

**Growth inhibitory activity of biflavonoids and
diterpenoids from the leaves of the Libyan *Juniperus
phoenicea* against human cancer cells**

Afaf Al Groshi, Hiba A. Jasim, Andrew R. Evans, Fyaz M. D. Ismail,
Nicola M. Dempster, Lutfun Nahar and Satyajit D. Sarker*

Centre for Natural Products Discovery, School of Pharmacy and Biomolecular Sciences,
Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3
3AF, United Kingdom

*Correspondence to: Satyajit D. Sarker, Centre for Natural Products Discovery, School of
Pharmacy and Biomolecular Sciences, Liverpool John Moores University, James Parsons
Building, Byrom Street, Liverpool L3 3AF, United Kingdom

Tel.: +44 (0)151 231 2096; Fax: +44 (0)151 231 2170

E-mail: S.Sarker@ljmu.ac.uk (S. D. Sarker)

Abstract

Three biflavonoids [cupressuflavone (**1**), amentoflavone (**2**) and sumaflavone (**3**)], four diterpenoids [13-*epi*-cupressic acid (**4**), imbricatholic acid (**5**), 3-hydroxy-sandaracopimaric acid (**6**) and dehydroabietic acid (**7**)] and one lignan [β -peltatin methyl ether (**8**)], were isolated from the cytotoxic fractions of the extracts of the leaves of the Libyan *Juniperus phoenicea* L. The structures of these compounds were elucidated by spectroscopic means. Cytotoxicity of the compounds **1-6** were assessed against the human lung cancer cell line A549 using the MTT assay. Compounds **1** and **3** showed cytotoxicity against the A549 cells (IC_{50} = 65 μ M and 77 μ M, respectively), whereas, compound **2** did not show any activity. Diterpenes **4-6** exhibited weak cytotoxicity against the A549 cells with the IC_{50} values of 159 μ M, 263 μ M and 223 μ M, respectively. The cytotoxicity of each compound was compared with the anticancer drug, etoposide (IC_{50} = 61 μ M). Cupressuflavone (**1**) was evaluated also for cytotoxicity against both the human PC3 cancer cell line and the normal prostate cell line (PNT2), and this compound revealed a high degree of cytotoxic selectivity towards the prostate cancer cells (PC3), with IC_{50} value of 19.9 μ M, without any evidence of cytotoxicity towards the normal prostate cell line (PNT2).

KEYWORDS:

Juniperus phoenicea, Cupressaceae, cytotoxicity, biflavonoids, diterpenes, MTT assay

Key Points

- Biflavonoids cupressuflavone (**1**) and sumaflavone (**3**) showed cytotoxic effect against the human cancer cell line A549.
- Diterpenes 13-*epi*-cupressic acid (**4**), imbricatholic acid (**5**) and 3-hydroxy-sandaracopimaric acid (**6**) were also cytotoxic to A549 cells.
- Cupressuflavone (**1**) exhibited a high degree of cytotoxic selectivity towards the prostate cancer cells (PC3), compared to its cytotoxicity to the normal prostate cell line PNT2.

1 INTRODUCTION

Juniperus phoenicea L. (Cupressaceae), commonly known as 'Phoenician Juniper or Arâr', is a well-known Libyan medicinal plant, which has been used in Libyan traditional medicine for the treatment of various human ailments including tumours and cancers (Al Groshi et al., 2018). Whilst previous phytochemical investigation on this plant revealed the presence of flavonoids, lignans, phenylpropanoids, sterols and terpenoids (Aljaiyash et al., 2014), bioactivity studies demonstrated its antidiabetic, antimicrobial, antiparasitic, contraceptive and cytotoxic properties (Sánchez de Medina et al., 1994; Qnais et al., 2005; Aljaiyash et al., 2014; Latif et al., 2014).

