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Original Research Article

Effect of *Salvadora persica* Linn root aqueous extract on oral epithelial dysplasia and oral cancer cell lines

Huda M Hammad[1]*, Khaled M Al-Qaoud[2], Mohammad M Hammad[3], Mariam A Mansi[4]

[1]Department of Oral Medicine and Surgery, Faculty of Dentistry, Jordan University of Science and Technology, PO Box 3030, Irbid 22110, [2]Department of Biological Sciences, Faculty of Science, Yarmouk University, PO Box 566, Irbid 21163, [3]Department of Preventive Dentistry, Faculty of Dentistry, Jordan University of Science and Technology, PO Box 3030, Irbid 22110, Jordan, [4]Department of Medical Laboratories, College of Applied Medical Sciences, Shaqra University, PO Box 1678, Al-Dawadmi 11911, Kingdom of Saudi Arabia

For correspondence:* ***Email:* *hmhammad@just.edu.jo;* ***Tel:*** *00962-795727340*

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Abstract

Purpose: *To evaluate the potential chemo-preventive and anti-oral cancer effects of Salvadora persica (S. persica) on oral epithelial dysplasia and oral squamous cell carcinoma cell lines.*
Methods: *Aqueous S. persica root extracts were prepared at concentrations up to 15.75 mg/mL and applied to oral epithelial dysplasia (DOK), oral squamous cell carcinoma (PE/CA-PJ15), and periodontal ligament fibroblast (PDL) cell lines. The effect of the extract on cell survival and proliferation was determined using MTT assay, while its effect on apoptosis in DOK and PE/CA-PJ15 lines were investigated by measuring apoptotic index using Hoechst stain.*
Results: *In DOK and PE/CA-PJ15 cell lines, cytotoxicity was significant at extract concentrations of 11.25, 13.50 and 15.75 mg/mL, while extract concentration of 13.50 mg/mL produced significant cytotoxic effects on PDL cell line ($p < 0.05$). The percentage of apoptotic cells significantly increased at extract concentration of 11.25 mg/mL for both DOK and PE/CA-PJ15 cell lines ($p < 0.05$).*
Conclusion: *Significant cytotoxic effects of aqueous root extract of S. persica appeared at a lower concentration in oral epithelial dysplasia and oral cancer cell lines than in normal PDL cell line. These results suggest the potential of S. persica for preventing oral cancer.*

Keywords: *Salvadora persica, Oral epithelial dysplasia, Oral cancer, Chemoprevention, Anticancer effect*

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INTRODUCTION

Cancers of the oropharyngeal region are the seventh most common cancers worldwide, and the ninth most common cause of cancer-related death. In 2012 alone, an estimated 529,000 new cases occurred worldwide, in addition to 292,000 deaths from oral cancer [1]. Despite advances in research and treatment modalities, the prognosis for oral cancer patients has not shown significant improvement [2]. Natural products constitute important sources of new medications, including chemo-preventive and chemotherapeutic agents for cancer. Some well-known examples of

clinically useful plant-derived anticancer compounds are the vinca alkaloids, podophyllotoxins, taxanes, and camptothecins, and their analogues [3].

Salvadora persica (S. persica) is a desert plant known in the Arab world as the *Araak* tree. Its branches and roots are the most common types of chewing stick or natural toothbrush (*Miswak* in Arabic) in the Middle East. Although it is used by Muslims as a religious rite, it had been used in other ancient civilizations as far back as the Babylonian era [4].

Several studies have been published on *S. persica*, mostly in relation to its use for oral hygiene. The antibacterial effect of extracts from various parts of the plant on bacteria causing dental caries and periodontal disease have been demonstrated in some studies [4,5].
Moreover, the antifungal, scolicidal, antimalarial, and antiviral effects of *S. persica* has been reported [4-7]. Ali *et al.* reported inhibitory effects of leaf extract of *S. persica* against HIV-1 tyrosine kinase TK $p56^{lck}$ [6]. Other biological effects attributed to *S. persica* include antidiabetic/hypoglycemic, hypolipidemic, antioxidant, anticonvulsant, sedative, anti-urolithiatic, wound-healing, and protective effects against gastric ulcers [4,5,7-9]. In addition, several studies have reported variable cytotoxicities of *S. persica* on various cultured cells [10-14].

In view of the cytotoxic effect attributed to *S. persica* extracts on various microbes and on some cultured cells, it is worthwhile to investigate whether it exerts a cytotoxic effect on oral cancerous and precancerous cells. Therefore, the aim of this study was to determine the effect of *S. persica* on oral epithelial dysplasia and oral cancer cell lines, so as to evaluate its potential chemo-preventive effect against oral cancer. A normal cell line derived from a periodontal ligament fibroblast was used as control (PDL cell line).

