Cytotoxic effect of *Salvadora persica* extracts on human gingival fibroblast cells

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**ABSTRACT**

Methods: This study was conducted between January and May 2012 in collaboration with Dental Caries Research Chair, College of Dentistry, King Saud University, Riyadh, Saudi Arabia. Extracts of *S. persica* were evaluated for their cytotoxic activity against HGFs using the 3 cytotoxic assays: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTS), lactic dehydrogenase (LDH), and crystal violet (CV). International standards for the evaluation of medical materials recommended cut-off value of cell survival >70% was used for interpretation of the results.

**Results:** Ethanol extract of *S. persica* at 0.5 mg/ml and 1 mg/ml and hexane extract of *S. persica* at 0.5 mg/ml were completely devoid of cytotoxic activity, hexane extract at 1 mg/ml in comparison with controls demonstrated some cytotoxicity with cell survival of 88% (p=0.045) in MTS, 86% (p=0.01) in LDH, and 88% (p=0.002) in CV assays. Similarly, ethyl acetate extract of *S. persica* at 0.5 mg/ml maintained cell viability of 91% in MTS, 81% in LDH, and 80% in CV assays. Maximum cytotoxicity against HGFs was observed with ethyl acetate extract of *S. persica* at 1 mg/ml with cell survival of 60% in MTS, 40% in LDH, and 66% CV assays (p=0.0001).

**Conclusion:** The acceptable level of cytotoxicity associated with *S. persica* ethanol and hexane extracts requires further evaluation to be used as irrigation solutions in endodontic treatment.

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**Methods.** This study was registered in the College of Dentistry Research Center, King Saud University for ethical approval, and conducted between January and May 2012 in the microbiology laboratory of the Dental Caries Research Chair, College of Dentistry, King Saud University, Riyadh Saudi Arabia.

**Cell culture.** After obtaining informed consent HGF cells were obtained from healthy gingival tissue of a human adult premolar that was excised during periodontal surgery. The fibroblasts were propagated in Dulbecco modified minimum essential medium (D-MEM) (Invitrogen Life Technologies, Bethesda, Maryland, MD, USA) supplemented with 20% fetal bovine serum, penicillin at concentrations of 250 i.u., streptomycin 250 µg, and fungizone (amphotericin B) 25 µg/ml (Sigma Chemical Co., St. Louis, MO, USA) in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37°C. At 70% confluence, the cells were sub-cultured by dislodging the monolayers using 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) (Invitrogen Life Technologies, Bethesda, MD, USA). The trypsinic activity of trypsin was neutralized by addition of culture media containing 10% fetal bovine serum (Invitrogen Life Technologies, Bethesda, MD, USA). The fifth passage of fibroblasts was re-suspended in fresh D-MEM containing 20% fetal bovine serum and 10% dimethyl sulfoxide (DMSO) (Invitrogen Life Technologies, Bethesda, MA, USA), and used for this experiment at a concentration of 1x10⁶/ml.

**Preparation of *S. persica* extracts.** The roots of *S. persica* were collected from Al-Mukwah, located in the Southern region of Saudi Arabia, in March 2010. The plant was identified by a taxonomist and a voucher specimen (#1745) was deposited at the herbarium, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia for future reference. The stock solution was prepared by extracting the fresh ground roots 3 times with the following solvents: hexane, ethylacetate, and ethanol. All extracts were prepared by percolating 100 g of dried powder in each solvent 3 times every 24-hour, with fresh solvent used each time. The extracts were freeze-dried to ensure that the remaining solvent was completely removed. All *S. persica* extracts were suspended in DMSO at a concentration of 100 mg/ml. The stock solution was kept in a freezer at -20°C. Working dilutions were made in physiological saline at pH 7.4.

