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ORIGINAL ARTICLE

Cytotoxic and Antitumor Properties of *Ziziphus spina-christi* Found in Al Bahah Region of Hejaz Area: An *In vitro* Study on Oral Cancer Cell Lines

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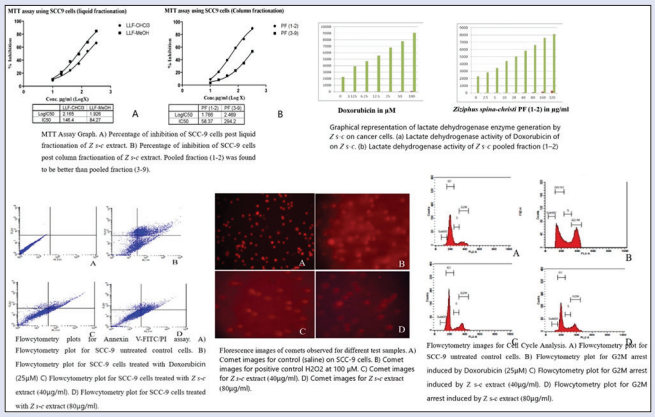
ABSTRACT

Background: *Ziziphus spina-christi* (*Z s-c*) is traditionally been used as an herbal medicine and is part of *Rhamnaceae* plants family. **Objectives:** The probable cytotoxic effect of *Z s-c*, found in Al Bahah region, on human oral cancer cell lines (SCC-9) was assessed in the present study. **Materials and Methods:** Bioactive compounds of *Z s-c* were evaluated using thin layer chromatography, analytical high-performance liquid chromatography and nuclear magnetic resonance spectroscopic analyses. The cytotoxic effect of *Z s-c* on SCC-9 cells was revealed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The mechanisms involved in the cytotoxicity of *Z s-c* were investigated in terms of apoptosis, mitochondrial membrane potential, and cell cycle using flow cytometry, while caspase-9 and *p53* activity was investigated using the Western Blot analysis. **Results:** *Z s-c* has potent variable cytotoxic effect on SCC-9 cells demonstrated through lactate dehydrogenase and comet assay. An effective cell cycle arrest was observed at the G2M phase with apoptotic induction. They significantly induce G2M cell cycle arrest and promote apoptosis in SCC-9 cell line. The expression of caspase-9 and *p53* was induced by *Z s-c* in a dose-dependent manner. **Conclusion:** *Z s-c* may be a novel candidate for the development of new natural product-based therapeutic agents against oral cancer.

Key words: Antitumor, apoptosis, cell cycle analysis, cytotoxicity, SCC-9 cell line, *Ziziphus spina-christi*

SUMMARY

- The aim of this research is to determine the cytotoxic impact of *Ziziphus spina-christi* (Z s-c) on the oral cancer cell line SCC-9
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay revealed that crude Z s-c had cytotoxicity with IC_{50} value of 180.4 μg/ml. The percentage inhibition of pooled fraction (1–2) was better compared to PF (3–9). *Z s-c* PF (1–2) was considered for further studies as its activity was better than other fractions
- Sample *Z s-c* PF 1–2 showed increased levels of lactate dehydrogenase at higher concentrations, i.e.,: 8111.18U/L at 320 μg/m when compared to control (untreated) which was 2276.26 U/L in SCC-9 cells
- Sulforhodamine B assay showed that sample *Z s-c* PF 1–2 has showed an IC_{50} value of 43.55 μg/ml inhibition in SCC-9 cells
- An effective cell cycle arrest was observed at the G2M phase with apoptotic induction. They significantly induce G2M cell cycle arrest and promote apoptosis in SCC-9 cell line. The expression of caspase-9 and *p53* was induced by *Z s-c* in a dose-dependent manner
- Z s-c may be a potential candidate to produce therapeutic agents against oral cancer based on new natural ingredients.



MTT Assay Graph. A) Percentage of inhibition of SCC-9 cells post liquid fractionation of *Z s-c* extract. B) Percentage of inhibition of SCC-9 cells post column fractionation of *Z s-c* extract. Pooled fraction (1-2) was found to be better than pooled fraction (3-9).

Graphical representation of lactate dehydrogenase enzyme generation by *Z s-c* on cancer cells. (a) Lactate dehydrogenase activity of Doxorubicin of on *Z s-c*. (b) Lactate dehydrogenase activity of *Z s-c* pooled fraction (1–2)

Flowcytometry plots for Annexin V-FITC/PI assay. A) Flowcytometry plot for SCC-9 untreated control cells. B) Flowcytometry plot for SCC-9 cells treated with Doxorubicin (25μM) C) Flowcytometry plot for SCC-9 cells treated with *Z s-c* extract (40μg/ml). D) Flowcytometry plot for SCC-9 cells treated with *Z s-c* extract (80μg/ml).

Florescence images of comets observed for different test samples. A) Comet images for control (saline) on SCC-9 cells. B) Comet images for positive control H2O2 at 100 μM. C) Comet images for *Z s-c* extract (40μg/ml). D) Comet images for *Z s-c* extract (80μg/ml).

Flowcytometry images for Cell Cycle Analysis. A) Flowcytometry plot for SCC-9 untreated control cells. B) Flowcytometry plot for G2M arrest induced by Doxorubicin (25μM) C) Flowcytometry plot for G2M arrest induced by *Z s-c* extract (40μg/ml). D) Flowcytometry plot for G2M arrest induced by *Z s-c* extract (80μg/ml).

Abbreviations used: DMEM: Dulbecco's modified eagle medium; DMSO: Dimethyl sulfoxide; DNA: Deoxyribose nucleic acid; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC: High-performance liquid chromatography; KSA: Kingdom of Saudi Arabia; LDH: Lactate dehydrogenase; MeOH: Methanol; MPT: Methoxypodophyllotoxin; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMR: Nuclear magnetic resonance; OD: Optical density; PBS: Phosphate-buffered saline; PF: Pooled fraction; RTPCR: Reverse transcription polymerase chain reaction; SPE: Solid phase extraction; SRB: Sulforhodamine B; TBST: Tris-buffered saline with tween; TLC: Thin-layer chromatography; *Z s-c: Ziziphus spina-christi.*

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INTRODUCTION

Oral cancer is Saudi Arabia's third most common cancer, accounting for about 26% of all cancers of the head and neck. Unfortunately, many cases of cancer are diagnosed terminally which necessitates robust and aggressive treatment strategies. This high incidence of oral cancer is limited primarily to Saudi Arabia's south-western region.[1] Prophet Mohammad (peace be upon him, 571–632 AD) promoted the use of

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plants in therapy; a practice known as Prophetic Medicine.[2] Many species of plants have been used since ancient times for ethnomedicine. This time was known as the golden age of evolution of ethnomedicine.

Ziziphus spina-christi (Z s-c) is commonly referred to as "Jujube" and is locally referred to as "Sidr" or "Nabuk." It is a multipurpose tree of the *Rhamnaceae* family. *Z s-c* grows in many parts of the world, such as European, African, Asian, and Central American countries. *Z s-c* is widely spread in Saudi Arabia across the southern and south-western regions. Popular in the folklore of Saudi Arabia, *Z s-c* leaves are known for their curative abilities in sexually transmitted diseases, dermatological disorders, and local inflammatory lesions. In addition, *Z s-c* leaves have been recorded to be used in folk medicine as an antidiabetic remedy.[3]

A cytotoxic effect against cervical, breast, and colon cancers was found from the aerial portion of *Z s-c*.[4] However, the cytotoxic and antitumor properties of the leaves of *Z s-c* grown in the south-western region of Saudi Arabia on oral cancer have not been elucidated yet.

