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

Hanan A. Balto, *BDS, MSc,* ***Khaled K. Al-Manei,*** *BDS,* ***Talal M. Bin-Mohareb,*** *BDS,* ***Zahid A. Shakoor,*** *MD, PhD,* ***Solaiman M. Al-Hadlaq,*** *MS, PhD.*

ABSTRACT

الأهداف: لتَقييم التَأثير السَام لمُستخلصات الأَراك عَلى خَلايا اللّثة اللّيفية للإنسان (HGFs).

الطريقة: أُجريت هذه الدراسة خلال الفترة ما بين يناير ومايو 2012م بالتَعاون مع كرسي بحث تَسوس الأسنان، كلية طب الأسنان، جامعة الملك سعود، الرياض، المملكة العربية السعودية. تم إذابة مُستخلصات الأَراك بالمُذيبات التَالية: الهكسان، والإيثيل أسيتات، والإيثانول، وأُستخدمَ تركيزان لكل وَاحد منهما 0.5 ملجم/مل والآخر 1 ملجم/مل وذَلك لتَقييم التَأثير السَام لها ضدَ خلايا اللّثة اللّيفية للإنسَان. ولإجراء هذَا التَقييم تمّ عَمل ثَلاثة فُحوصات مخبرية (4,5-3) ثنائي ميثيل الثيازول-2- ميثيل)-5،2- ثنائي فَينيل رباعي الزوليوم بروميد، رباعي الزول (MTS)، ونازعة اللبنيك (LDH)، والبلور البنفسجي (CV). و لتفسير النَتائج استخدمت المعايير العالمية لتقييم المَواد الطبّية، حيث تعتبر المواد غير سامة إذا بلغت نسبة الخَلايا الحيّة 70%<.

النتائج: أَظهَرت هذه الدراسة أَن مُستخلصات الأَراك في مُذيبات الإيثانول بتركيزيه 0.5 ملجم/مل، و 1ملجم/مل، والهكسان عند التركيز 0.5 ملجم/مل كانت خَالية من التَأثير السَام. في حين أَن 1 ملجم/مل لمذيب الهكسان عند مُقارنَته بالمَجموعات الضابطة أَظهر بَعض التَأثير السَام حيث كانت نسبة الخَلايا الحيّة MTS 88% (p=0.045)، وLDH 86% (p=0.01) ، وCV 88% (p=0.002) . إضافةً إلى ذلك كانت نسبة الخَلايا الحيّة لتركيز0.5 ملجم/مل من مستخلص الأَراك لمُذيب الإيثيل أسيتات عَلى النحو التَالي91% MTS ، و81% LDH، و80% CV. كما لُوحظ أنَ الحَد الأَعلى من التَأثير السَام ضدَ خَلايا اللّثة اللّيفية للإنسان كَانت عند تركيز 1 ملجم/مل لمُذيب الإيثيل أسيتات حيث كانت نسبة الخلايا الحيّة MTS 60%، وLDH 40%، وCV 66% (p=0.0001).

الخاتمة: المستوى المقبول للسمية المرتبطة بالإيثانول والهكسان، مستخلصات الأراك، يحتاج إلى مزيد من التقييم لاستخدامها كمحلول الري في المعالجة اللبية.

Objectives: To assess the cytotoxic potential of *Salvadora persica (S. persica)* extracts on human gingival fibroblast (HGF) cells.

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* using hexane, ethylacetate, and ethanol as solvents at concentrations of 0.5 mg/ml and 1 mg/ml 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.

Saudi Med J 2014; Vol. 35 (8): 810-815

From the Dental Caries Research Chair (Balto, Al-Hadlaq), Division of Endodontics (Al-Manei), Department of Restorative Dental Sciences, College of Dentistry, Department of Pathology (Shakoor), College of Medicine and University Hospitals, King Saud University, Security Forces Hospital (Bin-Mohareb), Riyadh, Kingdom of Saudi Arabia.

Received 17th April 2014. Accepted 24th June 2014.

