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Research Article

Anticancer and antioxidant activities of essential oils of *Chiliadenus iphionoides* from Jordan: *in vitro* and *in vivo* study

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Abstract

Medicinal plants have been used since ancient times and may even be considered the origin of modern medicine. Natural products are a potential source for drug discovery and development of cancer treatments. Several pharmacological therapeutic activities were reported for *Chiliadenus iphionoides*. This research was designed to evaluate the essential oil of *Chiliadenus iphionoides* antioxidant potential and antiproliferative activity both *in vitro* and *in vivo.* The essential oil of *Chiliadenus iphionoides* was extracted by hydrodistillation. The chemical composition analysis was performed using gas chromatography. The main compounds identified in *Chiliadenus iphionoides* essential oil were oxygenated monoterpenes, such as eucalyptol (36.08%). A dose-dependent inhibition of cell proliferation was observed after the treatment of various cell lines (A549 (human lung cancer adenocarcinoma), MDA-MB-231 (triple-negative breast cancer cell line), T47 (human breast cancer cell line), EMT6/P (mouse mammary sarcoma cell line)) with increasing concentrations of the essential oil (0.02–2.5 mg/mL). The essential oil of *Chiliadenus iphionoides* was more cytotoxic against EMT6 and T47 cells with IC_{50} values of 0.03±0.03 and 0.08± 0.009 mg/mL, respectively. The essential oil exhibited low cytotoxicity against a normal VERO cell line (IC_{50} value > 4 mg/ml). Balb/C mice were inoculated with EMT6/p breast cancer cells and then treated with (60 mg/kg/day intraperitoneal injection) of essential extract for ten days. Interestingly, a significant ($p < 0.05$) reduction occurred in the tumor size of the treated group compared to the control group. Treatment toxicity was evaluated by measuring liver and kidney parameters. The tumor-bearing mice treated with the *Chiliadenus iphionoides* extract showed normal serum levels of AST, creatinine, and slight elevation in ALT level. The results indicated that *Chiliadenus iphionoides* have anticancer properties both *in vitro* and *in vivo*. We suggest that eucalyptol, a major active component, is a promising candidate for use as an anticancer agent. However, further molecular investigation is required to understand the molecular bearings of *Chiliadenus iphionoides* activity.

Keywords

antitumor, *in vivo*, medicinal plants, natural products, essential oils

Introduction

Cancer is a major leading cause of mortality worldwide and a serious challenge affecting the health of all human societies (Zugazagoitia et al. 2016; Hassanpour and Dehghani 2017). Natural products have been used for thousands of years to prevent or treat various diseases. The importance of bioactive components of plants in pharmacy and medicine is well known since ancient times; the compounds of natural origin are often used as the basic skeleton in developing new anticancer drugs. They are considered promising options to improve treatment efficiency in cancer patients and decrease adverse reactions(Grigalius and Petrikaite 2017; Choudhari et al. 2020). Also, natural remedies and their derivatives strongly supply our understanding of cancer development and treatment mechanisms. In the last decade, more than 25% of new drug molecules were directly obtained from the plant mines, and another 25% were chemically tailored herbal substances(Grigalius and Petrikaite 2017).

In the Middle East countries, herbs are used both as a food source and for therapeutic purposes. *Chiliadenus iphionoides* (*C. iphionoides*), also known as *Varthemia iphionoides* (Asteraceae), is a bushy perennial plant that arises in the Mediterranean, Irano-Turanian, and Saharo-Arabian regions (Sbieh et al. 2022). Several pharmacological therapeutic activities have been reported for *C. iphionoides*, including antidiabetic, antioxidant, anti-inflammatory, anti-platelet, antimicrobial, and cardioprotective. Also, it exhibited cytotoxic activation against numerous types of cancer (Abbas et al. 2019). In folk medicine, *C. iphionoides* was used for abdominal pain, diabetes mellitus, weight loss, cold, and hyperacidity treatment. It is commonly used in Jordan for the treatment of gastrointestinal disorders (Haddad et al. 2016).

A previous study showed cytotoxic activity against breast cancer cell lines (EMT6, MCF-7, and T47D) using *C. iphionoides* dichloromethane extract. The extract induced apoptosis, inhibited VEGF (Vascular endothelial growth factor) expression and stimulated the immune system (Halees et al. 2019). In another study, the activity of *C. iphionoides* essential oil revealed apoptosis induction of several solid tumors, including prostate (PC3), breast (MCF7), and chronic myelogenous leukemia (K562) (Abbas et al. 2019).

