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CYTOTOXIC ACTIVITY OF *VARTHEMIA IPHIONOIDES* ESSENTIAL OIL AGAINST VARIOUS HUMAN CANCER CELL LINES

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Abstract*: Varthemia iphionoides* is a perennial plant that belongs to the Asteraceae family. This study investigated the cytotoxic effect of *V. iphionoides* essential oil on breast (MCF7), prostate (PC3), and chronic myelogenous leukemia (K562) and normal human fibroblast cell lines using MTT assay and flow cytometric analysis. In addition, GC-MS of the oil was carried out. The IC_{50} values for PC3, MCF7, K562, and fibroblast were 145.3, 188.8, 87.88 and 173.3 µg/mL, respectively. *V. iphionoides* essential oil was most effective against K562. Flow cytometric results for IC_{50} dose of *V. iphionoides* oil on K562 cells showed 32.2% apoptosis in 24 h. GC-MS analysis resulted in the identification of 25 compounds. 1,8-Cineole, borneol, and α-cadinol were the major constituents of *V. iphionoides* volatile oil. In conclusion, this study reveals for the first time the cytotoxic activity of *V. iphionoides* essential oil on K562 cell line which may occur through apoptosis induction.

Keywords: *Varthemia iphionoides*, cytotoxicity, anticancer, apoptosis

Cancer is a leading cause of death worldwide that accounted for 7.4 million deaths (about 13% of total deaths) in 2004. Deaths from cancer worldwide are projected to continue rising, with an estimated 11.5 million deaths in 2030 (1, 2). Chemotherapeutic agents used for treatment of cancers have many toxic effects on the normal cells and therefore the use of medicinal plants takes a great interest. Medicinal plant extracts have significant roles in human health care especially in developing countries (3)

Varthemia iphionoides Boiss. & Blanche (Syn. *Chiliadenus iphinoides*) is a perennial plant, 30-80 cm long that belongs to the Asteraceae family. It rises in rocky habitats such as the Sahara-Arabian, Mediterranean region, and Irano-Turanian regions (4). The base of this shrub is woody with many basal, unbranched stem. Leaves are oblong, simple, entire, sub-sessile, densely hairy, and grayish (5).

In folk medicine, it was used for abdominal pain, diabetes mellitus (6), weight loss (7), cold and hyperacidity treatment (8). The reported biological activities of *V. iphionoides* included inhibitory activity against α-amylase (4), anti-diabetic (9), antioxidant (10), anti-platelet activity (11), antifungal (12), antibacterial (13), antispasmodic (14) and anti-inflammatory (15).

Also, *V. iphionoides* extracts exhibited cytotoxic action on HepG2 human hepatocellular carcinoma cells (16), SKOV3 ovarian carcinoma cells (17, 18), BG melanoma cell line and A549 lung cancer cell line (18). Most importantly, *V. iphionoides* extracts exhibited a distinct cytotoxic effect on human myelocytic leukemia cell line (HL-60) (19). Up to our knowledge, no previous studies have investigated the cytotoxicity of *V. iphionoides* essential oil on cancer cell lines. Therefore, this study aims to determine the cytotoxic activity of *V. iphionoides* essential oil on different human cancer cell lines.

EXPERIMENTAL

Plant collection and extract preparation

V. iphionoides was collected during May 2015 from Batna/Salt, Amman, Hashemite Kingdom of Jordan. It was authenticated by Prof. Barakat E. Abu-Irmaileh, Faculty of Agriculture, University of Jordan. A voucher specimen was stored at Laboratory of Graduate Studies (Aster #5-2015), Al-Ahliyya Amman University, Jordan. The essential oil was prepared by hydrodistillation of fresh aerial parts using a Clevenger-type apparatus for 30 min and stored at -20°C until used. The percentage yield of oil was 0.97% from the fresh plant.

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Gas chromatography-mass spectrometric (GC-MS) analysis

Analysis was performed utilizing a Varian CP-3800 GC/MS/MS-220 (Saturn, the Netherlands) framework, prepared with a DB-5 GC capillary column (95% dimethyl polysiloxane, 5% diphenyl, 30 m × 0.25 mm i.d., 0.25 µm film 1 thickness) was used in this study according to the procedure mentioned Jaffal and Abbas (20). Identification of the chemical compounds in *V. iphionoides* oil was performed by built-in libraries including National Institution of Standards and Technology Co. and Wiley Registry of Mass Spectral Data and by comparing their calculated retention indices with literature values measured on columns of identical polarity and/or co-injection of pure authentic compounds.

