Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Anticancer Activity of *Juniperus procera* Grown in Southwestern Region of Saudi Arabia on Human Oral Squamous Cell Carcinoma Cell Lines

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Submitted: 31-Jan-2020 Revised: 15-Apr-2020 Accepted: 15-Jul-2020 Published: 20-Oct-2020

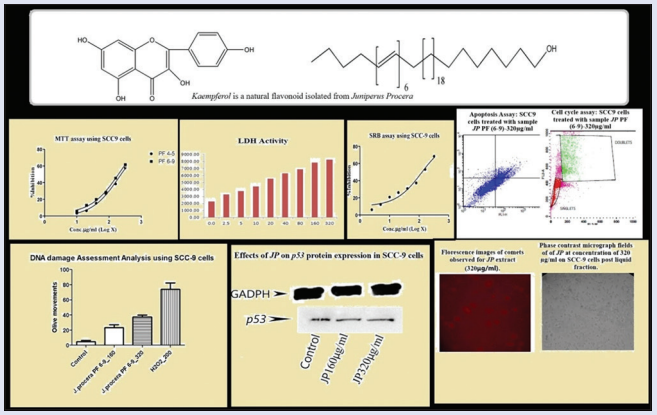
ABSTRACT

Background: One of the popular ancient plants used to treat various diseases such as hyperglycemia, hepatitis, jaundice, bronchitis, and pneumonia has been *Juniperus procera* (*JP*). *JP* is abundantly seen in the region of Al-Baha, Saudi Arabia, and is being used as a medicinal plant traditionally by local healers. **Objectives:** The objective was to evaluate the anticancer properties of *JP* from Al-Baha region on SCC-9 cells. **Materials and Methods:** Colorimetric assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, sulforhodamine B assay, and lactate dehydrogenase (LDH) assay were performed to check the cytotoxicity induced and cell viability. Assays evaluating cellular events such as apoptosis and cell cycle were also performed. **Results:** MTT assay revealed IC_{50} value of 201.6 µg/ml. The cancer cells primed with increasing concentrations of *JP* displayed an enhanced emission of LDH at elevated concentrations contrasted to cells which were not treated. The samples of *JP* pooled fraction (PF) (6–9) treated at 160 µg/ml and 320 µg/ml concentration showed 8.39% and 23.37% and 17.35% and 20.89% in early and late phases of apoptosis, respectively. Sample *P (PF 6-9)* at 160 µg/ml and 320 µg/ml has induced a G2M phase arrest of up to 21.06% and 26.94%. Deoxyribose nucleic acid damage was compared in tested concentrations of sample *JP* with untreated control cells in SCC9 cells. SCC9 cells that were treated with sample *JP* PF 6–9 showed the olive moments 23.22 and 37.30 at concentrations 160 µg/ml and 320 µg/ml, respectively. Studies of gene expression showed that increased concentrations of JP triggered the development of caspases and p53. **Conclusion:** The bioactive compounds found in *JP* were effective and potent against the SCC-9 cancer cells.

Key words: Antitumor, apoptosis, cell cycle analysis, cytotoxicity, *Juniperus procera*, SCC-9 cell line

SUMMARY

- The *in vitro* cytotoxic and antitumor capabilities of *Juniperus procera* were examined using oral SCC-9 cell lines
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay revealed crude extract of *JP* showed cytotoxicity with IC_{50} value of 208.7 µg/ml. Samples of *JP* after liquid-liquid extraction in methanol extract showed IC_{50} value of 274.9 µg/ml and $CHCl_3$ extract showed 77.65 µg/ml inhibition in SCC-9 cells. Post Column–Column extraction, sample pooled fraction (PF) 6–9 showed IC_{50} value of 201.6 µg/ml in SCC-9 cells. The sample *JP* PF (6–9) treated at 160 µg/ml and 320 µg/ml concentration has shown 8.39% and 23.37% early apoptotic cells and 17.35% and 20.89% of SCC-9 cells gated in the delayed programmed cell death phase contrasted to 0.00% of the controls
- Sample *P (PF 6–9)* at 160 µg/ml and 320 µg/ml has induced G2M phase arrest of up to 21.06% and 26.94% compared to the control cells having 12.74% arrest
- Deoxyribose nucleic acid damage was compared in tested concentrations of sample *JP* with untreated control cells in SCC9 cells. SCC9 cells that were treated with sample *JP* PF 6–9 showed the olive moments 23.22 and 37.30 at concentrations 160 µg/ml and 320 µg/ml, respectively
- Increased concentrations of *JP* activated pro-apoptotoc genes such as caspases and *p53*.



Abbreviations used: ABCG8: ATP-binding cassette sub-family G member 8; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CAR: Constitutive androstane receptor; CCl4: Carbon Tetrachloride; CYP2B10: Cytochrome P450 2B10; DMEM: Dulbecco's Modified Eagle Medium; DMSO: Dimethyl sulfoxide; DNA: Deoxyribose nucleic acid; ETO: Etofenamate; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; FXR: Farnesoid X receptor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC: High-performance liquid chromatography; INH: Isoniazid; *JP*: *Juniperus procera*; KSA: Kingdom of Saudi Arabia; LDH: Lactate dehydrogenase; MeOH: Methanol; MPT: Methoxypodophyllotoxin; MRP2: Multidrug resistance-associated protein 2; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NAE: Naturally available extracts; NMA: Normal melting agarose; NMR: Nuclear magnetic resonance; NSCLC: Nonsmall-cell lung carcinoma; OATP2: Organic anion transporting polypeptides; OD: Optical density; PBS: Phosphate-buffered

Cite this article as: AlShahrani A, AlShahrani I, Hosmani J, Togoo RA, Sakinatulain T, Alam T, *et al*. Anticancer activity of *Juniperus procera* grown in southwestern region of Saudi Arabia on human oral squamous cell carcinoma cell lines. Phcog Mag 2020;16:499-509.

saline; PF: Pooled fraction; PI3K/Akt: Phosphatidylinositol 3-kinase/protein Kinase B; PPT: Podophyllotoxin; RT-PCR: Reverse transcription–polymerase chain reaction; SPE: Solid-phase extraction; SRB: Sulforhodamine B; SULT2A: Hydroxysteroid sulfotransferase; TAA: Thioacetamide; TBST: Tris Buffered Saline with Tween; TLC: Thin-layer chromatography; TMZ: Temozolomide; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; UGT1A: UDP glucuronosyltransferase 1 family, polypeptide A cluster.

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DOI: 10.4103/pm.pm_28_20

Access this article online
Website: www.phcog.com
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INTRODUCTION

Tobacco is the world's leading cause of death and disability. Over 100,000 youth start smoking worldwide every day; most of them come from developing countries. The World Health Organization (WHO) reports 4.9 million deaths annually to tobacco use, and if the current trend continues, the exponential rise of new cases by the next decade has been predicted.[1] The WHO estimated that the prevalence of youth smoking in Saudi Arabia (KSA) was 25%. While the KSA is not directly involved in the cigarette manufacturing process, it currently imports 20,000 million cigarettes per year, costing approximately US$ 351.8 million. In terms of tobacco consumption, the KSA is now ranked eighth in the world; with a remarkable drop from 50-s in 1970–1972 to 23rd in 1990–1992. Oral cancer is Saudi Arabia's third most common cancer, and unfortunately, most cases are diagnosed in advanced stages which require palliative care.[2] Southwestern region of KSA has the highest number of clinically diagnosed oral cancers.[3]

Thanks to their antioxidant and free radical scavenging properties, naturally available extracts (NAEs) are pursued in the adjunctive cancer treatment. NAE has an underlying mechanism to prevent the proliferation of cancer cells.[4] Tribal and local people and herbal healers still have indigenous knowledge of traditional Saudi medicine. In the KSA, it is anticipated that >1200 of the total flowering plants (2250) will be of medicinal significance. The province of Al-Baha, located in southwestern Saudi Arabia, is well known for its numerous geographic regions–mountainous, flat, coastal, and high biodiversity. According to one ethno-pharmacological census of salutiferous flora in the region of Al-Baha, KSA, the most commonly used medicinal plants according to their user index were *Juniperus procera* (*JP*), *Rumex nervosus*, and *Ziziphus spina-christi*. These three herbs are prescribed mainly for dermatological disorders (42.6%) and soothing traumatic injuries.[5]

Despite the fact that conditions such as hyperglycemia, hepatitis, jaundice, bronchitis, and pneumonia have been cured traditionally utilizing *JP*,[6-8] it has never been tested for its prospective abilities to treat cancer of the oral cavity.