To the best of our knowledge, this study represents the first report on the cytotoxic effect of the essential oil of *C. iphionoides* on A549 (human lung cancer adenocarcinoma), MDA-MB-231 (triple-negative breast cancer cell line), T47 (human breast cancer cell line), EMT6/P (mouse mammary sarcoma cell line) VERO (healthy kidney epithelial cell line). The present study identified the chemical composition of the essential oil of the aerial parts and evaluated the antioxidant activity and the antiproliferative activity against different human cancer cell lines, *in vitro* and *in vivo*.

Materials and methods

Plant material

The aerial parts of *Chiliadenus iphionoides* were collected by the end of September from As-Subayhi, Al Balqa, Jordan. The botanical authentication and identification of the plant were performed by Dr. Hatem Taifour, Royal Botanic Garden, Jordan.

Essential oil extraction

The fresh aerial parts of *C. iphionoides* were subjected to hydrodistillation using a Clevenger-type apparatus. 250 gm of the fresh aerial parts were immersed in a 1000 mL water for 2 hrs. The essential oils obtained were stored in amber glass vials in a refrigerator at 4 °C.

GC-MS analysis

The chromatographic analysis of the essential oils was analyzed using a Varian 4000 GC–MS/MS system (Varian, Palo Alto, CA, USA). The compounds were separated on a 30 m × 0.25 mm × 0.25 µm HP–5 ms column (Agilent, USA). The column temperature was increased from 40 °C to 320 °C at a rate of 4 °C/min; injector temperature 250 °C; split ratio 1:100; injection volume 1 µL. The MS parameters were as follows: MSD detector, with transfer line temperature of 280 °C; ion source temperature, 250 °C; scan range, 30–650 m/z.

Determination of antioxidant activity

The antioxidant potential of the essential oils of the plant was determined using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) method, as described by Mustafa and colleagues (Mustafa et al. 2021). In a test tube, 4.5 mL of (0.1 mM) ethanolic DPPH solution and 0.5 mL of essential oil solution (in ethanol) were combined. After mixing, the solution was stored in the dark for 30 min at room temperature, and DPPH color was detected spectrophotometrically at 517 nm. The findings were represented as mg Trolox equivalent (TE)/kg dry weight (DW) using Trolox as the reference antioxidant.

Determination of antiproliferative activity

Cell lines and Cell culture Conditions

Five cell lines were used to examine the anticancer activity of *C. iphionoides* essential oil, namely: A549 (human lung cancer adenocarcinoma), MDA-MB-231 (triple-negative breast cancer cell line), T47 (human breast cancer cell line), EMT6/P (mouse mammary sarcoma cell line) VERO (healthy kidney epithelial cell line). The cell lines were obtained from the European

Collection of Cell Cultures (Salisbury, UK). The cells were cultured in a complete medium and incubated at 37 °C in a 5% CO_2, 95% humidity incubator. MDA-MB-231 and A549 cell lines were cultured in a complete DMEM medium. EMT6/P was cultured in a complete MEM medium. All culture media were supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin, and 0.1% gentamycin solution.

Antiproliferative assay

The antiproliferative activity of *C. iphionoides* essential oil was investigated using MTT (3-(4,5 Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) kit (Bioworld, UK). Cells were seeded in 96-flat bottom-well plates at a density of 3×10^3 cells/well for overnight incubation. Cells were incubated for 24 hrs at 37 °C, 5% CO_2. After incubation, the media was completely removed from each well, and the adherent cells were treated in triplicates with decreasing concentrations of *C. iphionoides* essential oils (2.5–0.02 mg/mL). Cells were incubated for 48 hrs. After that, 10 µL of MTT solution was added to each well and incubated for 3 hrs. Followed by the addition of 100 µL of DMSO and cultivated for an hour. A microplate reader (Biotek, Winooski, VT, USA) was used to measure the resulting color at 550 nm. Percentage cell survival was determined for all treatments and compared to the negative control (untreated cells).

In vivo proliferative assay

Experimental animal

Eighty adult healthy female Balb/c mice, weighing 23–25 g, were used in this study. Animal care and use were conducted according to standard ethical guidelines, and all experimental protocols were approved by the Research and Ethical Committee at the Applied Science University, Jordan (approval number: 2016PHA9). The mice were kept under standard conditions. Mice were kept separately in cages covered with wooden shavings and maintained under 12 hrs of light and 12 hrs of the dark cycle, 22–25 °C, 50–60% humidity, with constant ventilation.

