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Research Article

Cytotoxicity of Libyan *Juniperus phoenicea* against Human Cancer Cell Lines A549, EJ138, Hepg2 and MCF7

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ABSTRACT

Background: The current study was undertaken to assess the cytotoxicity of the leaves of Libyan *Juniperus phoenicea* (Cupressaceae) against human cancer cell lines.

Methods: The cytotoxicity of the *n*-hexane, dichloromethane (DCM) and methanol (MeOH) extracts of the leaves of *J. phoenicea* (JP), obtained from sequential Soxhlet extractions, was assessed against four human cancer cell lines: EJ138 (human bladder carcinoma), HepG2 (human liver hepatocellular carcinoma), A549 (human lung carcinoma) and MCF7 (human breast adenocarcinoma) using the MTT assay.

Results: The cell line A549 was the most sensitive to the JP extracts, with the highest level of cytotoxicity with the IC₅₀ values of 16, 13 and 100 µg/mL for the DCM, *n*-hexane and MeOH extracts, respectively. However, generally the most potent cytotoxic extract across the other cells tested was the *n*-hexane extract, followed by the DCM extract, whilst the MeOH extracts showed little or no cytotoxicity. The percentage of viability of cells decreased as the concentration of test compounds increased. The cytotoxicity of various chromatographic fractions from the extracts was also studied against the A459 cells. For the *n*-hexane fractions, the IC₅₀ values were 160, 62, 90, 30, 9.5 and 40 µg/mL for fractions 1 to 5 and 7, respectively. Fractions 4 and 5 showed the greatest effect. DCM fractions 2, 3 and 4 had the IC₅₀ values of 60, 92 and 19 µg/mL, respectively, and DCM fractions 5 to 8 were non-cytotoxic. Fractions 1 and 2 of the MeOH extract were non-cytotoxic, whereas cytotoxicity was observed for fractions 3 and 4 with IC₅₀ values of 50 and 85 µg/mL, respectively.

Conclusion: The outcome of the present study suggested that the JP leaves possess cytotoxic activities. The high level of cytotoxicity of the *n*-hexane and DCM extracts suggested that lipophilicity might affect the cytotoxicity of JP, where the less polar compounds had the strongest cytotoxicity.

Introduction

Juniperus phoenicea L. (Family: Cupressaceae) is a well-known Libyan medicinal plant, which has been used in Libyan traditional medicine for centuries for the treatment of various human ailments including tumours and cancers. In fact, in Egypt around 1500 BC was the first recorded use of using the *Juniperus phoenicea* medicinally, which was to relieve joint and muscle pain. This plant is used in folk medicine to treat rheumatism, gout, oedema, diarrhoea, poor appetite and urinary tract diseases. It is also reported to eliminate gastrointestinal bacteria and parasites.^{1,2} There are some reports on its use as a female contraceptive by some tribes.¹ The juniper berry was studied as a possible treatment for diet controlled diabetes mellitus.³

Several compounds, *i.e.*, terpenoids (monoterpenes: predominantly α -pinene, α -phyllandrene and δ -3-carene; sesquiterpenes: mainly δ -cadinene; diterpenes), phenolics

(*e.g.*, flavonoids and biflavones, phenylpropanoids and lignans), furanone glycosides, hydrocarbons, and sterols, have been isolated from the leaves and berries of *J. phoenicea* grown in different countries.⁴⁻⁸ Podophyllotoxin and deoxypodophyllotoxin were also found in the aerial parts of many *Juniperus* species including *J. phoenicea*.⁹

Various biological activities for *J. phoenicea* have been reported to date. The essential oils from this plant exhibited moderate antibacterial and antifungal activities.^{5-8,10-13} Elmhdwi *et al.*¹⁴ demonstrated that the extracts from *J. phoenicea* had antibacterial activity against both Gram-positive and Gram-negative bacteria including multiple-drug-resistant (MDR) strains. This activity was attributed to the abundance of α -pinene and the overall chemical constituents of these extracts. Antioxidant activity of this plant was detected using the ABTS radical-scavenging assay with the strongest

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antioxidant effect being found with the MeOH extract.⁶ The aqueous extract of *J. phoenicia* showed anti-diarrhoeal effect *in vivo* on experimental rat models of diarrhoea by reducing intestinal fluid accumulation and inhibiting intestinal motility.² Akkol *et al.*¹⁵ revealed remarkable anti-inflammatory and antinociceptive activities of five *Juniperus* species. Cairnes & Ekundayo⁹ showed that the ethanolic extract of *J. phoenicea* twigs and leaves and two isolated compounds, desoxypodophyllotoxin and *p*-peltatin-methyl ether, were cytotoxic in the KB cell cultures (human cervix carcinoma). A MeOH extract of *J. phoenicea* from Saudi Arabia and Indonesia displayed high cytotoxicity in Vero (normal monkey kidney) and HEP-2 (human laryngeal carcinoma) cell lines.¹⁶ In addition, this plant also displayed significant cytotoxicity against both MCF7 (human breast adenocarcinoma) and HCT-116 (human colon carcinoma) cell lines.¹