EXPERIMENTAL

The study was approved by Deanship of Research at Jordan University of Science and Technology (JUST).

Extraction of plant material

Miswak chewing sticks (*S. persica*) were purchased from a local market in Irbid, and were identified by a plant taxonomist from the Department of Biological Sciences at Yarmouk University. A voucher specimen (no. 10988) was deposited at the Yarmouk University Herbarium. An aqueous extract of *S. persica* was prepared according to the method of Darmani *et al*, but at a higher concentration [10]. The chewing sticks were ground into powder using an electric mill, and an aqueous extract was prepared by mixing 22.5 g of the powder with 100 mL of sterile, distilled, culture-grade water. The mixture was left for 48 h at 4 °C. Prior to use, the mixture was lyophilized using VaCo 2 freeze dryer (ZIRBUS Technology GmbH, Hilfe Gottes, Germany). Working concentrations were prepared in distilled water. The solutions were filtered through Whatman filter paper No. 1 (Whatman plc, Kent, United Kingdom), and then sterilized using Millipore 33 mm in diameter Sterile PVDF Syringe Filters of 0.22 µm (Millipore Corporation, Billerica, Massachusetts, USA).

Cell culture, maintenance and treatment

Human oral epithelial dysplasia (DOK) and oral cancer (PE/CA-PJ15) cell lines were purchased from the European Collection of Cell Cultures (ECCC) at Salisbury, Wiltshire, United Kingdom. Human periodontal ligament fibroblast cell line (PDL) was locally grown in our laboratory from the periodontal ligament of an extracted tooth of a healthy adult donor at JUST (IRB approval no. 20090066) [15]. The cells were maintained as exponentially growing cultures in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2 mM glutamine and 10 % fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria). Hydrocortisone (5 µg/mL) was added to the DOK cell line media. The cells were cultured at a temperature of 37 °C in an atmosphere of 5 % CO_2 and 95 % humidity. At confluence, the cells were trypsinized, washed, counted via trypan blue exclusion test, and seeded in 96-well plates at a density of 20×10^3 cells/well. After 24 h, 100 µL of the plant extract was added to separate wells at concentrations of 2.25, 4.5, 6.75, 9, 11.25, 13.5, and 15.75 mg/mL. These concentrations were selected based on results from a pilot study. A well without extract served as negative control. The cells were incubated with extracts for 24 h, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Six separate runs were performed for each concentration.

MTT assay

The incubation medium was removed through aspiration, and the cells were rinsed twice using sterile PBS buffer. The, 100 µL of fresh complete DMEM was added to each well in addition to 10 µL of 5 mg/mL MTT solution (Sigma, Saint. Louis, Missouri, USA). After incubation for 3 h at

37 °C, the plate was centrifuged at 2000 rpm for 10 min, and the supernatant was discarded. Then, the resultant formazan crystals were dissolved in 200 µL of ethanol/DMSO mixture (1:1, v:v), and the absorbance of the solution was read at 570 nm in a microplate reader.

Apoptotic index measurement

Following interpretation of the results of the MTT assay (see results section), apoptotic index measurement was performed on the DOK and PE/CA-PJ15 cell lines. The same methodologies for extraction of plant material, cell culture maintenance and treatment were employed, but slightly different concentrations were used for the apoptotic index measurement i.e. 1.12, 2.25, 6.75, 11.25, 13.5, and 15.75 mg/mL. The cells were incubated with the extract for 24 h prior to apoptotic index measurement. The floating and trypsinized adherent cells treated with the various extract concentrations were collected and rinsed using PBS. Then, the cells were incubated in Hoechst® 33258 (Acros Organics, Geel, Belgium) at a final concentration of 10 µg/mL at 37 °C for 30 min. Nuclear morphology was examined microscopically with Epi-fluorescence condenser III RS (Carl Zeiss, Oberkochen, Germany). The percentages of apoptotic cells were quantified by dividing the number of apoptotic cells with the total cell number counted from four random microscopic fields for each concentration at x400 magnification. At least a total of 200 cells were counted in the calculation of the apoptotic index.

Statistical analysis

The data obtained were analyzed using independent sample Student's *t*-test. Significance of the differences between the mean value for the negative control group and each of the mean values of OD at various concentrations was set at $p < 0.05$.

RESULTS

The *S. persica* extract, at concentrations of 2.25, 4.5 and 6.75 mg/mL produced positive but non-significant effect on the proliferation of PE/CA-PJ15 oral cancer cells, when compared to the control (p = 0.050, 0.096 and 0.755, respectively). Negative effects started to appear at extract concentration of 9 mg/mL, and became significant at concentrations of 11.25, 13.5, and 15.75 mg/mL (p = 0.033, 0.004, and 4.3E-05, respectively).