This research is intended to determine the cytotoxic impact of *Z s-c* on the oral cancer cell lines-SCC-9. The objective of this study is also to examine the nature of the cell cycle and the cell death mechanism induced by *Z s-c* on the SCC-9 cell line.

MATERIALS AND METHODS

Plant collection, identification, and extraction

Dried leaves of *Z s-c* were purchased from herbalists. Ground leaves of *Z s-c* (50 g) were taken in 250 ml of methanol and kept in water bath at 50°C for 4 h which as filtered using Whatman grade 1 qualitative paper. The procured methanol filtrate was kept for evaporation on water bath at 50°C. Postevaporation, obtained crude herbal extract is 3.2 g which is utilized for several assays.

For liquid-liquid (L-L) extraction chromatography, the methanol extract of *Z s-c* (500 mg) was dissolved in 10 ml of methanol. Chloroform, methanol, and hexane soluble fractions were separated using separating funnel. The yield received was 250 mg, 900 mg, and 46 mg, respectively, for methanol, chloroform, and hexane. The L-L fractions were later analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). For column chromatography 1.5 g methanol L-L chloroform fraction was dissolved in 4.5 ml of 5% methanol in chloroform. 4 g silica gel bed in chromatographic column (300 mm × 15 mm) in chloroform was prepared and the sample was loaded on the column. 5 ml to 15 ml fractions were collected in each test tube based on color. Fractions were next eluted with 10 ml methanol chloroform mixture (5%–40%). Fractions with similar pigment were pooled based on the TLC. Column fractions were kept for evaporation.

Thin layer chromatography

A TLC pane was designed using slender sheet of silica gel (2.5 mm) with fluorescent indicator F_{254}. 2.5 ml OF *Z s-c* samples were allowed to spot on this TLC pane. Later, one side of the pane was dipped into the solvent blend (chloroform: Methanol [9.5:0.5]). The blend inched to the other side of the pane and the sample spots separated into multitudinous constituents. The pane was withdrawn from the solvent blend postadequate development time. The multitudinous spots of the samples were identified using UV chamber and R_f value is calculated using a formula, R_f = Distance moved by compound/distance moved by solvent.

Analytical high-performance liquid chromatography

Chromatographic assessment of the *Z s-c* compounds were conducted using Shimadzhu LC-Prominence 20AT coupled with 6210 time of flight liquid chromatography/mass spectrometry (MS) detector and Agilent Poroshell 120 EC-C_{18} (C_{18} column 250 mm × 4.6 mm, 5u particle) column. Mobile phases A and B were HPLC grade methanol (50%) and HPLC grade water (50%). The flow rate was 1 mL/min and injection volume was 10 µL. The column temperature was adjusted to 35°C. The absorbance was at 254 nm.

Cell lines and culture conditions

The SCC-9 cell lines were cultured in Dulbecco's modified eagle medium (DMEM) media with additives like with 10% fetal bovine serum (FBS) and 2% penicillin. The concoction was rejuvenated twice a week.

Anti-proliferative 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

SCC9 cells were seeded in a 96 well plate at a concentration of 50,000 cells/well and incubated for 24 h at 37°C, 5% CO_2 incubator. The cells were treated with differing percentages (10, 20, 40, 80, 160, and 320 µg/mL) of *Z s-c*, for 24 h. Doxorubicin was taken as positive control and saline as negative control. After 24 h conjugation with *Z s-c*, 100 µl/well of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent at concentration of 5 mg/10 ml in 1% phosphate-buffered saline (PBS) was supplemented to the respective wells and incubated for 3–4 h. Postconjugation with MTT reagent, it was repudiated by pipetting without perturbing the cells. About 100 µl of dimethyl sulfoxide (DMSO) was combined to quickly solubilize the formazan. Optical density (OD) was gauged at 590 nm. The effective lethal concentration required for anti-proliferative effect was determined by plotting a graph and obtaining a curve with maximum number of cells killed and concentration of the *Z s-c* extract used. Formula used: (OD of control − OD of sample/OD of control) ×100. The inhibitory concentration or (IC_{50}) was ascertained using Graph Pad Prism 7 software (San Diego, CA, USA).[5]

Lactate dehydrogenase assay

The discharge of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium is exhibitive of membrane degeneration and is frequently inured as a measure of necrosis. The monolayer cell culture was trypsinized and the cell count was amended to 1.0×10^5 cells/ml utilizing corresponding media entailing 10% FBS. To every well of the 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000 cells/well) was aggregated. Following 24 h, as a fractional monolayer was produced, the supernatant was twitched off and awash with medium and 100 µl of separate test concentrations were interpolated on to the partial monolayer in microtiter plates which were incubated at 37°C for 24 h in 5% CO_2 atmosphere and test solutions in the wells were collected. Later, cell culture supernatant was incubated with assay reagents at 37°C for 1 min. The variations in absorbance/minute (OD/Min) during 3 min were calculated. The formula used to calculate: LDH activity (U/L) = (OD/min) ×16030.[6,7]

Sulforhodamine B assay

Sulforhodamine B (SRB 0.4% in 1% acetic acid, w/v) has been used in this study, as mentioned previously by Skehan *et al.* for 30 min.[8] After staining, the cells were rinsed four times with 1% acetic acid to remove unbound dye and the culture was air-dried. Bound dye was solubilised with 10 mM tris buffer (pH 10.5) for 5 min with shaking and the OD of both treated and untreated cells was read on an automated spectrophotometric plate reader at a single wavelength of 515 nm. The percentage growth inhibition (IC_{50}) was calculated as: ([OD of control − OD of sample]/OD of control) ×100.

Annexin V– fluorescein isothiocyanate/propidium iodide apoptosis assay

To evaluate the shift of phosphatidylserine (PS) from inner leaflets to outer leaflets of the plasma membrane, Annexin V– (fluorescein isothiocyanate [FITC])/PI apoptosis detection kit (Thermo Fisher scientific, India) was utilized. Procedure: Prior to induction of apoptosis, SCC-9 cells were plated as 1 × 106 cells per well in a 6-well plate using Dulbecco's modified eagle media (DMEM) cell culture. After 18 h, the wells were replaced with new culture media to the original volume. The cells were incubated with 40 and 80 µg/ml concentrations of *Z s-c* and incubated for 24 h. A rubber policeman was used to scrape and detach the cells from the dish followed by centrifugation. 500 µl of cell suspension was aliquoted and 10 µl of PI was added followed by 5 µL of Annexin V– FITC. Post-incubation, the cells were analyzed by a flow cytometer (BD Biosciences, San Jose, USA).[9-11]

