DOI: https://doi.org/10.31351/vol34iss4pp158-171

Exploring the Chemical Composition and Cytotoxic Effect of Hexane Extract of Aerial Portions of Iraqi *Amaranthus viridis* on A549 Cell-Line

Noor S Jaafar*,[1] and Enas J. Kadhim[1]

[1]Department of Pharmacognosy and Medicinal Plants, College of Pharmacy, University of Baghdad, Baghdad, Iraq.
***Corresponding author**

Received 18/4/2024, Accepted 19 /11/2024, Published 20/11/2025

Abstract

Amaranthus viridis (green amaranth), which is naturally grown in Iraq, belongs to the Amaranthaceae family and is considered a pseudocereal. This herb displays various secondary metabolites, including polyphenols, phytosterols, terpenes, and fatty derivatives. Anti-inflammatory, antioxidant, antidiabetic, cardioprotective, and hepatoprotective are among the evaluated and proven clinical activities. Recently, herbal remedies have emerged as a noteworthy new therapeutic option for several cancers. The most common cause of cancer mortality in Iraq is lung cancer. Despite its wide distribution, there is no previous study regarding this herb's phytochemistry and cytotoxic effect in Iraq. The n-hexane extract of *Amaranthus viridis* whole aerial parts was prepared using maceration and Soxhlet extraction methods. It was subjected to gas chromatography/ mass spectrometry and high-pressure liquid chromatography analysis to characterize its constituents. High-pressure liquid chromatography was used to determine phytosterol contents qualitatively and quantitatively. The extraction yield was 0.66 % and 1.017% for cold and hot hexane extracts, respectively. Based on gas chromatography/ mass spectrometry, the number of identified compounds was 15 and 18. Bis (2-ethylhexyl) phthalate was the major constituent in the cold hexane extract. Omega-3 (9,12,15-Octadecatrienoic acid, (Z,Z,Z)-) was only detected in the cold extract. 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- and squalene were identified in both extracts and at a higher proportion in the hot extract. 9,12-Octadecadienoic acid (Z,Z), homosalate were only detected in hot hexane extract, and for the first time in an Iraqi plant. High-pressure liquid chromatography analysis revealed the presence of campesterol, stigmasterol, spinasterol, and beta-sitosterol in greater amounts in the hot extract than cold extract. Campasterol is the least concentrated, while spinasterol is the most concentrated. Based on the results of gas chromatography/ mass spectrometry and high-pressure liquid chromatography, hot hexane extract was evaluated for its *in vitro* anticancer effect against the A549 cell line using (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. This assay showed a dose-dependent cytotoxic effect for hot hexane extract with a half-maximal inhibitory concentration (IC_{50}) value of 1393 µg/ml, possibly due to its antioxidant effect.

Keywords: *Amaranthus Viridis*, A549 Cell Line, Homosalate, Spinasterol, MTT Assay

Introduction

The Amaranthaceae family includes the genus Amaranthus, which is disseminated through tropical and subtropical areas. Genus Amaranthus contains about 60 species, about one-third of which are weeds, even though they are considered ornamentals, cereals, and leaf vegetables. *Amaranthus viridis (A. viridis)* is one of the widest-spreading weeds. *A*maranthus *viridis* is known as the slender amaranth, green amaranth, and Chinese spinach. It is an annual leafy slender herb that is cultivated and also considered an invasive, problematic weed[1-4]. Moisture, light, and warm conditions are the key requirements for its growth. *A. viridis* is naturally grown and distributed in Iraq[5]. Traditionally, this herb is used for pain management and fever; the whole plant is used for green and yellow dye production, and its infusion is used to purify blood. Dried or fresh leaves are used topically for infections such as orchitis and gonorrhea. [6]. *A. viridis* extract is used to increase the nutritional and antioxidant capacity through its polyphenolic and high amino acid contents; it is also considered a pseudocereal [2]. Earlier studies reported the existence of different classes of phytochemicals, terpenoids in their variable subclasses, fatty acids and saturated

hydrocarbons, and others. Yuenyong et al study reported the presence of the following fatty acids: palmitic acid, stearic acid, arachidic acid, oleic acid,and linoleic acid. Terpenoids such as squalene and cholecalciferol (Vitamin D3). Phytosterols such as stigmasterol, campesterol, and β-Sitosterol were present in Amaranthus seed oil [7,8]. 16,17,20,21-tetrahydro-16-(hydroxymethyl)-, methyl ester, (15.beta., 16E)- were identified in the ethyl acetate fraction of leaves extract using GC-MS[9]. Gas chromatography-mass spectrometry (GC-MS) is an interesting analytical tool that provides both qualitative and quantitative profiles of examined extract or mixture constituents [10].This technique incorporates two analytical methods. Mass spectroscopy identifies each component independently after gas chromatography separates the mixture's constituent parts.[11]. HPLC is a common tool for herbal product authenticity, fingerprinting, measurement, and quality control[12].Lung cancer is a common type of cancer that mostly affects males.[13], According to Alwan's study, lung cancer is the most common cause of cancer mortality in Iraq[14]. 5-fluorouracil (5-FU), docetaxel, paclitaxel, cisplatin, and other chemotherapeutics are common options for lung cancer. Resistance and side effects are the most emergent causes for alternative treatment options, including drugs obtained from natural sources such as plants[15]. The present work aims to use GC/MS to compare constituents of aerial parts of Iraqi *A. viridis* hexane extracts obtained by two extraction methods, to determine their phytosterol contents by HPLC, and to evaluate the cytotoxic effect of hot hexane extract on the A549 cell line.

Materials and Methods

Collection of plant materials

Amaranthus viridis whole aerial plant was collected from Baghdad city (Al-Taji) during spring 2023. The plant was authenticated by Assistant Prof. Dr. Sukaena Abass in the herbarium of the College of Science, University of Baghdad. The plant was gently washed with water, shade-dried for three weeks, and milled to a fine powder using an electrical miller.

Preparation of the n-hexane extract

Hexane extract was prepared using two methods.

1. By maceration (cold method)

A dried plant sample (200 gm) was macerated in 1500 ml n-hexane for 48 hours with continuous stirring using a magnetic stirrer. The solvent was evaporated under reduced pressure in a rotary evaporator, yielding a dark orange to brown extract, and the percentage yield was determined[16].

2. By Soxhlet (hot method)

Two hundred grams of desired parts were packed in a paper thimble, 1500 ml of hexane solvent was placed in a round-bottom flask, and the extraction was performed at 35℃ for 48 hours. The solvent was heated repeatedly and refluxed till complete exhaustion of the sample; the extract was filtered. Using a vacuum evaporator, the solvent was evaporated to get the dry extract. The percentage yield was determined[17].

Qualitative phytochemical analyses of n-hexane extracts

1. By Thin-layer chromatography (TLC)

Hexane extracts were analyzed by TLC for their phytosterol contents using standards (campesterol, stigmasterol, spinasterol, and beta-sitosterol). The used solvent system was toluene: methanol (18:2). The developed chromatogram was dried, sprayed with 5% ethanolic H2SO4, and placed in the oven at 110℃ for five minutes for colored spot development. Based on the TLC result, both extracts were subjected to GC/MS and HPLC analysis.

2. ***By Gas chromatography/Mass spectrometry (GC/MS) analysis***

Extracts obtained by both extraction methods were subjected to GC/MS analysis to investigate their constituents. The investigation was accomplished using an Agilent 7820A Gas Chromatograph coupled to a Mass detector. This instrument can analyze liquid, gas, volatile, and solid samples. Using the National Institute of Standards and Technology's (NIST) database, the mass spectrum of the GC/MS instrument was interpreted. Analysis was performed in the Ministry of Industry and Minerals/ Baghdad. Components were separated using a capillary column type Aglint HP-5ms Ultra lnei, its dimensions (30 m length x 250 µm inner diameter was 0.25 µm film thickness). Helium (99.99%) was used as a carrier gas. Pressure 11.933 psi. Injection volume 1 µl, Injection Type: Spitless, Scan Range: m/z 25-1000.GC Inlet Line Temperature: 250 ˚C, Aux heaters Temperature 320 ˚C. Injector Temperature: 250 ˚C. A certain temperature program was adopted for effective separation, which is shown in “Table 1”.

