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RESEARCH ARTICLE

Optimization of ultrasonic-assisted extraction and *in vitro* determination of the cytotoxic effect of ethyl acetate fraction of wild *Amaranthus viridis* on SKGT-4, AGS and A431 cell lines

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Abstract

Amaranthus viridis L. belongs to the Amaranthaceae family. It is a rich source of numerous phytochemicals and amino acids. The objective of this work was to optimize Ultrasound-Assisted Extraction (UAE) based on the extraction yield and Thin-Layer Chromatography (TLC) profile under different conditions, to compare the optimized UAE to the Soxhlet extraction method and evaluate the cytotoxic effects of the ethyl acetate fraction of the 80 % ethanolic extract on the SKGT-4 (human esophageal adenocarcinoma), AGS (human gastric adenocarcinoma) and A431 (human epidermoid carcinoma). A one-factor at a time experiment was carefully designed to assess the influence of the following factors on the extraction: time, frequency, solid-to-solvent ratio and aqueous ethanol concentration. Soxhlet extraction using 80 % aqueous ethanol was done for defatted plant material, then fractionation using chloroform, ethyl acetate and n-butanol. Cytotoxicity of ethyl acetate fraction was evaluated using the MTT assay on AGS, A431 and SKGT-4 cell lines. The results indicated that in the UAE, the solid-to-solvent ratio has the most significant effect on yield. Soxhlet extraction proved to be more efficient than UAE in terms of TLC profiles. The cytotoxicity of the ethyl acetate fraction exhibited cytotoxic activity against the tested cell lines in a concentration-dependent manner. Thus, selecting a particular extraction method depends on the target compounds. Soxhlet is preferred for gaining certain compounds that require heat for their extraction. The ethyl acetate fraction showed a cytotoxic effect on various cell lines related to cell components and their interactions with phytochemicals present in this fraction.

Keywords: A431; AGS; *Amaranthus viridis;* SKGT-4; UAE

Introduction

Earlier, thousands of years ago, people recognized the essential role of plants in various aspects of human life, mainly for nutrition and disease treatment. 80 % of the population uses medicinal plants for ailment management (1). The therapeutic potential of medicinal plants gave rise to the current medications made from pharmacologically active molecules isolated from these plants and used directly or after certain modifications to suit human use (2). *Amaranthus viridis* L. (family Amaranthaceae) is among the plants with multiple traditional uses for labor pain, venereal diseases, eye and respiratory problems and others (3). This herb gained attention as it is a rich source of amino acids and is used as a pseudocereal for individuals with grain allergies (4).

Extraction is the initial step for isolating and purifying bioactive compounds from their natural sources (5). This step is crucial in natural product research and ongoing efforts have been made to enhance extraction efficacy while simultaneously reducing the extraction time and cost (6).

Extraction is accomplished using traditional conventional methods and modern extraction techniques. Traditional methods such as maceration, digestion, infusion, decoction and Soxhlet. These extraction methods are simple but have drawbacks, including prolonged operational times, the need for large volumes of solvents and the degradation of heat-sensitive bioactive compounds in hot techniques. Modern methods represent the optimized solution for these problems (7). Modern extraction methods include supercritical fluid extraction, microwave-assisted extraction, ultrasonic-assisted extraction and pressurized liquid extraction. These methods save time and solvents and enhance extraction yield (8). UAE is a green extraction technique that causes cell membrane rupture and the diffusion of the cell contents into the surrounding medium. When US waves propagate through any media it creates a series of compression and expansion in the molecules of media such alternate pressure changes cause the formation growth and implosion of the air bubbles in the liquid media, that implosion is a violent collapse generate extremely high pressure and temperature at the surface of the cell membrane of plant matrix. As a result, cell thinning and distribution of the membrane create micro channels in the membrane; therefore, intracellular content becomes more available to solvent and solvent penetrates more easily through the microchannel toward target compounds, thus enhancing mass transfer and enhancing yield (9). The schematic illustration of the ultrasonic-assisted extraction mechanism is shown on Fig. 1.

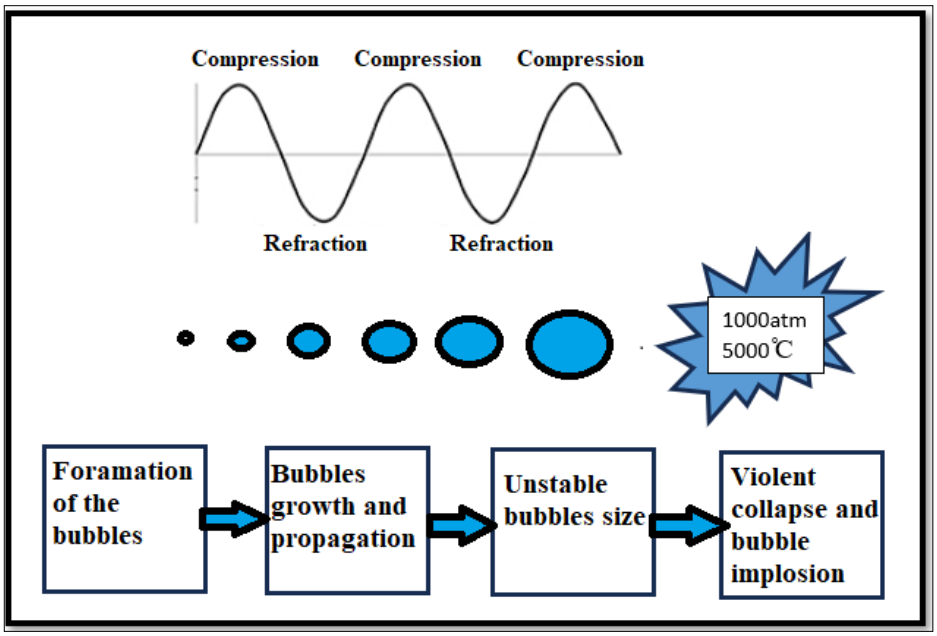


Fig. 1. The schematic illustration of the ultrasonic-assisted extraction mechanism.

