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RESEARCH ARTICLE

Antioxidant and cytotoxic potential of n-butanol fraction of *Amaranthus viridis*

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Abstract

Amaranthus viridis (F. Amaranthaceae), usually known as slender amaranth. It is a multifunctional vegetable that grows in tropical and subtropical areas, sustaining stressful conditions, especially drought, producing a wide array of secondary metabolites: phenolics, terpenes, carotenoids and others, which are responsible for various therapeutic effects. Aerial parts were defatted with hexane, then the defatted residue was extracted in the Soxhlet using 80 % aqueous ethanol. Ethanolic extract was sequentially and repeatedly partitioned with solvents of different polarities: chloroform, ethyl acetate and n-butanol. Then, the n-butanol fraction was hydrolyzed using reflex with 10 % aqueous HCl for 6 hrs. The unhydrolyzed and hydrolyzed n-butanol fractions were subjected to TLC and HPLC. The HPLC analysis was performed using three different eluents; eluent one composed of solvents A (distilled water with 0.1 % glacial acetic acid) and solvent B (acetonitrile with 0.1 % glacial acetic acid), eluent two, composed of solvent A (methanol: water: formic acid (10 : 88: 2 v/v)) and solvent B (methanol: water: formic acid (90: 8: 2 v/v)) and eluent three, consisting of methanol and 0.40 % phosphoric acid (49:51, V/V). According to TLC and HPLC results, the intact n-butanol fraction was evaluated for its antioxidant and cytotoxic effects using DPPH and MTT assays. The study revealed the presence of rutin, hyperoside, quercitrin, apigenin and naringenin only before hydrolysis. While gallic, ferulic, syringic, kaempferol, p. p-coumaric, vanillic, salicylic, protocatechuic acid, myricetin, isorhamnetin, luteolin, vanillin, caffeic acid, chlorogenic acid and quercetin were detected in both unhydrolyzed and hydrolyzed n-butanol fractions. In the DPPH assay, the intact n-butanol fraction showed slightly higher antioxidant activity than the ascorbic acid standard and a mild cytotoxic effect on the HRT-18 cell line with an IC_{50} equal to 302 µg/mL. In conclusion, prolonged hydrolysis time adversely affects the phenolic compound levels and a mild cytotoxic effect calls for further tests to confirm the anticancer potential.

Keywords: *Amaranthus viridis*; antioxidant; intact n-butanol fraction; MTT assay; quercetin

Introduction

Phenolics are a massive group of phytochemicals of plant and marine origin. Chemically, these compounds have aromatic rings with one or multiple hydroxyl groups (1, 2). Phenolics can be organized into two main classes: flavonoids and non-flavonoids. Flavonoids are sub-classified into flavone (luteolin), flavonols (quercetin), chalcone (butein), anthocyanins (delphinidin), flavanones (naringenin), isoflavones (daidzein) and neoflavanoids. Non-flavonoids are subdivided into phenolic acids, hydroxybenzoic acids (salicylic acid) and hydroxycinnamic acids (caffeic acid). The tannins are subdivided into hydrolyzable (gallo tannin) and condensed (proanthocyanin). Other subclasses of non-flavonoids include stilbenes (resveratrol), coumarins (scopoletin), lignins (lariciresinol), lignans (podophyllotoxin), xanthones (mangiferin), anthraquinones (emodin) and curcuminoids (curcumin). Other classes of phenolics include phenolic amides and ellagic acid (3-5). Phenolics are present in their native free form as aglycones and in bound form (6). Phenolic acids are bound by an acetal, ester and ether linkage to specific plant structural elements (lignin, protein, cellulose) or flavonoid or smaller-size moieties such as glucose, quinic, tartaric and maleic acids or other phytochemicals such as terpenes (7).

There are three forms of flavonoids: methylated derivatives, glycosides and aglycones. In nature, most flavonoids are glycosides linked to a sugar moiety in conjugated form as derivatives of mono-glycosidic, di-glycosidic and so on. Usually, the carbohydrate unit is galactose, arabinose, d-glucose, glucorhamnose, or l-rhamnose and the glycosidic link is found at position 3 or 7 (8).

Bound forms of phenolic compounds are susceptible to hydrolysis and structural characteristics, including the number of hydroxyl groups, affect stability; as the number of hydroxyl groups increases, the chemical stability of the phenolic compounds decreases; thus, kaempferol is more stable than quercetin and myricetin is the least stable due to the presence of three hydroxyl groups in ring B (6). The chemical structure of these flavonoids is shown in Fig. 1 (9, 10).

Amaranthus viridis, locally identified as slender amaranth, grows in tropical and subtropical areas. This herb is naturally grown in Iraq but has not been studied yet. Previous

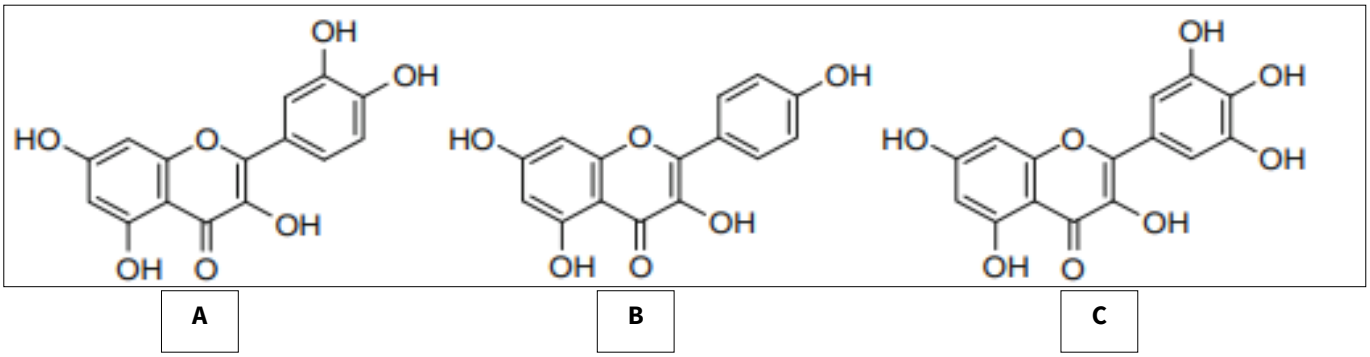

Fig. 1. Chemical structures of (A) Quercetin, (B) Kaempferol, (C) Myricetin (40, 41).