Table 1. GC Temperature Program Development.

Oven Program	Temperature
Ramp 1	65 ˚C
Ramp 2	60 ˚C to 180 ˚C 8 ˚C/min
Ramp 3	180˚C to 300˚C 7 ˚C/min
Ramp 4	300˚C hold to 5 min

Qualitative and quantitative HPLC identification of phytosterols from hexane extracts

HPLC separation was done on a SYKAMN HPLC chromatographic system equipped with a UV detector. The separation was performed in a C18-ODS column (25 cm x 4.6 mm). The mobile phase consists of acetic acid and acetonitrile using gradient elution mode, which is shown in "Table 2".

Table 2. HPLC mobile phase composition.

Mobile phase composition	Duration
A = 30 % acetonitrile, B= 5 % acetic acid	0 – 5 min
A = 50 % acetonitrile, B= 5 % acetic acid	6 –15 min

Analysis was done at room temperature. The wavelength of the UV detector was set to 220 nm, the flow rate was 1ml/min, and the injection volume was 0.1 µl for extracts and standards. Accurately weighted 10 mg solvent-free dried extracts were dissolved in 5 mL methanol (HPLC grade) to prepare a concentration of 2 mg /ml. The aliquot was then filtered through a 0.45 µm filter to get a clear solution before injection [18]. Regarding the HPLC profile, different phytosterols were used, including campesterol, β-sitosterol, stigmasterol, and spinasterol. The studied standards were prepared in methanol to get a concentration of 10 µg/ml. The existence of phytosterols was singled out by comparing the retention times for separated constituents to the retention times ′ standards. Since the area under a certain compound's peak in the HPLC method is proportionate to its concentration, a quantitative determination of each component in the chromatogram was computed by serially concentrating reference standards (5,10,15,20 ppm) to create a calibration curve between the concentration and its equivalent peak area. A linear equation was used to estimate concentrations.

"Y=a X +b"

Y: symbolized the response factor (AUC). a: is the slope of the curve (slop=y/x). x: designated the concentration in part per million (ppm), the y-intercept is shown by b. Using calibration curves of those standards, the concentration of those phytosterols was determined and expressed as µg /gm of dried sample.

Determination of the cytotoxicity of n. hexane extract on the A549 cell line

To assess the cytotoxic activity of n-hexane extract against lung cancer, A549 cells were employed as models[19]. Human adenocarcinoma alveolar basal epithelial cells, or A549 cells, were initially created in 1972 by D. J. Giard and colleagues. The targeted cells were extracted by isolating and cultivating malignant lung tissue from the removed tumor of a 58-year-old Caucasian man.

Maintenance of cell cultures

Maintaining A549 cells required incorporating 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 units/mL penicillin into Minimum Essential Medium (MEM). Once passing through trypsin-EDTA, the cells were reseeded twice a week at 50% confluence and cultivated at 37 °C.

Combination cytotoxicity assays

To assess the cytotoxic effect, 1×10^4 cells/well were utilized to seed the cell line. The cells were incubated at 37 °C for 72 hours till a monolayer confluence layer was obtained. The investigated substance (n. hexane extract was introduced to the cells using a series of concentrations (31.75, 62.5, 125, 500, 1000 µg/ml) and incubated for 24 hours. After eradicating the media and adding 28 µL of a 2 mg/ml MTT solution, an assessment of the cell viability was done. The cells were then incubated for three hours at 37 °C. After removing the MTT solution, 1،0 µL of dimethyl sulfoxide was added to the wells to solubilize the remaining crystals. The wells were then shaken and incubated for fifteen minutes at 37 °C. Using a microplate reader, the absorbency was established in triplicate at the test wavelength of 492 nm. The following formula was used to calculate the percentage of cytotoxicity, or cell growth inhibitory rate:

% Viability of cells = (Ab2-Ab1/Ab2) x 100%

% Cytotoxicity = 100 – cell viability

Ab 1: The treated cell's absorbance

Ab 2: Untreated cell absorbance [20].

Statistical analysis

The collected data were statistically examined using GraphPad Prism one-way ANOVA and multiple comparison Tukey's test with a level of significance α = 0.05. The values were shown as the triple measurements' mean ± standard deviation.

Result and Discussion

Certain medicinal components found in plants have amazing physiological effects; these components can be isolated and employed in the manufacture of drugs[21]. The % yield was 0.66 % and 1.017% for cold and hot methods, respectively. TLC result gave preliminary estimation for the presence of terpenoids, including phytosterols, "Figure 1". Hexane extracts of Iraqi *A. viridis* (from cold and hot methods) are evaluated by GC/MS "Figure 2, and 3". The identified compounds that exhibit 90% mass spectra similarity hits are displayed, whereas those that do not meet this threshold are not recognized. According to these, thirteen compounds were identified in the cold extract, while seventeen compounds were identified in the hot extract.

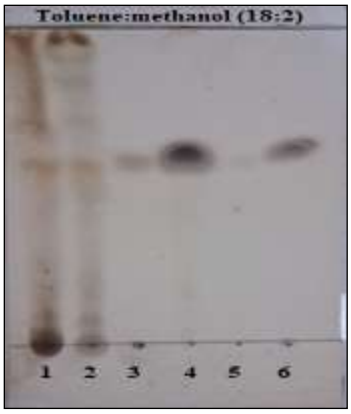


Figure1. TLC chromatogram for hexane extracts. 1: cold hexane extract, 2: Hot hexane extract, 3: Campesterol,4: β-sitosterol. 5: Spinasterol, 6: Stigmasterol. Detection after spraying with 5% ethanolic H_2SO_4 and heating for five minutes.

Bis(2-ethylhexyl) phthalate is the major constituent in the cold extract; meanwhile, 9,12-Octadecadienoic acid (Z, Z) is the main one in the hot extract. Linolenic acid (omega-3) is the only detected fatty acid in cold extract, and it is one of the major components, esters of fatty acids are also present in higher percentages and exert diverse actions as antimicrobial and antioxidant actions[22]. Saturated hydrocarbons, both linear and branched, were present as minor ingredients. In the hot extract, β-ionone (apocarotenoids) is a beta carotene cleavage product by the dioxygenase enzyme. Homosalate, salicylic acid ester (3,3,5-trimethylcyclohexenyl salicylate) was reported for the first time in the plant. This ester acts as a UV radiation absorber to protect the plant from sunlight, and it is principally present in desert plants[36].

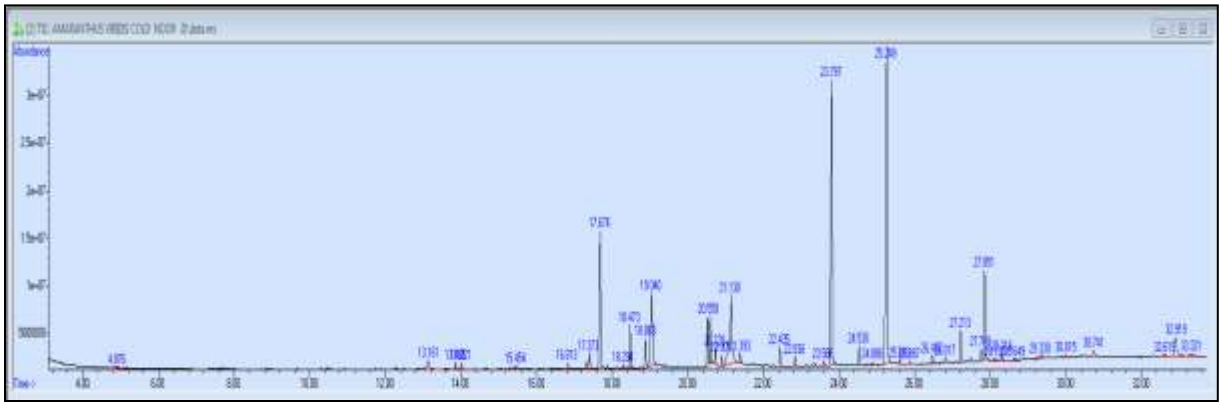


Figure 2. GC/ MS Chromatogram of *Amaranthus viridis* n-hexane extract obtained by maceration extraction method.