Cell cycle analysis

A cell cycle analysis was performed to analyze the cell cycle distribution of the SCC-9 cells. Working solution: 1 mg/ml Propidium iodide stock solution-Cat #P4864, (Sigma-Aldrich, USA) was prepared and a working solution of 0.05 mg/ml was obtained. Also prepared was a working solution of 0.05 mg/ml RNase A – Cat #109 169, (Bochringer Mannhein, Germany). Procedure: SCC-9 cells (1106 cells) were grown in a 6 well plate with a 4-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 2 ml of DMEM media. Cells are treated with J samples after a 24-h incubation period. Procera (160 µg/ml and 320 µg/ml concentration) and 1% DMSO as command in sample-free DMEM media with HEPES buffer and 24 h incubated. Collecting the SCC-9 cells and pelleting cells at room temperature at 1500 rpm for 5 min, the supernatant was discarded. The pellet was resuspended at 1X PBS in 200 µl and set at 70% ethanol in 2 ml overnight. Centrifugation was done at 4000 rpm for 10 min at 4°C after overnight fixation. The supernatant was discarded and with 2 ml of cold PBS the pellet was washed twice. Later, in 450 µl sheath fluid containing 0.05 mg/ml Propidium iodide (PI) and 0.05 mg/ml RNaseA, the cells were incubated at room temperature for 15 min. Fluorescence-activated cell sorting caliber (BD Biosciences, San Jose, USA) was used to assess the percentage of cells in controlled and untreated populations at different stages of the cell cycle.[12]

Comet assay

The comet assay is also known as single-cell gel electrophoresis assay. It is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Preparation of base slides: Low melting point agarose (LMPA) 0.5% (250 mg per 50 ml PBS) and 1% normal melting agarose (500 mg per 50 ml in Milli Q water) was prepared. Isolation of cells: The cells were scraped off into the mincing solution using a Teflon scraper to yield approximately 1 × 105 cells/ml. Five to ten microliters of the cell suspension was removed and mixed per 75 µl LMPA was processed with an addition of an equal amount of FBS. Electrophoresis under pH >13 alkaline conditions and neutralization of Microgel Slides: Buffer reservoirs were filled with freshly made Electrophoresis Buffer (pH 13) and the slides were placed in an alkaline buffer for 20 min and the current was adjusted to 300 milliamperes. Electrophoresis under pH >13 alkaline conditions and Neutralization of Microgel Slides: Buffer reservoirs were filled with freshly made electrophoresis buffer (pH > 13) and the slides were placed in an alkaline buffer for 20 min and the current was adjusted to 300 mA.

Staining and evaluation of DNA damage: The slides were stained with 80 µl of 1% ethidium bromide for 5 min. The slides were drained and kept in cold 100% ethanol for 20 min for dehydration. The slides were air-dried and placed in an oven at 50°C for 30 min. The DNA damage was visualized using a fluorescent microscope (Olympus BX 60, India). The length of DNA migration and the percentage of migrated DNA was analyzed using Image J software (version 1.47, NIH, United States) with open comet plugin. In general, 50–100 randomly selected cells were analyzed per sample. The amount of migration per cell and the number of cells with increased tails were compared. Normal saline was maintained as control and hydrogen peroxide (H_2O_2) was used as a positive control.[13]

Real-time quantitative polymerase chain reaction analysis

Reverse transcription-polymerase chain reaction (RTPCR; Applied Biosystems 7500 Fast, Foster City, CA, USA) was used to analyze the expression of apoptotic genes by using a real-time SYBR Green/ROX gene expression assay kit (QIAgen, Germany). The Fastlane® Cell cDNA kit (QIAGEN, Germany) was used to prepare cDNA directly from cultured cells. And the mRNA levels of Caspase-9 and t*p53* as well as the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were assayed using gene specific SYBR Green-based QuantiTect® Primer assays (QIAGEN, Germany). Quantitative real-time RT-PCR was performed in a reaction volume of 25 µL according to the manufacturer's instructions. The quantitative RT-PCR data were analyzed by a comparative threshold (Ct) method and the fold inductions of samples were compared with the untreated samples. GAPDH was used as an internal reference protein to normalize the expression of the apoptotic genes. The Ct cycle was used to determine the expression level in control cells and SCC-9 cells treated with *Z s-c* (PF 1–2) at concentration of 40 and 80 µg/ml. The gene expression level was then calculated as described by Yuan *et al.*[14] The results were expressed as the ratio of the reference gene to the target gene using the following formula: ΔCt = Ct (apoptotic genes)-Ct (GAPDH). To determine the relative expression levels, the following formula was used: ΔΔCt = ΔCt (Treated)-ΔCt (Control). Thus, the expression levels were expressed as n-fold differences relative to the calibrator. The value was used to plot the expression of apoptotic genes using the expression 2-ΔΔCt.

Relative protein expression of p53 (mutant) and glyceraldehyde 3-phosphate dehydrogenase by Western blotting

SCC-9 cells were cultured in DMEM expansion medium with 20% FBS and 15 mM HEPES, 100 units/ml penicillin G and 100 µg/ml streptomycin at 37°C, 5% CO_2 incubator. The cultured SCC-9 cells at a density of 1 × 10^6 cells were seeded and cultured for 24 h in a 6-well plate containing 2 ml of media. Cells were then treated with (*Z S-C* [PF 1–2] at 40 and 80 µg/ml) or without (control) desired concentrations of given samples with media and incubated for another 24 h. Cells were then harvested and centrifuged at 2000 rpm for 5 min at 4°C and supernatant was discarded carefully retaining the cell pellet. The cells, postharvesting, were washed twice using 1XPBS and then gently suspended in 200 µl of RIPA buffer with 1X Protease Inhibitor. This mixture was incubated for 30 min by gentle mixing every 5 min and the cells were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and stored in −20°C until further use. A total of 140 ug protein sample from each cell lysate was mixed with 5X loading dye and heated for 10 min at 95°C. The protein samples were loaded and separated on 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gel and run for 2 h at 100 volts. Polyvinylidene fluoride membrane (0.2 uM) was activated by immersing in 100% methanol for 1 min; membrane was washed with water for 1 min and prewet in

transfer buffer until transfer, Protein transfer was done for 20 min in Turbo Trans blot (Biorad, USA). Blot was blocked in 5% skim milk powder (SMP) + tris buffered saline with tween (TBST) overnight at 4°C on shaker. Membrane was washed thrice with TBST for 5 min at RT and followed by one wash with 1X PBS. Membrane was incubated with primary antibody at a dilution of 1: 1000 in 5% SMP + TBST for 2 h at R_T. Membrane was then washed then washed thrice with TBST for 5 min at R_T and followed by one wash with 1X PBS. Blot was incubated with secondary antibody (Anti-Rabbit horseradish peroxidase-immunoglobulin G) at a dilution of 1: 10,000 for 2 h at RT. The membrane was again washed three times with TBST for 5 min at R_T and followed by one wash with 1X PBS. Blotting membrane was incubated with ECL reagent for 5 min in dark and image was captured with Chemidoc MP imaging system (Biorad, USA).

Statistical analysis

All experiments were done in triplicate and denoted as mean ± standard error of the mean. ANOVA followed by *post hoc* test was applied for the comparison between different study groups. SPSS version 20 (IBM Corp, Armonk, NY) was used for the analysis of results.

RESULTS

Compound isolation and crude extraction

3.26 g of methanolic yield of *Z s-c* was received from the 50 g of raw dried and powdered leaves. The yield of *Z s-c* postliquid fraction in solvents CH_3OH, $CHCL_3$, and C_6H_{14} was 250 mg, 900, and 46 mg, respectively. Post-TLC and HPLC, the final pooled fraction PF 1 (1–2) was further considered for further evaluation of biological activity using SCC9 cells and characterization.

Nuclear magnetic resonance spectroscopic

The compound in its mass spectrum showed a peak at *m/z* 594.80 (electrospray ionization MS positive mode) suggesting a molecular weight of 594.0. In its IR spectra, it showed a broad absorption band at 3399/cm for a–OH group, characteristic bands at 1706/cm for C = O and at 1645 and 1612/cm for C = C group.