studies have shown that this plant is a rich source of phenolic compounds, terpenoids, carotenoids and others. The phenolic compounds content increases with drought (11). Phenolic compounds inhibit cancer growth and proliferation via their antioxidant, metal chelation, immune modulation and reduction of resistance to chemotherapeutic agents, enhancing endogenous defence mechanisms and induction of apoptosis (12, 13). *Amaranthus viridis* has an *in vitro* cytotoxic potential against MCF-7, HT-29 cells and HepG2 cell lines (11). In Iraq, colorectal cancer occupied the seventh position among the most common ten tumour types recognized (14). Multidrug resistance (MDR) is a problem associated with colorectal cancer that results in metastasis and treatment failure(15). HRT-18 (a rectal adenocarcinoma cell line) is used as an *in vitro* model to estimate the cytotoxic effect of compounds synthesized or obtained from natural sources. The current study aimed to recognize the type of phenolics present in the unhydrolyzed and hydrolyzed n-butanol fractions using TLC and HPLC to evaluate the unhydrolyzed fraction's antioxidant and anticancer effects on the HRT-18 cell line.

Materials and Methods

Chemicals and standards

Hexane, ethanol, chloroform, ethyl acetate, n-butanol, toluene, hydrochloric acid and formic acid were purchased from Alpha Chemika India. Ferric chloride, anhydrous sodium sulfate and potassium hydroxide were obtained from Sigma-Aldrich, USA. Hyperoside, naringenin, ferulic acid, gallic acid, caffeic acid, gentisic acid, Protocatechuic acid, p-coumaric acid, salicylic acid, syringic acid, vanillic acid, cinnamic acid, ferulic acid and vanillin were purchased from MACKLIN, China. Meanwhile, rutin, quercetin, quercetrin, apigenin, luteolin, isorhamnetin, kaempferol and finally chlorogenic acid were acquired from Biopurify, China. Trypsin/EDTA, RPMI 1641 and fetal bovine serum from Capricorn (Germany), DSMO from Santacruz Biotechnology (USA) and MTT stain from Bio-World (USA).

Collection and taxonomic identification of plant material

In April, fresh aerial parts of the plant were collected from Baghdad. Assist. Prof. Dr. Sukeyna Abass, a taxonomist from the University of Baghdad's Herbarium, recognized and authenticated the plant. The plant material was carefully cleaned and shade-dried for 2 weeks. A grinding mill was then used to produce a fine powder for the following extraction process.

Preparation of hydroalcoholic extract and fractionation

200 g of dried plants were defatted in a Soxhlet with 1500 mL of n-hexane for 48 hrs. The hexane extract was collected and dried for further analysis. The dried residue was extracted with 1500 mL of a hydroalcoholic solvent (80 % ethanol) at 45 °C until exhaustion. The filtered extract was evaporated on a rotary evaporator under vacuum to get a dry extract (extraction yield was 19.11 %).

Preliminary phytochemical examination was done for the hydroalcoholic extract and revealed the presence of flavonoids, phenolic acids, terpenoids and the absence of alkaloids (11, 16).

The dried hydroalcoholic extract was mixed with 250 mL of distilled water and stirred continuously in a water bath to achieve complete solubilization. Liquid-liquid extraction for 80 % ethanolic extract accomplished the initial separation of constituents using chloroform, ethyl acetate and n-butanol (250 x4 mL from each solvent). In each case, a rotary evaporator combined and dried the organic layers (16). The extraction yields for chloroform, ethyl acetate and n-butanol fractions were 24.59 %, 0.66 % and 14.55 %, respectively.

Hydrolysis of n-butanol fraction

2 g of n-butanol extract was reflexed with 150 mL 10 % HCl at 75 °C for 6 hrs. The mixture was cooled and the hydrolyzed phenolics were extracted by partitioning with ethyl acetate (150 mL x 3). Ethyl acetate layers were collected, dried over anhydrous sodium sulfate, filtration and dried by rotary evaporator (17).

TLC analysis of the unhydrolyzed and hydrolyzed n-butanol fractions

Thin-layer chromatography was performed to estimate the phenolic profile of n-butanol fractions. Silica gel GF_{254} precoated plates and UV light at 254 nm and 366 nm were used to identify phenolic compounds. 5 % alcoholic ferric chloride was used as a spray reagent for phenolic acid confirmation, while 5 % alcoholic KOH was implemented for flavonoid conformity (18). To identify glycosides (rutin, hyperoside, quercetrin) and chlorogenic acid in the unhydrolyzed n-butanol fraction (before hydrolysis), the mobile phases used are listed in Table 1 (section A). For the hydrolyzed n-butanol fraction, the mobile phases used are demonstrated in Table 1 (section B).

The standard compounds: syringic acid, vanillic acid, ferulic acid, cinnamic acid, caffeic acid, p-coumaric acid, protocatechuic acid, gallic acid, salicylic acid, gentisic acid,

Table 1. Solvent systems utilized in TLC analysis

	Solvent system name	Composition
Section A	**S_1**	Water: acetic acid: Ethyl acetate (3:3:14)
	S_2	Water: formic acid: acetic acid: ethyl acetate (2:1:1:18)
	S_3	Ethyl acetate: methanol: water (17:3:1.5).
Section B	**S_4**	Toluene: ethyl acetate: formic acid (12:5:3)
	S_5	Benzene: dioxane: acetic acid (18:5:1)
	S_6	Ethyl acetate: acetic acid: formic acid: water (50:3:3:4)

myricetin, kaempferol, quercetin, naringenin, isorhamnetin, apigenin, luteolin, vanillin and scopoletin were tested for their existence in n-butanol fraction after hydrolysis. All the standards were prepared by solubilizing 0.5 mg of each in 0.5 mL of analytical-grade methanol (19).