This study aimed at determining the impact of *JP* on SCC-9 cell lines. The purpose of this research is also to explore the essential attributes fuelled by *JP* on oral cancer cells.

MATERIALS AND METHODS

Compound isolation from *Juniperus procera*

Arid leaves of *JP* were purchased from herbalists. 50 g of raw *JP* leaves was taken in 250 ml of methanol (MeOH) and kept for 4 h extraction on a water bath at 50°C. The MeOH filtrate obtained was further kept for evaporation on a water bath at 50°C. Post evaporation, obtained crude herbal extract was weighed and stored for further studies.

Liquid–Liquid (L-L) extraction

MeOH extract was dissolved in 10 mL of MeOH (initially: 500 mg/10 ml, was used for liquid–liquid partition chromatography. Chloroform, MeOH, and hexane soluble fractions were separated using separating funnel. Later, L–L fractions were kept for evaporation on water bath at 50°C, followed by L–L fractions which were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) to check the purity.

Thin-layer chromatography for crude L–L fractions and column fraction

10 mg/ml samples were prepared and 2.5 µl of samples was spotted on the TLC plate and allowed to dry. A tray of TLC composed of a thin silica gel coat 0.25 mm with fluorescent indicator F_{254} with chloroform: MeOH (9.5:0.5) was used for TLC analysis. The tray was then placed with an edge dunking into the solvent blend and the zone of the sample not being immersed. The development of TLC trays takes place when the sample is partitioned into numerous segments as the solvent hauled onto other edges of the tray. Post optimal development time, the tray was withdrawn and withered. Later, the spots/zones were discerned employing UV chamber and R_f value is calculated using a formula, R_f = Distance moved by compound/distance moved by the solvent.

High-performance liquid chromatography

Test samples (10 mg/mL) were prepared from stock with HPLC grade MeOH, chloroform, and hexane [based on solubility, Tables 1 and 2] and used for HPLC analysis.

HPLC was performed on a Shimadzu LC-Prominence 20AT coupled with a photodiode array detector as per the manufacturer's instructions (Thermo Scientific, UK).

Cell lines and culture conditions

Cell lines: SCC-9 cells made available from ATCC, USA. Culture conditions: MEM (Sigma-Aldrich, USA) enriched with 4.5 g/l glucose, 2 mmol/l L-glutamine, 5% fetal bovine serum (FBS) (Sigma-Aldrich, USA), and 1% penicillin at 37°C in 5% CO_2 incubator.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The cells were treated with differing percentages (10, 20, 40, 80, 160, and 320 µg/mL) of *JP*, for 24 h. Doxorubicin was taken as a positive control and saline as a negative control. After 24 h conjugation with *JP*, 100 µl/well of the 3 (4,5dimethylthiazol2yl) 2,5 diphenyltetrazolium bromide (MTT) reagent at a concentration of 5 mg/10 ml in 1% phosphate-buffered saline (PBS) was supplemented to the respective wells and incubated for 3–4 h. Post conjugation with MTT reagent, it was repudiated by pipetting without perturbing the cells. About 100 µl of dimethyl sulfoxide was combined to quickly solubilize the formazan. Optical density (OD) was gauged at 590 nm. The effective lethal concentration required for antiproliferative effect was determined by plotting a graph and obtaining a curve with a maximum number of

Table 1: Fluorescence-activated cell sorting analysis of apoptosis detection in SCC-9 cells

Cell line	Sample µg/ml	FACS analysis of apoptosis detection in SCC-9 cells			
		Viable cells	Early apoptotic	Late apoptotic	Necrotic cells
SCC-9	Control	99.94	0	0	0.06
	JP PF (6-9)-160	69.9	8.39	17.35	4.36
	JP PF (6-9)-320	53.74	23.37	20.89	2
	Doxorubicin 25µM	22.02	20.62	49.9	7.46

FACS – Fluorescence-activated cell sorting; *JP* – *Juniperus procera*; PF – Pooled fraction; SCC – Squamous Cell Carcinoma

Table 2: Flow cytometry analysis of cell cycle arrest in SCC-9 cells

Cell line	Sample µg/ml	FACS analysis of cell cycle arrest in SCC-9 cells			
		SUBG0	G0/G1	S	G2M
SCC-9	Control	2.06	80.11	5.78	12.74
	JP (PF 6-9)-160	0.73	73.95	4.68	21.06
	JP (PF 6-9)-320	1.24	66.28	5.68	26.94
	Colchicine - 25µM	0	44.87	14.14	41.35

FACS – Fluorescence-activated cell sorting; *JP* – *Juniperus procera*; PF – Pooled fraction; SCC – Squamous Cell Carcinoma

cells killed and concentration of the *JP* extract used. The formula used was as follows: (OD of control–OD of sample/OD of control) ×100. The inhibitory concentration or (IC_{50}) was ascertained using GraphPad Prism 7 software (San Diego, California).[9]

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.[10] 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 was 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 was LDH activity (U/L) = (OD/Min) × 16030.[11]

Sulforhodamine B assay

A novel, quick, and economic colorimetric test to determine the cell damage, as developed by Skehan *et al.*,[12] was implemented in our study. The formula to calculate growth inhibition (IC_{50}) was tabulated by: [(OD of Control – OD of sample)/OD of Control)] x 100.

Apoptosis assay

Increases in phosphatidylserine asymmetry, observed by calculating the attachment of Annexin V to the cell membrane, were identified until morphological increases correlated with apoptosis occurred and the integrity of the membrane lost. Through conjugating fluorescein isothiocyanate (FITC) to Annexin V, apoptotic cells can be characterized and quantitated through flow cytometry on a single cell basis. Staining cells concurrently with FITC-Annexin V (green fluorescence) and propidium iodide (red fluorescence) allow early apoptotic and late apoptotic or necrotic cells to be discriminated.