In its ^{1}H-NMR spectra it exhibited signals

1. At 0.89 ppm for a methyl group
2. A broad singlet at 1.27 ppm, for a long chain of methylene groups
3. Signals at 1.61 and 2.06 ppm for methylene protons adjacent to double bonds.
4. At 3.5 ppm for protons under oxygen function.

In its ^{13}C-NMR spectra, it exhibited signals

1. Signal at 114.37 and 139.27 for the presence of a C = C group
2. A bunch of signals between 22.71 and 29.84 for the carbon atoms of the long chain methylene groups
3. Two signals at 14.12 ppm for the presence of methyl group carbon.

Anti-proliferative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Crude *Z s-c* showed cytotoxicity with IC_{50} value of 180.4 µg/ml, whereas standard Doxorubicin showed IC_{50} value of 23.61 µM in SCC9 cells. Samples *Z s-c* after L-L extraction, in MeOH extract showed IC_{50} value of 84.27 µg/ml and $CHCl_3$ extract showed 146.4 µg/ml inhibition in SCC-9 cells. After column-column extraction with $CHCl_3$, sample *Z s-c* showed IC_{50} value of 58.37 µg/ml and 294.2 µg/ml with sample pool fraction (Propidium iodide (PF)) (1–2) and PF (3–9) in SCC-9 cells, respectively. Chloroform extracts of *Z s-c* significantly and concentration dependently reduced viability of SCC-9 cells. The percentage inhibition of pooled fraction (1–2) was better compared to PF (3–9). *Z s-c* PF (1–2) was considered for further studies as its activity was better than other fractions [Figure 1].

Lactate dehydrogenase assay

Enhanced liberation of LDH was evident when SCC-9 cells were subjected to elevated levels of *Z s-c* extract. Sample *Z s-c* PF 1–2 showed increased levels of LDH at higher concentrations, i.e.,: 8111.18 U/L at 320 µg/m when compared to control (untreated) which was 2276.26 U/L in SCC-9 cells. Standard doxorubicin released 9072.98 IU/L of LDH at 100 µM of treatment in SCC-9 cells [Figure 2].

Sulforhodamine B assay

Analysis of SRB assay was done to identify the potential ability of Z s-c to inhibit cell growth of SCC-9 cells. Sample *Z s-c* PF 1–2 has showed an IC_{50} value of 43.55 µg/ml inhibition in SCC-9 cells when compared to Standard Doxorubicin which showed 26.95 µM inhibition [Table 1].

Annexin V – fluorescein isothiocyanate/propidium iodide apoptosis assay

The lower left quadrant displayed the double negative (Annexin V–/PI–) viable cells. Lower right quadrant displayed the early apoptotic cells (Annexin V +/PI–). The upper right quadrant displayed the late apoptotic/damaged cells (Annexin V–/PI +). The upper left quadrant represented the nonviable double positive (Annexin V +/PI +) necrotic/dead cells. The sample *Z s-c* PF (1–2) treated at 40 µg/ml and 80 µg/ml concentration has shown 7.60% and 20.62% early apoptotic cells and 12.17% and 15.72% of SCC-9 cells gated in the late apoptotic phase compared to the control having 0.00%. While Doxorubicin was taken as the standard and treated at 25 µM concentration has shown 49.90% late apoptotic cells. Early apoptosis was significantly noted as the concentration gradient of sample *Z s-c* increased to 80 µg/ml from 40 µg/ml [Figure 3 and Table 2].

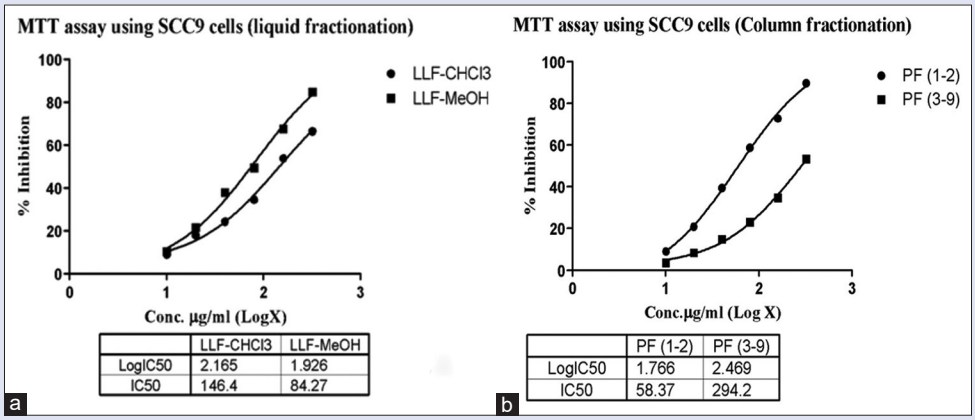
Cell cycle analysis

With aid of PI stain and flow cytometry analysis, the effect of *Z s-c* at PF (1–2) concentration was evaluated to check the dynamics of growth

Table 1: Sulforhodamine B assay of *Ziziphus spina christi* extract and doxorubicin on SCC-9 cells

Compound name	Concentration µM	OD at 590 nm	Percentage inhibition	IC_{50}
Control	0	0.519	0.00	
Doxorubicin	3.125	0.475	8.48	23.95
	6.25	0.426	17.92	
	12.5	0.354	31.71	
	25	0.249	52.02	
	50	0.171	67.05	
	100	0.091	82.47	
Control	0	0.519	0.00	
Z s-c PF	2.5	0.477	8.09	43.55
(1-2)	5	0.455	12.33	
	10	0.415	20.04	
	20	0.346	33.33	
	40	0.265	49.04	
	80	0.156	69.87	
	160	0.098	81.12	
	320	0.051	90.17	

SCC-9: The human tongue cancer cell line; *Z s-c: Ziziphus spina christi*; PF: Pooled fraction; IC_{50}: Half-maximal inhibitory concentration; OD: Optical density



	LLF-CHCl3	LLF-MeOH
LogIC50	2.165	1.926
IC50	146.4	84.27

	PF (1-2)	PF (3-9)
LogIC50	1.766	2.469
IC50	58.37	294.2

Figure 1: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay graph. (a) Percentage of inhibition of SCC-9 cells postliquid fractionation of *ziziphus spina-christi* extract. (b) Percentage of inhibition of SCC-9 cells postcolumn fractionation of *ziziphus spina-christi* extract. Pooled fraction (1–2) was found to be better than pooled fraction (3–9)

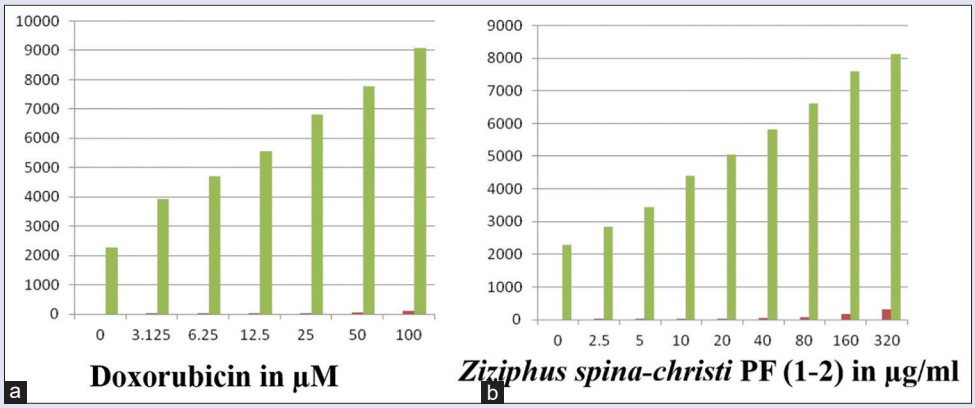


Figure 2: Graphical representation of Lactate dehydrogenase enzyme generation by *ziziphus spina-christi* on cancer cells. (a) Lactate dehydrogenase activity of doxorubicin of on *ziziphus spina-christi*. (b) Lactate dehydrogenase activity of *ziziphus spina-christi* PF (1–2)

cycle of SCC-9 cells. The samples of *Z s-c* showed effective cell cycle arrest. Sample *Z s-c PF (1–2)* at 40 µg/ml and 80 µg/ml has induced G2M phase arrest of up to 19.37% and 25.51% compared to the control cells having 12.74% arrest. While Colchicine treated at 25 µM has shown 41.35% of cells gated in G2M phase [Figure 4 and Table 3].

