Antioxidant, antibacterial, and anticancer properties of *Haloxylon salicornicum* extracted by microwave-assisted extraction

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Background
The wild plant *Haloxylon salicornicum* is a desert shrub species that is present in North America and some Middle Eastern countries and is used as a source of vegetation. The reports demonstrated the significance of its bioactive compounds by extracting them with different solvents, such as hexane, methanol, and water fractions using standard extraction methods and evaluating their biological functions as antioxidants, antibacterial, and anti-inflammatory.
Objective
To analyze the chemical compositions of *H. salicornicum* extracted by microwave-assisted extraction (MAE) using the antioxidant, antibacterial, and anticancer activities.
Materials and methods
In this research, ethanol solvent and simple MAE with gas chromatography–mass spectrometry analysis were used to classify the chemical compositions, and the ethanol extract was evaluated for antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl process, antibacterial activity, anticancer activity using liver cancer cells (HCAM) through apoptosis (AO/EB staining), and flow cytometry analysis for apoptosis and cell cycle arrest ratios.
Results and conclusion
The gas chromatography–mass spectrometry study revealed ~33 compounds. *H. salicornicum* antioxidant activity was IC_{50}=4.120 μg/ml as compared with vitamin C IC_{50}=4.898 μg/ml as a positive regulation. Antibacterial activity of *H. salicornicum* extract showed a significant inhibition toward *Staphylococcus aureus* and *Escherichia coli*. *H. salicornicum* cytotoxicity against liver cancer cells (HCAM) at 1000 μg/ml showed a significant inhibition ratio (42.35%). The AO/EB staining revealed DNA damage and apoptosis in the morphology of the cells. Early and late apoptosis were established, and the cell cycle was stopped in G1 phase. Our findings indicate that *H. salicornicum* is a valuable medicinal plant with biological applications. As a result, future research will focus on isolating the responsible natural molecules using MAE and mechanic studies.

Keywords:
flow cytometry, *Haloxylon salicornicum*, Iraqi wild plants, liver cancer cells, microwave-assisted extraction, natural antioxidants

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Introduction

Medicinal plants improve human health by strengthening the immune system and treating a wide range of diseases [1]. Traditional medicine made use of plants in China, India, Pakistan, and the Middle East [2,3]. Plant crudes are a rich source of antibacterial, antifungal, anti-inflammatory, anticancer, and other natural molecules [4]. In recent years, scientists have concentrated on discovering new plants with anticancer properties [5]. Many plants have been studied, and clinical trials for cancers, such as breast, cervical, colon, leukemia, and ovarian, have been conducted [6]. Furthermore, because of chemical components, such as flavonoids, saponin, phenols, alkaloids, and so on, conventional drugs have been used to treat a variety of diseases, including liver disease [7]. Plant-caused hepatotoxicity is unknown, and extensive clinical studies are required to assess hepatotoxicity of medicinal plants [8]. Because medicinal plants have the ability to cure liver diseases and cancer, it is critical to discover new plants to treat liver diseases. *Haloxylon salicornicum* is a desert plant in the Amaranthaceae family that grows in Jordan, Egypt, Saudi Arabia, Kuwait, Oman, the United Arab Emirates, Afghanistan, Pakistan, and Iraq [9]. According to Egyptian and Pakistani research, aerial parts of

H. salicornicum contain bioactive compounds such as alkaloids and terpenes with biological actions such as antituberculosis, hepatoprotective effect, and antifungal [10–13]. There is no information available in Iraq about the phytochemical composition or biological activities of *H. salicornicum*.

For the first time, this study used a microwave-assisted extraction (MAE) method and an ethanol solvent to classify the chemical composition of *H. salicornicum* by gas chromatography–mass spectrometry (GC–MS) analysis and evaluated its antioxidant, antibacterial, and anticancer properties via apoptosis and cell cycle arrest.

