	0.210	0.008	0.085	0.042	0.097	0.041	0.099	0.123	0.151	0.135	0.159

SD: Standard deviation

Table 3: Mean and SD results of various concentrations in72 h

	Negative control	Positive control	Orginal	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
N											
Valid	6	3	3	6	6	6	6	6	6	6	6
Missing	0	3	3	0	0	0	0	0	0	0	0
Mean	0.19817	0.00233	0.00233	0.02050	0.02133	0.01700	0.01533	0.05000	0.11083	0.13550	0.14217
SD	0.011805	0.000577	0.00577	0.005206	0.005750	0.006723	0.002251	0.039820	0.025230	0.028098	0.029963
Minimum	0.179	0.002	0.002	0.012	0.016	0.011	0.013	0.012	0.077	0.106	0.117
Maximum	0.213	0.003	0.003	0.027	0.030	0.026	0.019	0.094	0.137	0.176	0.171

SD: Standard deviation

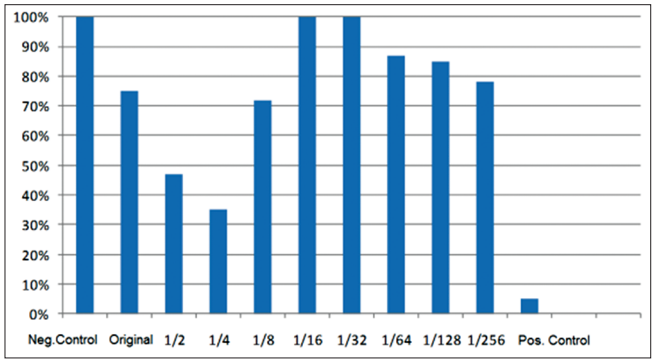

Diagram 1: Cell viability result in 1 h

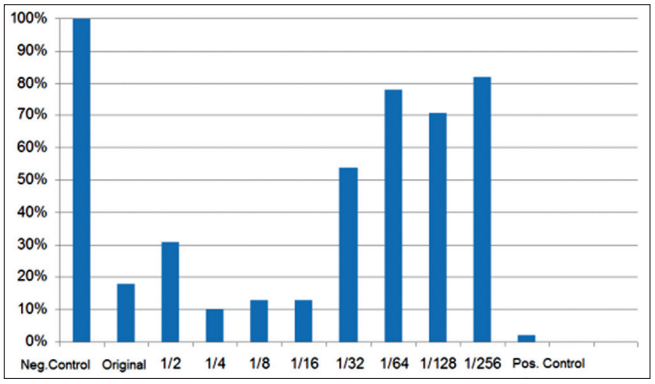

Diagram 2: Cell viability result in 24 h

and significant difference were reported in these four concentrations ($P < 0.05$).

DISCUSSION

There are different methods to assess cytotoxicity *in vitro*: Permeability assay, replication assay, morphologic studies, monolayer agar overlay and functional assay. MTT assay is a subgroup of the functional assay.[16] MTT assay evaluated levels of cell enzymes or cell mitochondrial function. MTT is a simple colorimetric assay that uses MTT salt to measure changes in mitochondrial enzyme succinyl dehydrogenase. The first salt is pale yellow. Mitochondrial succinyl-dehydrogenase enzymes break the salt and create a new dark blue product called formazan. Thus, only cells that contain vital mitochondria and active metabolism can reduce tetrazolium bromide salt and create formazan deposition. The resulting color change is directly related to cell metabolic activity.[17-19]

The data in this study showed no toxicity at the 1/64, 1/128 and 1/256 dilutions. At 1/32 dilution, toxicity was seen only after 72 h and in dilutions 1/8 and 1/16 toxicity was not observed in the 1st h.

Dilutions 1/2 and 1/4 however were cytotoxic. According to the results decreasing dilution, reduces toxicity.

Many studies have been done on a variety of mouthwashes such as CHx; Sanchez *et al*. showed that all bactericidal concentrations of CHx were lethal to embryonic fibroblasts

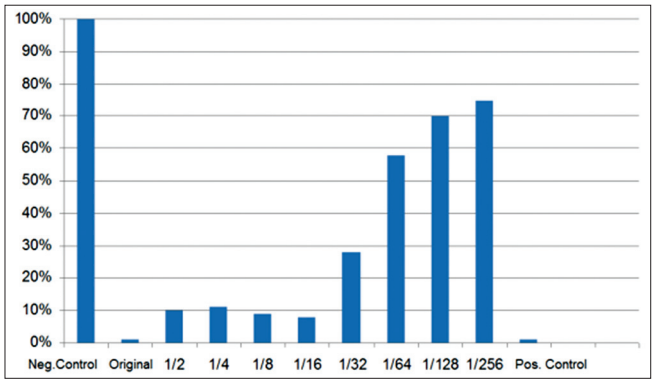

Diagram 3: Cell viability result in 72 h

in vitro.[20] Damour *et al*. and Fabreguette *et al*. suggested that therapeutic concentrations of antiseptics such as CHx are cytotoxic for fibroblasts and keratinocytes. They determined viability using the MTT test.[21,22] Boyce *et al*. demonstrated that CHx gluconate (0.05%) was uniformly toxic to both cultured human fibroblasts cells and microorganisms.[23] Chang evaluated effect of CHx on cultured human PDL cells *in vitro*. The result showed that CHx was cytotoxic to human PDL cells.[24]

In one study, we compared the antibacterial effect of Anbarnesa 0.2% and CHx 0.2% *in vitro* and result showed that both had similar inhibitory growth zones for different bacterial species which was significantly better than control specimens. CHx 0.2% induced higher minimal inhibitory concentration (MIC) values than Anbarnesa 0.2% for the *Streptococcus sanguinis* and *Enterococcus faecalis* species, while no significant differences were found between two agents regarding MIC values against the other bacteria. CHx 0.2% and Anbarnesa 0.2% showed higher growth inhibitory effects than control specimens against all bacteria except for *E. faecalis*. Hence, we concluded Anbarnesa 0.2% has some antibacterial properties, but it is not as efficacious as CHx 0.2% on some selected species, with no significant effect on the *E. faecalis* species.[12]

CONCLUSION

Given that satisfactory results of the Anbarnesa sbmu 1 solution that had no cytotoxicity at 1/64 dilution, it may be used as an alternative solution for plaque control with less common side-effects.

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