Comet assay

The SCC–9 cells treated with 40 µg/ml and 80 µg/ml concentrations of *Z s-c* showed increased DNA strand breaks and cytotoxicity. The results were statistically significant when compared to controls analyzed with graph pad prism software (version 7.04 CA, USA). The positive control H_2O_2 recorded a mean olive moment of 73.95 at 200 µM. The fluorescent images obtained indicated SCC9 cells that were treated with Sample *Z s-c* PF 1–2 showed the olive moments 9.30 and 23.18 at concentrations 40 µg/ml and 80 µg/ml. Dunnet's multiple comparison test showed significant differences between control and *Z s-c* at 80 µg/ml, whereas at 40 µg/ml, it was not significant [Figure 5].

Quantification of mRNA levels of apoptotic-related genes

The sample *Z s-c* (PF 1–2) at concentration of 40 µg and 80 µg treatment upregulated caspase-9 gene expression to 1.52 and 3.62 fold, respectively, and expression of *p53* gene was upregulated up to 2.64 and 4.66 folds compared to control (untreated) cells. Together, these data suggest that the expression of caspases and *p53* was induced by *Z S-C* in a dose dependent manner [Table 4].

Western blot analysis

Proapoptotic action of *p53 (mutant)* is fulfilled by downstream caspases: Caspase-9 and caspase-3. We next tested whether *Z s-c* activates caspase-9 to fulfil its proapoptotic potency. Western blot analysis revealed that stimulation with *Z s-c* triggered cleavage of caspase-9 in comparison to the control. The cells were treated with various concentrations of test sample PF 1–2 and the results suggest that the relative expression of *p53* was found to be 1.49 fold and 2.03 fold at 40 µg and 80 µg treatment, respectively, compared to control [Figure 6].

DISCUSSION

Genetic predisposition due to multifactorial changes in the cell signalling pathways could lead to cancer.[15] With about 6 lakh fresh cases diagnosed each annum, in whole world, cancer of oral cavity stood sixth and survival rates have not fared well in spite of rapid strides in treatment.[16] The standard chemotherapy for treating cancers has its own disadvantage in the form of its severe adverse effects on normal cells of the body and development of drug resistance by the cancerous cells. Medicinal plants have been studied as a potential new origin of cancer

Table 2: Fluorescence-activated cell sorting analysis of apoptosis detection in SCC-9 cells

Cell line	Sample µg/ml	Viable cells	Early apoptotic	Late apoptotic	Necrotic cells
SCC-9	Control	99.94	0	0	0.06
	Z s-c PF (1-2)-40 µg/ml	77.85	7.6	12.17	2.38
	Z s-c PF (1-2)-80 µg/ml	60.75	20.62	15.72	2.91
	Doxorubicin 25 µM	22.02	20.62	49.9	7.46

SCC-9: The human tongue cancer cell line; Z s-c: *Ziziphus spina christi*; PF: Pooled fraction

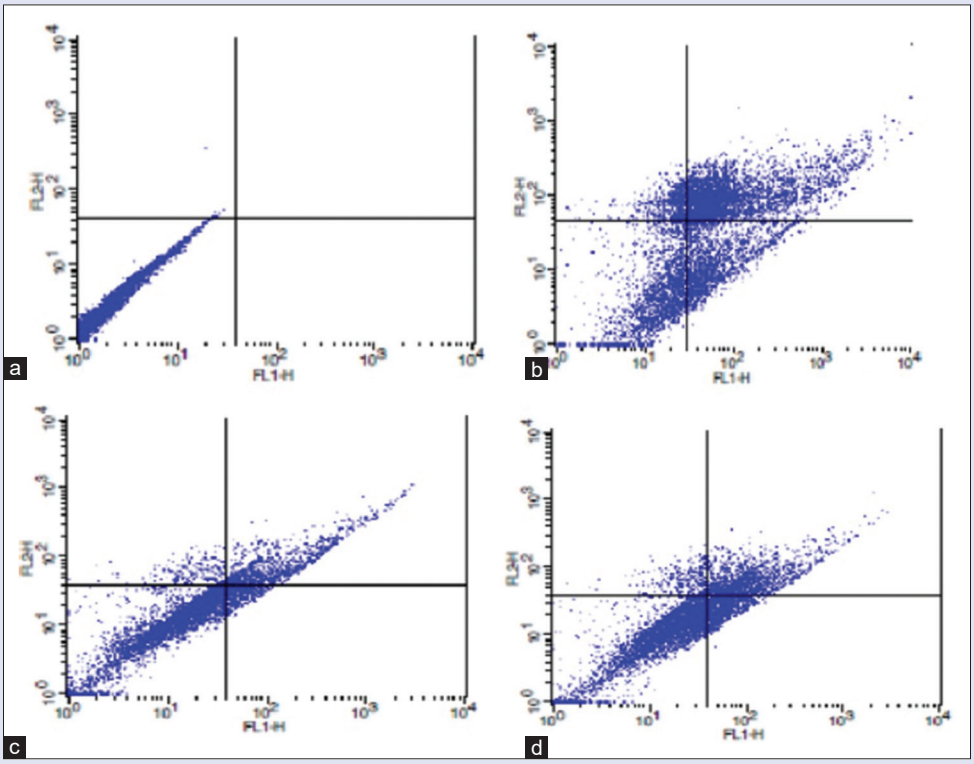


Figure 3: Flowcytometry plots for Annexin V-fluorescein isothiocyanate/PI assay. (a) Flowcytometry plot for SCC-9 untreated control cells. (b) Flowcytometry plot for SCC-9 cells treated with doxorubicin (25 µM) (c) Flowcytometry plot for SCC-9 cells treated with *ziziphus spina-christi* extract (40 µg/ml). (d) Flowcytometry plot for SCC-9 cells treated with *ziziphus spina-christi* extract (80 µg/ml)

therapy based on the ability to induce apoptosis and/or growth arrest in cancer cells without inducing cytotoxic results in healthy cells.[17]

