Research Article

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Induction of apoptosis and cell cycle arrest by chloroform fraction of Juniperus phoenicea and chemical constituents analysis

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Abstract: Different phytochemicals from various plant species exhibit promising medicinal properties against cancer. Juniperus phoenicea is a plant species that has been found to present medicinal properties. Herein, crude extract and fractions of J. phoenicea were examined to determine its anticancer properties against several cancer cells. The active fraction was chosen to assess its activity on cell cycle progression and apoptosis induction by annexin and propidium iodide (PI) biomarkers. Further, phytochemical screening for possible contents of active fraction using gas chromatography–mass spectrometry (GC-MS) analysis was conducted. It was demonstrated that cell proliferation was suppressed, and the MCF-7 cell line was the most sensitive to J. phoenicea chloroform fraction (JPCF), with the IC50 values of 24.5 μg/mL. The anti-proliferation activity of JPCF in MCF-7 cells was linked to the aggregation of cells in the G1 phase, increases in early and late apoptosis as well as necrotic cell death. Contents analysis of JPCF using GC-MS analysis identified 3-methyl-5-(2',6',6'-trimethylcyclohex-1'-enyl)-1-penten-3-ol (16.5%), methyl 8-oxooctanoate (15.61%), cubenol (13.48%), and 7-oxabicyclo[2.2.1]heptane (12.14%) as major constituents. Our present study provides clear evidence that J. phoenicea can inhibit cell proliferation, trigger cell cycle arrest, and induce apoptosis in tested cancer cells.

Keywords: Cupressaceae, Juniperus phoenicea, MTT assay, cancer, GC-MS

1 Introduction

Cancer continues to be a significant global public health risk factor, and cancer diagnoses are presumed to increase to 23.6 million new cases per year by 2030 [1]. Despite the progressive advance in conventional cancer treatments, medicinal plants with natural origins still offer promising options and have been recognized for their therapeutic effects with fewer adverse effects [2]. Juniperus (Family: Cupressaceae) is a genus of approximately 75 species of evergreen trees that are distributed throughout different regions of the world [3,4]. Juniperus species are well known as local remedies for several diseases. In Saudi Arabia, these species are commonly used to treat tuberculosis and jaundice [5]. Additionally, several species of this plant are used to treat bronchitis, hemorrhoids, and cold cough in Turkey [6]. Moreover, several recent studies have reported different pharmacological activities of Juniperus
essential oil and extracts which include cytotoxic, antimicrobial, hypoglycemic, and anti-inflammatory activities [4,7–9]. Among Juniperus species, Juniperus phoenicea, or “Arar” (local name), is a shrub that can grow up to eight meters tall and is widely distributed in southern and Mediterranean regions of Saudi Arabia [10]. In folk medicine, the leaves of J. phoenicea species are used to treat several ailments, including bronchopulmonary diseases, diabetes, and as a diuretic [11,12]. The antioxidant properties, antibacterial, and hepatoprotective effects of J. phoenicea have also been reported [13,14]. Numerous studies have revealed that J. phoenicea grown in different geographical regions are capable of reducing the proliferation of different cancer cells [15–18]. In this study, we have reported the cytotoxicity of J. phoenicea grown in Saudi Arabia against human lung, breast, and liver cancer cells as well as its chemical profile using gas chromatography–mass spectrometry (GC-MS). To the best of our knowledge, the current study is the first to report that J. phoenicea can induce cell cycle arrest and apoptosis induction in MCF-7 cancer cells.

2 Materials and methods

2.1 Plant collection and authentication

Fresh aerial parts of J. phoenicea were collected from Al-Madinah Al-Munawara, Saudi Arabia, in March, 2019. The plant material was authenticated by Professor Sami Zalat, Biology Department, College of Science, Taibah University, Saudi Arabia.

2.2 Crude extracts and fractions preparation

The aerial parts were thoroughly washed with water, kept in the dark for 15 days, and then powdered. Three hundred grams of powdered plant material was then mixed with 1,500 mL ethanol–water (70/30 v/v) for 48 h using a Soxhlet apparatus. Next, the blend was centrifuged at 2,500 × g, and the collected supernatant was concentrated under reduced pressure in a rotary evaporator. The hydroethanolic extract (crude extract) was then fractionated by different polarity solvents including n-hexane (n-Hex) chloroform (CHCl₃) and methanol (MeOH), and the residue was kept at −20°C for future use. The dry weight of each fraction was prepared at 20 mg/mL with DMSO cell culture grade.