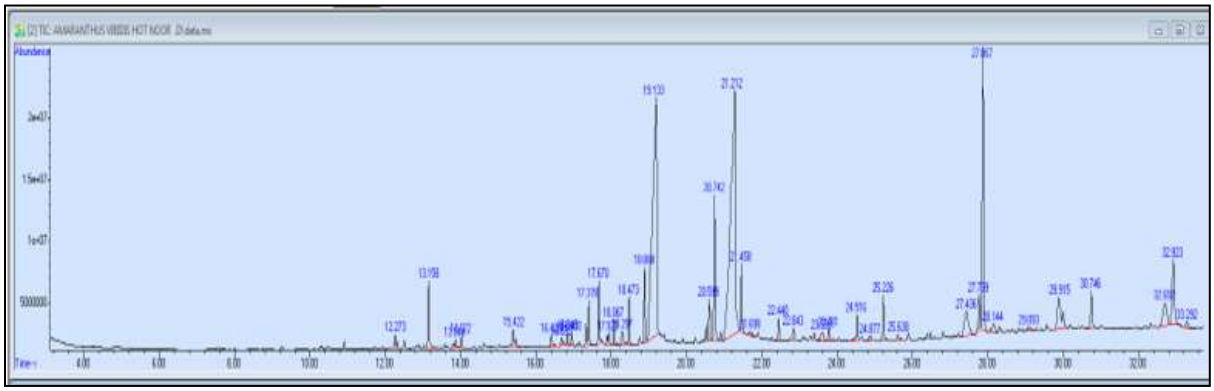


Figure 3. GC/ MS Chromatogram of *Amaranthus viridis* n-hexane extract obtained by Soxhlet extraction method.

Beta-sitosterol was the only detected phytosterol in the hot extract obtained by Soxhlet. Campasterol and spinasterol are not included in the NIST database. Terpenoids, including dihydroactinidiolide, squalene, and phytol, in addition to vitamin E, were detected in both extracts, all of them present in higher amounts in the hot extract. This may be attributed to the effect of temperature that increases compound solubility and enhances mass transfer and thus increasing the yield [37]. Previous studies reported

the presence of phytol in leaves and squalene in stems and leaves [38]. Acetic acid, chloro-, hexadecyl ester is another terpenoid detected in a small concentration of hot extract. The chemical constituents of cold and hot extracts are demonstrated in "Tables 3 and 4", respectively.

Table 3. GC/MS analysis of cold hexane extract.

Peak no	Retention time	Compound name and molecular formula	%Peak area	Compound nature	Biological activity
2	13.162	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- (Dihydroactinidiolide) $C_{11}H_{163}$	0.74	Volatile triterpene	Antimicrobial[27]
4	14.018	Tetradecane $C_{14}H_{30}$	0.29	Alkane hydrocarbon	Antibacterial and antifungal[28]
10	18.476	Hexadecanoic acid, methyl ester (methyl palmitate) $C_{17}H_{34}O_2$	2.37	Fatty acid methyl ester	potent antioxidant activity[29]
11	18.883	Dibutyl phthalate	1.59	Phthalates	Antibacterial activity [(30)
14	20.744	Phytol $C_{20}H_{40}O$	0.74	acyclic diterpene alcohol	potent anti-inflammatory effect [31]
15	20.899	Methyl stearate $C_{19}H_{38}O_2$	0.51	Fatty acid methyl ester	antimicrobial activity[32]
16	21.133	9,12,15-Octadecatrienoic acid, (Z,Z,Z)- linolenic acid (omega-3) $C_{18}H_{30}O_2$	8.94	Polyunsaturated fatty acid	neuroprotective, anti-inflammatory, and anti-depressant, satiation-inducing properties [33]
18	22.431	Hexacosane $C_{26}H_{54}$	1.01	Alkane hydrocarbon	Antimicrobial[34]
19	22.838	Heneicosane	0.50	Alkane hydrocarbon	Antimicrobial [35]
21	23.799	Hexanedioic acid, bis (2-ethylhexyl) ester (Diisooctyl adipate) $C_{22}H_{42}O_4$	19.18	Fatty acid ester	
22	24.526	2-methyl octacosane (Isononacosane) $C_{29}H_{60}$	1.06	Alkane hydrocarbon	antimicrobial[22]
24	25.253	Bis(2-ethylhexyl) phthalate $C_{24}H_{38O}$	25.11	Ester	antibacterial and larvicidal action[36]
27	26.465	2-methyl hexacosane $C_{17}H_{36}$	0.41	Branched alkane	Antimicrobial[22]
31	27.867	Squalene $C_{30}H_{50}$	4.98	Triterpene	Antioxidant, anti-inflammatory, and anti-atherosclerotic properties[37]
37	30.749	Vitamin E $C_{29}H_{50}O_2$	0.44	fat-soluble vitamin	Antioxidant[38]

Table 4. GC/MS analysis of hot hexane extract

Peak No	Retention time	Compound name and molecular formula	%Peak area	Compound nature	Biological activity
1	12.270	trans-beta-Ionone β-ionone $C_{13}H_{20}O$	0.55	Apocarotenoids	Antitumor, antifungal, antibacterial activities[39]
2	13.162	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- (Dihydroactinidiolide) $C_{11}H_{16}O_3$	2.06	Volatile triterpene	Antimicrobial[27]
4	14.019	Hexadecane (cetane) $C_{16}H_{34}$	0.27	Alkane hydrocarbon	antibacterial, antioxidant activities[40]
5	15.421	Not identified	0.85		
6	16.416	Tetradecanoic acid (Myristic acid) $C_{14}H_{28}O_2$	0.56	Saturated fatty acid	Nematicide, hypercholesterolemic, Lubricant[27]
8	16.840	Heneicosane $C_{21}H_{44}$	0.27	Alkane hydrocarbon	antimicrobial *[35]*
12	17.922	Acetic acid, chloro-, hexadecyl ester (Hexadecyl chloroacetate) $C_{18}H_{35}ClO$	0.58	Terpenoid	Not reported in the literature
13	18.069	Homosalate $C_{16}H_{22}O_3$	0.62	Salicylates	Use as sunscreen[41]
15	18.476	Hexadecanoic acid, methyl ester (methyl palmitate) $C_{17}H_{34}O_2$	1.20	Fatty acid methyl ester	Potent antioxidant activity[29]
16	18.883	Dibutyl phthalate $C_{16}H_{22}O_4$	1.90	Phthalates	Antibacterial activity, Natural anticancer agents inhibiting melanogenesis[11] [30]
19	20.744	Phytol $C_{20}H_{40}O$	4.04	Acyclic diterpene alcohol	Potent anti-inflammatory effect [31]
20	21.211	9,12-Octadecadienoic acid (Z,Z) (alpha-linoleic acid) $C_{18}H_{32}O_2$	27.30	unsaturated fatty acid	Antimicrobial activity[42]
23	22.440	2-methyloctacosane	0.55	Branched alkane	Antimicrobial[22]
24	22.838	Hexacosane $C_{26}H_{54}$	0.32	**Alkane** hydrocarbon	Antimicrobial activity[34]
29	25.227	Phthalic acid, di(2-propylpentyl) ester	1.27	Esters of phthalic acid	Allelopathic, antimicrobial, insecticidal effects[43]
30	25.634	**1,54**-Dibromotetrapentacontane $C_{54}H_{108}Br_2$	0.27	Halogenated hydrocarbon	
31	27.434	Beta-Sitosterol **$C_{29}H_{50}O$**	2.40	Phytosterol	Cholesterol-lowering action, anti-inflammatory action[44]
33	27.867	Squalene $C_{30}H_{50}$	9.01	Triterpene	Antioxidant, anti-inflammatory, and anti-atherosclerotic properties[37]
37	30.749	Vitamin E $C_{29}H_{50}O_2$	1.33	fat-soluble vitamin	Antioxidant[38]

HPLC identification of phytosterol

HPLC method (reversed-phase) combined with a UV-detector is a suitable technique for phytosterols analysis. According to previous literature, enhanced resolution and better peak shape can be achieved by the C-18 column [45]. HPLC chromatograms for cold and hot hexane extracts are demonstrated in "Figures 4 and 5", respectively. Full separation with great peak resolution was achieved for the phytosterol standards. In "Figure 6", the chromatogram is displayed in the coordinate's retention time-line intensity/ (minutes on x-axis)- (mA on y-axis). Campesterol appeared first at 3.45 min, followed by stigmasterol at 3.9 min, then spinasterol at 6.01 min, and finally β-sitosterol at 8.08 min "Table 5". For the quantitative assessment of particular phytosterols in comparison to phytosterol standards, the same chromatographic parameters were used. HPLC chromatograms of standards with their chemical structures are demonstrated in "Figure 6".