Address correspondence and reprint request to: Dr. Hanan Balto, Department of Restorative Dental Science, Division of Endodontics, College of Dentistry, King Saud University, PO Box 62645, Riyadh 11545, Kingdom of Saudi Arabia. Tel. +966 (11) 4677420. E-mail: h_balto@yahoo.com

Complete debridement during endodontic treatment is difficult to achieve by mechanical cleaning alone due to irregularities in the canal system, narrow isthmi, and apical delta.[1,2] Chemical debridement in the form of various irrigants is a useful adjunct to mechanical cleaning for effective removal of residual debris, necrotic tissue, and bacteria.[3] A number of root canal irrigating solutions are being applied currently in endodontic practice and none of the available solutions fulfills the criteria for being an ideal irrigating solution.[1] Properties of an ideal root canal irrigating solution include: broad spectrum antimicrobial activity, ability to dissolve the pulp tissue, non-cytotoxic to periradicular tissues, inactivation of endotoxin, prevention of smear layer formation during instrumentation, and removal of pre-existing smear layer.[4] Residual microbes along with the necrotic tissues after the root canal treatment can adversely affect the outcome of treatment.[5] Sodium hypochlorite (NaOCl) has been used as endodontic irrigating solution for decades due to its unique property of dissolving organic and necrotic tissues[6-9] and its anti-microbial activity against sessile endodontic pathogens organized in biofilms and microbes residing inside dentinal tubules.[10,11] The downsides of NaOCl; however, include its unpleasant taste, high toxicity,[12] and inability to remove the smear layer.[13,14] *Salvadora persica (S. persica)* a member of *Salvadoraceae* family is a small tree with soft whitish yellow wood that has been traditionally used in Africa, South America, the Middle East, and Asia as an effective tool for maintenance of oral hygiene.[15] Biological properties of *S. persica* extracts include antibacterial,[16] antifungal,[17] and antiplasmodial effects.[18] Aqueous and alcoholic extracts of *S. persica* have been shown to exert a potent anti-microbial activity against aerobic and anaerobic bacteria when applied as an irrigant in endodontic practice.[19,20] Moreover, *S. persica* extracts have also been shown to remove smear layer very efficiently.[21] Existing data regarding the biological properties of *S. persica* prompt evaluation of *S. persica* extract as a candidate for irrigating solution during endodontic treatment. This study was therefore performed to assess the cytotoxic potential of *S. persica* extracts on human gingival fibroblasts (HGFs) cells.

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% CO_2 and 95% O_2 at 37^{0}C. At 70% confluence, the cells were sub-cultured by dislodging the monolayers using 0.25% trypsine and 0.1% ethylenediaminetetraacetic acid (EDTA) (Invitrogen Life Technologies, Bethesda, MD, USA). The tryptic activity of trypsine 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 1x10^6/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 10% 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^{0}C. Working dilutions were made in physiological saline at a pH of 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^{0}C in 5% CO_2 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

Disclosure. Authors have no conflict of interests, and the work was not supported or funded by any drug company.

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[22] 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 490 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^{0}C in 5% CO_2 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

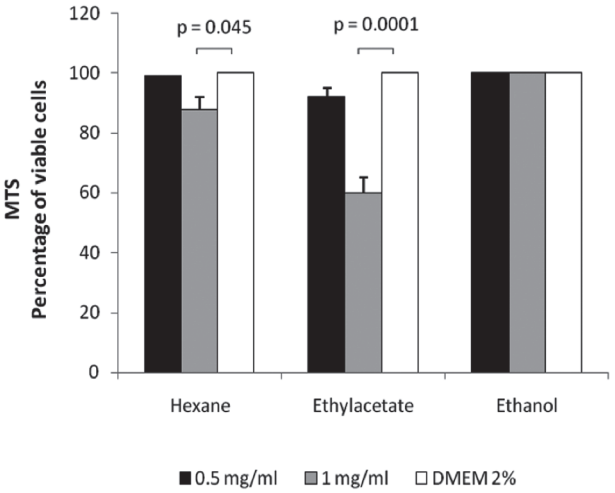

Figure 1 - Comparison of cytotoxic activities of hexane, ethyl acetate, and ethanol extracts of *Salvadora persica* using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTS) cytotoxic assay. D-MEM - Dulbecco modified minimum essential medium