HPLC analysis of the unhydrolyzed and hydrolyzed n-butanol fractions

Standards of phenolic acids and flavonoids were categorized into three groups. Group 1 (gallic acid, ferulic acid, apigenin, syringic acid, naringenin, kaempferol, rutin, p-coumaric acid, salicylic acid and vanillic acid). Group 2 (chlorogenic acid, genestic acid, protocatechuic acid, myricetin, isorhamnetin, luteolin, vanillin and caffeic acid). Group 3 (hyperoside, salicin, quercetrin, quercetin). The compounds with the tested fractions were analyzed using the HPLC model SYKAM (Germany) equipped with a UV detector, Column type C18-ODS and its dimensions (25 cm x 4.6 mm). Analysis was performed at 280 nm at room temperature, with a flow rate of 1mL/min. Before HPLC injection, the standards and the samples were dissolved in HPLC-grade methanol and filtered using a 0.45 µm filter. Different solvent systems and elution modes were utilized for each compound group. For group one, eluent one was used, which was composed of solvents A (distilled water with 0.1 % glacial acetic acid) and solvent B (acetonitrile with 0.1 % glacial acetic acid) (20) (Table 2). For group 2, eluent two was used, composed of solvent A (methanol: water: formic acid (10: 88: 2 v/v)) and solvent B (methanol: water: formic acid (90: 8: 2 v/v)) (21) (Table 3). For group three, eluent three, consisting of methanol and 0.40 % phosphoric acid(49:51, V/V), was employed, using isocratic elution mode (22).

Evaluation of the antioxidant effect on the unhydrolyzed n-butanol fraction

Antioxidant potential of n-butanol fraction was evaluated using a DPPH (1,1-diphenyl-2-picryl-hydrazyl-hydrate) assay. Serial concentrations of n-butanol fraction (1, 10, 20, 30 and 40 mg/mL in methanol) were prepared. In a 96-well microplate, DPPH solution (0.135 mM), 900 µL, was added to 50 µL of *A. viridis* n-butanol fraction and placed in a dark place for 30 minutes. The absorbance was read at 517 nm using a microplate reader. Methanol with DPPH was the blank and ascorbic acid was used as a positive control. The following equation calculates the ability to scavenge DPPH radical:

$$\text{Free radical scavenging activity} = 100 \times \frac{A_0 - A_S}{A_0}$$

A_0 absorption of DPPH solution, A_s Absorption of the sample (23). Minimum Inhibitory Concentration (IC_{50}) was used to express the result by plotting extract concentration against the percentage of antioxidant activity to generate the IC_{50} values from a dose-response curve using GraphPad Prism 8.

Determination of the cytotoxic effect of the unhydrolyzed n-butanol fraction against HRT-18

HRT-18: From the large intestine of a 67-year old male patient with adenocarcinoma, [HRT-18] cells were isolated. Research on toxicity and cancer uses HCT-8 [HRT-18].

Cell culture conditions and MTT assay

HRT-18 cell line was cultured in minimum essential media MEM (US Biological, USA) complemented with 10 % (v/v) fetal bovine serum (FBS), penicillin (100 IU) and streptomycin (100 IU). Incubation of the cells was done in a humidified atmosphere at 37 °C. Exponentially growing cells were employed to accomplish the experiment (24). MTT assay was performed (25). Using n-butanol fraction to evaluate its cytotoxic effect, the serial concentrations used were 31.2, 62.5, 125, 250, 500 and 1000 µg/mL.

Statistical analysis

The data were analyzed using GraphPad Prism 8 and Tukey's ANOVA multiple comparisons. Three separate measurements were taken and the mean ± SD of those results was displayed (26).

Table 2. Gradient elution mode for group one compounds (gallic acid, ferulic acid, apigenin, syringic acid, naringenin, kaempferol, rutin, p-coumaric acid, salicylic acid and vanillic acid)

Time/min	Solvent B
0-3.25	8-10 % B
3.25-8	10-12 % B
8-15	12-25 % B
15-15.8	25-30 % B
15.8-25	30-90 % B
25-25.4	90-100 % B
25.4-30	100 % B

Table 3. Gradient elution mode for group two standards (chlorogenic acid, genestic acid, protocatechuic acid, myricetin, isorhamnetin, luteolin, vanillin and caffeic acid)

Time/min	Solvent A
0-15	100 %
15-20	85 % A
20-30	50 % A
30-35	0 % A
36-42	100 % A

Results and Discussion

TLC analysis of unhydrolyzed and hydrolyzed n-butanol fractions

Unhydrolyzed and hydrolyzed n-butanol fractions were subjected to TLC and HPLC to predict the type of phenolic compounds present. TLC is the essential preliminary method for qualitatively determining the profile of phenolic compounds (27). Different solvent systems were used; the S1 solvent system, ethyl acetate: acetic acid: water (14:3:3), was the best mobile phase for unhydrolyzed n-butanol fraction, while the S4 solvent system, Toluene: ethyl acetate: formic acid (12:5:3), achieved efficient chromatographic separation for hydrolyzed n-butanol fraction. Based on the TLC results, rutin, hyperoside and chlorogenic acid were detected; quercitrin was not detected in the unhydrolyzed n-butanol fraction (Fig. 2).

In the S_4 solvent system, several compounds were separated on a TLC plate in the hydrolyzed n-butanol fraction. Regarding flavonoids, myricetin, quercetin, kaempferol, isorhamnetin, luteolin and apigenin were detected (Fig. 2). For phenolic acids, no distinguished spots were identified in this fraction after spraying with 5 % alcoholic $FeCl_3$ (Fig. 2). This might be related to these acids' partial degradation and low concentrations due to prolonged hydrolysis time (28).

