Chemical Composition and Cytotoxic Activity of *Pistacia atlantica var. kurdica* Fruits

Raad A. Kaskoos[1], Javed Ahamad[2], Subasini Uthirapathy[3]

[1]Department of Pharmacy, Al-Manara College for Medical Science, Amarah, Iraq
[2]Department of Pharmacognosy, Faculty of Pharmacy, Tishk International University, Erbil, Kurdistan Region, Iraq
[3]Department of Pharmacology, Faculty of Pharmacy, Tishk International University, Erbil, Kurdistan Region, Iraq

***Abstract*–*Pistacia atlantica var. kurdica* (Anacardiaceae) is a major subspecies found in the Kurdistan region of Iraq and has several beneficial bioactivities such as antioxidant, antibacterial, and antiviral. *P. atlantica* growing in the Kurdistan region is not yet studied phytochemically and pharmacologically. Hence, the goal of the present study is to characterize chemical compounds present in ethanolic extract of *P. atlantica* fruits by gas chromatography–mass spectroscopy (GC–MS) and to evaluate cytotoxic activity using A549 (human lung cancer) cell lines by (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) assay. The GC–MS analysis shows the presence of 33 chemical compounds which constitute about 99.95% of total compounds, and isospathulenol (14.63%), spathulenol (13.45%), α-terpineol (13.28%), limonene (10.92%), terpinolene (10.89%), β-myrcene (6.92%), ethyl pentadecanoate (6.15%), β-pinene (4.98%), and caryophyllene oxide (4.01%) were found as major chemical compounds. *P. atlantica* inhibits cell proliferation in A549 cell lines in a time (24 h) and dose-dependent manner (0.5–500 μg/mL). After 24 h of treatment with *P. atlantica,* the cell viability of A549 cell lines ranged from 93.01 ± 5.24 to 57.69 ± 4.15% for concentrations of 0.5–500 μg/mL, respectively. This study expands the knowledge of the chemical composition of *P. atlantica* fruits and provides scientific evidence for its possible use as an anticancer medicine. The substantial anticancer activity of *P. atlantica* fruits may be due to the presence of isospathulenol, spathulenol, α-terpineol, limonene, terpinolene, β-myrcene, ethyl pentadecanoate, β-pinene, and caryophyllene oxide.**

***Index Terms*–*Pistacia atlantica var. kurdica*; Anacardiaceae; Cancer; Gas chromatography–mass spectroscopy; A549 cell lines; (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) assay.**

I. Introduction

Pistacia atlantica Desf. (Family: Anacardiaceae) is the most common species of this genus and is abundantly found in Iraq, Syria, Iran, and Turkey (Sharifi, 2014). *P. atlantica* Desf. contains four prominent varieties or subspecies, and it includes *P. atlantica var. kurdica, P. atlantica var. cabulica, P. atlantica var. mutica,* and *P. atlantica var. atlantica* (Ahmed, et al., 2021). *P. atlantica var. kurdica* is native to Kurdistan Region of Iraq and mostly found wildly in Shaqlawa and Ranya districts of Kurdistan Region. *P. atlantica subsp. kurdica* is commonly known as *Dar qezwan* or *Dareben* in Kurdish and Atlas in English (Ahmed, 2017). *P. atlantica var. kurdica* is known as a potent antioxidant plant (Ben Ahmed, et al., 2016; Gourine, et al., 2010). It also has several bioactivities such as antimicrobial (Benhammou, Bekkara and Panovska, 2008; Sharifi and Hazell, 2012), antihyperglycemic (Kasabri, Afifi and Hamdan, 2011), cytotoxic (Hamelian, et al., 2018), and antiviral (Karimi, Moradi and Gafourian, 2020). Lung cancer has emerged as a major cause of cancer-related deaths worldwide, and smoking is considered as the primary cause. Adenocarcinoma, squamous cell carcinoma, and large cell carcinoma are the three types of lung cancer (Collins, et al., 2007). Lung cancer treatment varies based on the nature and stage of the tumor and can range from surgical resection to chemotherapy. Chemotherapy and surgical resection have both been linked to serious side effects. That's why the treatment of lung cancer needs palliative therapy along with chemotherapy (Jones and Baldwin, 2018). Medicinal plants are known to have anticancer activity in *in vitro, in vivo,* and clinical studies (Gezici and Şekeroğlu, 2019; Ahamad, et al., 2019). The present study is aimed to determine the chemical composition of ethanolic extract of *P. atlantica var. kurdica* fruits by gas chromatography–mass spectrometry method and also to evaluate its cytotoxic activity in A549 (human lung cancer) cell lines.