The day prior to the induction of apoptosis, 1×10^6 cells were plated per well for a 6-well plate using Dulbecco's Modified Eagle Medium (DMEM) cell culture medium. Post ~18 h, fresh culture medium was recouped. SCC-9 cells were treated with *JP* at varying concentrations to induce apoptosis and incubated for 24 h. Later, the cell culture medium was collected into 15-mL tubes. Utilizing cell spatula, the cells were loosened from the dish and 1 mL of medium was supplemented to every well, and finally, the contents were shifted to respective 15-mL tubes and centrifuged. The supernatant was castoff and the cells were washed with cold 1X PBS and then resuspended in 1 mL 1X Binding Buffer at a concentration of ~1×10^6 cells/mL. 500 µL of cell suspension was aliquoted and 10 µL of PI and 5 µL Annexin V were added. The suspension had been incubated in the dark at RT for 15 min. The cells were analyzed by flow cytometer as quickly as possible after incubation (within 1 h).[13-15]

Cell cycle analysis

1×10^6 SCC-9 cells were seeded and cultured for 24 h in a 6-well plate entailing 2 ml of media. Cells were then processed with desired concentrations of *JP* (160 µg/ml and 320 µg/ml concentration) with media and incubated for another 24 h. Cells were then gleaned and centrifuged at 2000 rpm for 5 min at 4°C and supernatant was junked raptly retaining the cell pellet. Cell pellet was washed by resuspending in 2 mL of 1X PBS. The washing was repeated another time with the same conditions. The supernatant was discarded retaining the pellet. Cells were set by resuspending in 300 µl sheath fluid accompanied by applying 1 mL of chilled 70% the chemical title for ethanol alcohol (EtOH) drop by drop with constant gentle shaking before inserting another 1 mL of chilled 70% EtOH at once. Instead, the cells were processed for or overnight at 4°C. Post fixing, the cells were centrifuged at 2000 rpm for 5 min at 4°C. The cell pellet was washed twice with 2 ml of cold 1X PBS. Cell pellet was then resuspended in 450 µl of sheath fluid containing 0.05 mg/ml PI and 0.05 mg/ml RNaseA and incubated for 15 min in dark. The percentage of cells in various stages of cell cycle in compounds treated and untreated populations was determined using fluorescence-activated cell sorting Caliber (BD Biosciences, San Jose, CA).[16]

Comet assay

The single-cell gel electrophoresis (SCGE/COMET ASSAY) blends the lucidity of biochemical procedures for discerning deoxyribose nucleic acid (DNA) single-strand breaks, alkali-labile sites, and cross-linking with the single-cell approach typical of cytogenetic assays.

Production of base slides

A clear slide is taken and cleaned with alcohol and allowed to dry. 0.5% Low Melting Point Agarose (LMPA) (100 mg/20 ml PBS) and 1.0% normal melting agarose (NMA) (200 mg/20 ml in Milli Q water) are prepared and heated until the agarose is dissolved. The LMPA vial is kept in 37°C dry/water bath to cool. While the NMA agarose is still hot, it is poured onto the slides evenly to cover the whole slide devoid of any air bubbles and coverslip is placed on the top. The underside of the slide is cleaned for any debris, and the slides are laid on the flat surface of the tray to dry. Post complete solidification of the gel, the coverslip is

carefully removed as to create a well on the slides. The slides are stored at room temperature until needed, avoiding high humidity conditions.

After at least 2 h at 4°C, slides were gently removed from the lysis solution and slides were kept next to each other on the horizontal gel box near one end, sliding them as close together as possible. The buffer reservoirs were filled with freshly made electrophoresis buffer (pH >13) until the liquid level completely covers the slides. Slides were left in the alkaline buffer for 20 min to allow for unwinding of the DNA and the elucidation of alkali-labile damage. Power supply is switched on to 18–24 V (~0.74 V/cm) and the current is regulated to 300 mA by raising or lowering the buffer level. The slides were electrophoresed for 30 min and the electricity was switched off. Delicately the slides were raised from the buffer and put on a drain tray. The slides were cleaned with chilled distilled water and left for 5 min and air-dried. The slides were washed once again with chilled 70% ethanol and left for 5 min and air-dried.

Assessment of deoxyribose nucleic acid degeneration

Slides were stained with 80 µl 1X ethidium bromide (EtBr) for 5 min and washed with cold PBS to remove excess stain. The slides were scored instantly after washing. The excess liquid on the back and edges of the slides was blotted before scoring.

For postulation of DNA damage, ascertainment was made of EtBr-stained DNA using a ×40 objective on a fluorescent microscope. The quantitative and qualitative extent of DNA damage in the cells was analyzed by measuring the length of DNA migration and the percentage of migrated DNA using ImageJ software with OpenComet plug-in. In general, 50–100 randomly selected cells were assessed per sample. Finally, the program calculated the tail moment by comparing the amount of migration per cell, the number of cells with increased migration, the extent of migration among damaged cells, and viability.

Polymerase chain reaction analysis

To evaluate the exhibition of apoptotic genes in the present study, we utilized a real-time SYBR Green/ROX gene expression assay kit (QIAGEN, Germany). The Fastlane® Cell cDNA kit (QIAGEN, Germany) was adapted to assemble cDNA exactly out of cultured cells, and the mRNA levels of Caspase-9, tp53, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were assayed using gene-specific SYBR Green-based QuantiTect® primer assays (QIAGEN, Germany). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out in a reaction volume of 25 µL according to the manufacturer's recommendation. The quantitative RT-PCR data were assessed by a comparative threshold (Ct) technique and the fold inductions of samples were correlated with the untreated samples. GAPDH was utilized as an internal reference gene to normalize the elucidation of the apoptotic genes. The Ct cycle was used to observe the expression level in control cells and SCC-9 cells treated with *JP* (pooled fraction [PF]: 6–9) at concentration of 160 and 320 µg/ml. The gene expression level was computed as described by Yuan *et al.*[17] Relative fold expression of the target gene was determined by the comparative CT Method (ΔΔ CT Method). Relative expression of the target gene in relation to the housekeeping gene (GAPDH) and untreated control cells were determined by the comparative CT method. Delta CT for each treatment was calculated using the formula: Delta Ct = Ct (target gene)−Ct (reference gene). To compare the individual sample from treatment with untreated control, delta CT of the sample was subtracted from control to get a delta–delta CT. Fold change in target gene expression for each treatment was calculated using the formula: fold change = 2^ (-delta-delta Ct).

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

SCC 9 cells were cultured in DMEM expansion medium with 20% FBS and 15 mM 4 (2 hydroxyethyl) 1 piperazineethanesulfonic acid, 100 units/ml penicillin G and 100 µg/ml streptomycin at 37°C in CO_2 (5%) 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. The SCC-9 cells were later treated with JP [PF 6–9] at desired concentrations of 160 and 320 µg/ml and incubated for another 24 h. Cells were then harvested and centrifuged at 2000 rpm for 5 min at 4°C and the supernatant was discarded carefully retaining the cell pellet. The cells, post harvesting, were washed twice using 1X PBS 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 140ug protein samples from each cell lysate were mixed with 5X loading dye and heated for 10 min at 95°C. The protein samples were loaded and separated on 12% SDS-PAGE gel and run for 2 h at 100 volts. PVDF membrane (0.2 µM) was activated by immersing in 100% MeOH for 1 min which was washed later with water for 1 min and was preserved in transfer buffer. 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 the shaker. The membrane was washed thrice with TBST for 5 min at RT and followed by one wash with 1X PBS. The membrane was incubated with primary antibody at a dilution of 1:1000 in 5% SMP + TBST for 2 h at RT. The membrane was then washed thrice with TBST for 5 min at RT and followed by one wash with 1X PBS. Blot was incubated with a secondary antibody (anti-rabbit HRP-immunoglobulinG) at a dilution of 1:10000 for 2 h at RT. The membrane was again washed three times with TBST for 5 min at RT and followed by one wash with 1X PBS. The blotting membrane was incubated with ECL reagent for 5 min in the dark and the image was captured with ChemiDoc MP imaging system (Bio-Rad, USA).