HPLC analysis of the unhydrolyzed and hydrolyzed n-butanol fractions

HPLC is an excellent tool for separating, qualitatively and quantitatively identifying phenolics in plant extracts (29). Table 4 demonstrates the retention time for the phenolic standards used in HPLC.

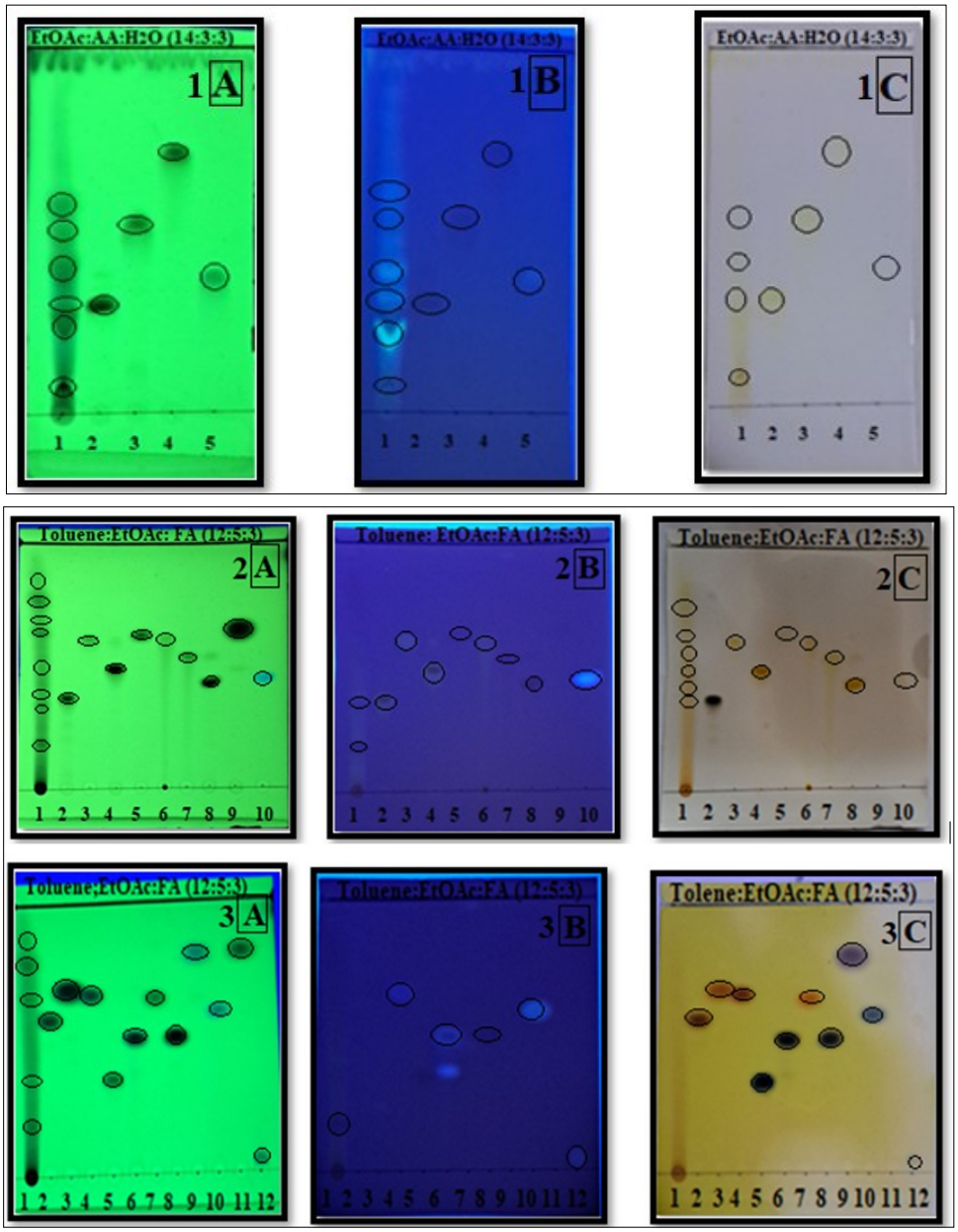

Fig. 2. 1A, 1B and 1C, TLC chromatograms developed in S_1 solvent system, 1: unhydrolyzed n-butanol fraction, 2: rutin, 3: hyperoside, 4: quercitrin and 5: chlorogenic. 2A, 2B and 2C, TLC chromatogram developed in S_4 solvent system, 1: hydrolyzed n-butanol fraction: myricetin, 3: kaempferol,4: quercetin, 5: naringenin,7: isorhamnetin, 7: apigenin,8: luteolin, 9: vanillin, 10: Scopoletin under UV light 254, 366 nm and after spraying with alcoholic KOH. 3A, 3B and 3C, TLC chromatogram developed in S_4 solvent system,1: hydrolyzed n-butanol fraction, 2: Syringic acid, 3: vanillic acid, 4: ferulic acid,5: gallic acid, 6: caffeic acid, 7: p-coumaric acid, 8: protocatechuic acid, 9: salicylic acid, 10: gentisic acid, 11: cinnamic acid, 12: chlorogenic acid. under UV light 254, 366 nm and after spraying with 5 % alcoholic ferric chloride.