Materials and methods

Plant collection

On March 19, 2019, a *H. salicornicum* plant was collected in Basrah Governate, Abo-Alkhaseeb region, AlSeeba district, Sihan village, southern Iraq. Dr Sarmad Ghazi of the Agriculture College at the University of Basrah in Iraq described the plant species. Within a week, 100 g of plant was dried, mechanically ground, and stored at 4°C. The study was conducted between March and December of 2020.

Methods

Haloxylon salicornicum extraction method

Five grams of *H. salicornicum* was added to 100 ml of absolute ethanol 98% and thoroughly mixed before being left for 20–30 min inside the MAE instrument. The extraction time was set to 5 min and the temperature was set to 50°C. The extracts were cooled for 10–20 min before being centrifuged for 15 min at 3000 rpm and filtered through Whatman filter paper 20 m. The ethanol extract was collected and measured the amount to be 100 mg, which was saved for future use [14].

Gas chromatography–mass spectrometry analysis of Haloxylon salicornicum extracted by microwave-assisted extraction

A GC coupled to an Agilent 7890B GC with 5977 A MSD and Mass Hunter workstation software was used for GC–MS analysis. The column temperature gradient was started at 40°C in a phenyl methyl siloxane 5% column under pressure of 6.0799 psi, and a linear gradient was obtained by raising the temperature from 50 to 280°C at a rate of 10°C/min. The injector was held at 290°C for 4 min, until the solvent was turned off. At a flow rate of 1 ml/min, helium was used as the carrier gas. In pulsed splitless injection, the molecular weight test range was 35–650 m/z, test rate 1562 (N2). The extracts were purified using syringe filters, and 1 µl of *H. salicornicum* ethanol extract was injected into the GC column and analyzed using the NIST library.

Antioxidant activity method

H. salicornicum was used to assess antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DDPH), as described by Mutlaq *et al*. [15], with some modifications. In a nutshell, different concentrations of *H. salicornicum* (10, 20, 30, 40, 50, 60, 70, and 80 µl) were applied to 100 µl of DPPH (1 mm) in a 96-wellplate and left in the dark for 30 min. The absorbance at 490 nm is then measured using a microplate reader (ELISA; Asyshitech, Manchester, UK). The following equation was used to calculate the antioxidant activity of *H. salicornicum* extract:

$$\text{Antioxidant activity}\% = \{1 - [A_s/A_c]\} \times 100.$$

where A_s=absorbance of HS+DPPH; A_c=absorbance of DPPH as control.

Antibacterial activity

The disc diffusion method was used to assess the zones of inhibition of the *H. salicornicum* extract against four Gram-positive and Gram-negative bacteria pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*). To summarize, 1000 µg/ml of the extract was applied to each plate (7-mm-diameter holes cut in the agar gel, 20 mm apart from one another). Under aerobic conditions, the plates were incubated for 24 h at 36°C. Confluent bacterial growth was observed after incubation. Inhibition zones were measured in millimeters (mm) [16].

Cytotoxicity assay

Human liver cancer cells (HCAM) were grown in a 10-cm plate containing RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified environment with 5% CO_2.

HCAM cells were grown in 96-well plates for 24 h before being treated with various concentrations (0–2000 µg/ml) of *H. salicornicum* for 24 h. Cell viability was assessed in a microplate reader at 620 nm (ELISA, Asyshitech) [17].

DNA damage detection by AO/EB staining

Trypsin fersin was added to HCAM cells by trypsinization process and then added medium RPMI-1640, placed a clean and sterilized slide on the planted cells' dish, and started planting 5000 of HCAM cells on the slide cover and then covered it

tightly by parafilm sheet for 24 h in the incubator 5% CO_2 at 37°C, according to Al-Shawi *et al.* [18] method and some modifications. After 24 h, discard the medium and add 1000 µg/ml of *H. salicornicum*, then close the dish tightly, and reincubate for 24 h. Next, lift the slide cover and place it in a clean slide, then add 70 µl of AO/EB stain, and immediately photograph the slide under a fluorescent microscope (Flourecent Microscope; Zeiss Axiolabe, CE, Germany).