Statistics

All assays were performed triple times and displayed as mean ± standard error of mean. Analysis of variance was applied for comparison between *JP* and controls used, followed by *post hoc* test. The findings were evaluated using SPSS version 20. (IBM Corporation, Chicago, IL, USA)

RESULTS

Compound isolation and crude extraction

2.56 g of methanolic yield of *JP* was received from the 50 g of raw dried and powdered leaves. The yield of *JP* post liquid fraction in solvents CH_3OH, $CHCI_3$, and C_6H_{14} was 300 mg, 1.27, and 28 mg respectively. Post TLC [Tables S1 and S2] and HPLC [Table 3], the final PF 2 (6–9) was further considered for further evaluation of biological activity using SCC9 cells and characterization.

Nuclear magnetic resonance spectroscopic analyses

The compound in its mass spectrum showed a peak at *m/z* 594.80 (electrospray ionisation mass spectrometry (ESI-MS) positive mode), suggesting a molecular weight of 593.0 amu (atomic mass units). In its infrared spectra, it showed a broad absorption band at 3032 cm^{-1} for a –OH group, characteristic bands at 1722 cm^{-1} for C = O, and at

1612 cm^{-1} for C = C group.

In its ^{1}H-nuclear magnetic resonance spectra it exhibited signals

1. At 0.88 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 4.05 ppm for protons under oxygen function.

Based on the following data, Figure S1 depicts the structure. However, ^{13}C nuclear magnetic resonance (NMR) [Table S3] used to describe the molecular structure revealed it to be kaempferol [Figure S1].

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Crude *JP* displayed cytotoxicity with IC_{50} value of 208.7 µg/ml, whereas standard doxorubicin showed IC_{50} value of 23.61 µM in SCC9 cells [Table S4]. Samples of *JP* after liquid–liquid extraction in MeOH extract showed IC_{50} value of 274.9 µg/ml and $CHCl_3$ extract showed 77.65 µg/ml inhibition in SCC-9 cells [Table S5]. After column–column extraction, sample *JP* showed IC_{50} value of 106.2 µg/ml and 65.34 µg/ml in PF 1–2 and PF 3, respectively, and sample PF 4–5 and PF 7–9 did not show any significant inhibition, hence IC_{50} was not calculated. Samples from *JP* PF (7–9) were further purified and samples PF 2–3 did not show significant activity, sample PF (4–5) showed IC_{50} value of 212.4 µg/ml, and sample PF 6–9 showed IC_{50} value of 201.6 µg/ml in SCC-9 cells [Figure 1]. Sample *JP* PF (6–9) was considered for rest of the assays included in the study, as its activity was found to be better than other fractions [Table S6]. Antiproliferative effect on treated and untreated cells with *JP* extract and doxurubicin is shown in Figure S2.

Lactate dehydrogenase assay

The cancer cells primed with *JP* sample concentrations displayed a greater release of LDH at higher concentrations than un-primed cells. Sample *JP* PF 6–9 showed increased levels of LDH at higher concentrations, i.e., 8271.48U/L at 320 µg/ml when compared to control (untreated) which was 2276.26U/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

Sulforhodamine B assay was used to estimate the antiproliferative effect of *JP* extract against SCC-9 cells. Sample *JP* PF 6–9 has showed an IC_{50} value of 120.2 µg/ml inhibition in SCC-9 cells when compared to standard doxorubicin which showed 26.95 µM inhibition [Figure 3 and Table S7].

Apoptosis assay

The sample *JP* PF (6–9) treated at 160 µg/ml produced an early and late apoptotic rate of 8.39% and 17.35% and with concentrations of 320 µg/ml, *JP* PF (6–9) produced 23.37% and 20.89% early and late apoptosis, respectively. Early apoptosis was significantly noted as the concentration gradient of sample *JP* increased to 320 µg/ml from 160 µg/ml. Doxorubicin which was taken as the standard and treated at 25 µM concentration showed 49.90% late apoptotic cells, whereas the control group showed no apoptosis [Figure 4 and Table 1].

Cell cycle analysis

The samples of *JP* showed effective cell cycle arrest. Sample *JP (PF 6–9)* at 160 µg/ml and 320 µg/ml has induced G2M phase arrest of up to 21.06% and 26.94%, respectively, compared to the control cells having 12.74% arrest. In the G_0/G_1 phase, a significant arrest of 73.95% was seen at the concentration of 160 µg/ml compared to 66.28% µg/ml at 320 µg/ml. While colchicine treated at 25 µM has shown 41.35% of cells gated in the G2M phase [Figure 5 and Table 2].

Comet assay

The cancer cells charged with JP concentrations of 160 µg/ml and 320 µg/ml displayed elevated breakages in the DNA strand and cytotoxicity. The findings were statistically significant relative to the controls evaluated. H_2O_2 used as a positive control recorded a mean olive moment of 73.95 at 200 µM. The fluorescent images obtained indicated SCC9 cells that were treated with sample *JP* PF 6–9 showed the olive moments 23.22 and 37.30 at concentrations 160 µg/ml and 320 µg/ml, respectively. Statistically significant differences between control and *JP* at 160 and 320 µg/ml were seen in Dunnett's multiple comparison test [Figure 6 and Table 4].

Expression of caspase- 9 and p53 genes in SCC-9 cells treated with *Juniperus procera*

The expression rates of several genes associated with cell death were analyzed to explore the dynamics at molecular level through which extracts of *JP*-induced apoptosis in the SCC-9 cells. Figures S3 and S4 illustrate the gene elucidation changes of caspase-9 and *p53*. *JP* enhanced the transcripts of caspase-9 and p53 by manifold. The sample PF 6–9 at the concentration of 160 µg and 320 µg treatment upregulated caspase-9 gene expression 1.41- and 4.82–fold, respectively, in SCC-9 cells. The PF 6–9 test at 160 µg and 320 µg treatment concentrations upregulated up to 2.45- and 6.17-folds of p53 gene expression relative to control (untreated) cells. Such data suggest, together, that *JP* triggered the elucidation of caspases and p53 as the dosage of the extract increased [Table 5].

Western blot analysis

Downstream caspases which perform the proapoptotic role of *p53* are caspase-9 and caspase-3. First, we checked whether *JP* stimulates caspase-9 to reach its proapoptotic potency. Western blot analysis revealed that *JP* stimulus caused caspase-9 cleavage as opposed to the control. With varied measures of the test sample PF 6–9, the results

Table 3: Summary of high-performance liquid chromatography analysis

Assay	Sample	Area (%)	Retention time (min)	Peak number
Crude	*JP* MeOH	53	1.7	3
		33	2.05	
Liquid fraction	LLF MeOH	45.2	1.65	4
		32	2.73	
	LLF $CHCl_3$	100	2.02	1
Column	PF2 (2-3)	99.9	1.8	2
	PF2 (4-5)	87.5	2.99	4
		11.5	1.99	
	PF 2 (6-9)	87	1.79	2
		13	1.43	

JP – Juniperus procera; PF – Pooled fraction; MeOH – Methanol

Table 4: Olive moments registered in *Juniperus procera* and controls

Sample	Mean±SD
Control	4.6302±14.1778
JP PF 6-9 160	23.2225±37.6615
JP PF 6-9 320	37.3036±24.8500
H2O2_200µM	73.9590±81.9025

Values are shown as mean±SD of 3 replicates per experiment. SD – Standard deviation; PF – Pooled fraction; *JP – Juniperus procera*