As a part of our continuing phytochemical and bioactivity studies on Libyan medicinal plants,¹⁷⁻²² we now report on the cytotoxicity of the extracts and fractions of the leaves of *J. phoenicea* growing in Libya against four human cancer cell lines: human bladder carcinoma cell line (EJ138), human hepatocellular carcinoma cell line (HepG2), human lung carcinoma cell line (A549) and human breast adenocarcinoma cell line (MCF7).

Materials and Methods

Plant material

The leaves of *Juniperus phoenicea* L. were collected from Al-Jabal Al Akhdar, Libya, in 2013. The plant material was identified at the Faculty of Science Herbarium, Tripoli-Libya, and a voucher specimen (no. D68122) has been retained there. Leaves of this plant were air-dried, powdered and kept in a tightly closed amber coloured container for subsequent studies.

Extraction

Ground leaves of *J. phoenicea* (86.5 g) were Soxhlet-extracted, sequentially, with *n*-hexane, DCM and MeOH, 800 mL each. Ten cycles were allowed for each extraction, and the temperature of the heating mantle for all extractions was kept constant at 60°C. The extracts were filtered and evaporated to dryness using a rotary evaporator at a temperature not exceeding 45°C under reduced pressure. All extracts were stored at 4°C.

Fractionation techniques

The crude extracts of *J. phoenicea* leaves were separated into various fractions using vacuum liquid chromatography (VLC) on silica gel for the *n*-hexane and DCM extracts, and solid-phase extraction (SPE) on reversed-phase C₁₈ silica for the MeOH extract.^{23,24} The *n*-hexane extract (4.6 g) was fractionated by VLC eluting with *n*-hexane-ethyl acetate mixtures of increasing polarity to yield seven fractions: F1: 82.5 mg, F2: 555 mg, F3: 998.7 mg, F4: 655.5 mg, F5: 118.8 mg F6: 22.1 mg, F7: 103.7 mg. Similarly, the DCM extract (1.289 g) was fractionated by VLC, starting elution with *n*-hexane-

DCM mixtures, then increasing polarity with MeOH to yield eight fractions: F1: 15 mg, F2: 20 mg, F3: 41 mg, F4: 134 mg, F5: 128.5 mg F6: 9.3 mg, F7: 2.7 mg and F8: 2.4 mg. The dried MeOH extract (1.4 g) was re-suspended in 10% MeOH in water, and subjected to solid phase extraction (SPE) using a cartridge (20 g) pre-packed with reversed-phase silica C₁₈ (ODS). A step gradient was applied starting with 20% MeOH in water, then 50%, 80% and 100% MeOH in water (200 mL each). Four fractions were collected: F1: 895 mg, F2: 135 mg, F3: 54 mg, F4: 25 mg, and these fractions were dried using a rotary evaporator and a freeze dryer.

MTT assay

The cytotoxic activity of the *n*-hexane, DCM and MeOH extracts of *J. phoenicea* were assessed against EJ138 (human bladder carcinoma), HepG2 (human liver hepatocellular carcinoma), A549 (human lung carcinoma) and MCF7 (human breast adenocarcinoma) cell lines. In addition, fractions obtained from *n*-hexane, DCM and MeOH extracts of *J. phoenicea* were also assessed for cytotoxicity against the cell line A549, as the extracts showed the highest level of activities against this cell line. The cells were seeded into 24-well plates (5 × 10⁴ cells/well) and incubated under 5% CO₂, 95% humidity at 37°C for 24 h. The different extracts or isolated fractions were diluted in medium containing DMSO (0.01% (v/v) (including the negative control), to achieve extract concentrations of (0, 0.8, 4, 20, 100 and 500 µg/mL) or isolated fraction concentrations of (0, 0.4, 2, 10, 50 and 250 µg/mL). These doses were then used to treat the cells for 24 h before assessment using the MTT assay²⁵ following the standard protocol. Briefly, each treatment was removed from the 24-well plates of cells and replaced with MTT solution (0.5 mg/mL MTT in medium (1 mL/well)). The cells were then incubated under 5% CO₂, 95% humidity at 37°C for 2 h. The MTT solution was then removed from each well of cells and replaced with isopropanol (0.5 mL/well) to lyse the cells, and to release and solubilise the blue formazan product. The absorbance reading (540 nm) of lysates from treated cells in each well on each occasion was recorded and was expressed as a percentage of the mean value of the control absorbance on each occasion. The results for each treatment and dose derived from at least 12 wells (n≥12) from three or more separate occasions. To determine IC₅₀ values, data were presented in EXCEL graphs with the dose value (µg/mL) on the X-axis on a log¹⁰ scale and the % viability compared to control on the Y-axis. The IC₅₀ values are determined from the trend line of the data points.