Table 4. Retention times for each group of standard compounds in its designated eluent

Group one standards		Group two standards		Group three standards	
Standard	**Retention time in minutes**	**Standard**	**Retention time in minutes**	**Standard**	**Retention time in minutes**
gallic acid	2.18	gentisic acid	3.7	hyperoside	2.1
chlorogenic acid	3.00	protocatechuic acid	4.2	quercetrin glycoside	5.9
ferulic acid	3.82	myricetin	5.22	quercetin	7.0
apigenin	4.15	isorhamnetin	6.12		
syringic acid	5.00	luteolin	7.08		
naringenin	5.75	vanillin	8.66		
kaempferol	6.11	caffeic acid	9.2		
rutin	8.01				
p-coumaric acid	10.20				
salicylic acid	11.3				
vanillic acid	12.11				

Three different solvent systems were used to obtain the retention time for standards and separated compounds in the analyzed fractions. Solvent system alteration and changing the bonded phase on the column packing (column functionality) were among the strategies used to improve separation and prevent coeluting or superimposed peaks, thus achieving considerable variations in retention time that minimize errors in recognizing separated compounds in the analyzed fractions (30). A qualitative HPLC analysis revealed that phenolic compounds in their free and conjugated form were identified in the unhydrolyzed n-butanol fraction. Meanwhile, only the free form of phenolics was present in the hydrolyzed fraction (31).

None of the analyzed fractions contained gentisic acid. However, previous study reported the presence of gentisic acid in some species of Amaranthus (*Amaranthus hypochondriacus*, *Amaranthus cruentus*, and *Amaranthus caudatus* (32). Before hydrolysis, rutin, quercitrin and hyperoside were detected only in the n-butanol fraction. Post-hydrolysis, just free-form flavonoids were recognized. This is attributed to the complete hydrolysis of the glycosidic linkage. A previous study reported that aglycones were produced efficiently from glycosides by refluxing the extract with 1.2 M HCl in 50 % aqueous methanol at 80 °C for 2 hrs (28).

HPLC chromatograms for the identified flavonoids and phenolic acids are shown in Fig. 3 and their names and AUC are listed in Table 5. Naringenin, apigenin, quercitrin, rutin and hyperoside were only detected before hydrolysis. The remaining flavonoids and phenolic acids are present in both

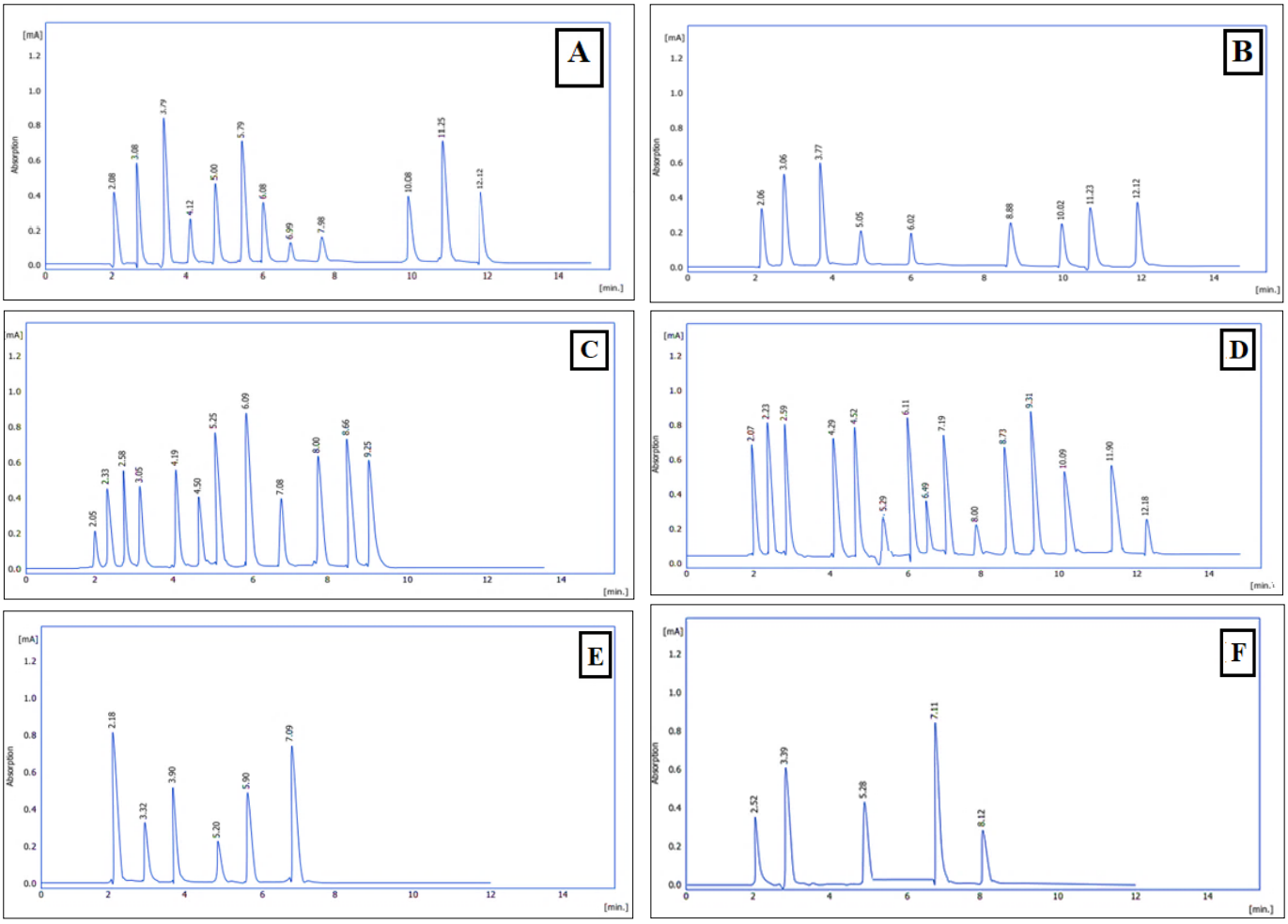

Fig. 3. Representative HPLC chromatogram for A: unhydrolyzed n-butanol fraction, B: hydrolyzed n-butanol fraction (group 1), C: unhydrolyzed n-butanol fraction, D: hydrolyzed n-butanol fraction (group 2), E: unhydrolyzed n-butanol fraction, F: hydrolyzed n-butanol fraction (group three).