II. Materials and Methods

A. Plant Materials and Chemicals

The fresh fruits of *P. atlantica var. kurdica* (1 kg) were collected in March 2021 from Shaqlawa, Kurdistan Region, Iraq. The authenticity of all the accession was ascertained by Dr. Raad A Kaskoos, Department of Pharmacy, Al-Manara College for

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Corresponding author's e-mail: javed.ahamad@tiu.edu.iq

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Medical Science, Amarah, Iraq. The plant sample was archived for future reference in the Faculty of Pharmacy, Tishk International University, Erbil, Iraq (voucher number: PRL/2021/05).

A549 (human lung cancer) cell lines were procured from National Centre for Cell Sciences, Pune, India. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and antibiotic solution were procured from Gibco (USA), whereas dimethyl sulfoxide (DMSO) and (3-4,5-dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (MTT) were from Sigma-Aldrich (USA), and HiMedia provided 1× phosphate-buffered saline (PBS) (India). Tarson provided a 96-well tissue culture plate and a wash beaker (India). All of the other chemicals and solvents were of analytical grade.

B. Preparation of Ethanolic Extract of P. atlantica

The fresh fruits of *P. atlantica* were pulverized in a mixed grinder and then extracted in an ultrasonicator (Elma, Germany). The pulverized fruits of *P. atlantica* were placed in a stoppered conical flask and extracted for 30 min with ethanol (250 mL) in an ultrasonicator at 30°C temperature. After filtering, the extract was treated with 5 g of activated charcoal and filtered again. The filtrate is concentrated in a rotary evaporator (Buchi, Switzerland) at 35°C. The concentrated ethanolic extract was then air-dried, and the dried ethanolic extract was kept in the refrigerator at 2–4°C until needed.

C. GC–MS Analysis and Identification of Chemical Constituents

The GC–MS method was used to analyze the chemical composition of an ethanolic extract of *P. atlantica* fruits. The test sample was analyzed on Agilent Bench Top GC–MS (Agilent Technologies, Wilmington, DE, USA) equipment using a DB-5 glass capillary column with specification of 30 m × 0.25 mm i.d.; film thickness of 0.25 µm. Helium was used as a carrier gas, and the flow rate was fixed at 1 mL/min. The oven temperature was set to 50°C for 1 min and then isothermally maintained at 320°C for 2 min, whereas the injector port temperature was kept at 280°C. The split ratio was kept at 1:5, and the 0.1 µL of *P. atlantica* ethanolic extract in DMSO was injected. Data were collected at 70 eV with 1.5 s scanning durations in the mass range of 50–1000 amu and a run time of 37 min. ChemStation software was used to handle the chromatography and mass spectra.

The individual chemical constituents were identified by comparing their Kovats index (K.I.) to those found in the literature and the mass fragmentation pattern of spectra obtained by GC–MS, and they were compared to those stored in the spectrometer database of NIST, NBS 54 K.L, WILEY8 libraries, and published literature for further identification of chemical constituents (Adams, 2007; Ali, 2001; Gourine, et al., 2010; Delazar, Reid and Sarker, 2004; Farhoosh, Tavakoli and Khodaparast, 2008). The area of the individual peaks was used to calculate the percent composition of each component.