The study conducted by Cairnes et al. (1980) revealed that the ethanolic extract of *J. phoenicea* twigs and leaves and two isolated compounds, desoxy-podophyllotoxin and β -peltatin-methyl ether, were cytotoxic in KB cell cultures (human cervix carcinoma). A methanol (MeOH) extract of this plant from Saudi Arabia and Indonesia displayed cytotoxicity in Vero (normal monkey kidney) and HEP-2 (human laryngeal carcinoma) cell lines (Abdul et al., 2014). In addition, this plant also exhibited cytotoxicity against both MCF7 (human breast adenocarcinoma) and HCT-116 (human colon carcinoma) cell lines (Aljaiyash et al., 2014). The cytotoxicity of the *n*-hexane, dichloromethane (DCM) and MeOH extracts of the leaves of the Libyan *J. phoenicea* was evaluated against four human cancer cell lines: EJ138 (human bladder carcinoma), HepG2 (human liver hepatocellular carcinoma), A549 (human lung carcinoma), and MCF7 (Al Groshi et al., 2018). The A549 appeared to be most sensitive to *J. phoenicea* extracts, with IC₅₀ values of 16, 13 and 100 μ g/mL for the *n*-hexane, DCM and MeOH extracts, respectively. As a part of our continuing cytotoxicity studies on this plant, we now report on the bioassay-guided isolation of compounds from the active fractions of the extracts of the leaves of the Libyan *J. phoenicea*, and cytotoxicity of the isolated biflavonoids (**1-3**) and diterpenoids (**4-6**) against the human lung cancer cell line A549, and selective cytotoxicity of cupressuflavone (**1**) against the human prostate cancer cell line PC3, compared to the normal prostate cell line (PNT2).

2 MATERIALS AND METHODS

2.1 General experimental procedures

Analytical HPLC was performed on a Dionex UPLC 3000 (Thermoscientific, UK) coupled with a photo-diode-array (PDA) detector (Thermoscientific). Extracts and fractions were analyzed on a Phenomenex C₁₈ column (150 × 4.6 mm, 5 μm, Phenomenex, USA). An Agilent 1200 Infinity series preparative HPLC system coupled with a PDA detector (Agilent, UK) was used to isolate compounds, both at semi-preparative (Luna C₁₈ semi-preparative column, 150 × 10 mm, 5 μm) and preparative (Hichrom ACE C₁₈ preparative column, 150 × 21.2 mm, 5 μm) scales. The column temperature was set at 25°C. The chromatogram was monitored at variable UV–vis wavelengths (205, 320 and 366 nm). NMR spectroscopic analyses were performed in CD₃OD or DMSO-d₆ solution on a Bruker AMX600 NMR spectrometer (600 MHz for ¹H and 150 MHz for ¹³C) or Bruker AMX300 NMR spectrometer (300 MHz for ¹H and 75 MHz for ¹³C). ESIMS analyses were performed on a Xevo G2-S ASAP or LTQ Orbitrap XL1 spectrophotometer or on an Agilent 6200 Series Accurate-Mass Time-of-Flight (TOF) LC/MS.

2.2 Plant materials

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

2.3 Extraction

Ground leaves of *J. phoenicea* (86.5 g) were subjected to Soxhlet extraction, sequentially, with *n*-hexane, DCM and MeOH (800 mL each). Ten cycles were employed for each extraction, and the heating mantle temperature 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 preserved at 4°C for further studies.

2.4 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 (Sarker et al., 2005; Al Groshi et al., 2018). All resulting fractions were subjected to the MTT assay using human cancer cell lines (Al Groshi et al., 2018), and comprehensive chromatographic analyses were conducted on the cytotoxic fractions to isolate compounds **1-8**.

2.5 Isolation of compounds

The active SPE fractions of the MeOH extract, fractions F3 (80% MeOH in water) and F4 (100% MeOH), 50 mg each, were analyzed by semi-preparative reversed-phase HPLC (Agilent) (Sarker and Nahar, 2012), using the mobile phase gradient 30-100% MeOH/water, both solvents had 0.1% trifluoro acetic acid (TFA), for 30 min and held for 10 min at 100% MeOH, 2 mL/min flow rate, monitored at 220, 254, 280 and 360 nm, to yield cupressuflavone (**1**, 12 mg, t_R = 20.0 min), amentoflavone (**2**, 2.5 mg, t_R = 21.9 min) and somaflavone (**3**, 1.5 mg, t_R = 26.3 min). The active VLC fractions of the *n*-hexane extract, fractions F2-F5, were chromatographed separately over silica gel normal column chromatography, eluting with ethyl acetate in *n*-hexane (2%, 4%, 6%.....20% at 2% increment) to afford 13-*epi*-cupressic acid (**4**, 8.2 mg, R_f = 0.6). The active VLC fraction of the DCM extract, fraction F4 (130 mg), was subjected to prep-HPLC, employing a linear gradient elution, 50-100% acetonitrile/water, both solvents had 0.1% TFA, for 30 min and held at 100% acetonitrile for 10 min, to produce β -peltatin methyl ether (**8**, 0.5 mg, t_R = 10.19), 13-*epi*-cupressic acid (**4**, 3.2 mg, t_R = 14.12), imbricatolic acid (**5**, 10.3 mg, t_R = 15.56) and 3-hydroxy sandaracopimaric acid (**6**, 3 mg, t_R = 17.5). Another active VLC

fraction of the DCM extract, fraction F6 (40 mg), was also analyzed by the same prep-HPLC system to isolate dehydroabietic acid (**7**, 1.3 mg, t_R = 29.5).