Table 5. Retention times and areas for identified compounds identified in the three eluents

Retention times and areas for the identified compounds developed in eluent one					
Retention time in minutes	**Area [mAU.s]**	**Identified compounds in the unhydrolyzed n-butanol**	**Retention time in minutes**	**Area [mAU.s]**	**Identified compounds in the hydrolyzed n-butanol**
2.08	5895.08	gallic acid	2.06	3256.98	gallic acid
3.08	7854.11	chlorogenic acid	3.06	5688.70	chlorogenic acid
3.79	15246.22	ferulic acid	3.77	10225.45	ferulic acid
4.12	3256.25	apigenin	5.05	2535.66	syringic acid
5.00	5321.00	syringic acid	6.02	2011.45	kaempferol
5.79	9856.65	naringenin	8.88	1895.80	-----
6.06	3214.56	kaempferol	10.02	2632.55	p-coumaric acid
6.99	985.65	-----	11.23	6521.45	salicylic acid
7.98	1002.55	rutin	12.12	2010.36	vanillic acid
10.08	3265.90	p-coumaric acid			
11.25	8520.11	salicylic acid			
12.12	3265.99	vanillic acid			
Retention times and areas for the identified compounds in eluent two					
2.05	18254.26	-----	2.08	26854.08	-----
2.33	25498.08	-----	2.2	39856.24	-----
2.58	44125.65	-----	2.59	63621.44	-----
3.05	50124.79	-----	3.03	60112.47	-----
4.19	65852.11	protocatechuic acid	4.2	39652.11	protocatechuic acid
4.5	26985.45	-----	4.58	112520.47	-----
5.25	80124.56	myricetin	5.29	10750.18	myricetin
6.09	98542.11	isorhamnetin	6.1	30652.14	isorhamnetin
7.08	33652.14	luteolin	6.4	54712.69	-----
8.00	74125.64	-----	7.1	22123.52	luteolin
8.66	77412.65	vanillin	8.02	105853.25	-----
9.25	65982.65	caffeic acid	8.7	112854.79	vanillin
			9.2	30652.12	caffeic acid
			10.05	24159.86	-----
			11.85	9652.14	-----
Retention times and areas for the identified compounds in eluent three					
2.18	125652.49	hyperoside	2.52	20125.65	-----
2.33	50125.66	-----	3.39	150324.58	-----
3.9	92541.15	-----	5.28	120325.99	-----
5.2	33621.04	-----	7.11	352102.65	quercetin
5.9	43652.80	quercetrin	8.12	46521.08	
7.09	112421.49	quercetin			

fractions (unhydrolyzed and hydrolyzed). Chlorogenic acid was present in both fractions, but its amount after hydrolysis was smaller. Chlorogenic acid is an ester of caffeic acid with quinic acid; basic hydrolysis is required to release caffeic acid (basic hydrolysis breaks the ester linkage), while acidic hydrolysis breaks the glycosidic linkage (33). In previous studies, syringic, vanillic, protocatechuic, sinapic, ferulic, cinnamic, p-coumaric and caffeic acids were the phenolic acids, while quercetin diglycoside, rutin and kaempferol glycoside were the flavonoids detected in *Amaranthus viridis* seeds (34). Another study reported rutin, quercetin and gallic acid in the leaves (35). The area or height of the peak can be used to determine or estimate the concentration of the separated compounds. If the peaks are sharp and the solvent flow rate is precisely controlled, then the peak area or height is proportional to the concentration (Kathryn Haas). The areas and, thus, the concentrations of all the separated compounds following hydrolysis were smaller than before hydrolysis; this may be attributed to the degradation of these compounds, especially sensitive flavonoids such as myricetin, in addition to phenolic acid degradation, since mixture reflexing was continued for six hours. Also, antioxidants, such as vitamin C, were not used during hydrolysis; thus, the degradation of phenolic compounds was enhanced (25). Also, acidic hydrolysis does not affect the bound form of phenolic acids(ester-linked phenolic acids) (33). The concentration of quercetin after hydrolysis was higher since quercetin is the aglycone part of rutin (36), quercitrin, hyperoside, in addition to other reported quercetin glycosides in *Amaranthus viridis* (34). Quercetin is also categorized as a flavonoid resistant to degradation (28). Fig. 4 shows the quercetin glycosides that, upon hydrolysis, gave quercetin aglycone.

Antioxidant effect of n-butanol fraction before hydrolysis

IC_{50} values express the concentration of extracted fractions that tend to quench 50 % of the free radicals. Lower IC_{50} values reflect excellent antioxidant activity, whereas higher IC_{50} values designate weak antioxidant activity (37). Table 6 demonstrates the percentage of scavenging activity of the n-butanol fraction and ascorbic acid at the selected doses. The maximum scavenging activity for the extract and control was achieved at 10 mg/mL. Fig. 5 demonstrates the IC_{50} value for the ascorbic acid and n-butanol fraction. The IC_{50} of the n-butanol fraction (28.18 mg/mL) is slightly higher than that of standard ascorbic acid (29.83 mg/mL).