Cell lines

The following cell lines were provided by Hamdi Mango Center for Scientific Research, University of Jordan: normal human fibroblast, MCF7, PC3, and K562 cells. The cell lines were grown in a humidified 5% CO_2 atmosphere incubator at 37°C and in RPMI 1640 medium and DMEM high glucose (Euroclone, S.p.A.) containing 10% FBS (Fetal Bovine Serum), 10 g/L penicillin-streptomycin and 10 g/L L-glutamine.

Cell proliferation assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (Promega, USA), is an assay, based on the ability of mitochondrial dehydrogenase to reduce MTT to a purple formazan product. This procedure was used to assess the antiproliferative activity of *V. iphionoides* essential oil in normal human fibroblast, MCF7, PC3, and K562 cells. The cells were suspended at a density of 2×10^4 cells/mL in media. Then 100 µL of each cell type was cultured into each well of 96-well microtiter plates and incubated for 24 h. Addition of

Table 1. Chemical composition of *V. iphionoides* essential oil obtained by GC-MS analysis.

No.	Retention index	Compound	%
1	906	Santolina triene	0.2
2	932	α-Pinene	0.3
3	969	Sabinene	1.0
4	974	β-Pinene	0.3
5	1000	Yomogi alcohol	0.9
6	1027	*p*-Cymene	3.1
7	1035	1,8-Cineole	28.9
8	1149	Camphor	0.7
9	1168	trans-Chrysanthemol	0.7
10	1177	Borneol	23.5
11	1182	1,4-Terpineol	6.2
12	1194	α-Terpineol	6.3
13	1232	Bornyl formate	0.9
14	1296	Bornyl acetate	1.2
15	1283	Chrysanthemic acid	1.8
16	1359	Neryl acetate	2.8
17	1398	cis-Jasmone	0.2
18	1452	Humulene	1.8
19	1456	Aromadendrene	0.2
20	1583	Caryophyllene oxide	0.6
21	1630	γ-Eudesmol	1.2
22	1639	τ-Cadinol	0.1
23	1352	α-Cadinol	12.0
24	1649	β-Eudesmol	3.7
25	1783	γ-Eudesmol acetate	0.1

Table 2. IC_{50} values for the studied cell lines.

Cell line	IC_{50} (µg/mL)	
	V. iphionoides essential oil	Doxorubicin
Normal human fibroblast	173.3	More than 58
PC3	145.3	3.66
MCF7	188.8	4.53
K652	87.88	More than 58

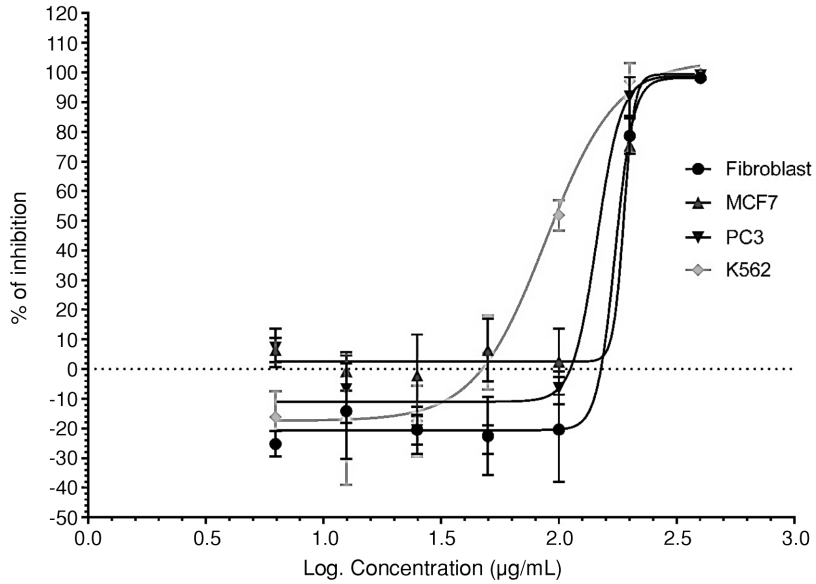

Figure 1. Percentage inhibition of growth by *V. iphionoides* essential oil against the studied cell lines

V. iphionoides essential oil dissolved in DMSO to the wells were completed in triplicate to a final concentration range from 400 µg/mL to 6.25 µg/mL in 2-fold serial dilutions and incubated at 37°C, 5% CO_2 for 24 h. Two controls were used; one contained medium with the vehicle (0.1% DMSO) and cells and the other contained *V. iphionoides* oil and medium without cells to check the effect of background colors. Doxorubicin was used as a positive control (21).

The test was performed according to the manufacturer guidelines. Briefly, 15 µL of MTT dye solution was added to each well and incubated for 4 h. Then, 100 µL of MTT stop solution was added to each well to stop the resulting MTT-formazan product, then the absorbance was measured at 590 nm using a microplate reader (Biotech, USA).