Antiproliferative activity of experimental animals

The mouse mammary tumor cells EMT6/P were collected by means of trypsinization, followed by centrifugation, rinsing, and re-suspension in minimal essential medium (MEM), resulting in a cell density of 1 million cells within a 100 µL volume. To evaluate cell viability, the trypan blue exclusion method was applied. Mice were injected subcutaneously into the abdominal area with a tumorigenic dose of 1×10^6 cells in 0.1 mL of MEM. After seven days, tumor size was measured using a digital caliper. Then, the tumor volume was calculated using the following formula: $(A \times B^2 \times 0.5)$, where A was the length of the longest aspect of the tumor, and B was the length of the tumor aspect perpendicular to A.

Tumor-bearing mice were randomly divided into two groups (n=9 for each group). The control group was injected intraperitoneally with a vehicle of olive oil. Then, the treatment group was injected intraperitoneally with 60 mg/kg/day with the essential oil (10% of the calculated LD_{50}) as mentioned in (Varthemia iphionoides and Pelargonium graveolens Extracts as a Treatment of Breast Cancer Implanted in Diabetic Mice). All treatments continued for seven days. At the end of the study, blood samples were withdrawn for each group, and tumors were measured. Then, mice were sacrificed, and their tumors were dissected and preserved in a 10% saline formalin solution for subsequent testing.

Assessment of liver and kidney toxicity

Liver and kidney toxicities were assessed after the treatment with *C. iphionoides*. Alanine transaminase (ALT), Aspartate transaminase (AST), and creatinine in serum samples were measured to evaluate liver and kidney functions by following the instructions of the relevant kits (BioMajesty, Germany).

Statistical analysis

Data were presented using the mean ± SEM (Standard Error of Mean). The statistical significance among the groups was determined using SPSS one-way analysis of variance (ANOVA) and student's t-test. Differences between groups were considered significant when the p-value was less than 0.05 ($p < 0.05$). IC_{50} values were calculated using non-linear regression in SPSS (Statistical Package for the Social Science, Chicago, Illinois, version 24).

Results

GC-MS analysis of essential oil of *C. iphionoides*

Twenty-three compounds were identified in essential oil through GC-MS (Table 1). The analysis of the essential oils revealed the presence of high concentrations of eucalyptol (42.6%), and trans-chrysanthemol (20.65%). Some other compounds were detected, including Yomogi alcohol (10.04%), γ- terpinene (4.09%), and o-cymene (3.40%).

Most of the essential oil compositions are terpenes, monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes. (Fig. 1).

Antioxidant activity

The antioxidant activity of the essential oil of *C. iphionoides* was 1.29±0.3 mg TE/g DW.

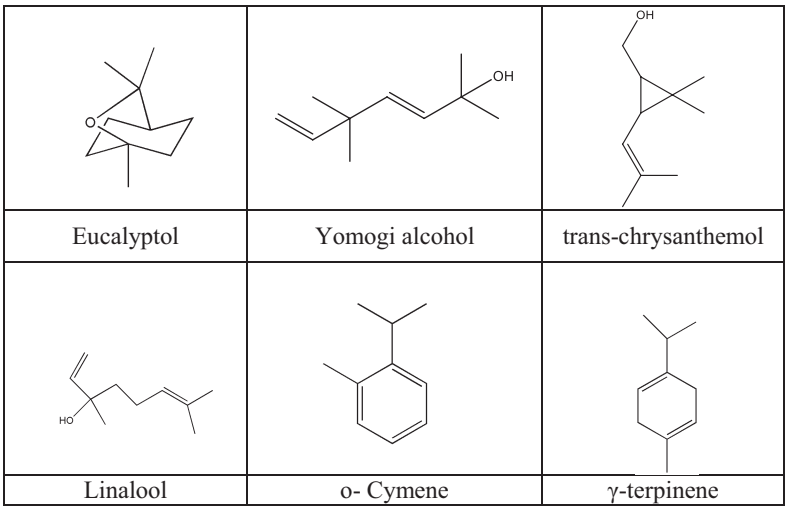


Figure 1. Major compounds identified in *C. iphionoides* essential oil by GC-MS analysis.