2.3 Cell culture

Human lung (A549), breast (MCF-7), and liver (HepG2) cancer cells were obtained from German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Braunschweig, Germany). Cells were maintained at 37°C in a humidified CO₂ incubator and were cultivated in DMEM medium (Gibco, US) supplemented with an FBS (10% final concentration) and 1% penicillin and streptomycin. Cells were subcultured when they reach 70% confluency and passage number of 25–30 was maintained for bioassay.

2.4 Cell viability (MTT assay)

MTT assay was used to assess the cell viability according to previous protocol [19]. Concisely, the cells were seeded in 24-well plates at a density of 1 × 10⁵ cells/well. Approximately 24 h after incubation, the cells were treated with a range of extract concentrations (10, 25, 50, 100, and 200 µg/mL), doxorubicin (positive control), and DMSO as a vehicle while untreated cells served as a negative control. After 48 h of incubation, 100 µL of MTT solution was added to each well; then, the plate continued to incubate in the CO₂ incubator for 4 h. Isopropanol–HCl solution (1,000 µL) was incorporated into each well and mixed thoroughly to dissolve the formazan product, which was measured using a microplate reader at 570 nm wavelength.

2.5 Cell cycle analysis

The cell phase distribution was performed according to the protocol outlined in [20,21] with slight alteration. MCF-7 cells were plated in 6-well culture plates at a density of 2 × 10⁵ cells/well. Adhered cells were serum-starved in 2% FBS for overnight. The cells were then washed, replaced with complete media, and exposed to the half maximal inhibitory concentration (IC₅₀) of J. phoenicea chloroform fraction (JPCF) for 24 h, while untreated cells served as a control. At the end of treatment, the cells were harvested, and the cell pellet was washed twice with ice-cold PBS and fixed in ice-cold absolute ethanol at −20°C for 4 h to overnight. The cells were then centrifuged, and the cell pellet was resuspended and incubated with RNaseA (100 µg/mL, 50 µL) for 15 min. Subsequently, cells were stained with propidium
iodide (PI) (0.5 mL, 50 µg/ml) for 30 min. The cell cycle stage was analyzed using a FACS flow cytometer (Cytomics FC 500; Beckman Coulter, Brea, CA, USA).

2.6 FITC annexin V/PI apoptosis detection

Apoptotic cells were detected using a FITC Annexin V Apoptosis Detection Kit (((BioLegend, CA, USA), according to the manufacturer’s instructions. Briefly, 24 h after treatment with JPCF, floating and adherent cells were collected from three wells and washed with cold PBS. The cell pellet was resuspended in 1× binding buffer (1 × 10⁶ cells/mL). The cells were then stained with 5 µL of FITC Annexin V and 5 µL PI and incubated for 15 min in the dark. Later, 400 µL of binding buffer was added, and cells were examined immediately after staining (within an hour) using a FACScan flow cytometer (Cytomics FC 500; Beckman Coulter, Brea, CA, USA).

2.7 GC-MS analysis

The phytoconstituents of JPCF were determined using a Perkin Elmer Clarus 600 GC-MS (PerkinElmer, Inc., Waltham, MA, USA) according to ref. [19]. In brief, JPCF aliquot was injected into the Elite-5MS column (30 m, 0.25 µm thickness, 0.25 µm internal diameter). The oven temperature was programmed to start at 40°C, held for 120 s, then increased to 200°C at a rate of 5°C min⁻¹ and held for 120 s. From 200°C, the temperature increased to 300°C at 5°C min⁻¹ and held for 2 min. The Adams [22] and Wiley GC-MS [23] compounds mass spectral libraries were used to compare similar mass spectra found for JPCF constituents. The characterizations of compounds were performed by comparing the RT (retention time) with genuine reference standards under the same above-indicated conditions [24].