2.6 Isolated compounds

2.5.1 Cupressuflavone (1) UV (MeOH): λ_{max} 204, 228, 274 and 330 nm; 1H NMR and ^{13}C NMR (600 MHz, DMSO- d_6) and ^{13}C NMR (75 MHz, DMSO- d_6): Supplementary Tables 1 and 2; ESIMS m/z 539 $[M+H]^+$, suggested the molecular formula $C_{30}H_{18}O_{10}$. All data were comparable to the published data (Alqasoumi et al., 2013).

2.5.2 Amentoflavone (2). UV (MeOH): λ_{max} 212, 270 and 332 nm; 1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): Supplementary Tables 1 and 2; ESIMS m/z 539 $[M+H]^+$, corresponding to the molecular formula $C_{30}H_{18}O_{10}$. All data were comparable to the published data (Markham et al., 1987; Terashima et al., 1999; Li et al., 2014; Bais & Abrol, 2016).

2.5.3 Sumaflavone (3). UV (MeOH): λ_{max} 224, 270 and 332 nm; 1H NMR (300 MHz, DMSO): Supplementary Table 1. ESIMS m/z 555 $[M+H]^+$, corresponding to the molecular formula $C_{30}H_{18}O_{11}$. All data were comparable to the published data (Markham et al., 1987).

2.5.4 13-Epi-cupressic acid (4). 1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Supplementary Tables 3 and 4; ESIMS m/z : 319 $[M-H]^-$, corresponding to the molecular formula $C_{20}H_{32}O_3$. All data were comparable to the published data (Su et al., 1994; Alqasoumi et al., 2013).

2.5.5 Imbricatolic acid (5). 1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Supplementary Tables 3 and 4; ESIMS m/z 321 $[M-H]^-$, corresponding to the molecular formula $C_{20}H_{34}O_3$. All data were comparable to the published data (Wenkert & Buckwalter, 1972; Su et al., 1994; Alqasoumi et al., 2013).

2.5.6 3-Hydroxy-sandaracopimaric acid (6). 1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): Supplementary Tables 3 and 4; ESIMS m/z 317 $[M-H]^-$, corresponding

to the molecular formula $C_{20}H_{30}O_3$. All data were comparable to the published data (Kozo & Takami, 1972; Alqasoumi et al., 2013).

2.5.7 Dehydroabietic acid (Callistricic acid) (7). 1H NMR (600 MHz, $CDCl_3$): δ_H 7.18 (1H, d, $J = 8.2$, H-1'), 7.00 (1H, dd, $J = 8.2$, 1.7, H-2'), 6.89 (1H, brd s, H-4'), 2.89 (1H, dd, $J = 5.6$, 16.6, H-7), 2.82 (2H, m, H-6'), 1.60 (2H, m, H-6), 1.27 (3H, s, H-12), 1.22 (6H, d, $J = 7.0$, H-7', H-8'), 1.1 (3 H, s, H-13); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 181.6 (C-19), 145.7 (C-9), 145.4 (C-13), 135.0 (C-8), 126.8 (C-14), 125.4 (C-11), 124.0 (C-12), 52.8 (C-5), 43.8 (C-4), 39.3 (C-3), 37.5 (C-1), 33.4 (C-15), 32.0 (C-7), 28.7 (C-18), 24.0 (C-6', C-7'), 21.7 (C-20), 20.9 (C-6), 19.9 (C-2). ESIMS m/z 301 $[M+H]^+$, corresponding to the molecular formula $C_{20}H_{28}O_2$. All data were comparable to the published data (Abad et al., 1991; González et al., 2010).