Table 6. Concentrations and related % scavenging activity of n-butanol fraction and ascorbic acid

Concentration mg/mL	% scavenging activity of n-butanol fraction	% scavenging activity of ascorbic acid
1	66.79	75.31
10	80.58	95.79
20	58.34	87.40
30	39.38	64.88
40	22.14	47.92

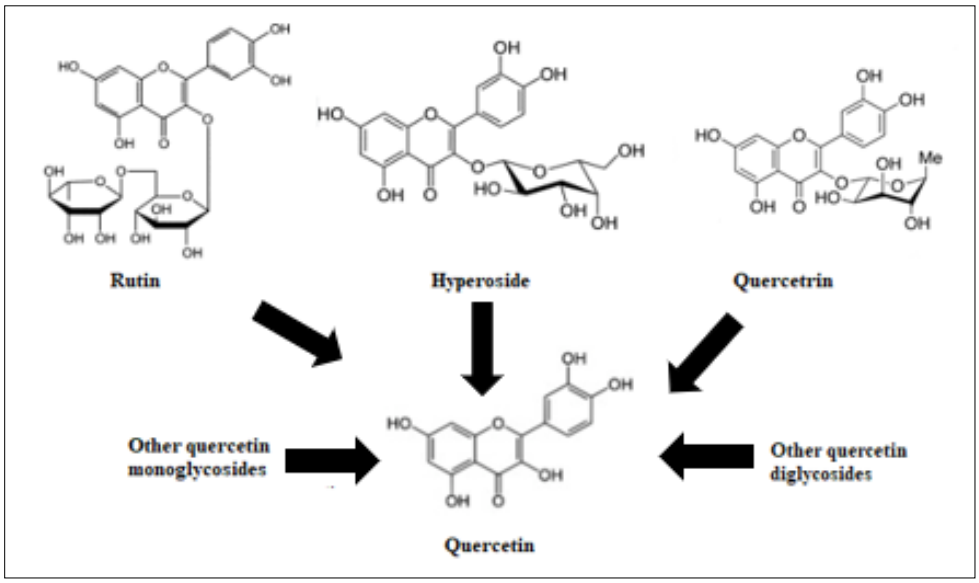

Fig. 4. Hydrolysis of quercetin glycosides.

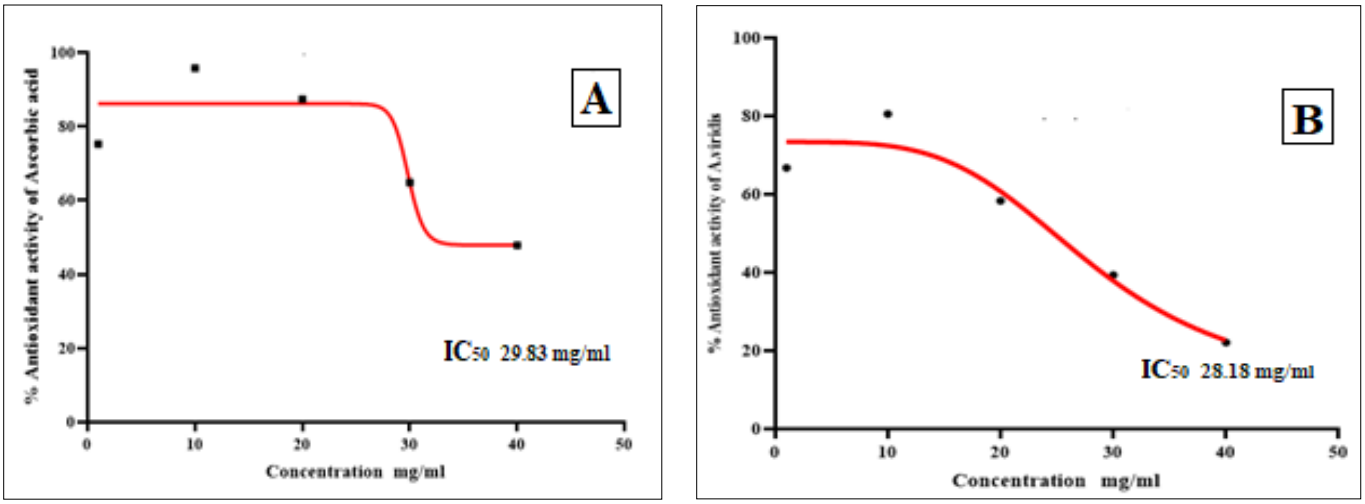

Fig. 5. Antioxidant activity and estimation of IC_{50} value of (A) ascorbic acid and (B) n-butanol fraction.

The obtained result was consistent with that demonstrated by Sunday's study (38). A previous study reported that the ethyl acetate leaf extract of *A. viridis* has higher antioxidant activity than the ethyl ether extract (39). The antioxidant effect is attributed to the phytochemicals, especially phenolic compounds, present in the n-butanol fraction, which scavenges and stabilizes DPPH free radicals, as demonstrated by TLC and HPLC on the n-butanol fraction.

Cytotoxic effect of n-butanol fraction before hydrolysis on HRT-18

Evaluation of cytotoxicity is a decisive initial step in recognizing the toxic effect of a substance, including crude plant extracts or isolated biologically active compounds obtained from natural sources, including plants (40). The cytotoxic potential of the n-butanol fraction of the 80 % ethanolic extract of *A. viridis* aerial parts demonstrated a mild cytotoxic activity against HRT-18 in a concentration-dependent pattern with an IC_{50} of 302 µg/mL. Fig. 6 shows explicitly the cytotoxic effect of the tested fraction on HRT-18 using consecutive doses of the extract starting from 31.2 to 1000 µg/mL. Fig. 7 shows the treated cells' morphological changes compared to the untreated ones.

As observed in the treated cell, the number of cells is reduced and changes in their morphological characteristics, such as shrinkage, production of apoptotic bodies, or membrane blebbing, indicate cell cytotoxicity (41). Our result declared a mild cytotoxic effect on HRT-18; however, a study published recently demonstrated no cytotoxic effect of aerial parts of methanolic extract of some samples of *Amaranthus viridis* on metastatic breast cancer (MDA-MB-231) and on the human liver ($HepG_2$), meanwhile other samples collected from different locations from Fayoum districts in Egypt demonstrated a powerful cytotoxic potential. The cytotoxic effect of these samples is attributed to the presence of alkaloids and appreciable quantities of guttiferic acid (42). In some instances, the absence of cytotoxic effect in some species of *Amaranthus viridis* could also be ascribed to the presence of high levels of antioxidant constituents such as ascorbic acid and polyphenols that scavenge or chelate free radicals in cancerous cells, mitigate apoptosis and thus inhibit cytotoxic effects (43). Also, the non-cytotoxic impact could be attributed to the absence of alkaloids and guttiferic acid in the analyzed fraction. The variation in types of phytochemicals results in variation in biological activities, including the cytotoxic effect, which may be related to the impact of climate, environmental, and ecological variations in addition to plant growth stage that affects the level and the types of secondary metabolites production and hence the pharmacological effect (44).