At a concentration of 2.25 mg/mL, the extract had a positive but non-significant effect on the proliferation of the DOK and PDL cell lines, when compared to the control (p = 0.211 and 0.974, respectively). Negative effects appeared at

Table 1: Effect of various concentrations of *S. persica* root extract on DOK, PE/CA-PJ1, and PDL cell lines

Concentration (mg/mL)	Cell line	Mean OD (proliferation)	SD	*t*
0	DOK	0.4857	0.0167	
	PE/CA-PJ15	1.0357	0.0405	
	PDL	1.0714	0.1710	
2.25	DOK	0.5110	0.0433	0.211
	PE/CA-PJ15	1.1323	0.0983	0.050
	PDL	1.0749	0.1137	0.974
4.5	DOK	0.4827	0.0295	0.833
	PE/CA-PJ15	1.0848	0.0517	0.096
	PDL	1.0288	0.0991	0.681
6.75	DOK	0.4725	0.0415	0.487
	PE/CA-PJ15	1.0512	0.1113	0.755
	PDL	0.9781	0.0745	0.355
9	DOK	0.4545	0.0540	0.206
	PE/CA-PJ15	1.0148	0.1103	0.673
	PDL	0.9130	0.0427	0.122
11.25	DOK	0.4305	0.0513	**0.031**
	PE/CA-PJ15	0.9605	0.0627	**0.033**
	PDL	0.6624	0.3890	0.103
13.5	DOK	0.3918	0.0544	**0.002**
	PE/CA-PJ15	0.9373	0.0507	**0.004**
	PDL	0.8040	0.0884	**0.032**
15.75	DOK	0.3545	0.0347	**8.1E-06**
	PE/CA-PJ15	0.8460	0.0540	**4.3E-05**
	PDL	0.6785	0.2799	0.054

Zero extract concentration = negative control. $p < 0.05$, compared to control (shown in bold font). Mean OD = mean optical density at 570 nm for 6 independent runs. SD = standard deviation

extract concentration of 4.5 mg/ml for both cell lines, and the effects became significant at concentrations of 11.25, 13.5, and 15.75 mg/mL for the DOK cell line (p = 0.031, 0.002, and 8.1E-06, respectively). For the PDL cell line, the effect became significant at extract concentration of 13.5 mg/mL (p = 0.032), but was not significant at 15.75 mg/mL. These results are shown in Table 1. As shown in Table 2 and Figure 1, the apoptotic effect of *S. persica* was evident in DOK and PE/CA-PJ15 cell lines at extract concentration of 11.25 mg/mL. The highest apoptotic effect on the two cell lines was seen at extract concentration of 15.75 mg/mL (11.3 and 26.8 % for DOK cell line and PE/CA-PJ15 cell line, respectively).

Table 2: Apoptotic index shown as percentage (%) of apoptotic cells from whole cells treated with different concentrations of *S. persica*

Cell line	Concentration mg/mL	Apoptotic index (%)
DOK	0	3.7
PE/CA-PJ15		5.3
DOK	1.12	7.5
PE/CA-PJ15		10.2
DOK	2.25	5.9
PE/CA-PJ15		8.6
DOK	6.75	6.1
PE/CA-PJ15		7.3
DOK	11.25	5.3
PE/CA-PJ15		23.7
DOK	15.75	11.3
PE/CA-PJ15		26.8

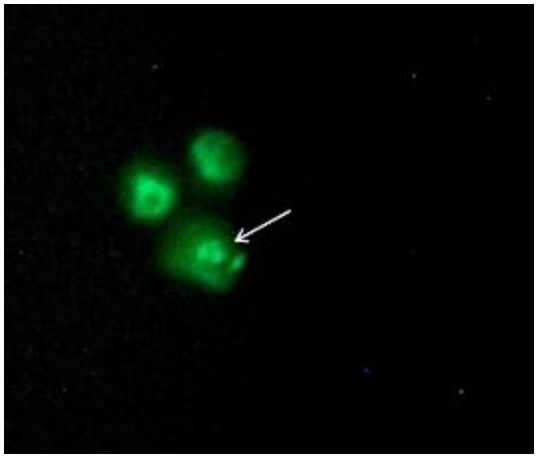

Figure 1: Morphological features of *S. persica*-treated cells stained with Hoechst® 33258. The arrow points to a cell showing nuclear fragmentation, a sign of apoptosis (x1000).