2.5.8 β -Peltatin methyl ether (8). 1H NMR (600 MHz, $CDCl_3$): δ_H 6.36 (2H, s, H-2, H-6), 6.28 (1H, s, H-3), 5.88 (2H, d, $J = 5.9$, O-CH₂-O), 4.58 (1H, d, $J = 4.3$ Hz, H-7), 4.47 (1H, dd, $J = 7.3$, 1.3 Hz, H-9), 4.07 (3H, s, CH₃O -6), 3.94 (1H, t, $J = 9.6$ Hz), 3.80 (3H, s, CH₃O-4), 3.76 (6H, s, CH₃O-3, CH₃O-5), 3.17 (1H, dd, $J = 5.1$, 16.8 Hz, H-7), 2.66 (1H, d, $J = 4.5$, H-8'), 2.64 (1H, m, H-8), 2.45 (1H, dd, $J = 10.6$, 17.1 Hz, H-7); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 175.5 (C-9'), 152.9 (C-3', C-5'), 148.6 (C-4), 145.1 (C-6), 138.1 (C-1', C-4'), 136.6 (C-5), 132.0 (C-2), 121.3 (C-1), 108.7 (C-2', C-6'), 104.8 (C-3), 101.3 (O-CH₂-O), 72.8 (C-9), 59.7 (CH₃O-6), 56.7 (CH₃O-3', CH₃O-5'), 55.7 (CH₃O-4') 47.7 (C-8'), 44.2 (C-7'), 32.7 (C-8), 27.9 (C-7). ESIMS m/z 429 $[M+H]^+$, corresponding to the molecular formula $C_{23}H_{24}O_8$. All data were comparable to the published data (Rojas-Sepúlveda et al., 2012).

2.7 MTT assay

The cytotoxic activity of the isolated compounds (**1-6**) from the active fractions of *J. phoenicea* leaves were assessed against the human lung carcinoma (A549) cell line. In addition, cupressuflavone **1** was assessed also for cytotoxicity against the human prostate cancer (PC3) cell line and the human prostate normal cell line (PNT2). The cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum and

seeded into 24-well plates (5×10^4 cells/well) and incubated under 5% CO₂, 95% humidity at 37°C for 24 h. The different isolated compounds were dissolved in DMSO and then diluted in medium containing DMSO to achieve a range of concentrations of each compound, all with a final treatment concentration of DMSO of 0.01% (including the negative control). These doses of each compound were then used to treat the cells for 24 h before assessment using the MTT assay (Mosmann, 1983; Nemati et al., 2013). Briefly, each treatment dose 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 release and solubilise the blue formazan product. Four samples (100 µL each) were taken from each well of the 24 wells of the assay plate and transferred to a 96-well plate. The absorbance of these samples was then measured at 570 nm on a Clario Star microplate reader. The average absorbance reading was calculated for each sample and was then expressed as a percentage of the mean value of the non-treated control absorbance readings on each occasion. The results for each treatment and dose are derived from at least 12 wells ($n \geq 12$) from three or more separate occasions.

2.8 Lactate dehydrogenase (LDH) assay

The membrane integrity of cells in response to cupressoflavone (**1**) treatment was assessed by estimating the amount of LDH released into the culture media. The release of the cytosolic enzyme LDH into the medium is indicative of membrane damage and is often used as a measure of necrosis. The A549 cells were seeded at a density of (1×10^5) cells/well in columns 1 to 9 of a 96-well plate (100 µL/well), while the columns 10 to 12 were filled with complete medium without cells (100 µL/well) as a negative control. The cells were given 24h to adhere and start to proliferate. Then the cells in columns 1 to 3 were treated with compound **1** at the concentration estimated to be its IC₅₀ value (65 µM) for 24h. Wells in columns 4 to 6 were untreated viable cells (low LDH release controls). After 24 h of treatment, a cell lysis buffer 10X (9% v/v Triton X-100 in distilled

H₂O, 10 µL/well) was added to the medium in wells in columns 7 to 9 and incubated for 15 min to allow complete lysis of the cells to occur (maximal LDH release controls). The LDH cytotoxicity detection kit (Roche) was used in accordance with the manufacturer's instructions by adding reaction mixture (1 mL lyophilized catalyst mixed with 11.25 mL of dye solution) into every well (100 µL/well) and incubated for a further 15 min at 37°C. After 15 min incubation, 50 µL/well stop solution (1M acetic acid), was added to each well of the 96-well plate (columns 1 to 12). The absorbance values were determined at two wavelengths, 490 nm and 690 nm and the 690 nm values were then subtracted from the 490 nm values. The intensity of colour is proportional to LDH activity and therefore the amount of LDH released from cells. The necrotic percentage was expressed using the formula: (sample value/maximal release) × 100% (Chan et al., 2013).