**Cell viability assays.** A 96-well tissue culture plates (NUNC, Roskilde, Denmark) were seeded with 50 µl cell suspension, and incubated for 48 hours at 37°C in 5% CO₂ and 75% relative humidity during which time confluent cell monolayers were formed. After incubation for 48 hours, 50 µl each of hexane, ethylacetate, and ethanol extracts of *S. persica* at

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concentrations of 0.5 mg/ml and 1 mg/ml were dispensed in a replicate of 4 wells and incubated for a further period of 24-hour under standard incubation conditions. As a negative control, cells were cultured in 2% of D-MEM. The viability of HGF was evaluated by 3 cytotoxic assays 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTS); lactic dehydrogenase (LDH), and crystal violet (CV). Each extract concentration was assessed twice in 2 independent experiments. Seventy percent cell survival was considered as a cut-off value in accordance with the recommendation of International Standards Organization for the evaluation of medical materials.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Mitochondrial dehydrogenase activity was measured by the ability of such enzymes from metabolically active cells to reduce reagent MTS into a soluble formazan salt. The stock solutions of MTS salt and phenazinemethosulfate (PMS) (Sigma Chemical Company, St. Louis, MO, USA) were prepared in phosphate buffered saline (PBS) (Invitrogen Life Technologies, Bethesda, MD, USA) at a concentration of 1 mg/ml and 125 μM respectively. The working solution of MTS: PMS was prepared by diluting 2.5 ml of stock solution in 7.5 ml of PBS and adding 10 μl of PMS. To each well 0.1 ml of working solution was dispensed into the well plates and incubated for 3 hours. After the incubation, the absorbance was read in the Microplate reader (Thermo Scientific, Bridgewater, NJ, USA) at a wave length of 590 nm.

Lactic dehydrogenase assay. Black-colored 96 well tissue culture plates (NUNC, Roskilde, Denmark) were seeded with 50 μl cell suspension and incubated for 48 hours at 37°C in 5% CO₂ and 75% relative humidity. After the formation of confluent cell monolayers, both concentrations of S. persica extracts were dispensed in test wells and the negative control wells. The plates were allowed to incubate at standard incubation conditions. Cytotox-one (Promega Scientific, Madison, WI, USA), commonly known as lactate dehydrogenase release bio-assay reagent, was used to determine the levels of cell lysis. Fifty micro liters of cytotox-one reagent was added to each well and plates were incubated in the incubator for 4 hours. Finally, the absorbance was read in a Microfluor reader (Thermo-Fisher, Bridgewater, NJ, USA) at 560 nm emission and 590 nm excitation.

Crystal violet assay. Transparent 96-well tissue culture plates (NUNC, Roskilde, Denmark) were used to evaluate the cell proliferation/cytotoxicity of S. persica extracts using the crystal violet stain (Sigma-Aldrich, St. Louis, MO, USA). After the formation of confluent cell monolayers in 96 well plates, the designated wells were exposed to the extracts along with the proper controls and plates were incubated for 24 hours. Monolayers were scored microscopically for any visible damage and wells were filled with PBS containing 1% formaldehyde for 3-hours to fix the monolayers. Fixative material was removed and the plates were left at room temperature to dry. Crystal violet stain was prepared as 2% in ethanol and water (1:1) and added to each well in a volume of 0.1 ml. Two hours later, cell monolayers were washed under tap water and wells were dried at room temperature after which ethylene glycol ether was added to each well in a volume of 0.2 ml and left for 10-15 minutes at room temperature. This step released all the residual stain and the plates were read in a Microplate reader (Thermo Scientific, Bridgewater, NJ, USA) at 520 nm wave length.

Statistical analysis. For this analysis, the Statistical Package for Social Sciences Version 16 for Windows (SPSS Inc., Chicago, IL, USA) was used. One-way ANOVA was used to compare the mean of absorbance values of S. persica extracts and negative control group for all cytotoxic assays. The Tukey post hoc test was used for pair-wise comparison. The level of significance was set at p<0.05.

Results. Figure 1 describes the comparison of cell viability data of hexane, ethylacetate, and ethanol extracts of S. persica with the controls in MTS assay using HGFs as substrate. Performance of ethanol extract at 1 mg/ml and 0.5 mg/ml concentrations were not significantly different from control with 100% cell viability. Ninety-nine percent of cells in the wells containing 0.5 mg/ml hexane extract and 88±4.1% cells in the wells with 1mg/ml hexane extracts were viable. Ethylacetate extract at 1 mg/ml exhibited maximum cytotoxicity with only 60±5.2% viable cells, whereas 92±2% HGFs survived at 0.5 mg/ml concentration of ethylacetate extract. Compared with the control wells, although statistically significant cytotoxicity was observed with hexane extract at 1 mg/ml (p=0.045), it was higher than the International Standards Organizations (ISO) recommended cut-off value as opposed to significantly higher cytotoxicity exhibited by ethylacetate extract at 1 mg/ml (p=0.0001).