Flow cytometric analysis

Apoptosis

Flow cytometric analysis was used to predict early and late apoptosis using the Khan *et al.* [19] system with some modifications. HCAM cells were treated for 24 h with or without 1000 µg/ml *H. salicornicum*. The cells were harvested, rinsed twice with PBS, and labeled with 5 ul of FITC-conjugated annexin V, as directed by the manufacturer. The samples were immediately analyzed on a flow cytometer (Beckman Coulter, Epics XL, Flowcytometry, PARTEC; model: CyFlow, Föreningsgatan, Landskrona, Germany; software FLOWMA) after being incubated in the dark for 10 min and then labeled with PI.

Cell cycle arrest

For 24 h, HCAM cells were treated with and without 1000 µg/ml *H. salicornicum* extract. The cells were then washed with PBS and fixed in 70% ice-cold ethanol overnight at 4°C. After washing twice with PBS, cells were stained for 30 min in the dark at room temperature with a solution containing 50 µg/ml PI and 100 µg/ml RNase A. Flow cytometry (Beckman Coulter, Epics XL) was used to examine the stained cells [19].

Statistical analysis

The cytotoxicity and antioxidant experiments were replicated thrice. GraphPad Prism 8.1 was used to estimate the IC_{50} value of antioxidant activity using a one-way variable equation.

Results and discussion

Gas chromatography–mass spectrometry analysis of *Haloxylon salicornicum* extracted by microwave-assisted extraction

GC is a popular technique for analyzing chemical compositions in medicinal plants using polar and nonpolar solvent extracts. It hastens the precompositions and provides an idea of the chemical components in the extract. Thus, many methods are used to manufacture chemical components from herb crudes, such as solvent extraction, distillation, pressing, and sublimation. However, solvent extraction is the most commonly used process, according to the extraction theory. Aside from that, the effectiveness of extraction techniques varies, depending on a few factors such as heating, time, and energy type [20,21]. MAE is a low-cost and quick extraction technique that has demonstrated high efficiency in the extraction of medicinal plants, especially with ethanol solvent [22]. In this research, we used *H. salicornicum* for the first time with ethanol solvent and MAE to obtain the ethanol extract, and we analyzed the chemical compositions using GC to classify the form of compounds according to the NIST library. Table 1 displayed the ~33 chemical compounds found by GC–MS, some of these compounds had a high area percent in the ethanol extract. The compound 9,12,15-Octadecatrienoic acid (Z,Z,Z) had the highest peak (RT=25.078 min) followed by compound n-hexadecanoic acid (RT=22.43 min), n-Tetracosanol-1 (RT=30.005 min), 9-Octadecenamide (Z) (RT=25.913 min), Z-8-Methyl-9-tetradecanoic acid (RT=25.286 min), Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl) ethyl ester (RT=27.080 min), Cis-Vaccenic acid (28.477 min), and the remaining compounds, each with its own set of peaks and retention periods. In some countries, the chemical constituents of *H. salicornicum* have been identified, for example, Ashraf *et al.* [23] who conducted research in Pakistan discovered that the plant stem and leaves were high in essential biological components such as alkaloids, saponins, tannins, glycosides, and cardiac glycoside. Ullah *et al.* [24] conducted a study in Saudi Arabia using aqueous extract of *H. salicornicum* and analyzing the chemical constituents, which revealed that the majority of compounds differed from GC–MS analysis in this study. These differences of compounds can be related to the polarity of the extraction solvent and the microwave-assisted technique since they both have high selectivity. In addition, the type of soil and environment influences the chemical constituents of *H. salicornicum*.