2.9 Statistical analysis

All MTT assays were carried out in triplicate on separate occasions. One-way ANOVA of multiple comparisons was applied (between control and different concentrations) using Dunnett's multiple comparison tests (GraphPad Prism version 7.04). The differences between groups were considered statistically non-significant (ns) at a P value ≥ 0.05, significant (*) at a P value < 0.05, very significant (**) at a P value < 0.01, highly significant (***) at a P value < 0.001. The results were mean values ± standard error of the mean derived from n ≥ 12 from three separate occasions.

3 RESULTS AND DISCUSSION

In this study, bioassay-guided fractionation was carried out on the *J. phoenicea* cytotoxic fractions of the leaves that were evaluated previously (Al Groshi et al., 2018). The VLC fractions of the *n*-hexane extract, F2 to F5, and of the DCM extract, F4 and F6, and the SPE fractions of the MeOH extract, F3 and F4, were all considered cytotoxic. The most potent fraction appeared to be F5 of the *n*-hexane extract with an IC₅₀ value of 10

$\mu\text{g/mL}$, then F4 of the DCM extract (IC_{50} value $19 \mu\text{g/mL}$) and F3 and F4 of the MeOH extract (IC_{50} values 50 and $85 \mu\text{g/mL}$) (Al-Groshi et al., 2018).

Several phytochemicals, *e.g.*, alkaloids, flavonoids, lignans, phenols, steroids and terpenes, possess cytotoxic properties against cancer cells (Fernando & Rupasinghe, 2013). Notably, all these groups of phytochemicals have previously been isolated from the leaves and the berries of *J. phoenicea* and other *Juniperus* species grown in different countries (Cairnes et al., 1980; Comte et al., 1997; Barrero et al., 2004; Aboulela et al., 2005).

Biflavonoids comprise a group of dimeric flavonoids and possess different biological activities of high importance, such as anticancer, antioxidant, antimicrobial, antinociceptive and anti-inflammatory (Mercader & Pomilio, 2012). These compounds strongly affect the cancer cells with little effect on normal cell proliferation, suggesting a therapeutic potential against cancer (Mercader & Pomilio, 2012). Three biflavonoids, cupressoflavone (**1**), amentoflavone (**2**) and sumaflavone (**3**), were obtained from the active fractions (F3 and F4) of the MeOH extract of the leaves of *J. phoenicea*, and their structures (Figure 1) were elucidated based on spectral analyses (UV-Vis spectroscopy, NMR and MS spectrometry); all data were comparable to the respective published data.

The three biflavonoids (**1-3**) were tested for cytotoxicity against the human lung cancer cell line A549. Here, compounds **1** and **3** were tested for the first time against this cell line; both showed moderate cytotoxicity against these cancer cells with the IC_{50} values of $65 \mu\text{M}$ and $77 \mu\text{M}$, respectively, and compared with the positive standard etoposide displaying an IC_{50} value of $61 \mu\text{M}$ (Table 1) whereas, compound **2** did not show any activity against these cancer cells. Compound **2** was previously reported as a weak cytotoxic agent against lung cancer cells A549. However, it was shown to possess potent cytotoxic effects against both MCF-7 and HeLa cells, with the IC_{50} values of 25 and $20 \mu\text{M}$, respectively (Lee et al., 2012).

Cupressoflavone (**1**) was previously reported from *Juniperus turbinata* (Venditti et al., 2018), and it was shown to possess cytotoxic activity against the human cancer cell lines: breast adenocarcinoma (MDA-MB 231), malignant melanoma (A375) and colon carcinoma (HCT116), with IC₅₀ values of 16.1, 12.7 and 19.3 μ M, respectively, after 72 h incubation, using the MTT assay. However, on the present study compound **1** displayed a significant decrease in the percentage viability of lung cancer cells A549 at concentrations higher than 0.33 μ M (Table 1). The LDH assay was also carried out on compound **1** against the lung cancer cell line (A549); the results suggested that cytotoxicity might result in cellular necrosis (Figure 2). The necrotic percentage was calculated as 56.3 %. As compound **1** was isolated in good amounts, and was the major compound among the active compounds, it was also tested on the prostate cancer cell line (PC3) and the normal prostate cells (PNT2). It is of interest that the prostate cancer cells (PC3) were more sensitive to compound **1** (IC₅₀ = 19.9 μ M) (Figure 3) than normal prostate cells (PNT2) (did not show any cytotoxicity) (Figure 4).