Figure 2 compares data for hexane, ethylacetate, and ethanol extracts of S. persica induced cytotoxicity with the controls in LDH assay using HGFs as substrate. Ethanol extract at both concentrations of 0.5 mg/ml and 1 mg/ml performed well, and no cytotoxic effects were observed. Hexane extract of S. persica at 1 mg/ml supported 86±4.3% cell viability, which was significantly less (p=0.01) than the control wells (100%) though...
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Higher than the 70% ISO cut-off value. Ethylacetate extract at 1 mg/ml concentration supported only 40±6.2% cell viability, which was significantly lower than the control (*p*<0.0001). Ethylacetate extract of *S. persica* at 0.5 mg/ml supported 81±3.1% cell viability that was less than (*p*=0.001) the controls, but it was within the acceptable range.

**Figure 3** shows data comparing the cytotoxic effect of hexane, ethylacetate, and ethanol extracts of *S. persica* with the controls in CV assay using HGFs as substrate. Ethanol extract of *S. persica* at 0.5 mg/ml and 1 mg/ml concentration again was shown to be completely devoid of any cytotoxic effect and no different from the controls. Cell viability in wells containing hexane extract of *S. persica* at 1 mg/ml was 88±6.7% although less than the control wells (*p*=0.002) with 100% viable cells were within the acceptable range. Ethylacetate extract of *S. persica* at 1 mg/ml again exhibited significant cytotoxicity with only 66±7.4% viable cells (*p*=0.0001) when compared with the control cells. Viability of cells in wells containing 0.5 mg/ml ethyl acetate extract was 80%, which was significantly lower than the control wells (*p*=0.001).

**Discussion.** The ethanol extract of *S. persica* was found to be completely devoid of cytotoxic effects on HGFs in 3 cytotoxic assays consistently. Ethanol as a solvent for *S. persica* has been tested for toxic effects by intra-peritoneal injections in varying concentrations and was shown to be well tolerated by experimental animals. Similarly, the safety of ethanol as a solvent has been well documented in a study investigating cytotoxic effects of various mouth-rinse on HGF. Moreover, ethnolic extracts of *S. persica* in different concentrations have also been assessed for their cytotoxic effects on L929 cell line derived from mouse fibroblasts. Whereas, neat and 50% concentrations of ethnolic *S. persica* were able to induce morphological changes suggestive of cytotoxicity 25% extract of *S. persica* was

**Figure 1** - Comparison of cytotoxic activities of hexane, ethyl acetate, and ethanol extracts of *Salvadora persica* using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTS) cytotoxic assay. D-MEM - Dulbecco modified minimum essential medium

**Figure 2** - Comparison of cytotoxic activities of hexane, ethyl acetate, and ethanol extracts of *Salvadora persica* using lactic dehydrogenase (LDH) cytotoxic assay. D-MEM - Dulbecco modified minimum essential medium

**Figure 3** - Comparison of cytotoxic activities of hexane, ethyl acetate, and ethanol extracts of *Salvadora persica* using crystal violet (CV) assay cytotoxic assay. D-MEM - Dulbecco modified minimum essential medium
found to be the optimal concentration.\textsuperscript{25} As opposed to the existing evidence supporting safety of \textit{S. persica} one hour exposure to a low concentration of \textit{S. persica} solution has been shown to induce cytotoxic effects on cells involved in wound healing and HGF, which could be neutralized by addition of fetal calf serum (FCS).\textsuperscript{26} The neutralizing ability of FCS was attributed to serum proteins binding to potent toxic components in the mouthwash. The lack of cytotoxicity on human gingival fibroblasts observed in the present study in 3 different cytotoxic assays not only indicates that ethanol extract of \textit{S. persica} is non-toxic, but also prompts further evaluation of this extract as an irrigation solution for endodontic treatment.