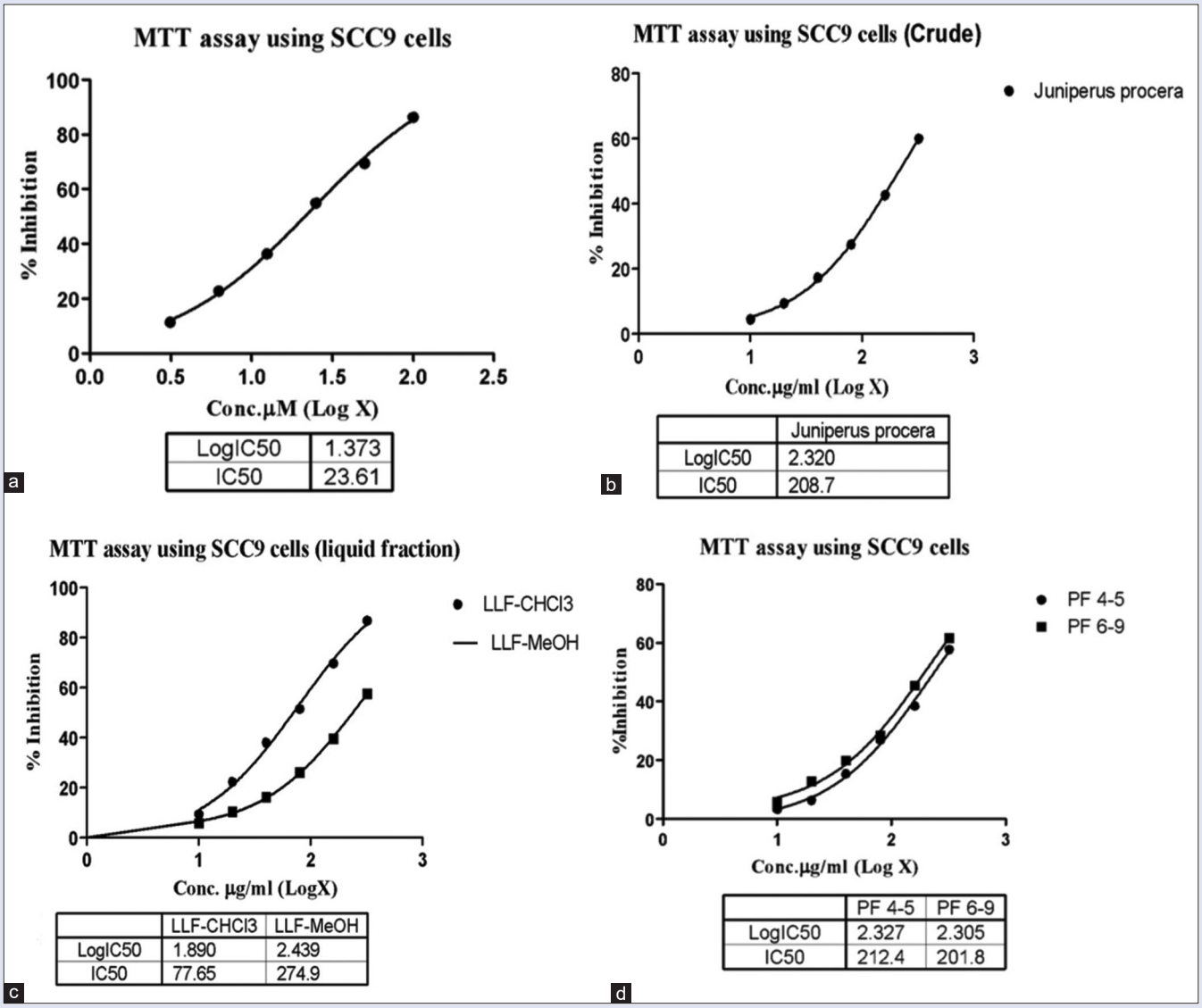


Figure 1: Effect of *Juniperus procera* extract on the viability of SCC-9 cells based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and IC_{50} value. (a) Effect of Standard (Doxorubicin drug) on SCC-9 cells. (b) Effect of crude extract of *Juniperus procera* on SCC-9 cells. (c) Effect of Liquid extraction of *Juniperus procera* on SCC-9 cells. (d) Effect of Column fractions of *Juniperus procera* pooled fraction 4–5 and pooled fraction 6–9 on SCC-9 cells

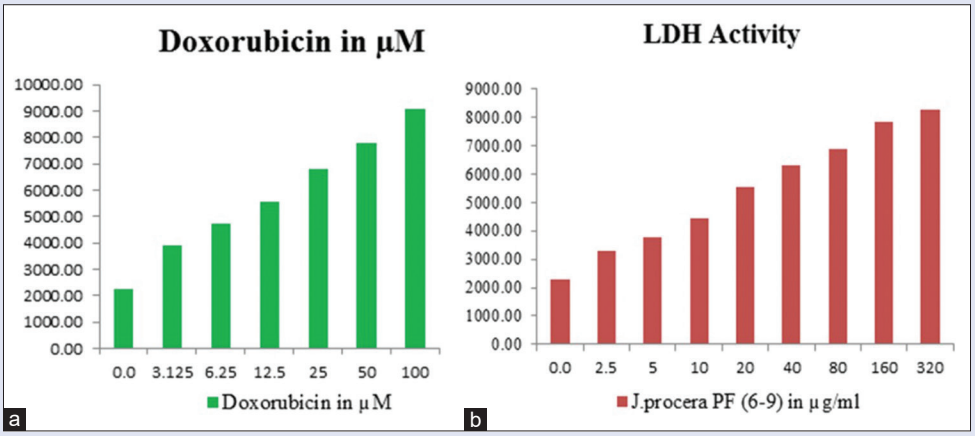


Figure 2: Graphical representation of lactate dehydrogenase enzyme generation by *Juniperus procera* on cancer cells. (a) Lactate dehydrogenase activity of Doxorubicin of on *Juniperus procera*. (b) Lactate dehydrogenase activity of *Juniperus procera* pooled fraction (6–9)

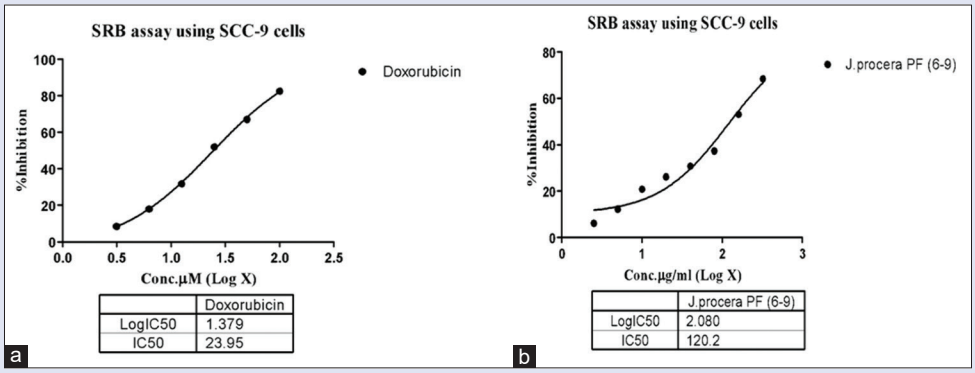


Figure 3: Dose response curve of Doxorubicin and *Juniperus procera* extract against SCC-9 cells. (a) Percentage of inhibition of SCC-9 cells as the concentration of Doxorubicin increases. (b) Percentage of inhibition of SCC-9 cells as the concentration of *Juniperus procera* extracts increases

Table 5: Relative expression of caspase-9 and *p53* gene in SCC-9 cells treated with *Juniperus procera*