Apoptosis assay

K562 Cells were plated in 6-well plates (5×10^4/well) and were treated with the inhibitory concentration of 50% of cells (IC_{50}) of *V. iphionoides* oil in 24 h. Negative control was treated with DMSO. Apoptosis was monitored using the TACS Annexin V–FITC Apoptosis Detection Kit (R&D Systems, USA) following the kit protocol. The percentage of apoptotic cells was measured by flow cytometric analysis using a FacsCalibur flow cytometer (BD Biosciences/USA).

Statistical analysis

All data were expressed as a mean ± standard deviation ($\chi \pm SD$). All analyses, IC_{50} calculation and graphics were performed using GraphPad Prism version 6.01, 2012 (GraphPad Software, San Diego, USA).

RESULTS

Twenty-five compounds were identified in essential oil through GC-MS analysis (Table 1). 1,8-Cineole (28.86%), borneol (23.53%) and α-cadinol (12.0%) were the major constituents of *V. iphionoides* essential oil.

V. iphionoides essential oil exhibited concentration-dependent growth inhibition. The percentage inhibition of growth by *V. iphionoides* essential oil

against the studied cell lines is shown in Figure 1. The IC_{50} values and the percentage viability are shown in Table 2.

Flow cytometric results for IC_{50} dose of *V. iphionoides* oil on K562 cells, showed 32.2% late apoptosis while 60% showed necrosis in 24 h as shown in Figure 2. that was significantly higher than untreated and DMSO treated cells.

DISCUSSION AND CONCLUSION

In a recent study conducted in the United States, prostate cancer between men and breast cancer between women were the most incident cancers in all racial and ethnic groups. Lung and bronchus cancer and colorectal cancers follow (22). Siegel et al. (2), suggested that 50% of the newly diagnosed cancers in men will be prostate, lung and bronchus, and colorectal cancers. Prostate cancer may count for 27% of detected cases. While breast, lung and bronchus, and colorectal were the most diagnosed types of cancer among women in 2014. Research on the application of essential oils as anticancer agents is not new as reviewed in Raut and Karuppayl (23). However, this study represents the first report on the cytotoxicity effect of *V. iphionoides* oil.

GC-MS analysis of *V. iphionoides* essential oil resulted in the identification of 25 compounds. 1,8-Cineole, borneol and α-cadinol were the major constituents. This agrees with the previous study of the volatile oil of Jordanian *V. iphionoides* in which borneol was the main component in fresh aerial parts. Among other compounds, 1,8-Cineole (eucalyptol) and camphor were present (24). In another study, mostly all identified component of the essential oils of *V. iphionoides* -collected from different geographical regions- were the same as in this study, with interpopulation variation in the percentage of these components (25).

In our study, the essential oil of *V. iphionoides* was rich in the monoterpenes 1,8 Cineole (28.86%) and borneol (23.53%). The anticancer activity of *V. iphionoides* can be attributed at least partially to borneol. The cytotoxicity of borneol and its DNA damaging effects were studied in malignant HepG2 hepatoma cells, malignant Caco-2 colon cells, and nonmalignant human VH10 fibroblasts. Borneol showed cytotoxicity in all cell lines and did not cause DNA strand breaks. Su and collaborators (26) demonstrated that borneol potentiates selenocystine induced apoptosis by enhancement of cellular uptake and activation of reactive oxygen species