Table 5. Retention time, height, and area of phytosterols standards.

Phytosterol standard	Retention time (min)	Height (mAU)	Area (mAU.s)
Campesterol	3.45	764.80	1920.11
Stigmasterol	3.9	791.58	1865.08
Spinasterol	6.01	774.13	854.11
β-sitosterol	8.08	585.44	690.48

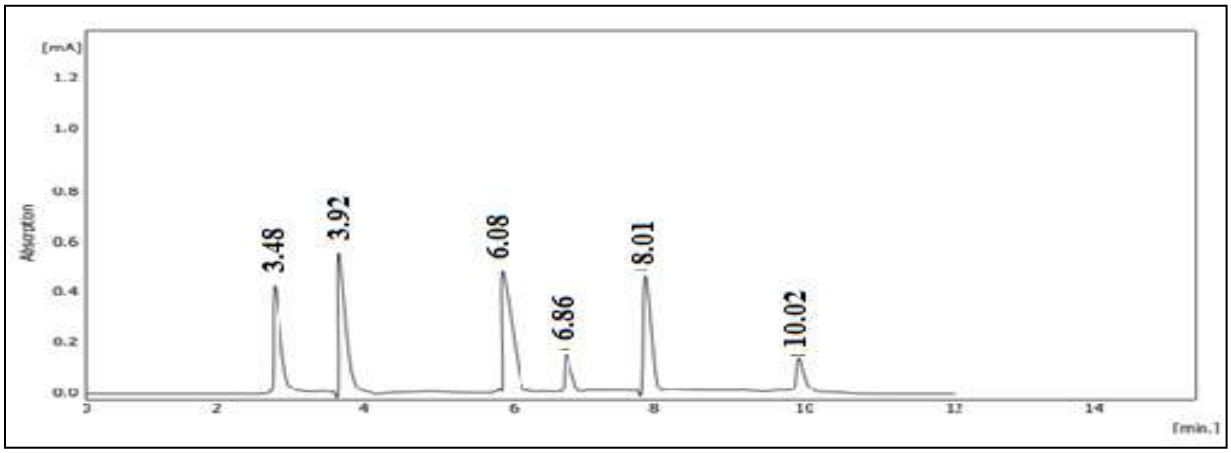


Figure 4. HPLC chromatogram of n-hexane extract obtained by the cold method.

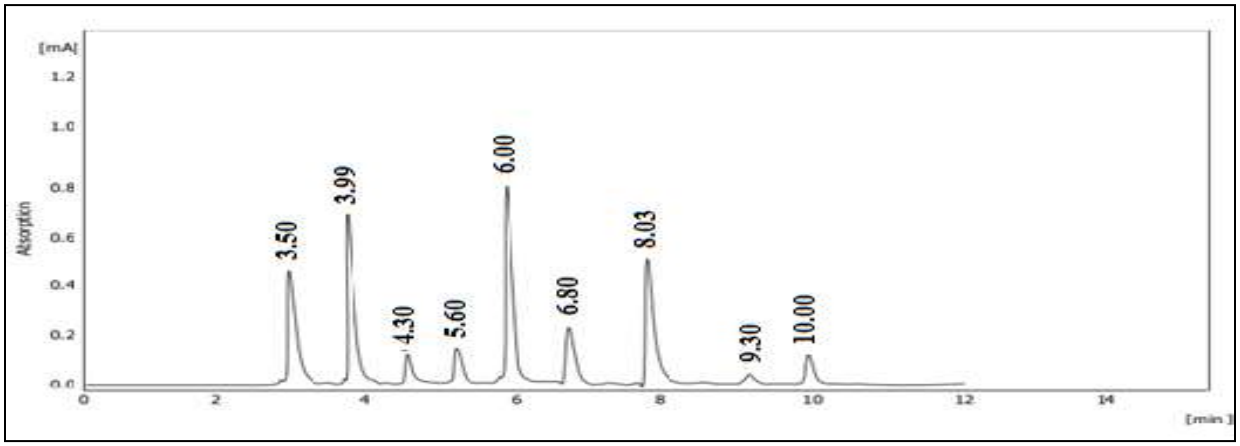
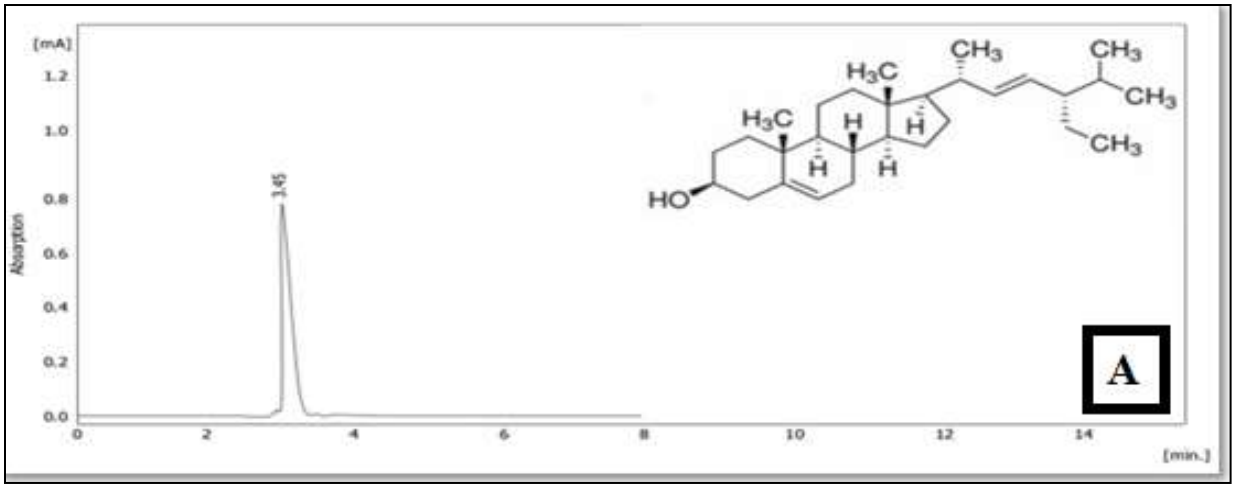
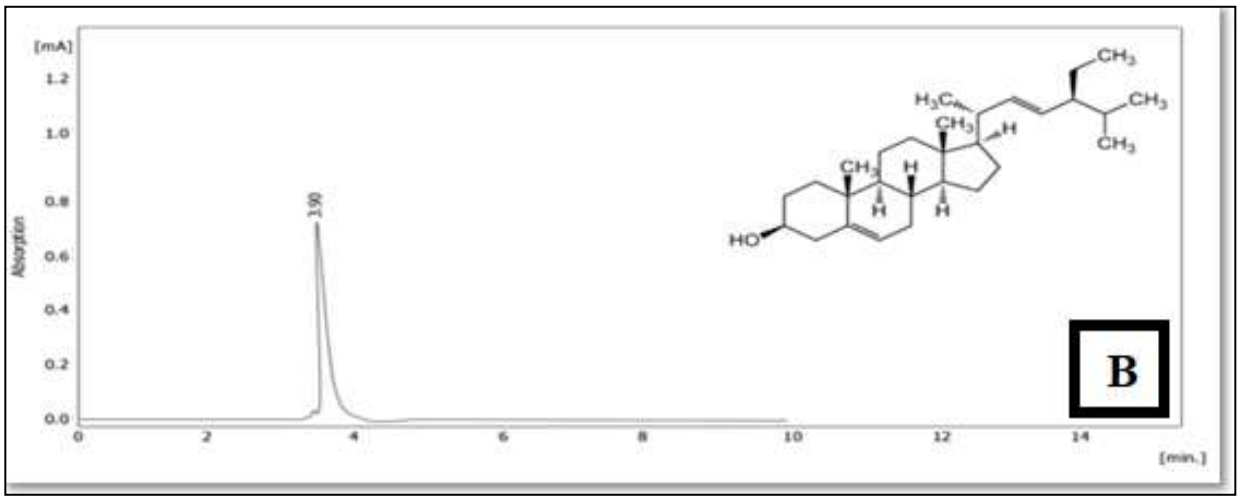
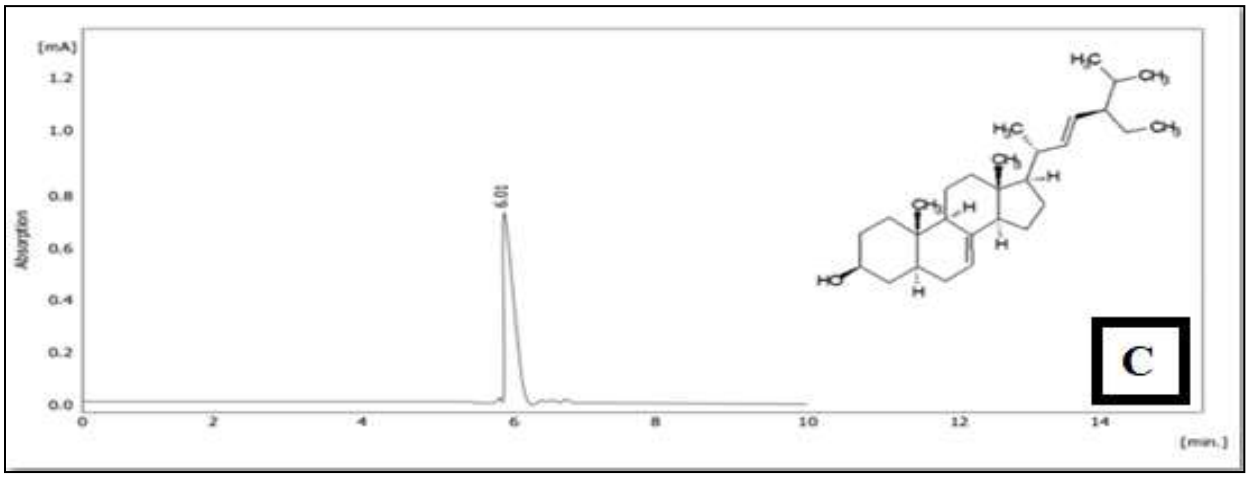