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

No	Rt	Compound	Percentage (%)	Method of Identification
1	8.55	Santolina triene	0.44	GC-MS
2	9.38	α-Phellandrene	0.06	GC-MS
3	10.2	Camphene	0.21	GC-MS
4	11.11	Sabinene	0.99	GC-MS
5	12.06	Yomogi alcohol	10.04	GC-MS
6	12.79	Carene	3.15	GC-MS
7	13.08	o-cymene	3.40	GC-MS
8	13.43	Eucalyptol	42.60	GC-MS
9	14.37	γ-Terpinene	4.09	GC-MS
10	15.46	trans-Linalool oxide	0.55	GC-MS
11	15.97	Linalool	1.37	GC-MS
12	17.45	Pinocarveol	1.87	GC-MS
13	18.23	trans-chrysanthemol	20.65	GC-MS
14	19.53	α-terpineol	0.84	GC-MS
15	20.32	trans-carveol	0.40	GC_Ms
16	24.8	Geranyl acetate	0.33	GC-MS
17	25.48	p-Mentha-1,5-dien-8-ol	0.28	GC-MS
18	27.09	Caryophyllene	0.96	GC-MS
19	33.2	Humulenol-II	3.54	GC-MS
20	33.9	Isoaromadendrene epoxide	3.15	GC_MS
21	34.1	α-cadinol	1.08	GC_MS

Rt: retention time.

Antiproliferative activity

A dose-dependent inhibition of cell proliferation was observed after treatment of various cell lines with increasing concentrations (0.02–2.5 mg/mL) of *C. iphionoides* essential oil (Fig. 2).

The essential oil tested on the normal fibroblast cells showed safety and selectivity. The essential oil of *C. iphionoides* was more cytotoxic against EMT6 and T47 cells with IC_{50} values of 0.03±0.03 and 0.08± 0.009 mg/mL, respectively (Table 2).

In-vivo *antiproliferative effect*

Tumor size in mice treated with *C. iphionoides* essential oil was significantly ($p < 0.05$) decreased compared to the negative control (Figs 3, 4). Essential oil showed significant inhibition of tumors where a reduction in tumor size was recorded (-57.2%) compared with the untreated control group (+76.4%). For both groups, there was no death recorded. The percentage of mice with no detectable tumor in the essential oil-treated group was 44%, and mice showed normal activity with no side effects.

Table 2. IC_{50} (mg/mL) of *C. iphionoides* essential oil on different cell lines.

Cell line	IC_{50} (mg/mL)
MDA-MB-231 (triple-negative breast cancer cell line)	1.51± 0.04
T47 (human breast cancer cell line)	0.08± 0.009
A549 (human lung cancer adenocarcinoma)	1.18± 0.04
EMT6/P (mouse mammary sarcoma cell line)	0.03± 0.03
VERO (healthy kidney epithelial cell line)	Nontoxic

Results are represented as mean ± SD, n = 3.

Effects on serum level of ALT, AST, creatinine

The plasma level of AST for the tumor-bearing mice group was comparable to normal mice. However, the mildest elevation of ALT was observed in the experimental group treated with the *C. iphionoides* essential oil (Fig. 5A). The creatinine level for tumor-bearing mice groups was comparable to normal mice (Fig. 5B).

Discussion

The present study was conducted to examine the phytochemical composition and potential biological activities of the essential oil of *C. iphionoides*. The antioxidant and anticancer activities of the essential oils were investigated both *in vitro* and *in vivo*. The extraction of essential oil was performed using the aerial parts of the plant, and the phytochemical composition was analyzed with GC-MS in a previously developed and validated method. The essential oil showed potent antioxidant activity, which might be behind the ability of our essential oil to reduce cancer cell viability and promote apoptosis.