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	18.00	0.02
GAPDH	PF 6-9 160	N/A	N/A	N/A	19.46	0.22
GAPDH	PF 6-9 320	N/A	N/A	N/A	20.41	0.15
Caspase 9	Control	1.00	1.41	1.41	36.38	0.19
Caspase 9	PF-40	1.41	1.60	1.60	37.34	0.25
Caspase 9	PF-80	4.82	2.98	2.98	36.52	0.21
GAPDH	Control	N/A	N/A	N/A	18.28	0.19
GAPDH	PF 6-9 160	N/A	N/A	N/A	20.58	0.47
GAPDH	PF 6-9 320	N/A	N/A	N/A	20.02	0.29
p53	Control	1	1.41	1.41	33.20	0.55
p53	PF-40	2.45	1.85	1.85	34.21	0.03
p53	PF-80	6.17	2.67	2.67	32.31	0.24

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase; PF – Pooled fraction; SEM – Standard error of mean; N/A – Not available; SCC – Squamous Cell Carcinoma

Table 6: Western blot data on analysis of expression of *p53* protein

Sample	Concentration µg/ml	Band intensity proteins		Normalized	Relative gene expression
		GAPDH	*P53*		
Control	0	48521.957	12118.007	0.25	1.00
PF 6-9	160	46831.045	27911.643	0.60	2.39
PF 6-9	320	36007.388	37920.291	1.05	4.22

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

were suggestive that the relative expression of p53 was found to be 2.39-fold and 4.22-fold at 160 µg and 320 µg, respectively, compared to control [Figure 7 and Table 6].

DISCUSSION

The need to develop new medicines of innovation is strong and urgent modes of action to combat cancer. The inability of hybrid, genetic, and realistic methods to lead chemical structures and the recent resurgence of natural product chemistry as a major contributor to the drug discovery cycle illustrate the value of biodiversity testing of new chemical objects with novel biological activities.[18]

Most of the recent studies published on the medicinal benefits of *JP* focused on its hepatoprotective, antidiabetic, antimalarial, antimycobacterial, and antioxidative properties.[7,19-23] The strong cytotoxic compound known to cause liver damage, cholestasis, and colon cancer is lithocholic acid secreted by the flora. Alkhedaide AQ tested the preventive effect on hepatotoxicity caused by exposure to lithocholic acid of experimental mice of *JP* extract administration. The serum levels of the liver function parameters, prevented by *JP* co-administration, were significantly increased with lithocholic acid toxicity. The mRNA downregulation of ATP-binding cassette sub-family G member 8, organic anion transporting polypeptides, hydroxysteroid sulfotransferase, constitutive androstane receptor, farnesoid X receptor, Cytochrome P450 2B10, multidrug resistance-associated protein 2, and UDP glucuronosyltransferase 1 family, polypeptide A cluster was been prevented by *JP* extract. Histopathological studies of hepatic tissue showed that lithocholic acid exhibited severe hepatotoxicity, with areas of irregularly distributed necrosis with inflammatory infiltration. The co-treated community of *JP* showed a minor change in liver tissue, a decline in necrotic areas, and limited inflammatory infiltration.[23]

In male albino mice, the effect of *JP* and *Olea oleaster* leaves' extracts and their combination on hepatic cirrhosis caused by thioacetamide (TAA) was investigated. It was shown that mice were being treated with *O. oleaster* as well as *JP* leaves extracts, and their combination has mitigated the physiological and histopathological changes caused by the administration of TAA. In addition, the majority of improvements were seen in mice supplemented with *O. oleaster* leaves extract followed by *JP* leaves extract and their combination.[22]

The fraction of petroleum ether *JP* demonstrated significant hepatoprotective activity when investigating carbon tetrachloride-

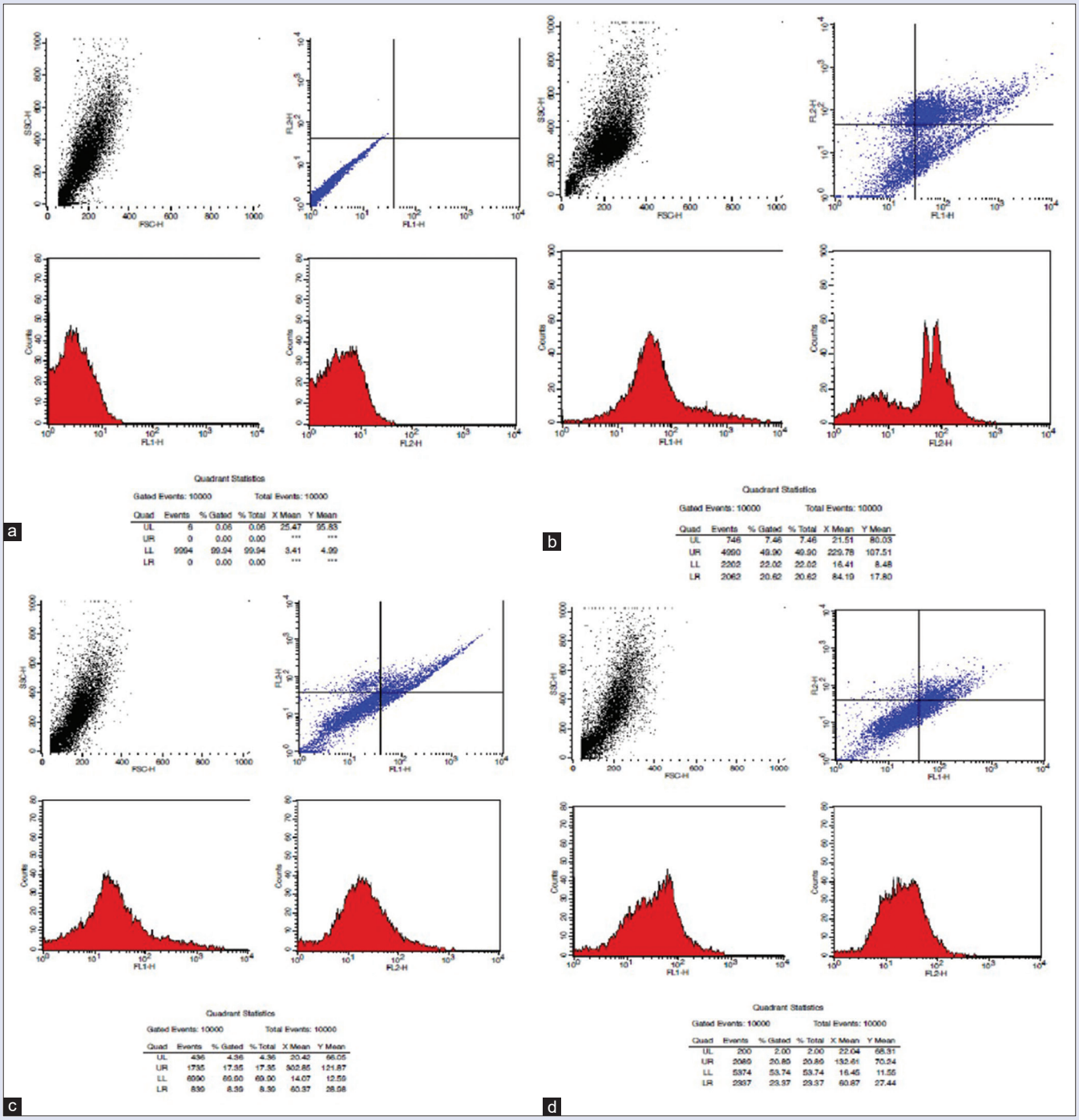


Figure 4: Flowcytometry plots for Annexin V-fluorescein isothiocyanate/PI assay. (a) Plots for SCC-9 untreated cells used as control. (b) SCC-9 cells treated with Doxorubicin (25 μM) (c) SCC-9 cells treated with *Juniperus procera* extract (160 μg/ml). (d) SCC-9 cells treated with *Juniperus procera* extract (320 μg/ml)

induced Wistar male rats liver injury. The hepatoprotective activity was evaluated and confirmed using histopathology analysis by quantifying biochemical parameters.[6]

The essential oil of *JP* (*Cupressaceae*) showed that *JP* could serve as a promising larvicide herb as a protection from *Anopheles arabiensis* in both laboratory and semi-field conditions with lower doses of LC_{50}.[21]

The collegial action of antimycobacterial elements from *JP* has been assessed against four atypical species in association with isonicotinic acid hydrazide (isoniazid [INH]). When checked with a subtoxic concentration of totarol derived from JP, the INH potency was increased fourfold, using an *in vitro* checkerboard procedure, against each mycobacterium.[20]

Till date, the effectiveness of *JP* on oral cancer cell lines has been not evaluated, and our study is the first comprehensive approach in evaluating the *in vitro* outcomes of the *JP* derivatives on oral SCC-9 cells.