An exhaustive search of the literature failed to yield any previous reports of hexane extract of \textit{S. persica} evaluation for cytotoxicity using HGF as a substrate. Although hexane extract of \textit{S. persica} at a concentration of 1 mg/ml displayed some cytotoxic activity against HGF, it was considered safe according to ISO guidelines.\textsuperscript{23} Higher cytotoxic activity detected for hexane extract of \textit{Mallotus philippensis} (Lam.) Muell. Arg. root against leukemic cells has been attributed to a higher concentration of polyphenolic compounds in the extract.\textsuperscript{27} Although polyphenolic compounds have been detected in \textit{S. persica}, the concentration of these compounds in \textit{S. persica} is not as high as concentrations of polyphenolic compounds found in other plants.\textsuperscript{28} It is possible that the cytotoxic activity of \textit{S. persica} observed at a higher concentration in the present study could be due to hexane mediated efficient extractions of polyphenolic compounds in \textit{S. persica} though present at a lower concentration. Despite being cytotoxic to a relatively lesser degree, it appears that the hexane extract can be used as a safe irrigant solution during endodontic treatment. Hexane extract of barbados cherry (a fruit of Malpighia emarginata DC) has been shown to exhibit a significantly higher cytotoxic activity against tumor cell lines derived from human oral squamous cell carcinoma and human submandibular gland carcinoma compared with HGF.\textsuperscript{29} Collectively, these observations indicate that hexane extract \textit{S. persica} appears to be non-toxic to HGFs, and further investigations are needed to validate the findings of the present study.

Cytotoxicity data regarding ethyl acetate extract of \textit{S. persica} are scarce. A significantly higher cytotoxic activity against HGFs by ethyl acetate extract of \textit{S. persica} was observed in the present study particularly at 1 mg/ml concentration. Using MTT assay, ethylacetate extract of \textit{S. persica} has been evaluated for its cytotoxic effects on various human carcinoma cell lines including human hepatocellular carcinoma, human breast adenocarcinoma, human colon adenocarcinoma, lung carcinoma, and green African monkey kidney.\textsuperscript{30} Although ethyl acetate extract of \textit{S. persica} was shown to be cytotoxic against all carcinoma cell lines, but the extent of cytotoxicity was less than the cytotoxic effects observed consistently in all the cytotoxic assays applied in the present. Of particular interest among the assays used in the present study were the results obtained by LDH assay as the enzyme remains stable for up to 36 hours after the cell death; thus, virtually eliminating the possibility any false negative results.\textsuperscript{31} The observed discrepancies in the cytotoxic activities of ethyl acetate extract of \textit{S. persica} could be due to the different substrates used as HGFs may be more sensitive to the toxic effects of ethyl acetate extract.

The use of HGFs in the present study appears to be beneficial as the primary culture of human cells of dental origin is considered to be more relevant to cytotoxicity studies of endodontic materials than established cell lines.\textsuperscript{32} Moreover, primary cells can be cultured with a low number of passages, which minimizes cellular changes that can result from cell culture manipulation.\textsuperscript{33} On the other hand, the limited lifespan of primary cell lines and the genomic instability in later passages led to the use of immortalized gingival fibroblasts as substrate in cytotoxicity studies.\textsuperscript{34} Based on previous findings\textsuperscript{16,21,35,36} and the current results, ethanol and hexane extracts of \textit{S. persica} appear to have potential to be used as an effective endodontic irrigation solution as far as the cytotoxic activity is concerned. Nevertheless, other aspects of \textit{S. persica} extracts, such as tissue dissolving capacity and residual effect require further investigation to support their clinical application in endodontics.

In conclusion, ethanol followed by hexane extracts of \textit{S. persica} appear to be the optimal concentration. As opposed to the existing evidence supporting safety of \textit{S. persica} solution has been shown to induce cytotoxic effects on cells involved in wound healing and HGF, which could be neutralized by addition of fetal calf serum (FCS).\textsuperscript{26} The neutralizing ability of FCS was attributed to serum proteins binding to potent toxic components in the mouthwash. The lack of cytotoxicity on human gingival fibroblasts observed in the present study in 3 different cytotoxic assays not only indicates that ethanol extract of \textit{S. persica} is non-toxic, but also prompts further evaluation of this extract as an irrigation solution for endodontic treatment.

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