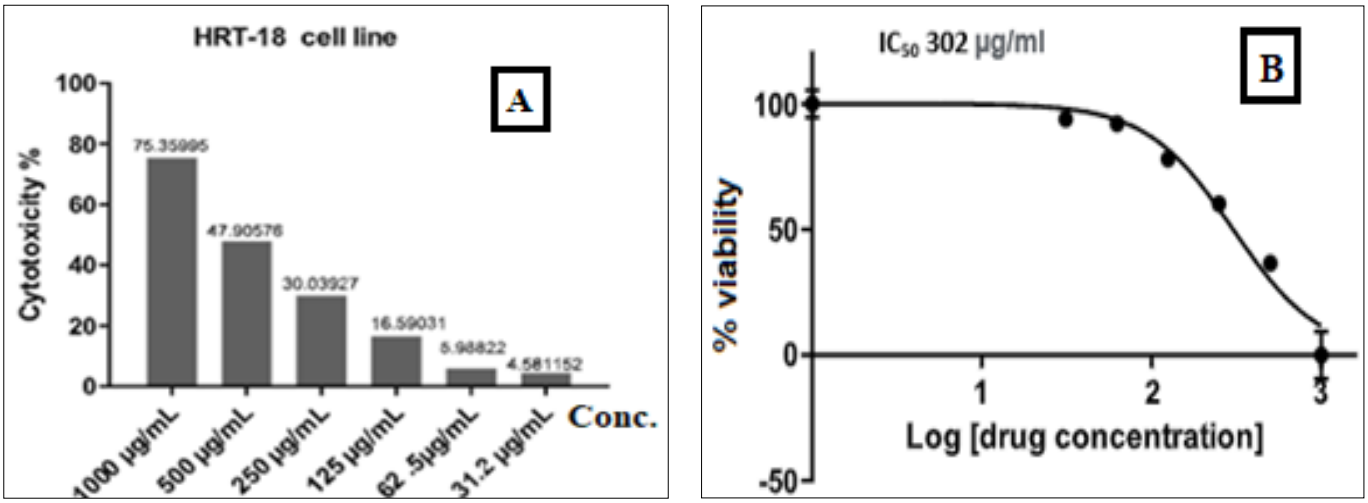

Fig. 6. Illustrative image for morphological features in HRT-18 before and after treatment with n-butanol fraction.

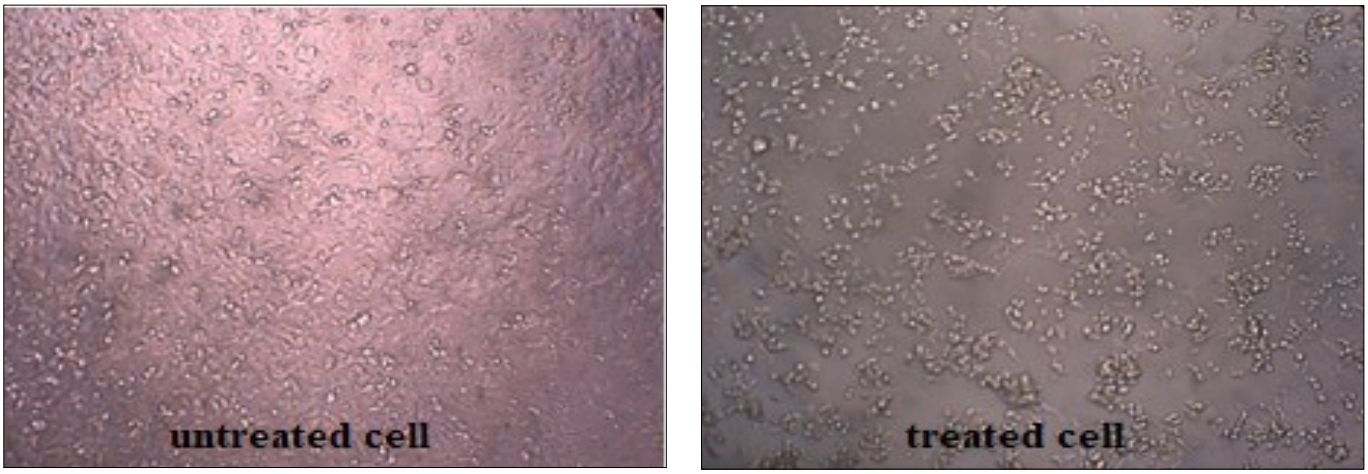

Fig. 7. Illustrative image for morphological features in HRT-18 before and after treatment with n-butanol fraction.

Conclusion

The present study demonstrated the phenolic profile of Iraqi *Amaranthus viridis* unhydrolyzed and hydrolyzed n-butanol fractions. Quercetin is present in significant amounts after hydrolysis. Prolonged hydrolysis time adversely affects the phenolic compound levels, highlighting the importance of optimizing hydrolysis parameters, including time, for preservation of phenolic compounds qualitatively and quantitatively. The plant demonstrated antioxidant activity and a mild cytotoxic effect on the HRT-18 cell line, calling for further tests to confirm the anticancer potential, focusing attention on the necessity of multi-assay lines and profound mechanistic studies.

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Authors' contributions

NSJ and EJK completed the literature review, designed and performed the experiments, wrote and edited and finally reviewed the manuscript. All authors read and accepted the final manuscript.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflict of interest to declare.

Ethical issues: None

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