Most of the anticancer/antiproliferative studies were on different species of *Juniperus*, namely *Juniperus communis*, *Juniperus phoenicea*, *Juniperus turbinata* Guss, and *Juniperus horizontalis*. We are comparing our results

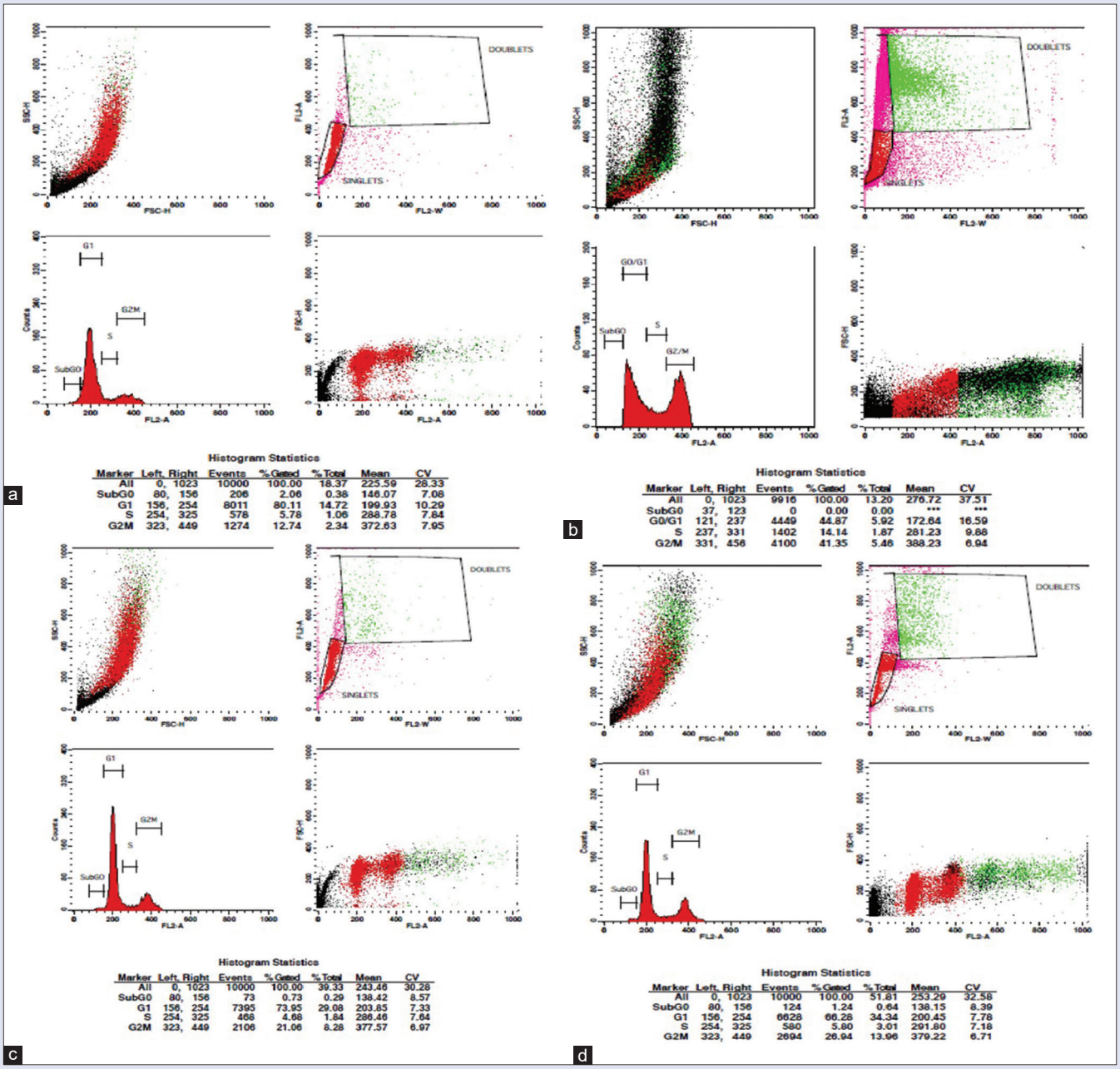


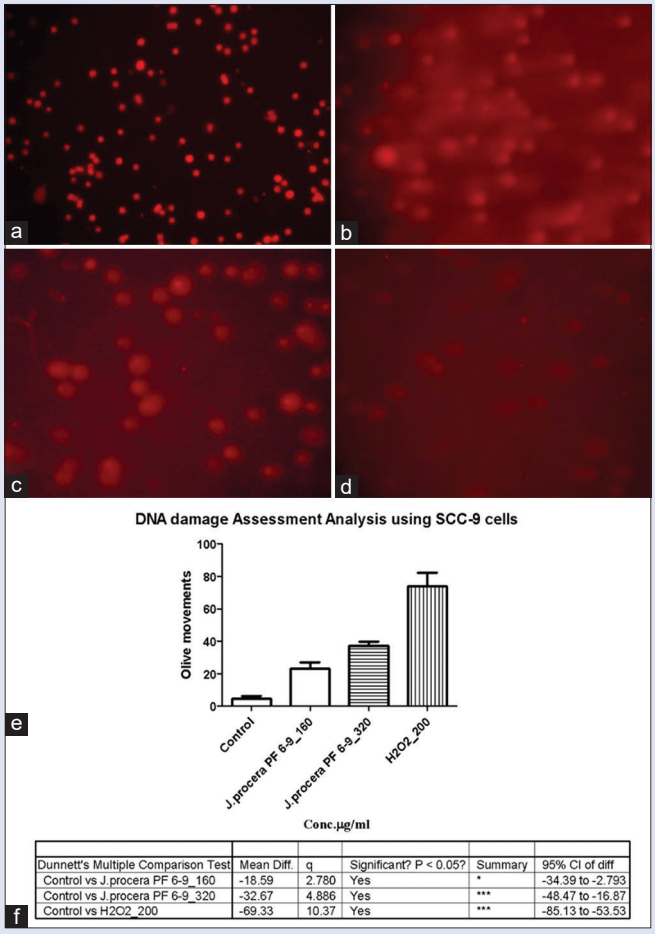
Figure 5: Flowcytometry images for Cell Cycle Analysis. (a) SCC-9 untreated cells used as control. (b) G2M arrest induced by Doxorubicin (25 µM) (c) G2M arrest induced by *Juniperus procera* extracts (160 µg/ml). (d) G2M arrest induced by *Juniperus procera* extracts (320 µg/ml)

with some of the similar kind of studies on different Juniperus species.