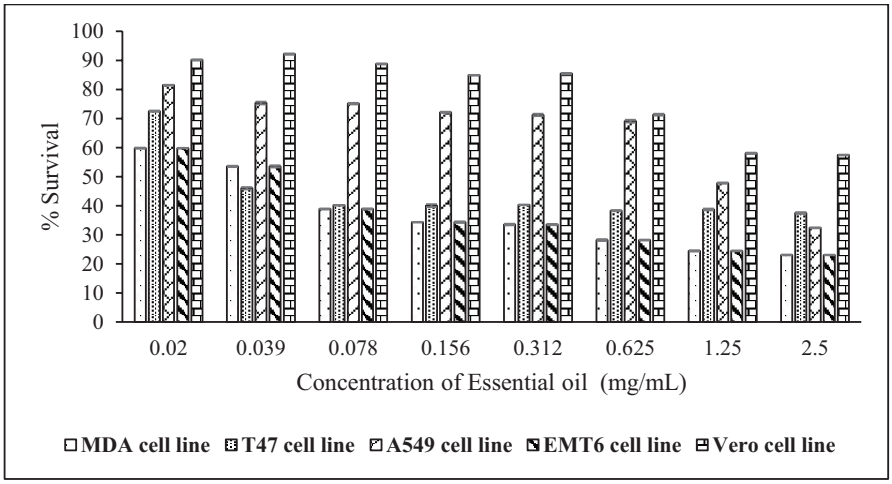


Figure 2. Antiproliferative activity of essential oil of *C. iphionoides* on MDA-MB- 231, T47, A549, EMT6, Vero cell lines

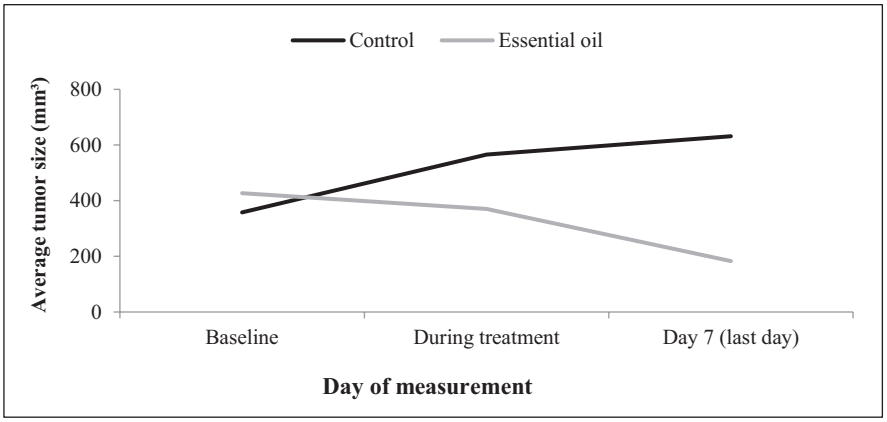
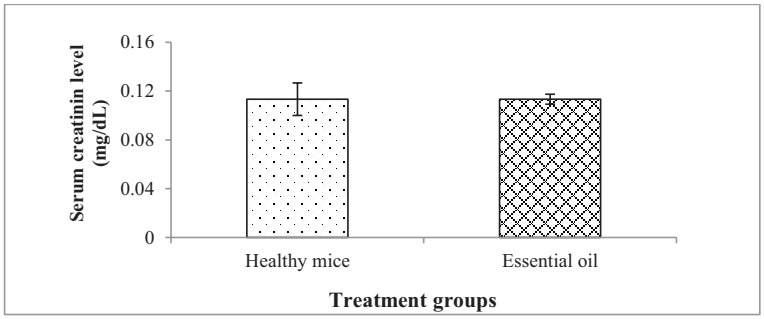


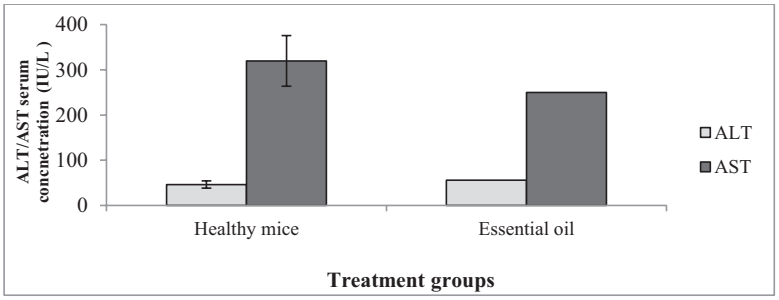
Figure 3. A plot of change in average tumor size (mm^3) vs. time in (days) of treatment iEMT6/P ($p < 0.05$) compared to the control group

Figure 4. Effect of C. *iphionoides* essential oil on tumor size and cure percentage. Treatment with *C. iphionoides* essential oil reduced tumor size and increased cure percentage compared to the negative control. (N = 9 mice) in each group.