Figure 5. HPLC chromatogram of n-hexane extract obtained by the hot method.


[mA]
Absorption
3.45
[min.]
A


[mA]
Absorption
3.90
[min.]
B


[mA]
Absorption
6.01
[min.]
C

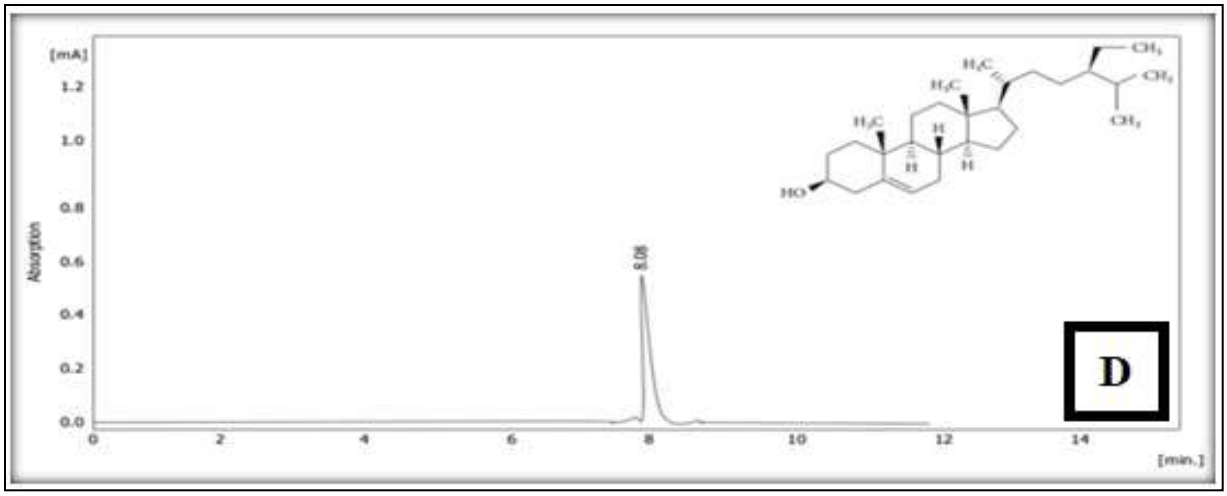


Figure 6. HPLC chromatogram of standards (A): Campesterol standard and its chemical structure., (B): Stigmasterol and its chemical structure., (C): Spinasterol and its chemical structure., (D): Beta-sitosterol and its chemical structure.

HPLC chromatogram of cold and hot hexane extracts revealed the presence of six and nine compounds, respectively, by comparing the retention time of the observed peaks of HPLC chromatograms with the retention time of standards (campesterol, stigmasterol, spinasterol, and β-sitosterol). Calibration curves of standards with their straight-line equations are shown in "Figure 7". The concentrations were determined using the obtained equation for each standard and the area under the curve for each identified phytosterol in extracts obtained from cold and hot methods. Phytosterol concentrations were determined, and according to the results demonstrated in "Tables 6, and 7", all the detected phytosterols were present in higher concentrations in the hot extract. In both extracts, campesterol has the lowest concentration; meanwhile, spinasterol has the highest concentration. The total phytosterol content in Amaranthus oil in a published study was about 2326 µg/g [7].

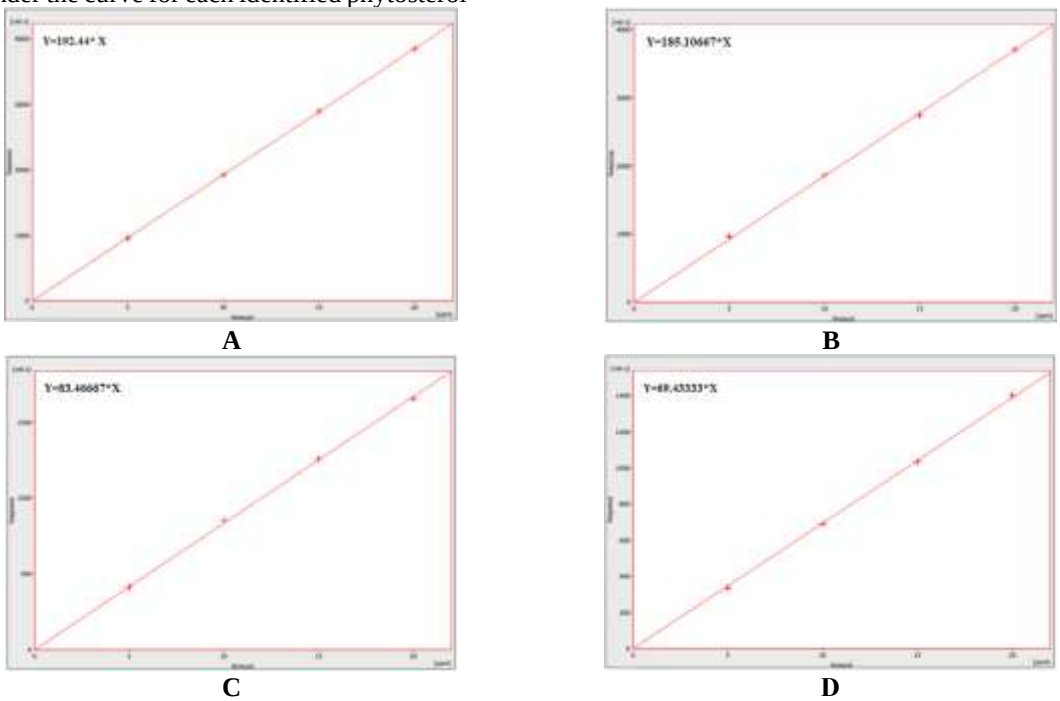


Figure 7. Calibration curves with their straight-line equations for A: Campesterol, B: Stigmasterol, C: Spinasterol, D: Beta-sitosterol.