The efficacy of *J. communis* extracts on human melanoma cells *in vitro* and *in vivo* spelled out that they were harmless to physiologically normal cells of the renal system, cells of endothelial origin, and fibroblasts and demonstrated nearly twice the selectivity of B16/F10 melanoma cells.[24] In our study, the crude extract of *JP* had IC_{50} values of 208.7 µg/ml, whereas the purified extract of *JP* subjected to column–column extraction yielded a PF 6–9 IC_{50} value of 201.6 µg/ml in SCC-9 cells. Doxorubicin, an anticancer drug, had IC_{50} value of 23.61 µM in SCC9 cells. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay was conducted which revealed that *JCo* derivatives effected caspase cascade activation, via evaluation of the levels BAX and AIF, which had elevated, whereas Bcl-2 and Procaspase-9 diminished, which, in turn, triggered melanoma cell apoptosis.[24] In our study, apoptosis activation was seen in a dose-dependent manner, and as the dose increased from 160 to 320 µg/ml, early apoptosis was markedly increased to 23.37% from 8.39% in SCC-9 cells.

Cell cycle study of cancer cells found that therapy resulted in time-dependent cell cycle detention at the initial phase: 57.33% ± 0.39%; 67.59% ± 0.74%; 69.95% ± 0.38%; 81.83% ± 0.19%; and 65.05% ± 1.85%. The extract caused a majority of cells to arrest during the initial process, with a corresponding decline in the synthesis phase from 6 to 24 h ($P < 0{:}05$). A significant decline of B16/F10 melanoma cells which entered the mitosis phase was seen when treated with extracts of *JCo*.[24] In our study, *JP* extract induced cell cycle arrest as the concentration

increased. Sample *JP* (*PF* 6–9) at 160 µg/ml and 320 µg/ml has induced G2M phase arrest of up to 21.06% and 26.94% compared to the control cells having 12.74% arrest. In the G_0/G_1 phase, a significant arrest of 73.95% was seen at the concentration of 160 µg/ml compared to 66.28% µg/ml at 320 µg/ml. These results of our study determined that *JP* extract effected cell cycle termination via the modulation of p53 signaling pathway.



Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs J.procera PF 6-9_160	-18.59	2.780	Yes	*	-34.39 to -2.793
Control vs J.procera PF 6-9_320	-32.67	4.886	Yes	***	-48.47 to -16.87
Control vs H2O2_200	-69.33	10.37	Yes	***	-85.13 to -53.53

Figure 6: Florescence images of comets. (a) Comet images for control (saline) on SCC-9 cells. (b) Comet images for positive control H_2O_2 at 100 µM. (c) Comet images for *Juniperus procera* extract (160 µg/ml). (d) Comet images for *Juniperus procera* extract (320 µg/ml). (e) Graphical representation of deoxyribose nucleic acid damage expressed in olive moments. (f) Statistical evaluation of olive moments by Dunnett's multiple comparison tests. *Juniperus procera* extract (320 µg/ml) showed maximum deoxyribose nucleic acid strand breakage capacity

A study on Libyan *J. phoenicea* via bioassay-guided isolation of compounds revealed that bioflavonoid cupressuflavone and sumaflavone had a cytotoxic effect against the A549 cells. Cupressuflavone was effective on PC3 cells and being relatively safe on PNT2 cells.[25] The possible bioflavonoid as per the ^{13}C-NMR analysis in our study was kaempferol (3,4',5,7-tetrahydroxyflavone) isolated from *JP*. Kaempferol's cytotoxic activity against SCC-9 cells in our study could be due to the presence of an aromatic hydroxyl group at C-5.

A study on the water extracts of *J. communis* L (JE) conducted by Raasmaja *et al*. proved to be highly effective in activating the tumor suppressor genes, limiting the cancer cell proliferation and inducing apoptosis. It was noted that extracts of herbs such as Juniperus when used in combination with anticancer drugs yielded better results.[26]

The scope of studying the synergism of natural *JP* extract and cytostatic drugs remains wide open, and studying the role of p53 activation and Akt inhibition on *JP* would yield beneficial results in cancer research.

Tsai *et al*. effectively denoted that the *JCo* derivatives were effective in treating glioma via the stoppage of blood vessel formation. The derivatives of this herb were superior in causing cell damage of glioma cells in contrast to the effects of the drug temozolomide. The *JCo* extract efficiently halted the progression of cell cycle indicated by the presence of more number of cells in sub G_1 phase.[27] In our study too, it was found that in G_0/G_1 phase, significant arrest of 73.95% was seen at the concentration of 160 µg/ml compared to 66.28% µg/ml at 320 µg/ml. This suggested that *JP* extract effectively blocked SCC-9 in the G_0/G_1 phase.

Medicinal plants such as Juniperus are known to be rich in analogs of podophyllotoxin (PPT). PPT is known to be an important agent in one of the popular anticancer drugs.[28]A study carried out by Doussot *et al*. revealed that two varieties of *Juniperus* contained large quantities of DPT and PPT aryltetraline, the largest concentration found in J. Virginian plant sample, which also measured a significant amount of methoxypodophyllotoxin. Plant extracts have been tested against four separate human cancer cell lines to determine their *in vitro* antiproliferative effects. Development inhibitory effects on different cancer cell lines with extracts from species with high aryltetraline content have been achieved as predicted.[29]

HPLC analysis to describe the complete lignin profile and presence of any PPT analogs from the species of *JP* belonging to different parts of the world will pave away essential function in the identification and production of antitumor medications.

CONCLUSION

To summarize, according to our understanding, the naturally available vegetation of *JP* in the Al-Baha region harbors potent constituents which proved to be effective in limiting the proliferation of the oral cancer cells *in vitro*. The results of all the assays performed in our study could open

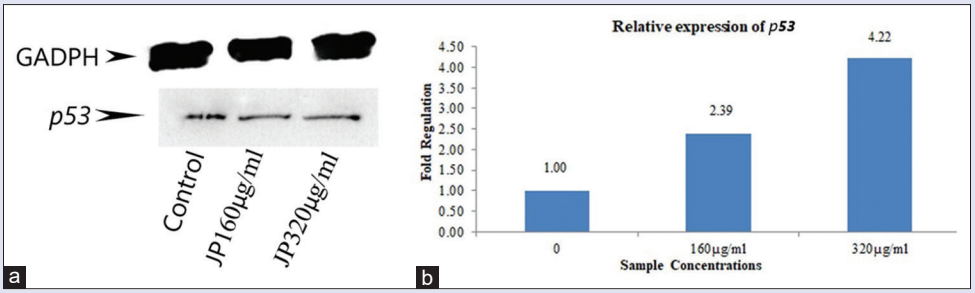


Figure 7: Effects of *Juniperus procera* on p53 protein expression in SCC-9 cells. Cells were treated with *Juniperus procera* (160 and 320 µg/ml) for 24 h. (a) Total cell protein were extracted and subjected to western blot assay using antibodies against p53, with Glyceraldehyde 3-phosphate dehydrogenase (GADPH) used as a housekeeping gene internal control. (b) Results are expressed as the mean ± standard deviation ($n = 4$). $^{**}P < 0.01$ versus control

up several promising vistas for further researches exploring therapeutic properties of regional vegetations on oral cancer.

Acknowledgements

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University, Abha, Kingdom of Saudi Arabia for funding this work through the General Research Project under grant number (279/1440)."

Financial support and sponsorship

This work was supported by Deanship of Scientific Research (Year: 1440; Research No. 279), King Khalid University, Abha, Kingdom of Saudi Arabia.

Conflicts of interest

There are no conflicts of interest.

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