Cancer is one of the leading causes of death in the world. Because the current chemotherapeutic treatments are causing serious side effects, researchers are searching for a novel candidate with less harmful side effects and more effective action against cancer. Although *C. iphionoides* has long been used to treat a wide range of illnesses, insufficient studies investigated how effective it is at fighting cancer. Moreover, to the best of our current knowledge, our work is the first to report the antioxidant activity of *C. iphionoides* essential oil and their effects on the cell lines tested: A549 (human lung



(A)



(B)

Figure 5. Effect of *C. iphionoides* treatment on serum levels of (**A**) Creatinine and (**B**) ALT, AST. Serum creatinine level is expressed in mg/dL, and concentrations of ALT and AST are expressed by IU/L. Mice were treated with (60 mg/kg/ day) of essential oil. Results are expressed as means (bars) ± SEM (lines). ALT, alanine transaminase; AST, aspartate transaminase.

cancer adenocarcinoma), MDA-MB-231 (triple-negative breast cancer cell line), T47 (human breast cancer cell line), EMT6/P (mouse mammary sarcoma cell line). In this investigation, *C. iphionoides* exhibited a dose-dependent capacity to suppress the development of cancer cells with potent antioxidant activity. The results of the phytochemical screening of *C. iphionoides* essential oil showed several monoterpenes, and sesquiterpenes that align with previous studies, except for borneol (Avato et al. 2004; Abbas et al. 2019; Al-Tawarah et al. 2020; El Yaagoubi et al. 2021). Previously, the essential oil was found to contain eucalyptol and borneol as the main constituents. It is well known that the composition of essential oil can change with the time of the year and, consequently, the time of collection (Figueiredo et al. 2008).

Essential oils detected in the aerial part of *C. iphionoides* are a vital class of secondary metabolites that own various pharmacological activities (de Sousa et al. 2023). In the same context, numerous investigations have revealed that monoterpene exhibits anticancer properties (Al-Tawarah et al. 2020). The anticancer activity of *C. iphionoides* can be attributed at least partially to eucalyptol. The cytotoxicity of eucalyptol was proven against hormone-refractory prostate cancer and drug-resistant human lung cancer (Boukhatem et al. 2020). Other studies indicated that eucalyptol can induce apoptosis in human colon cancer cell lines HCT116 and RKO. Eucalyptol treatment was associated with inactivation of survivin and Akt and activation of p38, finally causing apoptosis (Cha et al. 2010; Murata et al. 2013). In a study by Murrata and colleagues, eucalyptol showed significantly inhibited tumor progression in xeno-transplanted SCID mice compared to a control group (Murata et al. 2013). On the other hand, other studies showed that eucalyptol antiproliferative properties are mediated by inducing apoptosis, by increasing cellular uptake and triggering reactive oxygen species (ROS), which cause DNA damage, amplifying the effects of selenocysteine-induced apoptosis (Sampath et al. 2017; Abbas et al. 2019; Tiwari et al. 2023).

Tumor size was measured at the beginning of the experiment, at the middle, and at the end and compared to the control group findings. Tumor size in mice treated with *C. iphionoides* essential oil was significantly decreased compared to the negative control, and interestingly, the percentage of mice with no detectable tumor in the essential oil-treated group was 44%. Such a decrement in tumor size could result from the presence of effective anticancer agents. In the literature, no studies were available to evaluate the effect of essential oil of *C. iphionoides in vivo*. In this study, essential oil significantly suppressed tumor growth. This can be attributed to the ability of essential oil to induce apoptosis. However, various studies have disclosed that some constituents of essential oils can be highly effective in shrinking tumors while exhibiting low toxicity (Estanislao Gómez et al. 2016; Mohamed Abdoul-Latif et al. 2023).

Therefore, the action of essential oil on cancer cells is the result of the effect of each individual compound modulated by the potential action of the synergistic effect. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine were measured to assess the possibility of liver and kidney toxicity development after treatment with the essential oil of *C. iphionoides*. No significant differences were observed in the ALT, AST, and creatinine concentration of the treated group compared to

the control group or to the normal mice values. Accordingly, the essential oil of *C. iphionoides* at the used concentration of 60 mg/kg/day can be considered safe.

Conclusion

Exploring the anticancer properties of essential oil is a current research area that needs to be studied in parallel with conventional chemotherapy. The compositions of the aroma profile and hydrodistilled essential oils of the aerial parts of *C. iphionoides* grown in Jordan were investigated for antiproliferative activity *in vivo* for the first time. Hydrocarbon monoterpenes, oxygenated monoterpenes, and oxygenated sesquiterpenes dominated the hydrodistilled oil. The oil exhibited promising antiproliferative activity with an excellent safety profile. It is recommended to evaluate the antiproliferative activity of the essential oil using other cancer cell lines.

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