Table 6. Phytosterol compounds present in the n-hexane fraction obtained by the cold method

Peak no	Peak ID	Retention time (min)	Height (mAU)	Area (mAU.s)	Concentration µg/0.35gm dry extract	Concentration µg/100gm dry plant
1	Campesterol	3.48	400.12	105985.44	550.7	157354.33
2	Stigmasterol	3.92	560.80	130254.87	703.7	201149.76
3	Spinasterol	6.08	530.90	245718.14	2943.9	838542.86
5	Beta-Sitosterol	8.01	520.11	111268.14	1602.5	457877.14

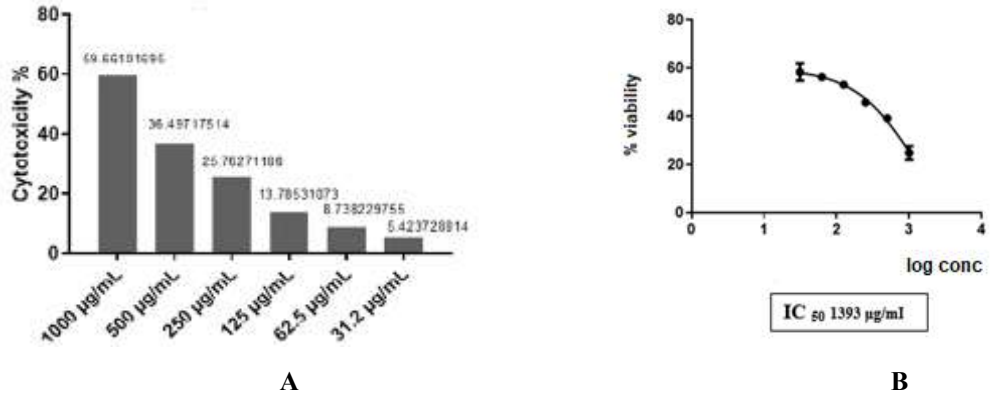
Table 7. Phytosterol compounds present in the n-hexane fraction obtained by the hot method.

Peak no	Peak ID	Retention time (min)	Height (mAU)	Area (mAU.s)	Concentration µg/0.35gm dry extract	Concentration µg/100gm dry plant
1	Campesterol	3.5	430.25	250458.99	1353.1	386600
2	Stigmasterol	3.99	684.18	364158.80	1967.3	562085.71
5	Spinasterol	6.00	800.95	587415.99	7037.73	2011780
7	Beta-Sitosterol	8.03	490.44	190854.14	2748.7	785342.86

Ragasa CY *et al* reported the isolation of spinasterol from Amaranthus stems and roots [38]. A previous study reported that the Soxhlet method provides higher phytochemical contents, including phytosterols[46]. The higher temperature applicable in the Soxhlet extractor improves the kinetics of the process. Also, the continued contact of plant material with the desired solvent increases mass transfer and ultimately increases the extraction yield [47].

Cytotoxic effect of hot n-hexane extract

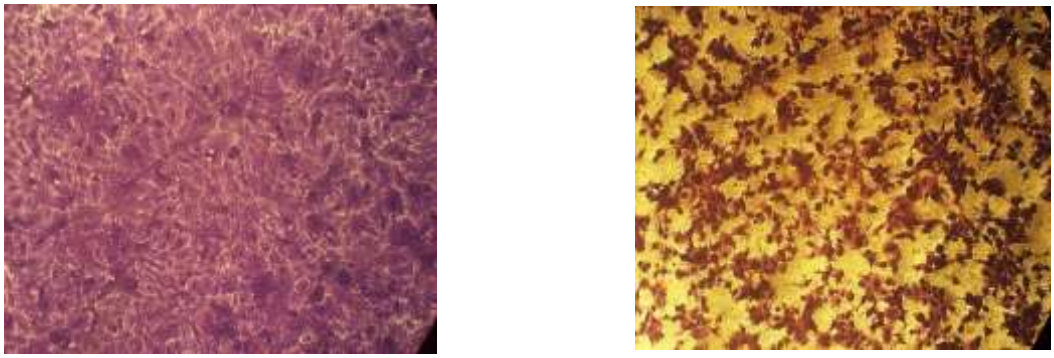
Hexane extract obtained by Soxhlet was evaluated for its cytotoxic potential against A549 lung cancer cells using rising concentrations for IC_{50} value determination. MTT assay based on the color change of tetrazolium salt. In viable cells, NADH and NADPH caused the reduction of yellow-soluble tetrazolium to insoluble purple formazan crystals[48]. According to the result demonstrated in the Figure. 8" The cytotoxic effect is concentration-dependent, as the extract concentration increases, the number of viable cells decreases, and the maximum cytotoxic effect is observed at the highest concentration of about 59.6%. Log dose-response curve used to obtain IC_{50}. IC_{50} is the log concentration of the tested sample that inhibits fifty percent of cell viability[49]. The effect of n. hexane fraction over A549 cell growth and IC_{50} of the tested fraction on the A549 cell line is demonstrated in Figure 8. The morphological features of treated and untreated cells are stated in "Figure 9".



A **B**

Figure 8. (A): Effect of n-hexane fraction on A549 cell growth. The result is stated as % of cytotoxicity, (B): IC_{50} of hot n-hexane fraction on the A549 cell line.

The value of IC_{50} is 1393 µg/ml, and a concentration-dependent cytotoxic effect was produced. However, previous studies demonstrated a significant cytotoxic effect of the ethanolic leaf extract of *A. viridis* on the MCF-7 breast cancer cell line (IC_{50} of 18.33 µg/ml)[50]. Additionally, ethyl acetate extract from the whole plant has stronger anticancer properties against HepG2 and HT-29 cancer cells[51]. Several mechanisms induce the cytotoxic effect. One mechanism of cytotoxicity involves increasing the oxidative stress, leading to the interference of DNA replication and proliferation of the neoplastic cells, thus inducing apoptosis through reactive oxygen species formation in cancer cells**.**



A **B**

Figure 9. The morphological image of the A549 lung cancer cell line *in vitro*, seen at 10x magnification, shows (a) control cells and (b) cells treated with n. hexane fraction.

In neoplastic cells, antioxidants can decrease the reactive oxygen species to a level that promotes cancer growth, proliferation, and migration while decreasing some detrimental effects, like DNA damage [52].n -hexane extract of *A. viridis* contains significant amounts of antioxidants such as phytosterols, squalene, vitamin E, and other natural antioxidant compounds that terminate the effect of reactive oxygen species and thus modulate the extract's cytotoxic effect. The other possible explanation for this effect is that A549 is an adenocarcinoma cell line that might have a weak response to certain chemotherapeutic agents, such as pemetrexed and cisplatin. When tumors resist chemotherapeutic agents, they generally become cross-resistant to other anticancer drugs [53, 54].

Conclusion

The plant is a rich source of terpenoids, especially phytosterols and squalene, particularly when the hot extraction method is used. The obtained data demonstrated the presence of homosalate only in the hot hexane extract and in a small concentration**.** **T**he present study shows that the hot hexane extract of *Amaranthus viridis* exhibits a dose-dependent cytotoxic effect against the lung carcinoma (A-549) cell line. Further studies are required to evaluate the *in vitro* cytotoxic effect using other tests and higher concentrations.

Acknowledgment

The authors appreciate the role of the pharmacognosy and medicinal plants department / College of the Pharmacy / University of Baghdad for providing the opportunity to accomplish this study.

Conflict of Interest

The authors declare there is no conflict of interest.

Funding

Self-funded

Author Contributions

The manuscript was designed, performed, and written by NSJ, and reviewed by Pro. Dr. EJK.

References

1. Francischini A, Constantin J, Oliveira Jr R, Santos G, Braz G, Dan H. First report of *Amaranthus viridis* resistance to herbicides. Planta daninha. 2014;32:571-8.
2. Chikane T, Patil S, Bhushette P, Sonawane SK. Comparative assessment, optimization, and characterization of bioactive constituents from Amaranthus species: An indigenous lesser-known vegetables of India. Food Science and Applied Biotechnology. 2021;4(2):183-93.
3. Omodamiro O, Jimoh M, Ezurike P. In vitro, antioxidant activity and hypolipidemic effects of an ethanol extract of *Amaranthus viridis* leaves on hyperlipidemic Wistar albino rats. British Journal of Pharmaceutical Research. 2016;9(6):1-12.
4. Amin SFM, Karim R, Yusof YA, Muhammad K. Effects of enzymatic liquefaction, drying techniques, and wall materials on the physicochemical properties, bioactivities, and morphologies of Zinc-amaranth (*Amaranthus viridis* L.) powders. International Journal of Food Science. 2021;2021:1-13.