In our study, sumaflavone (**3**) was isolated for the first time from *J. phoenicea*. However, it was previously reported from *Rhus coriaria* (Anacardiaceae) (Van Loo et al., 1988 and *Selaginella tamariscina* (Selaginellaceae) (Yang et al., 2006). Its effect on inducible nitric oxide synthase (iNOS) gene expression was studied and the results revealed both its inhibition on NO production and its blockage of the lipopolysaccharide (LPS)-induced expression of iNOS (Yang et al., 2006). Another study was carried out by Lee et al. (2008) and the results suggested that compound **3** could be developed as a potential preventive or therapeutic agent of skin aging by examining its effect on MMP-1 production and MMP-2 enzymatic activity in human dermal fibroblasts (HDFs) exposed to UV irradiation. MMPs are enzymes that are responsible for the degradation of ECM (extracellular matrix) components such as collagen and elastin.

Our results revealed a highly significant cytotoxic effect of compound **3** against the A549 cell line at the concentration of 135.3 μ M, while the lower concentrations were found to be non-significant. The presence of the aromatic hydroxyl group at C-5 in **3** might have played a role in the potentiation of the cytotoxic activity, whereas, the lack of this

hydroxyl group in compound **2** might have had an inhibitory effect on this activity. It has previously been reported that the substitution of the aromatic hydroxyl group at C-5 in the A ring is essential for inhibition of cell growth of human promyelocytic leukaemia cells (HL-60) (Chen et al., 2014).

There has been much research over the recent years highlighting the cytotoxic/anticancer activity of diterpenoids, either *in vitro* against a variety of mammalian cancer cell lines, or *in vivo* in animal models of cancer (Greay & Hammer, 2015). However, lignans are secondary metabolites, which exhibited prominent cytotoxic, antioxidant and antitumor activities. Cairnes et al. (1980) showed that the ethanolic extract of *J. phoenicea* twigs and leaves in addition to two isolated compounds, desoxy-podophyllotoxin and β -peltatin-methyl ether, were cytotoxic to the KB cell cultures (human cervix carcinoma).

The VLC fraction (F3) of the *n*-hexane extract and those (F4 and F6) of the DCM extract led to the isolation of four diterpenes (Figure 1). The ^1H and ^{13}C NMR spectroscopic data indicated that they could be two bicyclic diterpenes labdanes (13- *epi*-cupressic acid **4** and imbricatolic acid **5**), two tricyclic diterpenes (the pimarane 3-hydroxy sandaracopimaric acid **6** and the abietane dehydroabietic acid **7**). The three diterpenes (**4-6**) were tested for their cytotoxicity against the A549 cell line and exhibited weak cytotoxicity with IC_{50} value of 159 μM , 223 μM and 263 μM , respectively (Table 1). It has been suggested that compound **5** induces cell cycle arrest in the CaLu-6 cell line cell (human, Caucasian, lung, adenocarcinoma) (De Marino et al., 2011), but no cytotoxic effect of compound **6** on lung cancer cells has previously been reported.

The VLC fraction F4 of the DCM extract also yielded two lignans. The ^1H and ^{13}C NMR spectroscopic data indicated that they belong to the furan subgroups. The presence of 3, 4, 5-trimethoxyphenyl, which are the most frequently occurring aromatic rings found in lignans (Tsopmoa et al., 2013), suggesting that they are deoxypodophyllotoxin and β -peltatin methyl ether **8** (Figure 1). Deoxypodophyllotoxin was impure, but its presence in F4 with the other lignan **8** indicates the high potency of this fraction (IC_{50} = 19 $\mu\text{g/mL}$).

Deoxypodophyllotoxin mixture was also tested against A549 and it showed potent cytotoxicity with an IC₅₀ value of 1.11 µg/mL. The reference anticancer drug, etoposide was cytotoxic against the A549 lung cancer cells in this study at IC₅₀ value = 61 µM.