D. Cytotoxic activity of P. atlantica var. kurdica

The potential cytotoxic activity of *P. atlantica* ethanolic extract (for concentration range 0.5–500 µg/mL) against A549 cell lines (human lung cancer) was assessed using the MTT assay. The assay was performed by the method described by Marquez, et al. (2020). A549 cell lines were cultured in liquid medium (DMEM) supplemented with 10% FBS, 100 µg/mL penicillin, and 100 µg/mL streptomycin, and kept at 37°C in a 5% CO_2 atmosphere. Trypsinization was used to extract the cultured A549 cells, which were then pooled in a 15 mL tube. The cells were then plated at a density of 1×10^5 cells/mL/well (200 µL) in a 96-well plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24–48 h at 37°C. In a serum-free DMEM medium, the cells were rinsed with sterile PBS and treated with *P. atlantica* ethanolic extract. Each sample was replicated 3 times, and the cells were cultured for 24 h at 37°C in a humidified 5% CO_2 incubator. MTT (20 µL at conc. of 5 mg/mL) was added to each well after the incubation period, and the cells were incubated for another 2–4 h until purple precipitates were visible under an inverted microscope. Finally, the medium was aspirated out of the wells together with MTT and rinsed with 1× PBS. DMSO (100 µL) was also added to dissolve formazan crystals, and the plate was agitated for 5 minutes. Using a microplate reader (Thermo Fisher Scientific, USA), the absorbance of each well was measured at 570 nm. The percent cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD test}}{\text{OD control}} \times 100$$

III. Results and Discussion

A. GC–MS Analysis of P. atlantica var. kurdica

The GS–MS analysis was performed to identify different chemical constituents present in ethanolic extract of *P. atlantica var. kurdica* found in Iraqi Kurdistan. The GC–MS analysis of ethanolic extract of *P. atlantica* yielded 33 chemical compounds which constitute about 99.95% of total chemical compounds (Table 1 and Fig. 1). The major chemical compounds of *P. atlantica var. kurdica* were identified as isospathulenol (14.63%), spathulenol (13.45%), *α*-terpineol (13.28%), limonene (10.92%), terpinolene (10.89%), *β*-myrcene (6.92%), ethyl pentadecanoate (6.15%), *β*-pinene (4.98%), and caryophyllene oxide (4.01%). The other chemical compounds present in *P. atlantica var. kurdica* were *cis*-limonene oxide (2.18%), isobutyl hexanoate (2.19%), epiglobulol (2.02%), phytol (1.90%), and globulol (1.29%). The minor chemical compounds which are less than 1% also listed in Table 1. As *P. atlantica* is found in different parts of the world, its chemical composition has been analyzed by several researchers. Delazar, et al. (2004), studied the chemical composition of oleoresin from *P. atlantica var. mutica* by GC–MS method growing in Iran, and the results show the presence of *α*-pinene (70%) limonene oxide (9%), citral (5.72%), and myrtenol (5.31%) as major constituents. Gourine, et al. (2010), studied 34 samples of *P. atlantica* Desf. from different locations of Algeria by GC–MS and found *α*-pinene (5.54–66.61%), camphene (0.75–20.85%),

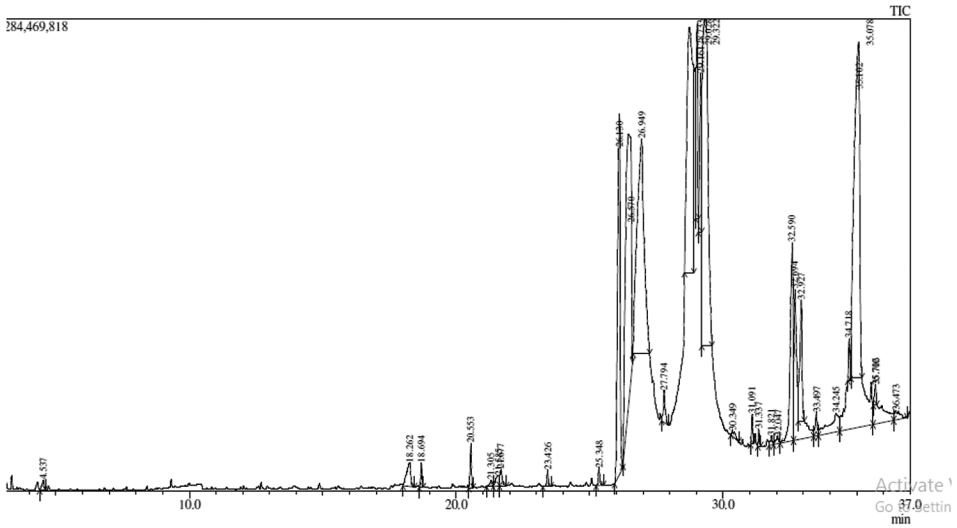

Fig. 1. Gas chromatography–mass spectroscopy spectra of ethanolic extract of *Pistacia atlantica var. kurdica* fruits.