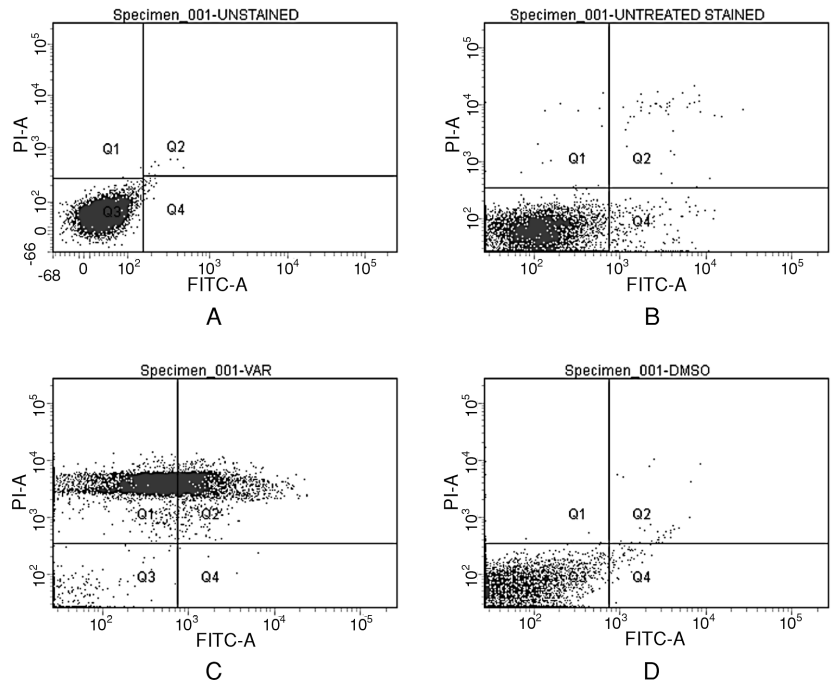

Figure 2. Flow cytometric results for IC_{50} of *V. iphionoides* oil on K562 cells in 24 h. (A) Untreated, unstained cells showed 0.1% apoptotic cells. (B) Untreated stained cells showed 0.4% apoptotic cells. (C) *V. iphionoides* oil-treated cells showed 32% apoptotic cells (cells in upper right quadrant are in late apoptosis). (D) Vehicle-treated cells showed 0.3% apoptotic cells

(ROS) which mediated DNA damage in human hepatocellular carcinoma cells. Another active constituent in *V. iphionoides* oil is 1,8-Cineole. n-Hexane extract of *Callistemon citrinus* containing 1,8-Cineole revealed potential anticancer activity in a dose-dependent manner. Also, cell death was induced through ROS-mediated apoptosis by this extract (27). 1,8-Cineole also induced apoptosis in human leukemia Molt 4B and HL-60 cells (28). Therefore, the apoptotic effect of *V. iphionoides* oil is mainly due to the presence of 1,8-Cineole and borneol.

Among the most common types of cancer in Jordan are breast, prostate, and leukemia which account for 30% of pediatric cases (29). This study was conducted using cell lines related to these cancers. In our study, 200 µg/mL of *V. iphionoides* oil produced 82.8% inhibition on chronic myelogenous leukemia cell line K562, which was the highest among all experimental cell lines. Also, it exerted a higher inhibition rate than doxorubicin in K562 only. Up to our best knowledge, this study represents the first report on the cytotoxic effect of the essential oil of *V. iphionoides* on MCF7, PC3, and K562 cell lines. Also, based on the results of flow cytometry the present study suggested that the mechanism of the cytotoxicity of *V. iphionoides* oil on K562 could be due to the induction of cell death by late apoptosis and/or necrosis mechanism. The more confirmatory experiment suggested being done in the future.

Despite differences in chemical composition between the essential oil and extracts of *V. iphionoides*, different extracts of *V. iphionoides* utilized in other studies also showed promising cytotoxic activity on cancer cell lines. Hexane, chloroform and ethanol extracts inhibited the growth of human myelocytic leukemia (HL-60) cells by 89.0, 68.4 and 62.3%, respectively, at a concentration of 200 µg extract/mL (19). According to Yarmolinsky et al. (18), *V. iphionoides* ethanolic extract exhibited *in vitro* anticancer effects on human leukemia HL-60 cells higher than on other tested human cancer cell lines including SKOV3 ovarian carcinoma cells, BG melanoma cells and A549 lung cancer cells. Al-Dabbas et al. (30) attributed the anticancer activity of *V. iphionoides* to the synergistic antiproliferative effects of flavones. Two 3-methoxyflavones were isolated from this plant and inhibited human leukemia HL-60 cells (30). Similarly, Yarmolinsky et al. (18) found that the flavonoid fraction, but not the polyphenol-rich fraction, of the ethanolic extract of *V. iphionoides,* exhibited the cytotoxic effects.

The proliferation inhibition activity of *V. iphionoides* oil in the present study against studied solid cancer cell lines (MCF7) was less than K562 Leukemia cell line. A concentration of 50 µg/mL of *V. iphionoides* oil in the present study resulted in only 6.43% inhibition of proliferation against MCF7 (93.57% viability). Similar results were obtained by Abu-Dahab and Afifi (31) in which 50 µg/mL of ethanolic extract of leaves and flowers of *V. iphionoides* resulted in 84.95%. viability of MCF7. On the other hand, the effect of *V. iphionoides* essential oil on normal human fibroblasts proliferation was biphasic, cytotoxic at high doses while it promoted proliferation at lower doses, still the calculated IC_{50} for fibroblast was significantly higher than cancer cell lines studied other than MCF7 Also, Budovsky et al. (17) found that *V. iphionoides* ethanolic extract displayed a dual dose-dependent effect on cell growth of primary cultures of human foreskin fibroblasts (HFF) and human dermal fibroblasts (HDF).

In conclusion, this study showed for the first time the cytotoxic activity of *V. iphionoides* essential oil against several solid tumor cell lines as well as K562 cell line which showed the apoptotic effect at the late phase.

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Conflict of interests

No conflict of interest to declare.

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