CONCLUSIONS

The outcome of this study suggested that the cytotoxicity observed with the less polar extracts and fractions of *J. phoenicea* leaves was contributed by various cytotoxic lignans and terpenes, while the cytotoxic activity revealed in the more polar MeOH extract and fractions was due to the presence of cytotoxic biflavonoids. Cupressoflavone **1** revealed a high degree of selectivity against prostate cancer cells, which might suggest that this compound could be an ideal candidate for further studies towards developing it as a potential anticancer therapeutic agent.

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CONFLICT OF INTEREST

The authors declare that there are no competing interests.

ORCID

Afaf Al Groshi ID: <https://orcid.org/0000-0003-4664-914X>

Andrew R. Evans ID: <https://orcid.org/0000-0002-2756-6683>

Fyaz M. D. Ismail ID: <https://orcid.org/0000-0002-3595-6665>

Hiba A. Jasim ID: <https://orcid.org/0000-0002-3637-3090>

Nicola M. Dempster ID: <https://orcid.org/0000-0001-8532-4845>

Lutfun Nahar ID: <https://orcid.org/0000-0002-1157-2405>

Satyajit D. Sarker ID: <https://orcid.org/0000-0003-4038-0514>

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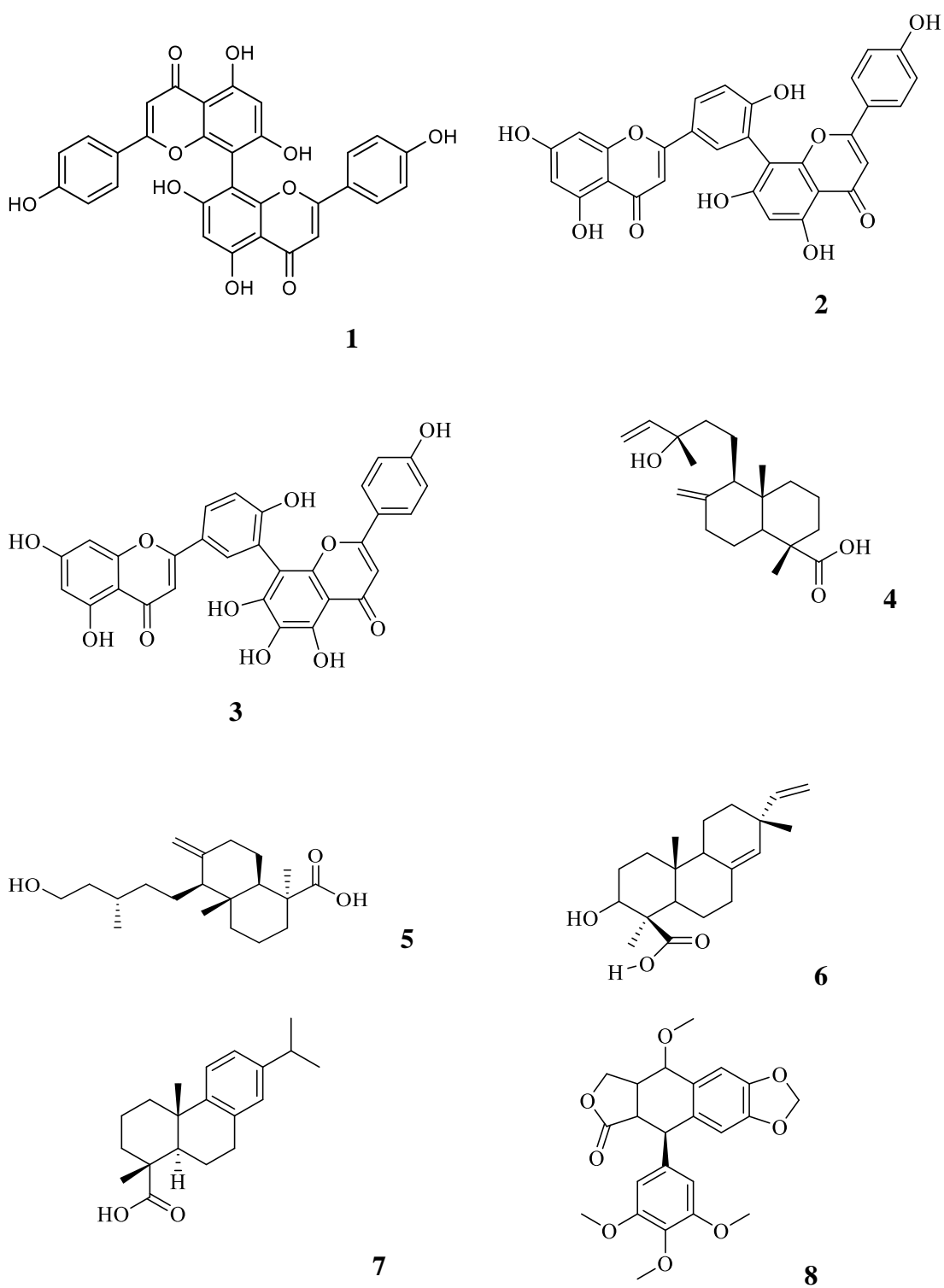