2.8 Statistical analysis

OriginPro 8.5 software was used to perform statistical analysis. All data were reported as mean ± SD of three experiments and were analyzed with unpaired t-tests. Differences are considered as statistically significant if p < 0.05.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results

3.1 Cytotoxicity of *Juniperus phoenicea* against different cancer cell lines

The crude and three fractions of *J. phoenicea* were screened for their potential cytotoxic activity. MTT assay results indicated that *J. phoenicea* crude extract and fractions suppressed the proliferation of all three examined cancer cell lines in a dependent manner with the JPCF showing promising activity (Figure 1). The cytotoxic activity of *J. phoenicea* fractions in terms of IC₅₀ values is listed in (Table 1). Our results indicated that MCF-7 was most sensitive to JPCF with IC₅₀ of 24.5 µg/mL in comparison to other tested cells (Table 1). Therefore, it was chosen to complete further experiments.

3.2 JPCF induces G1 cell cycle arrest

Next, we evaluated the influence of JPCF on the cell cycle of MCF-7 cells using flow cytometry. Cell cycle analysis indicated that IC₅₀ induced G1 phase cell cycle arrest (Figure 2). JPCF indicated a significant increase in the proportions of G1 phase (increased from 59 ± 0.4% to 75.6 ± 0.4%) and a reduction in the proportion of S and G2M phases (decreased by approximately 9 and 8%, respectively) compared with corresponding proportions in control cells (Figure 2).

![Figure 1: Antiproliferative effect of JPCF on A549, HepG2, and MCF-7. Cells were treated with *J. phoenicea* chloroform fraction (JPCF) at different concentrations for 48 h followed by the measurement of cell proliferation by MTT assay. Data represent as % of cell survival.](image-url)
3.3 JPCF induces apoptosis of MCF-7 cells

Double staining with annexin V-FITC and PI dyes was performed to recognize cells undergoing apoptosis events using a flow cytometer. As shown in (Figure 3), JPCF exhibited apoptotic induction at $IC_{50}$ concentration. The early apoptosis cell population increased from 2.1 ± 0.3% to 13.1 ± 0.5% ($p \leq 0.05$), while the late apoptosis cell percentage increased from 2.3 ± 0.2% to 21.5 ± 0.5% ($p \leq 0.01$). In addition, a remarkable increase in the number of necrotic cells was also observed (Figure 3). Our flow cytometric data suggest that JPCF mediated inhibition of MCF-7 cancer cells through apoptosis as well as necrosis induction.

3.4 Chemical composition of the JPCF

Since JPCF was the most active fraction, it was profiled using GC-MS analysis (Figure 4). The chemical content,

### Table 1: $IC_{50}$ values of the *J. phoenicea* crude extract and various fractions against lung, liver, and breast cancer cells

| Cell type     | Crude  | $n$-Hex | CHCl₃ | MeOH  | Doxorubicin |
|---------------|--------|---------|-------|-------|-------------|
| A549 (lung)   | 146.2 ± 1.5 | 80 ± 1.2 | 34.2 ± 0.5 | 186.1 ± 1.8 | 1.2 ± 0.2 |
| HepG2 (liver) | 98.2 ± 0.9  | 76.4 ± 1.1 | 57.6 ± 0.9 | 160.4 ± 0.9 | 1.1 ± 0.3 |
| MCF-7 (breast)| 65.4 ± 0.5  | 55.1 ± 0.6 | 24.5 ± 0.5 | 130.4 ± 0.5 | 1.3 ± 0.4 |

*Figure 2: Chloroform fraction of *J. phoenicea* induces G1 cell cycle arrest in MCF-7 cells. MCF-7 treated cells with the test fraction at $IC_{50}$ for 24 h and were then stained with PI. Representative histogram that was obtained using a flow cytometer where quantitation was obtained from (a). $p \leq 0.05$, $**p \leq 0.01$, or $***p \leq 0.001$. 
retention times, and area percentages of JPCF are displayed in Table 2 according to their elution on the HP Innowax column. GC-MS analysis identified approximately 19 phytoconstituents. The most abundant constituent was 3-methyl-5-(2′,6′,6′-trimethylcyclohex-1′-enyl)-1-penten-3-ol (16.5%), methyl 8-oxooctanoate (15.61%), cubenol (13.48%), and 7-oxabicyclo[2.2.1]heptane (12.14%). The remaining compounds in JPCF are listed in Table 2.