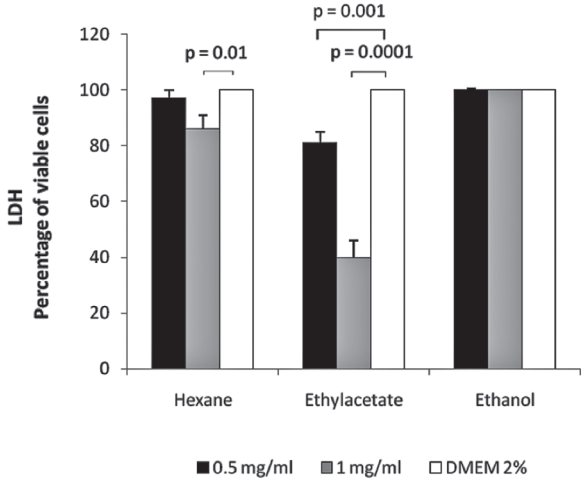

Figure 2 - Comparison of cytotoxic activities of hexane, ethyl acetate, and ethanol extracts of *Salvadora persica* using lactic dehydrogenase (LDH) cytotoxic assay. D-MEM - Dulbecco modified minimum essential medium

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*

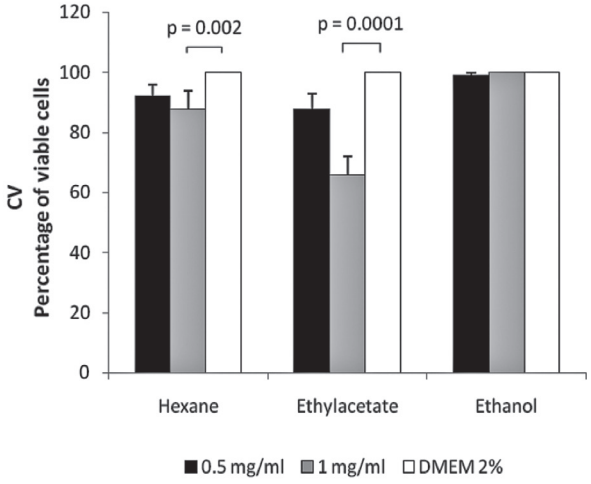

Figure 3 - Comparison of cytotoxic activities of hexane, ethyl acetate, and ethanol extracts of *Salvadora persica* using crystal violet (CV) assay cytotoxic assay. D-MEM - Dulbecco modified minimum essential medium

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.[23] Similarly, the safety of ethanol as a solvent has been well document in a study investigating cytotoxic effects of various mouth-rinses on HGF.[24] 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 ethanolic *S. persica* were able to induce morphological changes suggestive of cytotoxicity 25% extract of *S. persica* was

found to be the optimal concentration.[25] As opposed to the existing evidence supporting safety of *S. persica* one hour exposure to a low concentration of *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).[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 *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 *S. persica* evaluation for cytotoxicity using HGF as a substrate. Although hexane extract of *S. persica* at a concentration of 1 mg/ml displayed some cytotoxic activity against HGF, it was considered safe according to ISO guidelines.[22] Higher cytotoxic activity detected for hexane extract of *Mallotusphilippensis (Lam.) Muell. Arg.* root against leukemic cells has been attributed to a higher concentration of polyphenolic compounds in the extract.[27] Although polyphenolic compounds have been detected in *S. persica,* the concentration of these compounds in *S. persica* is not as high as concentrations of polyphenolic compounds found in other plants.[28] It is possible that the cytotoxic activity of *S. persica* observed at a higher concentration in the present study could be due to hexane mediated efficient extractions of polyphenolic compounds in *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.[29] Collectively, these observations indicate that hexane extract *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 *S. persica* are scarce. A significantly higher cytotoxic activity against HGFs by ethyl acetate extract of *S. persica* was observed in the present study particularly at 1 mg/ml concentration. Using MTT assay, ethylacetate extract of *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.[30] Although ethyl acetate extract of *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.[31] The observed discrepancies in the cytotoxic activities of ethyl acetate extract of *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.[32] Moreover, primary cells can be cultured with a low number of passages, which minimizes cellular changes that can result from cell culture manipulation.[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.[34]