Antioxidant and antibacterial activities of *Haloxylon salicornicum* extracted by microwave-assisted extraction

The antioxidant role of medicinal plants has increased the importance of their use in traditional medicine. This role is concerned with the chemical composition of medicinal plants, such as flavonoids and phenolic compounds [25,26]. In this report, the antioxidant activity of *H. salicornicum* ethanol extracted by MAE was evaluated for the first time. It demonstrated a high significant antioxidant function with an IC_{50} value of 4.120 µg/ml as compared with vitamin C IC_{50} value of 4.898 µg/ml, Fig. 1; the IC_{50} values suggested the extract good antioxidant function.

Table 1 According to the NIST library, the chemical compositions of *Haloxylon salicornicum* extracted by microwave-assisted extraction using gas chromatography–mass spectrometry analysis revealed ~33 bioactive compounds

Numbers	RT (min)	Name of compound
1	6.415	Glycine, N,N-dimethyl-, methyl ester
2	8.006	Glycine, N,N-dimethyl-, ethyl ester
3	9.007	2-Furancarboxaldehyde, 5-methyl-
4	10.542	Octan-8-ol, 2,5-diaza-2,5-dimethyl-
5	13.127	dl-Phenylephrine
6	14.913	Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate
7	15.754	2-Cyclohexylpiperidine
8	17.130	Ascaridol
9	17.331	1,4-Dimethoxy-2,3-dimethylbenzene
10	17.414	3(N,N-Dimethylaurylammonio) propanesulfonate
11	19.728	1-Tetradecanamine, N,N-dimethyl
12	20.326	Tetradecanoic acid
13	20.465	6-hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one
14	21.153	Phytol, acetate
15	21.584	1,2-15,16-Diepoxyhexadecane
16	22.431	n-Hexadecanoic acid
17	23.786	Phytol
18	24.078	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-
19	24.266	Octadecanoic acid
20	24.391	Hexadecanamide
21	25.287	Z-8-methyl-9-tetradecanoic acid
22	25.913	9-Octadecenamide, (Z)-
23	26.121	Octadecenamide
24	26.941	Z-8-methyl-9-tetradecanoic acid
25	27.08	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl) ethyl ester
26	27.539	9-Octadecenamide, (Z)-
27	28.477	Cis-Vaccenic acid
28	28.616	Octadecanoic acid, 2-hydroxyl-1-(hydroxylmethyl) ethyl ester
29	30.005	n-Tetracosanol-1
30	31.472	Gamma-tocopherol
31	32.000	Octacosanol
32	34.293	(E)-Labda-8(17),12-diene-15,16-dial
33	35.057	Gamma-sitosterol

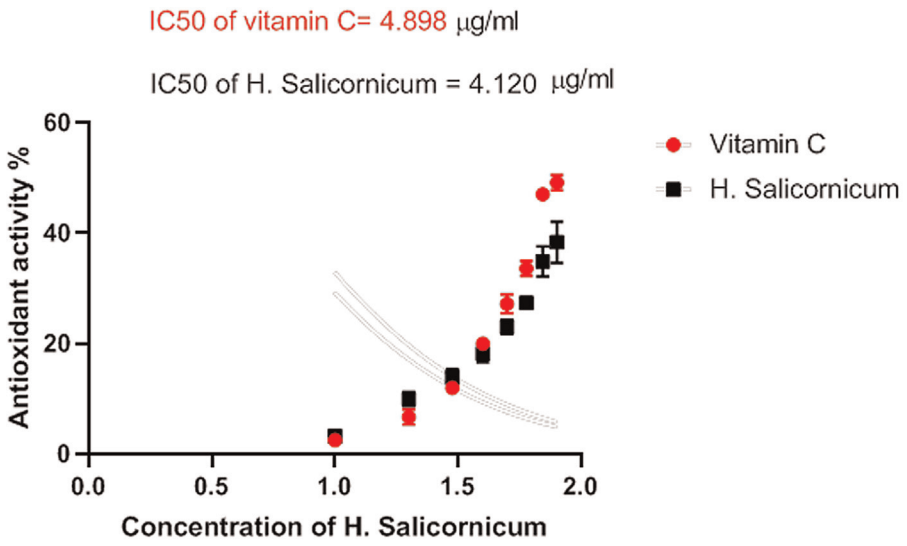
Antibacterial activity of *H. salicornicum* extracted by MAE against four Gram-positive and Gram-negative bacteria pathogens (*S. aureus*, *E. coli*, *K. pneumonia*, and *P. aeruginosa*) has yet to be identified. As a result, we used a disc diffusion method with 1000 µg/ml of ethanol extract to target the infect bacteria pathogen. Table 2 shows the zones of inhibition for two pathogens, Gram-positive *S. aureus* and Gram-negative *E. coli* (both 9 mm). When compared with positive standard antibiotics, the antibacterial activity was weak. These high antioxidant and weak antibacterial activities paved the way for research to examine the anticancer properties of *H. salicornicum* extracted by MAE using liver cancer cells (HCAM).