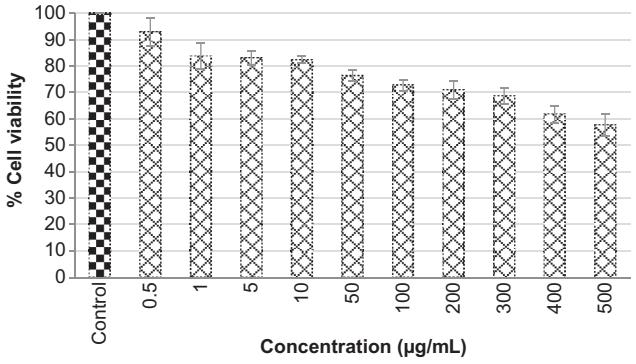

Fig. 2. The cell cytotoxicity produced by ethanolic extract of Pistacia atlantica var. kurdica fruits (represented as % cell viability) (data were presented as mean of triplicate determinations ± SD).

β-pinene (1.09–13.12%), *p*-cymene (0.39–10.19%), and terpinene-4-ol (0.42–15.97%) as major chemical constituents. Mecherara-Idjeri, et al. (2008), studied the chemical composition of leaf, fruit, and gall essential oils of *P. atlantica* Desf. from Algeria, and the major chemical constituents were found as α-pinene (32.6–54.7%) and *β*-pinene (8.0–20.2%). The above research studies were performed on *P. atlantica var. mutica* and other subspecies of it, and also plants were studied from different geographical locations such as Iran and Algeria. The current study is the first study on *P. atlantica var. kurdica* growing in Iraqi Kurdistan and shows the presence of isospathulenol, spathulenol, α-terpineol, limonene, terpinolene, *β*-myrcene, ethyl pentadecanoate, *β*-pinene, and caryophyllene oxide as major chemical constituents. The variation in chemical constituents and their amounts may be due to differences in geographical locations, altitude, soil, rainfall, etc. (Ahamad and Uthirapathy, 2021a and b).

B. Cytotoxic Activity of P. atlantica var. kurdica

The cytotoxic activity of *P. atlantica var. kurdica* was assessed using A549 (human lung cancer) cell lines by MTT test. Figs. 2 and 3 show the results of the MTT assay.

Table I
Chemical Composition of Ethanolic Extract of *Pistacia atlantica var. kurdica* Fruits

Name of chemical compound	RT	RI	% composition
4-Hydroxyhexan-3-one	4.537	846	0.15
Ethylene glycol monoisobutyl ether	18.262	878	0.72
Heptanal	18.694	904	0.24
Tricyclene	20.553	920	0.43
α-Thujene	21.305	925	0.12
α-Pinene	21.585	934	0.32
Camphene	21.677	946	0.27
Verbenene	23.426	960	0.16
Sabinene	25.348	976	0.20
β-Pinene	26.130	980	4.98
β-Myrcene	26.570	992	6.92
Limonene	26.949	1032	10.92
cis-Ocimene	27.794	1039	0.44
Terpinolene	28.733	1088	10.89
cis-Limonene oxide	29.028	1137	2.18
Isobutyl hexanoate	29.161	1160	2.19
α-Terpineol	29.322	1180	13.28
3Z-Hexenyl 3-methylbutanoate	30.349	1231	0.15
Tetradecane	31.091	1402	0.26
β-Caryophyllene	31.337	1432	0.14
α-Humulene	31.821	1458	0.13
β-Curcumene	32.047	1517	0.16
Caryophyllene oxide	32.590	1573	4.01
Ethyl pentadecanoate	32.694	1991	6.15
Epiglobulol	32.927	2026	2.02
Methyl elaidate	33.497	2080	0.33
Globulol	34.245	2092	1.29
Viridiflorol	34.718	2100	0.37
Spathulenol	35.078	2140	13.45
Isospathulenol	35.102	2241	14.63
(*E*)9-Hexadecen-1-ol	35.706	2413	0.24
Phytol	35.712	2620	1.90
Palmitic acid	36.473	2915	0.31

Where, RT: Retention time and KI: Kovats index

P. atlantica inhibits cell proliferation of A549 cell lines in a time (24 h) and dose (0.5–500 µg/mL) dependent manner. After 24 h of treatment with *P. atlantica* extract,