Based on previous findings[16,21,35,36] and the current results, ethanol and hexane extracts of *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 *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 performed well in 3 cytotoxic assays as far as their cytotoxic activity against HGFs is concerned. In the backdrop of the already well documented antimicrobial potential of *S. persica* extracts, the findings of this study highlight the need for further evaluation of ethanol and hexane extracts of *S. persica* as effective irrigation solutions for endodontic treatment.

Acknowledgments. *The authors express their appreciation to Mr. Yunus Siddiqui for his technical assistance during the study. The authors would like to thank the College of Dentistry Research Center (NF2280) and the Deanship of Research, King Saud University, Riyadh, Kingdom of Saudi Arabia for their support in this research.*

References

1. Singla MG, Garg A, Gupta S. MTAD in endodontics: an update review. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2011; 112: e70-e76.
2. Peters O, Schönenberger K, Laib A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. ***Int Endod J*** 2001; 34: 221-230.
3. Haapasalo M, Shen Y, Qian W, Gao Y. Irrigation in endodontics. ***Dent Clin N Am*** 2010; 54: 291-312.
4. Zehnder M. Root canal irrigants. ***J Endod*** 2006; 32: 389-398.
5. Engström B, Lundberg M. The correlation between positive culture and the prognosis of root canal therapy after pulpectomy. ***Odontologisk Revy*** 1965; 16: 193-203.
6. Naenni N, Thoma K, Zehnder M. Soft tissue dissolution capacity of currently used and potential endodontic irrigants. ***J Endod*** 2004; 30: 785-787.
7. Clarkson RM, Moule AJ. Sodium hypochlorite and its use as an endodontic irrigant. ***Aus Dent J*** 1998; 43: 250-256.
8. Craig Baumgartner J, Mader CL. A scanning electron microscopic evaluation of four root canal irrigation regimens. ***J Endod*** 1987; 13: 147-157.
9. Grossman LI, Meiman BW. Solution of pulp tissue by chemical agents. ***J Endod*** 1982; 8: S10-S12.
10. Spratt D, Pratten J, Wilson M, Gulabivala K. An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. ***Int Endod J*** 2001; 34: 300-307.
11. Orstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. ***Dent Traumatol*** 1990; 6: 142-149.
12. Spangberg L, Engström B, Langeland K. Biologic effects of dental materials: 3. Toxicity and antimicrobial effect of endodontic antiseptics in vitro. ***Oral Surg Oral Med Oral Pathol*** 1973; 36: 856-871.
13. Goldman LB, Goldman M, Kronman JH, Lin PS. The efficacy of several irrigating solutions for endodontics: a scanning electron microscopic study. ***Oral Surg Oral Med Oral Pathol*** 1981; 52: 197-204.
14. McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. ***J Endod*** 1975; 1: 238-242.
15. Fine DH. Chemical agents to prevent and regulate plaque development. ***Periodontol 2000*** 1995; 8: 87-107.
16. Ababneh H. The effect of the extract of the miswak (chewing sticks) used in Jordan and the Middle East on oral bacteria. ***Int Dent J*** 1995; 45: 218-222.
17. Al-Bagieh N, Idowu A, Salako N. Effect of aqueous extract of miswak on the in vitro growth of *Candida albicans*. ***Microbios*** 1994; 80: 107-113.
18. Ali H, König G, Khalid S, Wright A, Kaminsky R. Evaluation of selected Sudanese medicinal plants for their in vitro activity against hemoflagellates, selected bacteria, HIV-1-RT and tyrosine kinase inhibitory, and for cytotoxicity. ***J Ethnopharmacol*** 2002; 83: 219-228.