4 Discussion

The use of medicinal plants has highlighted the value of plants as a valuable source of therapeutic agents. Several antitumor agents from natural sources are broadly used in chemotherapy. Both traditional and current medicines have been recommended as promising methodologies to discover and bring new plant-derived compounds to market [25,26]. Therefore, many studies focus on plants as they have been used to prevent several chronic diseases, including cancer. In this study, we have shown for the first time that Saudi Arabian J. phoenicea exerts anticancer activities through cell cycle arrest and apoptosis induction.

The cytotoxicity of the Juniperus genus against different cancer types has been reported in several studies [27–29]. Various reports on J. phoenicea species grown in different regions confirm that they also display strong antiproliferative activities. In line with our obtained results, Maamoun et al. (2016) found that crude extract of Egyptian J. phoenicea leaves exerted strong cytotoxicity against various carcinoma cell lines [18]. Additionally, various fractions from Libyan J. phoenicea also displayed a potent effect on breast MCF-7 cancer cells [15,30], with a slight difference in IC_{50} values. However,

![Figure 3: Induction of apoptosis and necrosis in MCF-7 cells by J. phoenicea chloroform fraction. MCF-7 cells were treated with the test fraction at IC_{50} for 24 h, then stained with FITC-Annexin V/PI. Dot Plots of JPCF-treated and untreated MCF-7 cells obtained using flow cytometer where a1 = necrosis, a2 = late apoptosis, a3 = viable, and a4 = early apoptosis. The percentage (%) of cell distribution. *p ≤ 0.05, **p ≤ 0.01.](image-url)
Figure 4: GC-MS chromatogram and chemical structures of some constituents from JPCF.

Table 2: GC-MS analysis of JPCF

| Compound name                                           | Chemical formula | MW (g/mol) | RT (min) | Area % |
|----------------------------------------------------------|------------------|------------|----------|--------|
| trans-Caryophyllene                                      | C_{15}H_{24}     | 204.35     | 10.70    | 0.600  |
| alpha-Humulene                                           | C_{15}H_{24}     | 204.35     | 11.09    | 1.430  |
| delta-Cadinene                                           | C_{15}H_{24}     | 204.35     | 11.65    | 2.530  |
| 1s-cis-calamenene                                        | C_{15}H_{22}     | 202.33     | 11.71    | 3.640  |
| beta-Cedrene                                             | C_{15}H_{24}     | 204.36     | 11.82    | 0.700  |
| Nerolidol                                                | C_{15}H_{32}O    | 222.37     | 11.99    | 0.640  |
| Caryophyllene oxide                                      | C_{15}H_{24}O    | 220.35     | 12.36    | 0.510  |
| Humuladienone                                            | C_{15}H_{24}O    | 220.35     | 12.62    | 0.330  |
| Cubenol                                                  | C_{15}H_{26}O    | 222.37     | 12.74    | 13.480 |
| tau-Muurolol                                             | C_{15}H_{32}O    | 222.37     | 12.89    | 3.320  |
| 3-Ethenyl-3-methyl-2-(1-methylenyl)-6-(1-methyethyl)-cyclohexanol | C_{19}H_{34}O   | 222.37     | 13.22    | 1.210  |
| 7-Acetyl-2-hydroxy-2-methyl-5-isopropylbicyclo[4.3.0]nonane | C_{18}H_{25}O_2 | 238.37     | 13.68    | 2.150  |
| Citronellyl acetate                                       | C_{15}H_{25}O_2  | 198.3      | 14.19    | 0.600  |
| Ethyl ester of heptadecanoic acid                        | C_{18}O_2        | 298.5      | 14.53    | 1.140  |
| Methyl 8-oxoctanoate                                     | C_{15}H_{26}O    | 172.22     | 15.18    | 15.610 |
| Ethyl ester of heptadecanoic acid                        | C_{19}H_{34}O_2  | 284.5      | 15.28    | 3.480  |
| 3,7,11,15-Tetramethyl-2-hexadecen-1-ol                   | C_{20}H_{40}O_2  | 296.5      | 16.08    | 1.360  |
| 3-Methyl-5-(2′,6′,6′-trimethylcyclohex-1′-enyl)-1-penten-3-ol | C_{19}H_{34}O_2  | 222.37     | 17.26    | 16.500 |
| 7-Oxabicyclo[2.2.1]heptane                               | C_{9}H_{12}O     | 98.14      | 16.84    | 12.140 |
According to our survey, this is the first report that examined the effect of *J. phoenicea* extracts on the cell cycle and cell death mode on MCF-7 cells.