DISCUSSION

The results of this study show that at high concentrations, the aqueous extracts of *S. persica* exerted overall cytotoxic effects on the oral epithelial dysplasia DOK, oral cancer PE/CA-PJ15, and the PDL fibroblast cell lines. The cytotoxic effects became significant at extract concentration of 11.25 mg/mL and above, for the first two lines, but only at 13.5 mg/mL for the third (normal) cell line. These results are in agreement with the results of several studies that showed cytotoxic effects of *S. persica* on various microbes and some cultured cells, as indicated in the introduction section.

The phytochemical composition of various parts of *S. persica* has been investigated by different methods in a number of studies. Many compounds were reported, but of interest in this context are saponins, terpenes and terpenoids, tannins, polyphenols (including flavonoids), sterols (especially beta-sitosterol), vitamin C, alkaloids, glucotropaeolin and its breakdown product, benzyl-isothiocyanate, and benzyl-amides such as butanediamide and benzylurea [5,16,17]. These compounds and compound categories, whether from plants or synthetic sources, have been found to possess antioxidant and/or antineoplastic properties [16,18-22]. It is highly likely that in the present study, some of these compounds were present in the aqueous extract.

The extent to which these findings apply to the daily use of *miswak*, and whether these phytochemicals do exert cancer-preventive effects in the oral cavity or elsewhere in the body when swallowed, are areas that should be investigated. Concerns were expressed by Rajabalian *et al* in their study on a mouthwash containing *S. persica* extract, regarding possible cytotoxic effect of a high concentration of the extract or any of its components on normal oral tissues [11]. However, these concerns may not necessarily apply to the use of the *S. persica* stick as a toothbrush, since the concentration of materials leached from the stick during brushing is likely to be very low. Rajabalian *et al* [12] suggested that the use of the mouthwash should be avoided in patients with fresh open oral wounds, in view of its cytotoxic effect on macrophages, epithelial, fibroblastic and osteoblastic cells, all of which are components of the wound healing process.

However, it should be noted that the epithelial and osteoblastic cells in their study were of neoplastic rather than normal lineage, and that the mouthwash also contained extracts from *Achillea millefolium,* which reportedly has antiproliferative properties [23]. In contrast, in a study by Darmani *et al* on a mouse fibroblast cell line, the pure aqueous extract of *S. persica* was found to produce markedly enhancing rather than

cytotoxic effects at all concentrations used, when compared to its cytotoxic effect on cariogenic bacteria at the same concentrations [10]. Similarly, an enhancing effect on the PDL fibroblast cell line was found in this study at the low concentration of 2.25 mg/mL. However, cytotoxic effects were produced at higher concentrations, being significant only at 13.5 mg/mL.

Moreover, Balto *et al* [12] studied the cytotoxic effects of hexane, ethyl acetate, and ethanol extracts of *S. persica* on human gingival fibroblasts at concentrations of 0.5 and 1 mg/mL. The results showed that the ethanol extract at concentrations of 0.5 and 1 mg/mL, and the hexane extract at concentration of 0.5 mg/mL were devoid of cytotoxic effects, while the other extracts demonstrated some cytotoxicity. Thus, the level of cytotoxicity with ethanol and hexane extracts were deemed acceptable, and further evaluation of the extracts for use as endodontic irrigation solutions was suggested [12]. In another study by the same authors [13], the efficacy of hexane and ethanol extracts of *S. persica* in preserving human foreskin fibroblasts was compared to that of other media, to determine their suitability for storage of avulsed teeth before re-implantation. None of the tested solutions caused significant decrease in viable cell counts [13].

A study by Tabatabaei *et al* [14] found that aqueous extract of *S. persica* enhanced proliferation of dental pulp stem cells at a concentration of 2.87 mg/mL, a finding similar to that in the current study with respect to the effect of extract concentration of 2.25 mg/ml on the PDL fibroblast cell line. However, in the study [14], the threshold for significant cytotoxicity was lower, at a concentration of 5.75 mg/mL. It should be emphasized that the results of a study on tissue culture only give a preliminary idea on the effects of certain compounds, and it should not be considered as an alternative to an *in vivo* study. The protective mechanisms and the interactions of various factors *in vivo* may sometimes give different results.

CONCLUSION

This study has demonstrated that the aqueous root extract of *S. persica* produces cytotoxic effects on oral epithelial dysplasia, oral cancer, and normal human periodontal ligament fibroblast cell lines. The overall results of the study suggest a potential role of *S. persica* in cancer prevention. However, more studies, including *in vivo* investigations, are recommended to confirm this potential.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. HM Hammad conceived and designed the study. KM Al-Qaoud provided major advice in the study design, and the tissue culture facility. MM Hammad contributed to the conceptualization and design of the project. MA Mansi provided advice on the study design and performed the experiments. All authors contributed to the writing of the manuscript. All authors read and approved the manuscript for publication.

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