5. Akbar S, Rahman R, Mushtaq A, Azeem MW, Al Mahruqi ZMH. Slender Amaranth: A review on botany, chemistry, pharmacological importance and potential benefits 2017;12.152-156

6. Pooja R, Ajay B, Bharathi D. Geomorphology, Phytochemistry, Ethnomedical and Pharmacological activities of *Amaranthus viridis* Linn. Asian Journal of Pharmaceutical Research. 2023;13(1):41-4.

7. Yuenyong J, Pokkanta P, Phuangsaijai N, Kittiwachana S, Mahatheeranont S, Sookwong P. GC-MS and HPLC-DAD analysis of fatty acid profile and functional phytochemicals in fifty cold-pressed plant oils in Thailand. Heliyon. 2021;7(2).

8. Pulipati S, Babu P, Narasu L. Phytochemical and pharmacological potentials of *Amaranthus viridis* L. International journal of phtytomedicine. 2014;6:322-26.

9. Akbar M, N Sherazi I, Khalil T, S Iqbal M, Akhtar S, N Khan S. Identification of antifungal compounds from slender amaranth. Planta Daninha. 2020;38:1-8.

10. Muzahid AA, Sharmin S, Hossain MS, Ahamed KU, Ahmed N, Yeasmin MS, et al. Analysis of bioactive compounds present in different crude extracts of *Benincasa hispida* and *Cucurbita moschata* seeds by gas chromatography-mass spectrometry. Heliyon. 2023;9(1): e12702.

11. Thamer FH, Thamer N. Gas chromatography–Mass spectrometry (GC-MS) profiling reveals newly described bioactive compounds in *Citrullus colocynthis* (L.) seeds oil extracts. Heliyon. 2023;9(6):e16861.

12. Nag M, Kar A, Chanda J, Mukherjee PK. RP-HPLC analysis of methanol extract of Viscum articulatum. Journal of Ayurveda and Integrative Medicine. 2020;11(3):277-80.

13. Hameed HA, Naser AY, Talib NM. Clinical and therapeutic characteristics and medical cost of managing cancer patients in Al-Anbar, Iraq: A cross-sectional study.Saudi Pharmaceutical Journal. 2022;30(12):1802-8.

14. Al Alwan NA. General oncology care in Iraq. Cancer in the Arab World: Springer Singapore Singapore; 2022. p. 63-82.

15. Khafagy ES. Cytotoxic and Apoptotic Effect of *Rubus chingii* Leaf Extract against Non-Small Cell Lung Carcinoma A549 Cells. Processes. 2022;10(8):1-14.

16. Al-Tameemi ZS, Hamad MN. Isolation of Three Secondary Metabolites from the *Eucalyptus camaldulensis* Dehnh. Plant for the First Time.2022;12(1):55-62

17. Ravi R, Zulkrnin NSH, Rozhan NN, Nik Yusoff NR, Mat Rasat MS, Ahmad MI, et al. Chemical composition and larvicidal activities of *Azolla pinnata* extracts against Aedes (Diptera: Culicidae). PloS one. 2018;13(11):e0206982.

18. Maji AK, Pandit S, Banerji P, Banerjee D. A validated RP-HPLC method for simultaneous determination of betulin, lupeol and stigmasterol in *Asteracantha longifolia* nees. International journal of pharmacy and pharmaceutical sciences. 2014;6(5):691-5.

19. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. Journal of the National Cancer Institute. 1973;51(5):1417-23.

20. Salman MI, Emran MA, Al-Shammari AM, editors. Spheroid-formation 3D engineering model assay for in vitro assessment and expansion of cancer cells. AIP Conference Proceedings; 2021: AIP Publishing. 2021 2372(1):030011.

21. Jaafar NS, Hassan AF, Hamad MN. Evaluation of the Genotoxicity of the Aerial Parts of Iraqi *Euphorbia cyathophora* on Bone Marrow and Spleen Cells in Mice. Iraqi Journal of Pharmaceutical Sciences. 2023;32(2):41-5.

22. Rhetso T, Shubharani R, Roopa M, Sivaram V. Chemical constituents, antioxidant, and antimicrobial activity of *Allium chinense* G. Don. Future Journal of Pharmaceutical Sciences. 2020;6(1):1-9.

23. A H. bioeduscience Pharmaceutical Activity of Medicinal Plant *Amaranthus Viridis* Linn.Due to its Chemical Constituents: A Review BIOEDUSCIENCE, 2023;7(2):143-8.

24. Matsunaga S, Guenther A, Potosnak M, Apel E. Emission of sunscreen salicylic esters from desert vegetation and their contribution to aerosol formation. Atmospheric Chemistry and Physics. 2008;8(24):7367-71.

25. Lee N, Yunus M, Idham Z, Ruslan M, Aziz A, Irwansyah N, editors. Extraction and identification of bioactive compounds from agarwood leaves. IOP Conference Series: Materials Science and Engineering; 2016: IOP Publishing.2016;162(1):012028.

26. Ragasa CY, Austria J, Subosa A, Torres O, Shen C-C. Chemical constituents of *Amaranthus viridis*. Chemistry of Natural Compounds. 2015;51:146-7.

27. Mujeeb F, Bajpai P, Pathak N. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of Aegle marmelos. BioMed research international. 2014;2014:1-11.

28. Nasr ZS, El-shershaby H, Sallam KM, Abed N, Ghany A-E, Sidkey N. Evaluation of Antimicrobial Potential of Tetradecane Extracted from *Pediococcus acidilactici* DSM: 20284-CM Isolated from Curd Milk. Egyptian Journal of Chemistry. 2022;65(3):705-13.

29. Hamed A, Mantawy E, El-Bakly W, Abdel-Mottaleb Y, Azab S. Methyl palmitate: the naturally occurring cardioprotective agent. Archives of Pharmaceutical Sciences Ain Shams University. 2020;4(1):47-62.

30. Shobi T, Viswanathan M. Antibacterial activity of di-butyl phthalate isolated from *Begonia malabarica*. Journal of Applied Biotechnology & Bioengineering. 2018;5(2):97-100.

31. Olofsson P, Hultqvist M, Hellgren LI, Holmdahl R. Phytol: A chlorophyll component with anti-inflammatory and metabolic properties. Recent Advances in Redox Active Plant and Microbial Products: From Basic Chemistry to Widespread Applications in Medicine and Agriculture. 2014:345-59.

32. Nakaziba R, Amanya SB, Sesaazi CD, Byarugaba F, Ogwal-Okeng J, Alele PE. Antimicrobial bioactivity and GC-MS analysis of different extracts of *Corchorus olitorius* L. leaves. The Scientific World Journal. 2022;2022:1-9.

33. Blondeau N, Lipsky RH, Bourourou M, Duncan MW, Gorelick PB, Marini AM. Alpha-linolenic acid: an omega-3 fatty acid with neuroprotective properties—ready for use in the stroke clinic? BioMed Research International. 2015;2015:1-9

34. Rukaiyat M, Garba S, Labaran S. Antimicrobial activities of hexacosane isolated from *Sanseveria liberica* (Gerome and Labroy) plant. Adv Med Plant Res. 2015;3(3):120-5.

35. Vanitha V, Vijayakumar S, Nilavukkarasi M, Punitha V, Vidhya E, Praseetha P. Heneicosane—A novel microbicidal bioactive alkane identified from Plumbago zeylanica L. Industrial Crops and Products. 2020;154:112748.

36. Javed MR, Salman M, Tariq A, Tawab A, Zahoor MK, Naheed S, et al. The Antibacterial and Larvicidal Potential of Bis-(2-Ethylhexyl) Phthalate from Lactiplantibacillus plantarum. Molecules. 2022;27(21):7220.

37. Lou-Bonafonte JM, Martínez-Beamonte R, Sanclemente T, Surra JC, Herrera-Marcos LV, Sanchez-Marco J, et al. Current insights into the biological action of squalene. Molecular nutrition & food research. 2018;62(15):1800136.