19. Nawal A, Sabawi A, Khalik A, Sheikh A, Mahmoud Y. The antimicrobial activity of *Salvadora persica* solution (Miswak-Siwak) as root canal irrigant (a comparative study). ***University of Sharjah Journal of Pure And Applied Sciences*** 2007; 4: 69-91.
20. Salman T, Moataz G, Shaekh A, Osama M. The antimicrobial effect of water extraction of *Salvadora persica* (Miswak) as a root canal irrigant. ***Al-Rafidain Dental Journal*** 2005; 5: 33-36.
21. Balto H, Ghandourah B, Al-Sulaiman H. The efficacy of *Salvadora persica* extract in the elimination of the intracanal smear layer: A SEM study. ***Saudi Dental Journal*** 2012; 24: 71-77.
22. International Standards Organization. Biological evaluation of medical devices-Part 5: Tests for in vitro cytotoxicity. Geneva (CH): International Standards Organization; 2009.
23. Al-Bayaty FH, AI-Koubaisi AH, Ali NAW, Abdulla MA. Effect of mouth wash extracted from *Salvadora persica* (Miswak) on dental plaque formation: A clinical trail. ***J Med Plants Res*** 2010; 4: 1446-1454.
24. Wilken R, Botha S, Grobler A, Germishuys P. In vitro cytotoxicity of chlorhexidine gluconate, benzydamine-HCl and povidone iodine mouthrinses on human gingival fibroblasts. ***SADJ*** 2001; 56: 455-460.
25. Al-Samh A, Dua'a A, Al-Nazhan S. In vitro study of the cytotoxicity of the miswak ethanolic extract. ***Saudi Dental Journal*** 1997; 9: 125-132.
26. Rajabalian S, Mohammadi M, Mozaffari B. Cytotoxicity evaluation of *Salvadora persica* mouthwash on cultured human and mouse cell lines in the presence and absence of fetal calf serum. ***Indian J Dent Res*** 2009; 20: 169.
27. Khan M, Qureshi RA, Hussain M, Mehmood K, Khan RA. Hexane soluble extract of *Mallotus philippensis (Lam.)* Muell. Arg. root possesses anti-leukaemic activity. ***Chem Cent J*** 2013; 7: 1-6.
28. Noumi E, Snoussi M, Trabelsi N, Hajlaoui H, Ksouri R, Valentin E, et al. Antibacterial, anticandidal and antioxidant activities of *Salvadora persica* and *Juglans regia* L. extracts. ***Journal of Medicinal Plants Research*** 2011; 5: 4138-4146.
29. Motohashi N, Wakabayashi H, Kurihara T, Fukushima H, Yamada T, Kawase M, et al. Biological activity of barbados cherry (acerola fruits, fruit of Malpighia emarginata DC) extracts and fractions. ***Phytother Res*** 2004; 18: 212-223.
30. Ibrahim AY, El-Gengaihi SE, Motawe HM. Phytochemical and cytotoxicity investigations of *Salvadora persica* bark extracts. ***JASMR*** 2011; 6: 127-133.
31. Jahn K, Stoddart MJ. Viability assessment of osteocytes using histological lactate dehydrogenase activity staining on human cancellous bone sections. ***Methods Mol Biol*** 2011; 740: 141-148.
32. Al-Nazhan S, Spangberg L. Morphological cell changes due to chemical toxicity of a dental material: an electron microscopic study on human periodontal ligament fibroblasts and L929 cells. ***J Endod*** 1990; 16: 129-134.
33. Karimjee C, Koka S, Rallis D, Gound T. Cellular toxicity of mineral trioxide aggregate mixed with an alternative delivery vehicle. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 2006; 102: e115-e120.
34. Illeperuma RP, Park YJ, Kim JM, Bae JY, Che ZM, Son HK, et al. Immortalized gingival fibroblasts as a cytotoxicity test model for dental materials. ***J Mater Sci Mater Med*** 2012; 23: 753-762.
35. Sote E, Wilson M. In-vitro antibacterial effects of extracts of Nigerian tooth-cleaning sticks on periodontopathic bacteria. ***Afr Dent J*** 1995; 9: 15.
36. Wolinsky L, Sote E. Inhibiting effect of aqueous extracts of eight Nigerian chewing sticks on bacterial properties favouring plaque formation. ***Caries Res*** 1983; 17: 253-257.