Statistical analysis

All experiments were carried out in triplicate on separate occasions. Data were expressed as means ± standard error of the mean. The graphs were plotted using nonlinear regression with the use of Microsoft Excel version 2013.

Results and Discussion

In the present study, the cytotoxic effect of *J. phoenicea*

leaf extracts against four human cancer cell lines, MCF7, HepG2, EJ138 and A549, was determined using the MTT assay. Although cytotoxicity of *J. phoenicea* against MCF7 was reported previously,⁴ to the best of our knowledge, this is the first report on the assessment of cytotoxicity of *J. phoenicea* leaf extracts and fractions against HepG2, EJ138 and A549 cell lines. The extracts of *J. phoenicea* showed the highest level of cytotoxic activity against the human lung adenocarcinoma cell line A549 with the IC₅₀ values of 16, 13 and 100 µg/mL, respectively, for the *n*-hexane, DCM and MeOH extracts (Table 1). Whilst the DCM extract appeared to be the most cytotoxic extract against the A549 cells, the *n*-hexane extract was the most cytotoxic across the board against HepG2, EJ138 and MCF7 having respective IC₅₀ values of 10, 40 and 14 µg/mL. The DCM extract also resulted in cytotoxicity in all cell lines tested with IC₅₀ values of 50 µg/mL for EJ138 cells, 42 µg/mL for HepG2 cells, 16 µg/mL for MCF7 cells and 13 µg/mL for A549 cells. The MeOH extract showed low-level of cytotoxicity in EJ138 (IC₅₀ 130 µg/mL), HepG2 (IC₅₀ 900 µg/mL), A549 (IC₅₀ 100 µg/mL) and MCF7 (IC₅₀ >1000 µg/mL) (Table 1). According to Sahranavard *et al.*,²⁶ plant extracts are only considered to be cytotoxic to cells if the IC₅₀ value is <100 µg/mL, therefore these MeOH extract results were regarded as non-cytotoxic to each of the cell lines tested. The higher level of cytotoxic activity of the *n*-hexane and DCM extracts suggested that lipophilicity might have an impact on the cytotoxic activity of *J. phoenicea*, where the less polar (high lipophilic) compounds showed the strongest cytotoxic effect. However, it is known that lipophilicity is one of the major factors that influences the transport, absorption, and distribution of chemicals in biological systems. Bioassay-guided phytochemical and biological fractionation is an approach whereby a crude extract of plant is fractionated and re-fractionated, continually monitored fractions by appropriate bioassay(s), until a pure biologically active fraction/compound is found.²⁴ To adopt this approach, *n*-hexane, DCM and MeOH extracts were further fractionated by two different techniques: solid-phase extraction for MeOH extract and VLC for the *n*-hexane and DCM extracts to get different fractions

(four fractions from MeOH extract, seven fractions from *n*-hexane extract and eight fractions from DCM extract). All fractions were tested for cytotoxicity against the A549 cell line, as these cells appeared to be the most sensitive to the all three extracts. Taken that IC₅₀ values >100 µg/mL are considered as non-cytotoxic, *n*-hexane fractions 1 (F1) and 6 (F6), DCM fractions 1, and 5 to 8 (F1, F5, F6, F7 and F8) and MeOH fractions 1 and 2 (F1 and F2) were all regarded as non-cytotoxic in the A549 cells. However, *n*-hexane fractions 2 to 5 and 7 (F2, F3, F4, F5 and F7) were cytotoxic. In addition, DCM fractions 2 to 4 (F2, F3 and F4) and MeOH fractions 3 and 4 (F3 and F4) were also considered cytotoxic (Table 2). The most potent fractions appear to be *n*-hexane fraction 5 (F5) with an IC₅₀ value of 10 µg/mL and DCM fraction 4 (F4) with an IC₅₀ value of 19 µg/mL.

Whilst the MeOH fraction 1 (F1), which was the most polar fraction of all, was found to be non-cytotoxic, an interesting observation was that doses 2, 10 and 50 µg/mL did appear to trigger proliferation in the A549 cells with approximately a 20% increase in viability compared to the control (0 µg/mL) (Figure 1). However, further interrogation of these data indicated that only the 50 µg/mL dose resulted in a statistically significant difference compared to the control (P<0.05). There was high variability in the data resulting from fraction 1 (F1), and this might have contributed to this anomaly.