Several reports on efficacy of naturally available extracts on oral cancer cell lines have been published recently. Anticancer properties present in earthworm coelomic fluid of Eudrilus eugeniae, Eiesnia foetida, and perionyx excavates on cell lines of oral cancer were reported by Augustine *et al*. It was noted that earthworm coelomic fluid had the ability to abort the cell cycle in G2M phase and initiate apoptosis. LDH, comet, and clonogenic assay revealed that earthworm coelomic fluid of these three species had a variable lethal effect and SCC-9 cells. Earthworm coelomic fluid of Eudrilus eugeniae was found to be superior in its antitumor properties compared to others.[18] The methanolic extracts of marine algae, Enteromorpha compressa were evaluated for their antiproliferative potency on Cal33 cell line (tongue SCC) by Pradhan *et al*. They reported that methanolic algal extract boosted intrinsic apoptosis against OSCC by downregulating protective antioxidant enzymes. Initiation of autophagy to promote apoptosis in oral cancer cells was also noted.[19] The nexus between cell death as well as destruct in of damaged cellular constituents induced by extracts of *Terminalia bellirica* in relation to reactive oxygen species was established in a study conducted by Patra *et al*. The gallic acid present in the extract was responsible for the arrest in cell multiplication. It was highlighted that gallic acid causes oxidative perturbation in oral cancer cells and stimulate apoptosis.[20]

In Saudi Arabia, *Z s-c* is wildly distributed across the southern and south-western provinces and is often used as a decorative and shade plant. It is a tall tree that can attain a height of 20 m and is greatly respected by Muslims since it is mentioned in the Sunnah and the Holy Quran twice. The leaves of *Z s-c* have been used by local Saudi healers to treat diabetes, dermatological disorders, and chronic inflammatory conditions. In ancient Chinese medicine too, *Z s-c* was used to improve the cardiac output and biliary system. The fruits and stem barks of Zs-c also have been used for their medicinal value in Bedouin. Secondary metabolites such as tannins, flavonoids, terpernoids, saponin glycosides, and alkaloids were the biological active compounds identified in *Z s-c* and these were the one responsible for its medicinal properties. Alcoholic and aqueous extracts from the various parts of *Z s-c* tree contained these bioactive compounds which were tested for their medicinal value.[21]

Elucidation of 10 dammarane-type saponins as well as 12 known polyphenols was carried out in one study published by Bozicevic A *et al*. on *Z s-c* leaves extract. Comparisons were drawn with respect to saponins from *Z s-c* samples from four different origins. Eight saponins (3–10) were reported for the first time. Lactogenin glycosides

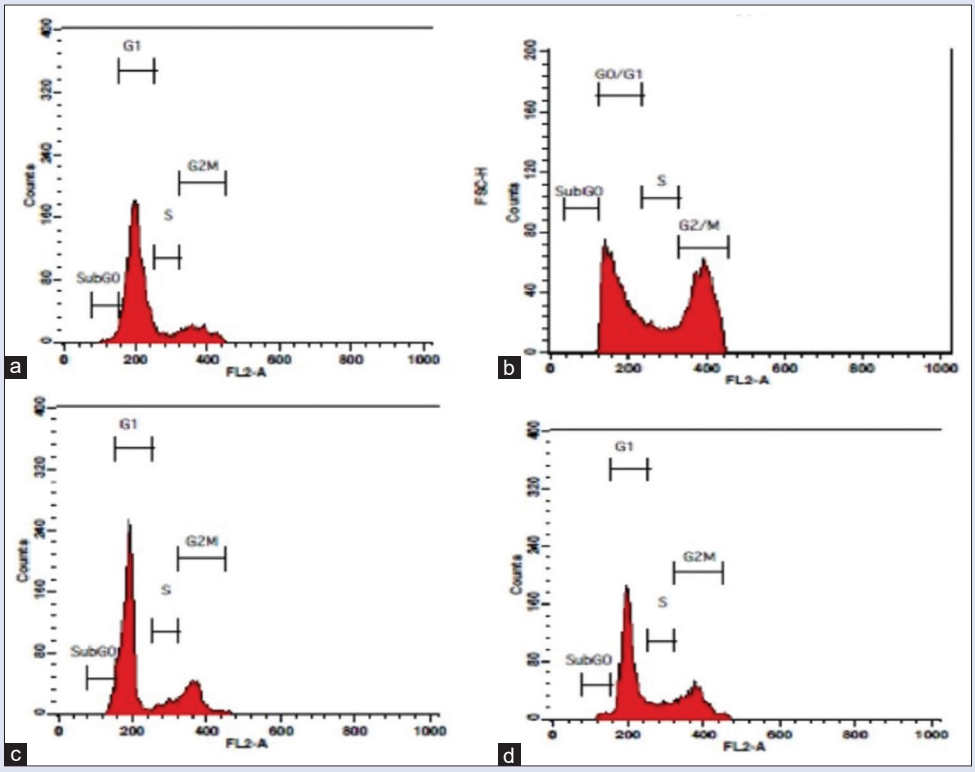


Figure 4: Flowcytometry images for cell cycle analysis. (a) Flowcytometry plot for SCC-9 untreated control cells. (b) Flowcytometry plot for G2M arrest induced by doxorubicin (25 µM) (c) Flowcytometry plot for G2M arrest induced by *ziziphus spina-christi* extract (40 µg/ml). (d) Flowcytometry plot for G2M arrest induced by *ziziphus spina-christi* extract (80 µg/ml)

Table 3: Flow cytometry analysis of cell cycle arrest in SCC-9 cells

Cell line	Sample µg/ml	SUBG0	G0/G1	S	G2M
SCC-9	Control	2.06	80.11	5.78	12.74
	Z s-c PF (1-2)-40 µg/ml	4.89	69.79	7.15	19.37
	Z s-c PF (1-2)-80 µg/ml	1.52	60.46	13	25.51
	Colchicine_25µM	0	44.87	14.14	41.35

SCC-9: The human tongue cancer cell line; *Z s-c: Ziziphus spina christi*; PF: Pooled fraction

were the predominant saponins in all four *Z s-c* samples. A biosynthetic pathway directing to three formerly sapogenins was also reported.[22]

Tuenter *et al*. conducted an experiment to isolate and elucidate the chemical structure from *Ziziphus nummularia* and *Z s-c* by HPLC-DAD and HPLC-PAD-(HRMS)-SPE-NMR. Seven cyclopeptide alkaloids were identified. Spinannine-B(3) and Spinannine-C(4) were the two novel compounds which were isolated for the first time form *Z s-c*. In addition, nummularine-D(5), nummularine-E(6) and amphibine-D(7) were also elucidated for the first time in *Z s-c*.[23]

In our current study, the biochemical characteristics of *Z s-c* vis-à-vis TLC, MS, and nuclear magnetic resonance were carried out and presented. Unfortunately, the type of compound isolated could not be identified.