Anticancer activity of *Haloxylon salicornicum* extracted by microwave-assisted extraction

To the best of our knowledge, no study has been conducted to examine the anticancer activity of *H. salicornicum* extract. As a result, this is the first study to look at the anticancer properties of *H. salicornicum* extracted by MAE and using ethanol solvent toward liver cancer cells (HCAM).

The prescreening of *H. salicornicum* extract revealed that the concentration 1000 µg/ml has a major toxicity on liver cancer cells (HCAM) with a killing activity of 42.35%. This concentration was chosen over the others because it was less toxic to normal cells while being more toxic to liver cancer cells, and it was used to detect apoptosis morphology in HCAM cells using AO/EB staining. The AO/EB staining method is used to visualize nuclear changes and apoptotic body formation, which are hallmarks of apoptosis. To quantify apoptosis, cells are counted under a fluorescence microscope. The green color of cells represents untreated live cells, the green-to-orange color represents early apoptosis, the orange color represents late apoptosis (nuclear fragmentation), and necrotic cells have an orange color but a nuclear

Figure 1



IC_{50} value of antioxidant activity of *Haloxylon salicornicum* extracted by MAE, using GraphPad Prism software analysis for windows. MAE, microwave-assisted extraction.

Table 2 Zones of inhibition of *Haloxylon salicornicum* extracted by microwave-assisted extraction, using four bacteria pathogen (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*)

Bacterium name	Type	Ampicillin[a]	Gentamycin[b]	Zones of inhibition (mm)
Staphylococcus aureus	Gram positive	28	–	9
Escherichia coli	Gram negative	–	23	9
Pseudomonas aeruginosa	Gram negative	–	–	–
Klebsiella pneumonia	Gram negative	–	–	–

[a,b]Standard.

morphology similar to that of viable cells, with no condensed chromatin, as shown in Fig. 2. AO/EB staining revealed that *H. salicornicum* has an effect on liver cancer cells, which was confirmed by flow cytometry.