Figure 1: Isolated compounds from the Libyan *J. phoenicea* leaves

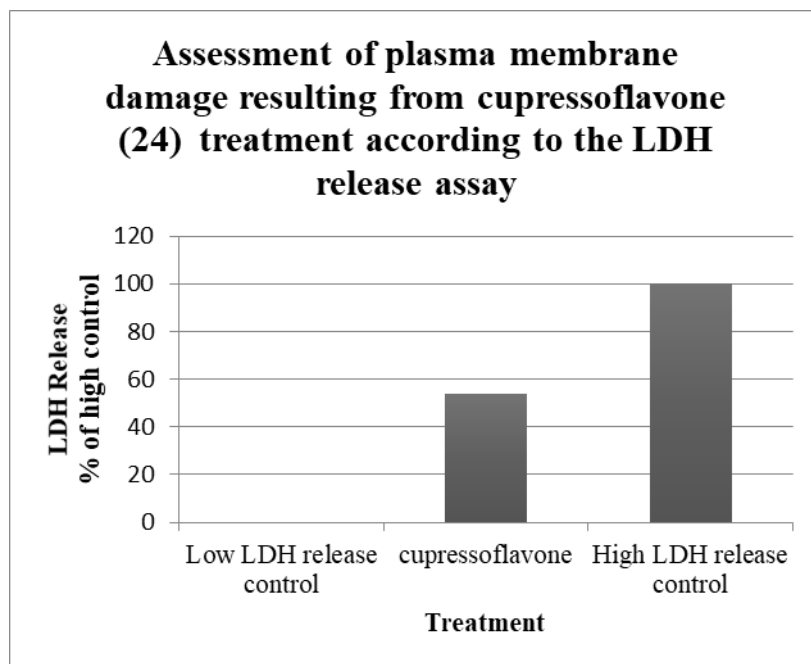


Figure 2: LDH release result for cupressuflavone (1) against the lung cancer cell line (A549)

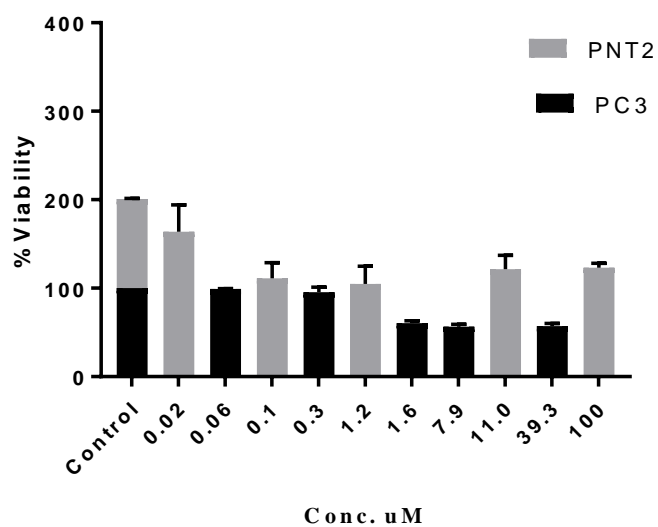


Figure 3: The cytotoxic activity of cupressoflavone (1) against prostate cancer cell line (PC3) and the human prostate cell line (PNT2)

Table 1: The IC₅₀ (μM) of different isolated compounds from *J. phoenicea* leaves on A549 lung cancer cells.

Pure compound	IC ₅₀ μM ± S.E
Cupressoflavone (1)	65 ± 1.55
Amentoflavone (2)	No activity
Sumaflavone (3)	77 ± 1.64
13- <i>Epi</i> -cupressic acid (4)	159 ± 1.16
Imbricatolic acid (5)	263 ± 2.45
3-Hydroxy-sandaracopimaric acid (6)	223 ± 2.47
Etoposide	61 ± 1.56