In one study, the cytotoxic effects of different extracts of *Z s-c* on Hela and MDA-MB-468 tumor cell lines, using MTT assay showed good relationship between absorbance and number of cell. In order to fractionize compounds with different polarities in *Z s-c*, chloroform, chloroform-methanol (9:1), butanol, methanol, methanol-water (7-1), and water were used for extraction. All tested extracts showed significant cytotoxicity against both cell lines used but they were more effective against MDA-MB-468 cells. The highest order of potency of the extracts against both cell lines was seen in Chloroform-methanol extract.[4]

In our study, crude extraction was carried out in methanol. For L-L extraction of the methanolic extract, the solvents used were chloroform, methanol, and hexane. IC_{50} was 146.4 µg/ml for LLF (CHCl3) and 84.27 µg/ml for LLF (MeOH). Postcolumn-column fraction, pooled fraction 1–2 showed better percentage of inhibition of cancer cells as the concentration of *Z s-c* increased from 10 to 320 µg/ml. Extraction of flavonoids, alkaloids, and glycosides from *Z s-c* is better when chloroform and methanol solvents are used. The cytotoxic effects of *Z s-c* could be explained by the presence these flavonoids, alkaloids, tannins, terpernoids, and glycosides.[21]

Bark from *Z s-c* collected from the hail region was evaluated for its cytotoxic properties against cancer cell lines derived from the colon and breast. Predominant phytochemical, phytol was found in the bulk and it possessed potent cytotoxic effects. The Alkaline Ethyl Acetate extract of phytol was highly effective in its anticancer abilities.[21]

The total extract of ethanol, ethanol-aqueous (1:1) as well as aqueous fractions of *Z. s-c* leaves from Dashtestan-Bushehr, Iran, were evaluated for cytotoxicity, cell cycle inhibition and apoptosis against the human breast adenocarcinoma MCF-7 cell line (C135).[23]

Z s-c plants from different altitudes varied in their genetic and chemical composition. RAPD, ISSR, GC–MS and HPLC analysis of *Z. s-c* plants at Abha (2227.86 m), Dala Valley (1424 m), Rakhma Valley (1000 m), Raheb Valley (505 m) and Al-Marbh (147 m) to identify the genetic and constituents was performed. *Z s-c* plants present at almost similar altitudes matched with respect to the chemical and genetic makeup as per RAPD and ISSR analysis. *Z s-c* possessed regional uniqueness which was

revealed by GC-MS analysis. *Z s-c* found in high altitudes of Abha had a unique bioactive constituents compared to *Z s-c* grown elsewhere.[24]

New betulin derivative having anticancer activity was found in *Z s-c* native to UAE. The anticancer activity was measured by MTT assay on MDA-MB-231for breast cancer cells, A549 for lung cancer cells, HEPG2 for hepatic cancer cells and U-87 for brain cancer cells. The survival rate of all tested cancer cells lines was low when stem of *Z s-c* was used compared to thorns and leaves. The study clearly indicates that different environmental climates lead to production of variable metabolites from a plant.[25]

Leaves of *Z s-c* used in the current study were from the plants grown in Al Bahah region. Al Bahah region is located in the Southwest of the Kingdom of Saudi Arabia. The town is surrounded by natural tree and farming plateaus. The province is renowned for its lovely trees, birds, valleys and mountains. Al Bahah city experiences mild climate with temperatures ranging between 12°C and 23°C (53.6°F–73.4°F). Due to its location at 2500 m (8200 ft), the climate is moderate in summer and cold in winter above sea level. Humidity ranges from 52% to 67%. Annual rainfall in the mountainous region ranges between 229 and 581 mm.[26] The Methanolic plant stems extract was solvent fractionated into chloroform, methanol, and hexane fractions in our study. Chloroform fraction was more likely the target fraction since it showed selective activity on oral SCC-9 cells with no activity on untreated cells. *Z s-c* leaves chloroform fraction was fractionated into further 10 fractions on TLC. The chloroform fraction was further fractionated into 10 fractions. Fractions 1–2 with the target anticancer activity was subjected to repeated chromatographic purification using preparative TLC and HPLC to afford a pure compound with the target activity. The chemical and genetic composition of *Z s-c* found in Al Bahah used in the current study is different from the ones grown in different provinces of KSA. Comparative studies on the phytochemical and the pharmacological properties of different parts of *Z s-c* from various provinces of KSA will identify potential chemicals and potent compounds having medicinal value.

Medicinal value of several plants and shrubs grown in natural habitats of KSA needs to be unearthed. The drive to popularize traditional medicine available in KSA is being currently implemented by drafting organizing acts and drafting the laws by Saudi Ministry of Health. One such medicinal plant is *Z s-c* grown in south and south-western region of the country.[27,28] Our study demonstrated that *Z s-c* leaves extract from Al Bahah region of KSA is a promising antitumor agent for OSCC and lead to new preventive or therapeutic options and enhanced understanding of the interaction between phytochemicals of *Z s-c* and gene regulation in SCC-9 cells. However, our study had certain limitations which we intend to address in our further experiments. First, we could not identify the compound isolated. Second, we utilized only a single cell line in our experiments. In future, we will extend our experiments to another cancer cell line of the same origin and compare our data with a normal cell derived from the same tissue.

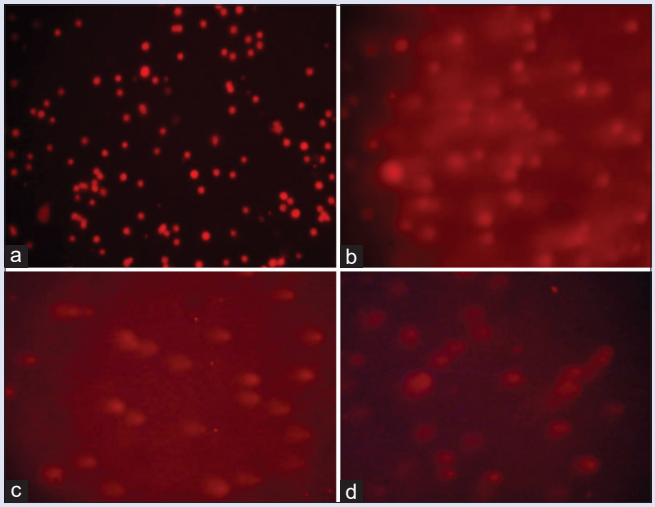


Figure 5: Florescence images of comets observed for different test samples. (a) Comet images for control (saline) on SCC-9 cells. (b) Comet images for positive control hydrogen peroxide at 100 µM. (c) Comet images for *Ziziphus spina-christi* extract (40 µg/ml). (d) Comet images for *Ziziphus spina-christi* extract (80 µg/ml)

CONCLUSION

To conclude, multiparametric analysis of the phytocomponents derived from *Z s-c* revealed that *Z s-c* inhibits oral cancer cells in the dose-dependent manner by initiating cell death and inhibiting cell proliferation. Our future endeavors should focus in identifying potential benefits of different parts of *Z s-c* tree grown worldwide.

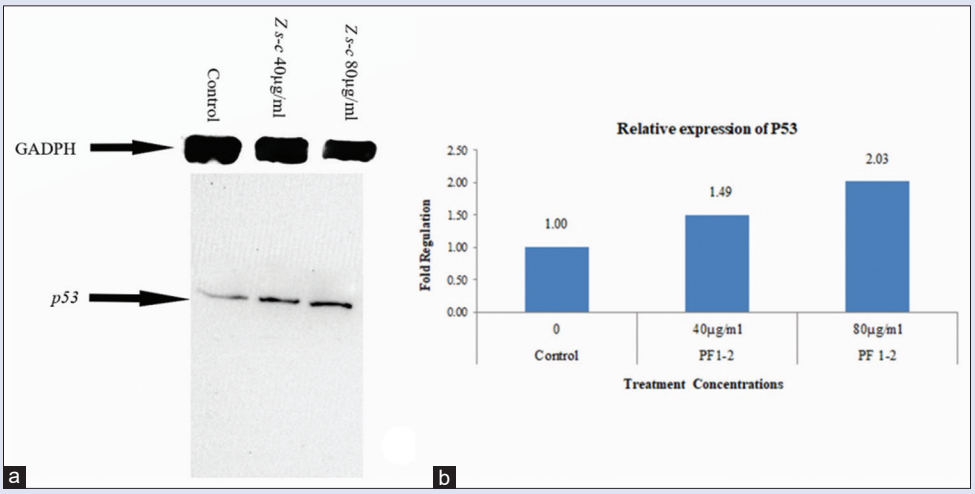


Figure 6: Effects of *Ziziphus spina-christi* on p53 protein expression in SCC-9 cells. Cells were treated with *Ziziphus spina-christi* (40 and 80 µg/ml) for 24 h. (a) Total cell protein were extracted and subjected to Western blot assay using antibodies against *p53*, with Glyceraldehyde 3-phosphate dehydrogenase (GADPH) used as a housekeeping gene internal control. (b) Results are expressed as the mean ± standard deviation ($n = 4$). $^{**}P < 0.01$ versus control