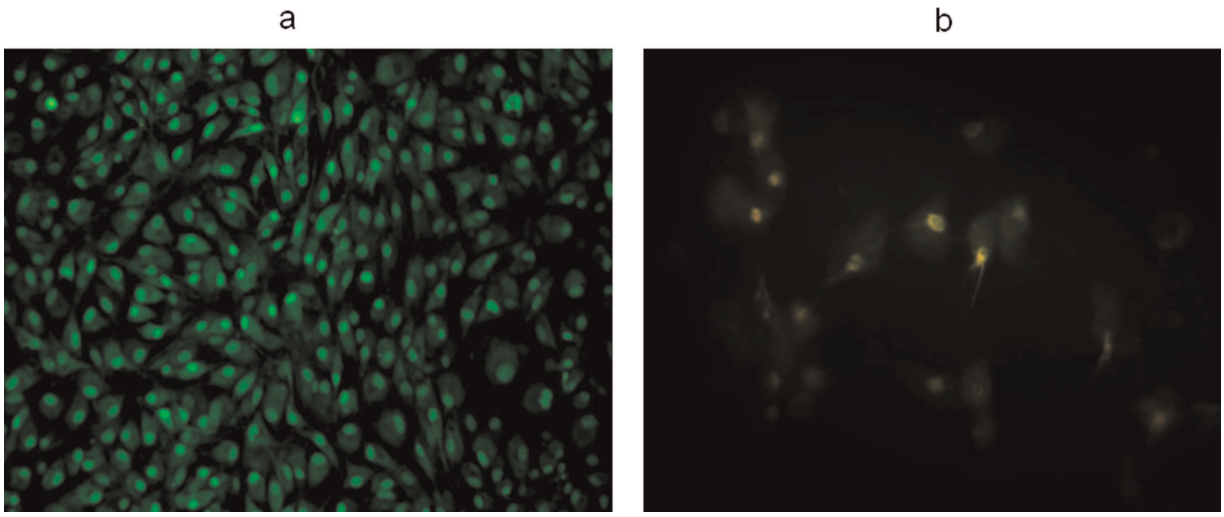
Figure 3a and b demonstrated a major apoptosis effect of *H. salicornicum* on HCAM cells as compared with the control. Furthermore, flow cytometry analysis revealed that early apoptosis (Q1) ratio was 17.8%, late apoptosis (Q2) ratio was 65%, necrosis (Q3) ratio was 3.84%, and live cells' (Q4) ratio was 13.4%; as compared with control (cells treated with DMSO), early apoptosis (Q1) ratio was 14.7%, late apoptosis (Q2) ratio was 42.1%, necrosis (Q3) ratio 11.4%, and live cells' (Q4) ratio 31.8%.

Furthermore, with the same concentration of *H. salicornicum* extract, the cell cycle of HCAM cells was established, with the results displaying G1, S, and G2 phases. The control value of G1 is smaller than the treated value, while the control value of S and G2 is higher than the treated value. This phase-change behavior suggested that *H. salicornicum* extract arrested HCAM cells in the G1 phase (Fig. 3c and d). The chemical composition of *H. salicornicum* extracted by MAE might play a role in the antioxidant, antibacterial, and fighting liver cancer, which may improve its properties in the future research.