In the same manner, a recent study reported on the strong cytotoxic activity of Algerian *J. phoenicea* essentials oils against two human breast adenocarcinoma (MCF-7 and T-47D) cells. However, the IC₅₀ values were also different from what we found here; this result could be due to the different components in the same species as a consequence of different geographical origins and environmental factors [31,32].

According to criteria defined by the American National Cancer Institute regarding the cytotoxic activity of crude plant extracts (IC₅₀ < 30 µg/mL) [33], JPCF was the most efficient fraction of all fractions from *J. phoenicea* (IC₅₀ = 24.5 µg/mL). Therefore, it was selected to assess its activity on the cell cycle and apoptosis induction at the corresponding IC₅₀ value.

It is well known that cell proliferation is controlled by a highly regulated cell cycle process, and the dysregulation of the cell cycle is one feature of cancer cells [34,35]. Natural compounds that can disrupt cell cycle progression are considered among the most commonly used anticancer drugs [36]. Hence, the effect of JPCF on cell cycle progression was explored. Our findings showed that JPCF caused a significant cell cycle arrest in the GO/G1 phase suggesting the involvement of JPCF constituents in mediating this activity. Some studies have described the prevention of cell cycle progression for compounds and plant extracts belonging to the genus Juniperus [29,37]. Apoptosis evasion is also one of the most important characteristics of cancer cells, and targeting this pathway is a critical therapeutic approach to cancer therapy [38,39]. Therefore, we examined the capacity of JPCF to initiate apoptosis using the FTIC-Annexin V/PI method. In this method, early apoptotic, late apoptotic/necrotic, and dead cells are differentiated and quantitatively analyzed via flow cytometry [40]. The present study showed that JPCF mediated a significant antiproliferative effect that was associated with apoptosis and necrosis cell death. In fact, it has been noted that several conventional chemotherapeutic agents exerted various forms of cell death, the most important of which are apoptosis and necrosis [41]. Additionally, several constituents and extracts from the Juniperus genus were found to mediate apoptosis in different cancer cells [42–45]. GC-MS is one method for characterizing the constituents in plant extracts. In this study, we have documented different constituents found in JPCF. In partial agreement with what we found in this study, Keskes et al. reported the presence of alpha-humulene (16.9%) and alpha-cubebene (9.7%) in the hexane extract of Tunisian *J. phoenicea* [46]. In contrast, the obtained GC-MS profile was varied from those reported for Egyptian *J. phoenicea* [18]. These differences could result from several factors such as geographical location, the plant part used, and the extraction methodologies and solvents used.

The resulting GC-MS analysis showed the presence of some compounds in JPCF that were previously known to exert antiproliferative activities. Delta-cadinene was found among these compounds and is one of the most widely occurring plant sesquiterpenes. Hui et al. reported that delta-cadinene had potent anticancer effects on OVCAR-3 (human ovarian cancer cells) through the induction of apoptosis, sub-G1-phase cell cycle arrest, and caspase activation [47]. The cytotoxic activity of cubenol, which represents 13.48% of all constituents of JPCF, also exhibited strong cytotoxicity against the retinoblastoma cancer cell line (NCI-H187) [48]. Alpha-humulene (β-caryophyllene), a sesquiterpene that has anticarcinogenic effect and is widely distributed in different plant species [49], was also detected in our GC-MS data. Taken together, the presence of these components in JPCF may synergistically exert the anticancer potential of *J. phoenicea* reported in this study.

5 Conclusions

This study displayed that the extracts obtained from *J. phoenicea* species have antiproliferative effects against lung, liver, and breast cancer cells. In particular, we found that JPCF exerted cell death on MCF-7 cells through blocking the cell cycle in the G1 phase. Apoptotic and necrotic effects of JPCF on MCF-7 cancer cells were also observed. In parallel, GC-MS analysis revealed the presence of some anticancer compounds such as cubenol, delta-cadinene, and alpha-humulene. Based on this phytochemical depiction, we can conclude that some of these compounds, along with the remaining constituents of JPCF, are capable of initiating cell death in human cancer cells. These outcomes propose that JPCF may be an excellent source of active phytochemicals for cancer treatment. Future studies should aim to explore the anticancer activity of each compound, its detail mechanism, and its synergistic effects.

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