Table 4: Relative expression of caspase-9 gene and tumor protein gene in SCC-9 cells treated with *Ziziphus spina christi*

Target gene	Sample	Expression (normalized)	Expression SEM	Corrected expression SEM	Mean Cq or Ct	Cq or Ct SEM
GAPDH	Control	N/A	N/A	N/A	19.57	0.26
GAPDH	PF (1-2) 40 µg/ml	N/A	N/A	N/A	19.12	0.17
GAPDH	PF (1-2) 80 µg/ml	N/A	N/A	N/A	21.52	0.23
Caspase-9	Control	1.00	1.82	1.82	37.51	0.04
Caspase-9	PF (1-2) 40 µg/ml	1.52	1.93	1.93	36.46	0.69
Caspase-9	PF (1-2) 80 µg/ml	3.62	2.60	2.60	37.61	0.80
GAPDH	Control	N/A	N/A	N/A	20.14	2.16
GAPDH	PF (1-2) 40 µg/ml	N/A	N/A	N/A	21.40	0.08
GAPDH	PF (1-2) 80 µg/ml	N/A	N/A	N/A	21.48	0.45
p53	Control	1	1.41	1.41	33.07	0.55
p53	PF (1-2) 40 µg/ml	2.64	1.90	1.82	32.93	0.67
p53	PF (1-2) 80 µg/ml	4.66	2.37	1.94	32.19	0.24

Cq: Quantification cycle; Ct: Threshold cycle; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; SEM: Standard error of the mean; *P53*: Tumor protein; N/A: Not applicable; PF: Pooled fraction

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Patil S, Alamir AW, Arakeri G, Awan KH, Bhandi SH, Aljabab A, *et al.* The relationship of shammah (Arabian snuff) chewing to the risk of oral cancer and oral potentially malignant disorders. J Oral Pathol Med 2019;48:425-32.
2. Rabia AA. Key plants in fighting cancer in the middle east. Chin Med 2015;6:124-35.
3. Al-Ghamdi AA, Shahat AA. Antioxidant, hypoglycemic and anti-diabeticactivities of *Ziziphus spina-christi* (L) Willd (Rhamnacae) leaf extract. Trop J Pharm Res 2017;16:2601-10.
4. Jafarian A, Zolfaghari B, Shirani K. Cytotoxicity of different extracts of arial parts of *Ziziphus spina-christi* on Hela and MDA-MB-468 tumor cells. Adv Biomed Res 2014;3:38.
5. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
6. Chan FK, Moriwaki K, De Rosa MJ. Detection of necrosis by release of lactate dehydrogenase activity. Methods Mol Biol 2013;979:65-70.
7. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89:271-7.
8. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107-12.
9. Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, *et al.* Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995;182:1545-56.
10. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994;84:1415-20.
11. van Engeland M, Ramaekers FC, Schutte B, Reutelingsperger CP. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 1996;24:131-9.
12. Jackman J, O'Connor PM. Methods for synchronizing cells at specific stages of the cell cycle. Curr Protoc Cell Biol 2001;Chapter 8:Unit 8.3.
13. Glei M, Schneider T, Schlörmann W. Comet assay: An essential tool in toxicological research. Arch Toxicol 2016;90:2315-36.
14. Yuan JS, Reed A, Chen F, Stewart CN Jr. Statistical analysis of real-time PCR data. BMC Bioinformatics 2006;7:85.
15. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: Oncogene and non-oncogene addiction. Cell 2009;136:823-37.
16. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al.* Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86.
17. Abraham M, Augustine D, Rao RS, Sowmya SV, Haragannavar VC, Nambiar S, *et al.* Naturally available extracts inhibiting cancer progression: A systematic review. J Evid Based Complementary Altern Med 2017;22:870-8.
18. Augustine D, Rao RS, Anbu J, Chidambara Murthy KN. *In vitro* cytotoxic and apoptotic induction effect of earthworm coelomic fluid of *Eudrilus eugeniae, Eisenia foetida*, and *Perionyx excavatus* on human oral squamous cell carcinoma-9 cell line. Toxicol Rep 2019;6:347-57.
19. Pradhan B, Patra S, Behera C, Nayak R, Patil S, Bhutia SK, *et al.* Enteromorpha compressa extract induces anticancer activity through apoptosis and autophagy in oral cancer. Mol Biol Rep 2020;47:9567-78.
20. Patra S, Panda PK, Naik PP, Panigrahi DP, Praharaj PP, Bhol CS, *et al. Terminalia bellirica* extract induces anticancer activity through modulation of apoptosis and autophagy in oral squamous cell carcinoma. Food Chem Toxicol 2020;136:111073.
21. Abdel-Zaher AO, Salim SY, Assaf MH, Abdel-Hady RH. Antidiabetic activity and toxicity of *Zizyphus spina-christi* leaves. J Ethnopharmacol 2005;101:129-38.
22. Bozicevic A, De Mieri M, Di Benedetto A, Gafner F, Hamburger M. Dammarane-type saponins from leaves of *Ziziphus spina-christi*. Phytochemistry 2017;138:134-44.
23. Tuenter E, Foubert K, Staerk D, Apers S, Pieters L. Isolation and structure elucidation of cyclopeptide alkaloids from *Ziziphus nummularia* and *Ziziphus spina-christi* by HPLC-DAD-MS and HPLC-PDA-(HRMS)-SPE-NMR. Phytochemistry 2017;138:163-9.
24. Farmani F, Moein M, Amanzadeh A, Kandelous HM, Ehsanpour Z, Salimi M. Antiproliferative evaluation and apoptosis induction in MCF- 7 cells by *Ziziphus spina christi* leaf extracts. Asian Pac J Cancer Prev 2016;17:315-21.
25. Moustafa MF, Hesham AE, Quraishi MS, Alrumman SA. Variations in genetic and chemical constituents of *Ziziphus spina-christi* L. populations grown at various altitudinal zonation up to 2227 m height. J Genet Eng Biotechnol 2016;14:349-62.
26. Soliman S, Hamoda AM, El-Shorbagi AA, El-Keblawy AA. Novel betulin derivative is responsible for the anticancer folk use of *Ziziphus spina-christi* from the hot environmental habitat of UAE. J Ethnopharmacol 2019;231:403-8.
27. Awadh Ali NA, Al Sokari SS, Gushash A, Anwar S, Al-Karani K, Al-Khulaidi A. Ethnopharmacological survey of medicinal plants in Albaha Region, Saudi Arabia. Pharmacognosy Res 2017;9:401-7.
28. Aati H, El-Gamal A, Shaheen H, Kayser O. Traditional use of ethnomedicinal native plants in the Kingdom of Saudi Arabia. J Ethnobiol Ethnomed 2019;15:2.