Conclusion

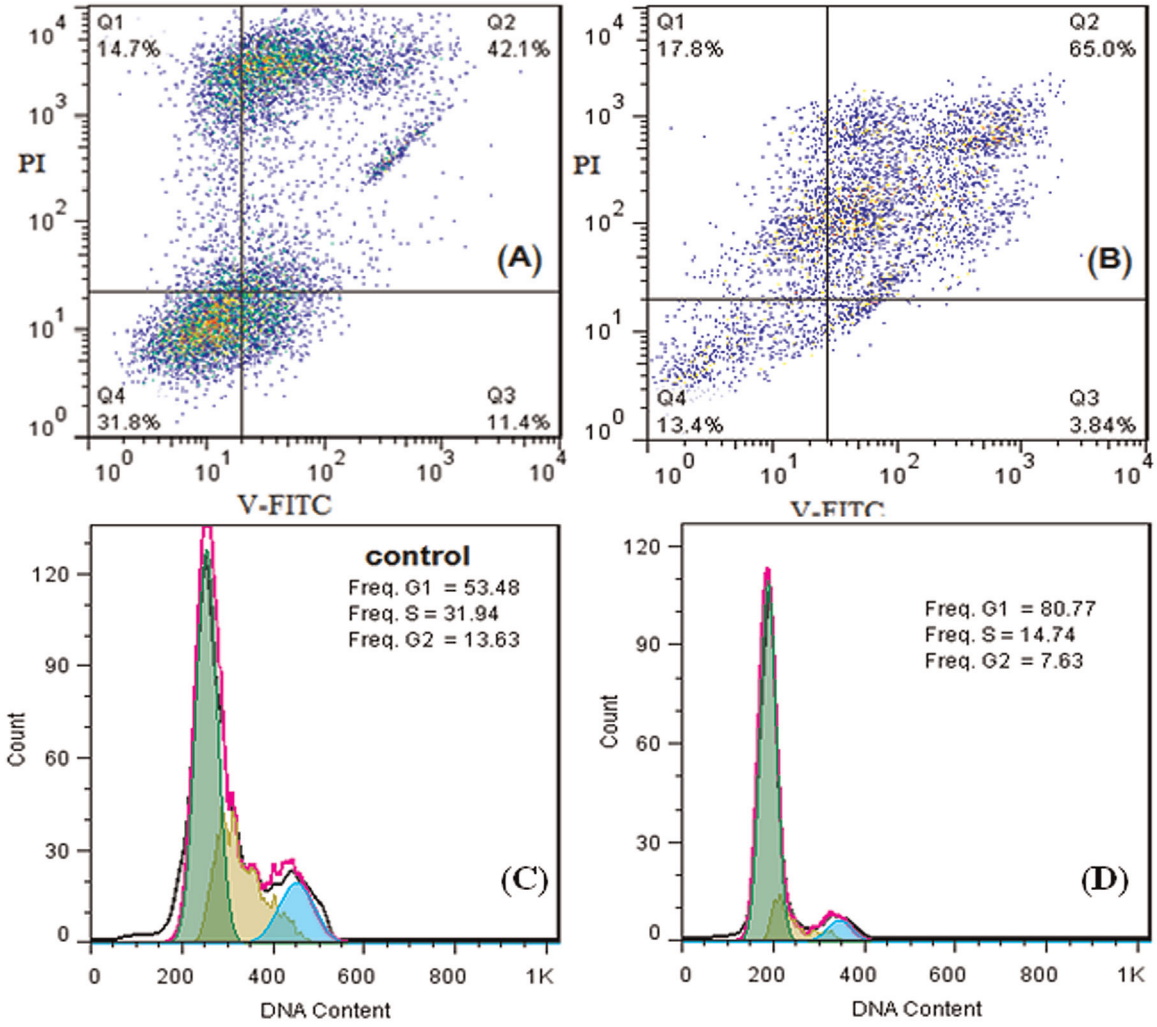
H. salicornicum is an edible desert plant with biological properties. However, environmental conditions and soil type can have an effect on their chemical composition for primary and secondary metabolism. Investigating the significance of this desert plant will pique the interest of researchers in developing medical applications for it. As a result, we evaluated the biological functions of ethanol extract using MAE. The findings of ethanol extract as antioxidant, antibacterial, and anticancer activities increase the medicinal value of this plant and could advance research in isolating and identifying the responsible molecules of biological functions. In terms of liver cancer activity, the extract entered the cell in the G1 process and reduced reactive oxygen species and late apoptosis, implying that regular doses of the extract

Figure 2



AO/EB staining showed the DNA damage of liver cancer cells: (a) untreated cells appear in green color. (b) Treated cells with *Haloxylon salicornicum* appear in orange color of late apoptosis.

Figure 3



The apoptosis and cell cycle of liver cancer cells affected by *Haloxylon salicornicum* extracted, A and C: treated cells. B and D: Untreated cells.

could minimize or prevent liver cancer and diseases. As a result, *H. salicornicum* plant is beneficial to animal nutrition by supporting liver functions and may improve human liver functions by lowering the risk of liver cancer and diseases.

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Authors' contribution: A.A.Y.: prepared the plant for extraction process using microwave-assisted extraction. M.F.H.: analyzed DPPH and antibacterial experiments. A.A.A.A.: designed the article experiments and wrote the article. All the authors contributed to reading and approving the article.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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