38. Rizvi S, Raza ST, Ahmed F, Ahmad A, Abbas S, Mahdi F. The role of vitamin E in human health and some diseases. Sultan Qaboos University Medical Journal. 2014;14(2):e157.

39. Paparella A, Shaltiel-Harpaza L, Ibdah M. β-Ionone: Its occurrence and biological function and metabolic engineering. Plants. 2021;10(4):754.

40. Yogeswari S, Ramalakshmi S, Neelavathy R, Muthumary J. Identification and comparative studies of different volatile fractions from *Monochaetia kansensis* by GCMS. Global Journal of Pharmacology. 2012;6(2):65-71.

41. Wolverton SE, Wu JJ. Comprehensive dermatologic drug therapy: Elsevier Health Sciences; 2019.

42. Manilal A, Sujith S, Kiran GS, Selvin J, Shakir C. Cytotoxic potentials of red alga, *Laurencia brandenii* collected from the Indian coast. Global J Pharmacol. 2009;3(2):90-4.

43. Huang L, Zhu X, Zhou S, Cheng Z, Shi K, Zhang C, et al. Phthalic acid esters: Natural sources and biological activities. Toxins. 2021;13(7):495.

44. Azeez RA, Abaas IS, Kadhim EJ. Isolation and characterization of β-sitosterol from *Elaeagnus angustifolia* cultivated in Iraq. Asian J Pharm Clin Res. 2018;11(11):442-6.

45. Khonsa K, Setyaningrum D, Saputro A, Amelia T, Ibrahim S, Damayanti S. Analysis of β-sitosterol in supplement using high-performance liquid chromatography: development and validation. methods. 2022;15(3):1997-2003.

46. Ramli SN, Aminudin NI, Ahmad F, Susanti D. Comparison of extraction techniques for three Calophyllum species and their antioxidant activity. Malaysian Journal of Analytical Sciences. 2019;23(4):586-94.

47. Stéphane FFY, Jules BKJ, Batiha G, Ali I, Bruno LN. Extraction of bioactive compounds from medicinal plants and herbs. Nat Med Plants. 2021; 1338:1-39

48. Ziska R, Agustina A, editors. Cytotoxic activity assay of n-hexane extract of *Solanum nigrum* L. fruits fermented by kombucha against MCF-7 breast cancer cell line. Journal of Physics: Conference Series; 2019: 1-9

49. Jaafar NS, Alshammaa DA, zuhair Abdul-lalil T, Ibrahem NM. Quantitative Determination and

Cytotoxic Effect of Oleanolic Acid from *Olea europaea* Leaves Extract Cultivated in Iraq. Iraqi Journal of Pharmaceutical Sciences (P-ISSN 1683-3597 E-ISSN 2521-3512). 2022;31(2):244-50.

50. Malicdem ACM, Aducal MLO, Cuasay JC, Dalisay DAA, Mendoza AI, Napiza GME, et al. In-Vitro Anti-Proliferative Effect of Flavonoid Extract of *Amaranthus viridis* (Kolitis) Leaves Against MCF-7 Breast Adenocarcinoma Cell Line. European Journal of Molecular & Clinical Medicine. 2020;7(2):4129-40.

51. Jin Y-S, Xuan Y, Chen M, Chen J, Jin Y, Piao J, et al. Antioxidant, anti-inflammatory, and anticancer activities of *Amaranthus viridis* L. extracts. Asian Journal of Chemistry. 2013;25(16):8901.

52. Yang H, Villani RM, Wang H, Simpson MJ, Roberts MS, Tang M, et al. The role of cellular reactive oxygen species in cancer chemotherapy. Journal of Experimental & Clinical Cancer Research. 2018; 37:1-10.

53. Gao Y, Dorn P, Liu S, Deng H, Hall SR, Peng R-W, et al. Cisplatin-resistant A549 non-small cell lung cancer cells can be identified by increased mitochondrial mass and are sensitive to pemetrexed treatment. Cancer cell international. 2019;19:1-14.

54. Kuo W-T ,Tu D-G, Chiu L-Y, Sheu G-T, Wu M-F. High pemetrexed sensitivity of docetaxel-resistant A549 cells is mediated by TP53 status and downregulated thymidylate synthase. Oncology Reports. 2017;38(5):2787-95.

استكشاف التركيب الكيميائي والتأثير السام للمستخلص الهكساني للأجزاء الهوائية من نبات عرف الديك البري على سلالة الخلايا A549

نور صباح جعفر*[1] و ايناس جواد كاظم[1]

[1]قسم العقاقير والنباتات الطبية، كلية الصيدلة، جامعة بغداد، بغداد، العراق

الخلاصة

ينتمي نبات عرف الديك البري (الأمارانث الأخضر) إلى عائلة الأمارانثوس، وهو ويعتبر من الحبوب الكاذبة. يُظهر هذا العشب العديد من المستقلبات الثانوية، بما في ذلك البوليفينولات، والفيتوستيرولات، والتربينات، والمشتقات الدهنية. ومن بين الأنشطة السريرية التي تم تقييمها وإثباتها أنه مضاد للالتهابات، ومضاد للأكسدة، ومضاد للسكري، وواقي للقلب، وواقي للكبد. ظهرت مؤخرًا العلاجات العشبية كخيار علاجي جديد جدير بالملاحظة للعديد من أنواع السرطان. السبب الأكثر شيوعًا لوفيات السرطان في العراق هو سرطان الرئة. في العراق، لا توجد دراسة سابقة حول الكيمياء النباتية والتأثير السام للخلايا لهذا العشب على الرغم من انتشاره الواسع. تم تحضير مستخلص ن.هكسان من الأجزاء الهوائية الكاملة من نبات الأمارانثوس فيريديس عن طريق النقع واستخراج سوكسليت. تم إخضاعه للكروماتوغرافيا الغازية / مطياف الكتلة وتحليل الكروماتوغرافيا السائلة عالية الضغط لتوصيف مكوناته. تم استخدام كروماتوغرافيا السائل عالي الضغط لتحديد الفيتوستيرولات نوعيًا وكميًا. كان محصول الاستخلاص ٠,٦٦٪ و ١,٠١٧٪ لمستخلص الهكسان البارد والساخن على التوالي. في كروماتوغرافيا الغاز / مطياف الكتلة، كان عدد المركبات التي تم تحديدها 15 و 18. كان ثنائي (٢-إيثيل هكسيل) فثالات هو المكون الرئيسي في مستخلص الهكسان البارد. تم الكشف عن أوميغا ٣ (حمض ٩،١٢،١٥-أوكتاديكاترينويك، Z)، Z، (Z-) فقط في المستخلص البارد. تم تحديد ٢ (H٤)- بنزوفورانون، ٥،٦،٧،٧a-تتراهيدرو-٤،٤،٧a-ثلاثي ميثيل-، (R)- والسكوالين في كلا المستخلصين وبنسبة أعلى في المستخلص الساخن. تم الكشف عن حمض ٩،١٢-Octadecadienoic acid (Z,Z) والهوموسالات فقط في مستخلص الهكسان الساخن ولأول مرة في مصنع عراقي. كشف تحليل كروماتوغرافيا السائل عالي الضغط عن وجود كامبسترول وستيغماستيرول وسبيناسترول وبيتا سيتوستيرول بكميات أكبر في المستخلص الساخن منه في البارد. كامباسترول هو الأقل تركيزًا، بينما سبيناسترول هو الأكثر تركيزًا. بناءً على النتائج، تم تقييم مستخلص الهكسان الساخن لتأثيره المضاد للسرطان في المختبر ضد خط الخلايا A549 باستخدام اختبار (٣- (٤، ٥-ثنائي ميثيل ثيازوليل-٢)-٢، ٥-بروميد ثنائي فينيل تترازوليوم). أظهرت نتائج هذا الاختبار تأثيرًا سامًا يعتمد على الجرعة لمستخلص الهكسان الساخن بقيمة نصف التركيز المثبط الأقصى (IC50) البالغة ١٣٩٣ ميكروجرام / مل، وقد يكون ذلك بسبب تأثيره المضاد للأكسدة.

الكلمات المفتاحية ، ***Amaranthus viridis*** ، **خط الخلية A549، هوموسالات، سبيناستيرول، MTT**