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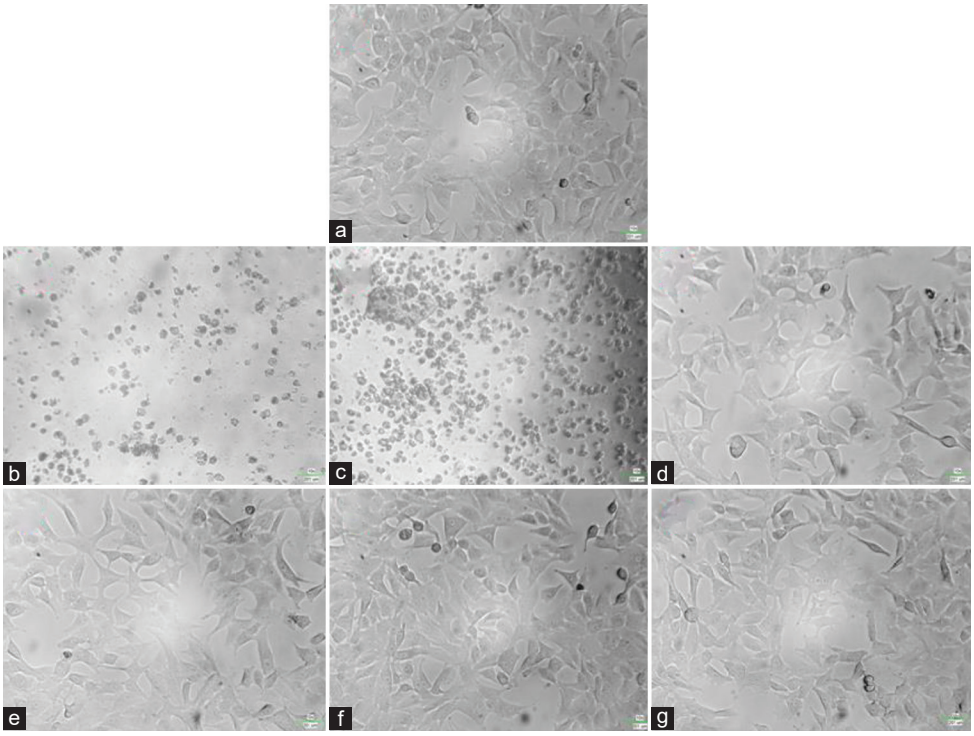

Fig. 3. Cell cytotoxicity produced by ethanolic extract of *Pistacia atlantica var. kurdica* fruits against human lung cancer A549 cell lines. Where, figure (a): Control cells; and figure b to g: *P. atlantica var. kurdica* ethanolic extract: ([b]: 500 µg/mL, [c]: 300 µg/mL, [d]: 100 µg/mL, [e]: 50 µg/mL, [f]: 10 µg/mL, and [g]: 0.5 µg/mL).

the cell viability of A549 cell lines ranged from 93.01 ± 5.24 to 57.69 ± 4.15% at concentrations of 0.5–500 µg/mL, respectively (Figs. 2 and 3). For A549 cell lines, the IC_{50} value of *P. atlantica* ethanolic extract was 21.91 µg/mL after 24 h. Even when the incubation was extended to 24 h, the untreated A549 cells maintained their original morphology and intimate contact with each other, as shown in Fig. 3A. After 24 h' treatment with ethanolic extract of *P. atlantica,* the A549 cells lost their natural form. The elongated spindle-shaped morphology of the A549 cell lines had vanished. Suspension cells (dead cells) were observed after the treatment was extended to 48 h, and more suspension cells were observed after 24 h (Fig. 3B-G). The MTT test for cell cytotoxicity has long been used to screen medicinal plants with possible anticancer activity (Ahamad, et al., 2019).

IV. Conclusion

The GC–MS analysis of ethanolic extract of *P. atlantica var. kurdica* fruits shows the presence of 33 chemical constituents, and isospathulenol, spathulenol, *α*-terpineol, limonene, terpinolene, *β*-myrcene, ethyl pentadecanoate, *β*-pinene, and caryophyllene oxide were found as major chemical constituents. The ethanolic extract of *P. atlantica var. kurdica* shows time- and dose-dependent inhibition of A549 cell lines. The present study explores the chemical composition of *P. atlantica var. kurdica* fruits and provides the scientific basis for its potential use as an anticancer medicine.

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