Several phytochemicals, *e.g.*, alkaloids, flavonoids, lignans, phenols, steroids and terpenes have been demonstrated to possess prominent cytotoxic properties against cancer cells.²⁷

Notably, all these groups of phytochemicals have previously been isolated from the leaves and berries of *J. phoenicea* and other *Juniperus* species grown in different countries.^{9,28-30} Podophyllotoxin, which is also present in *J. phoenicea* at a low level,³¹ is a well-known cytotoxic compound against cancer cell lines. Its cytotoxicity is mediated by inhibition of microtubule formation and this compound serves as a unique starting compound for the semisynthesis of anticancer drugs that are known to inhibit topoisomerase II such as etoposide, teniposide, or etopophos.³¹

Table 1. The IC₅₀ (µg/mL) of *n*-hexane, DCM and MeOH extracts of *J. phoenicea* on a selection of four cancer cell lines.

Cell type	IC ₅₀ values (µg/mL)		
	<i>n</i> -Hexane	DCM	MeOH
Human bladder carcinoma cells (EJ138)	40	50	130
Human hepatocellular carcinoma cells (HepG2)	10	42	900
Human lung carcinoma cells (A549)	16	13	100
Human breast adenocarcinoma cells (MCF7)	14	16	> 1000

The IC₅₀ values for the *n*-hexane, DCM and MeOH extracts of *J. phoenicea* different on the cell lines of four different cancer types. Values greater than 100 µg/mL were considered as non-cytotoxic.

Table 2. The IC₅₀ (µg/mL) of *n*-hexane, DCM and MeOH fractions of *J. phoenicea* fractions on A549 (human lung carcinoma) cells.

Extracts	IC ₅₀ values (mg/mL) of the fractions							
	F1	F2	F3	F4	F5	F6	F7	F8
<i>n</i> -Hexane	160	62	90	30	9.5	>1000	40	NA
DCM	>1000	60	92	19	>1000	>1000	>1000	>1000
MeOH	>1000	244	50	85	NA	NA	NA	NA

The IC₅₀ values for the *n*-hexane, DCM and MeOH fractions of *J. phoenicea* on A549 human lung carcinoma cells. Values greater than 100 µg/mL were considered as non-cytotoxic. NA = Not applicable.

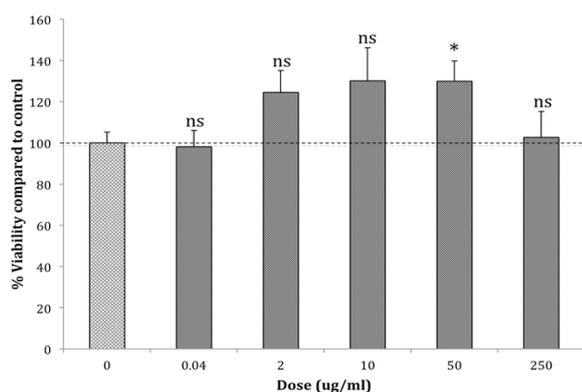


Figure 1. The cytotoxic activity of *J. phoenicea* MeOH fraction 1 (F1) against A549 human lung carcinoma cell line. The asterisk * indicates a significant difference when compared to control ($P < 0.05$) and ns indicates no significant difference to control. The results are mean values \pm SEM derived from $n \geq 12$ from three separate occasions.

Ren *et al.*³² detailed the anticancer effects of the flavonoids, which were shown to inactivate carcinogen, inhibit proliferation, arrest the cell cycle, induce apoptosis and differentiate, inhibit angiogenesis, prevent oxidation and reverse multidrug resistance. Previous studies had shown that many flavonoids and polyphenolic compounds were present in some *Juniperus* species.³³⁻³⁵

Conclusion

The outcome of the present study suggested that the *J. phoenicea* leaves possess cytotoxic activities. The high level of cytotoxic activities of the *n*-hexane and DCM extracts suggested that lipophilicity might affect the cytotoxic activity of *J. phoenicea*, where the less polar (high lipophilic) compounds might have the strongest cytotoxic effect. As terpenes are the main lipophilic compounds previously reported from less polar extracts and essential oils of *J. phoenicea*, it is reasonable to assume that the cytotoxicity observed with the less polar extracts was contributed by various cytotoxic terpenes as well as the lignans, podophyllotoxin and deoxy-podophyllotoxin.

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Conflict of interests

The authors claim that there is no conflict of interest.

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