Globally, cancer is responsible for one out of six deaths and it is the second leading cause of mortality (10). Esophageal and gastric cancer are the most common cancers in Asia (11). Skin cancer also affects millions of people annually. Despite surgical intervention being the standard option for cancer management, there is an increasing demand for new antineoplastics, particularly those derived from nature. Herbs and medicinal plants were tried and could be considered an option for the management of GIT cancer, including gastric and esophageal cancer, besides skin cancer (10,12). In Iraq, these cancers are of particular concern. The selected cell lines represent models for these tumors, aiding in demonstrating the effect of the same extract components on cancers of different origins. The primary purpose of the current research was to use one factor at a time experiment in UAE to evaluate various factors' influence, including extraction time, frequency, solvent concentration and solid-to-solvent ratio on extraction yield, compare it to the Soxhlet extraction method and to evaluate the cytotoxic effect of ethyl acetate fraction on SKGT-4, AGS and A431 cell lines.

Materials and Methods

Gathering and authentication of herbal plant material

Fresh aerial parts of the *Amaranthus viridis* plant were gathered from Baghdad during the spring of 2023. Assistant Professor Dr. Sukaena Abass verified the plant at the herbarium of the College of Science, University of Baghdad. The plant was gently washed with water, allowed to dry in the shade for three weeks and then ground to a fine powder using an electric mill.

Extraction by UAE

Before performing UAE, the powdered plant materials (10 g each) were defatted with 75 mL of hexane using the Soxhlet apparatus until exhaustion. The temperature was set at 35 °C. This procedure was repeated several times to get the required defatted portions of plant materials; then, the defatted samples were subjected to UAE using a prob ultrasonic device (QSONICA sonicators). The extraction was performed at room temperature.

Experimental design using one factor at a time experiment

A one-factor-at-a-time experiment was meticulously designed to evaluate the influence of each factor. The defatted sample (10 g) and a specified amount of solvent (aqueous ethanol) were placed in a 250 mL beaker. Four factors were evaluated: time, frequency, solid-to-solvent ratio and concentration of the aqueous ethanol.

Three selected factors remain constant during the extraction, while one factor undergoes alteration. The comprehensive conditions for each run were as listed in Table 1. The prob runs in pulse mode. The pulse duration was 20 sec, while the pulse interval was 10 sec during the cycle time. After each extraction run, the extract was filtered and dried. The extraction yield was determined. Extraction yield and TLC were used as an index for extraction efficiency (13). In TLC, a mobile phase composed of toluene, ethyl acetate and formic acid (12:5:3) was used (14). Extraction was performed again using the optimized parameters (Table 2) and the extraction yield was determined and compared to the Soxhlet method.

Statistical analysis

The results (extraction yields) were analyzed using SPSS and Graph Prism 8. The statistical method includes multiple linear regression. Level of significance $\alpha = 0.05$.

Extraction by a conventional Soxhlet extractor

About 10 g of the desired plant was packed in a thimble and defatted with 75 mL of n-hexane at 35 °C until exhaustion. The hexane filtrate was concentrated for further analysis. The defatted mark was dried and then repacked in a new thimble. Extraction was performed at 50 °C using 80 % aqueous ethanol (75 mL) till exhaustion. The extract was then filtered and dried to obtain the desired dry extract. The extraction yield was determined; TLC was done to compare the phytochemical profile for dry extracts obtained from both extraction methods using a mobile phase composed of toluene: ethyl acetate and formic acid in proportions of (12:5:3). Based on the TLC result, Soxhlet method was done on a large scale using 200 g of plant material. The extract obtained by this method was subjected to fractionation (14) where it was reconstituted in distilled water (250 mL) with continuous stirring in a water bath until partial solubilization. A reconstituted extract was

Table 1. Fixed and changed factors (variables) used in a one-factor experiment at a time in the four selected conditions

Condition I		**Condition II**	
Fixed variables	**Changed variable**	**Fixed variables**	**Changed variable**
Time (15 min)	Solid to solvent ratio 1:5 1:7.5 1:10	Solid to solvent ratio 1:7.5	Frequency (kHz) 40 50 60
Frequency (50 kHz)		Time (15 min)	
% ethanol concentration (80 %)		% ethanol concentration (80 %)	
Condition III		**Condition IV**	
Fixed variables	**Changed variable**	**Fixed variables**	**Changed variables**
Solid to solvent ratio 1:7.5	% Ethanol concentration 70 % 80 % 90 %	Solid to solvent ratio 1:7.5	Time (min) 10 15 20 25
Frequency (50 kHz)		Frequency (50 kHz)	
Time (15 min)		% Ethanol concentration (80 %)	

Table 2. Optimized extraction factors were achieved in UAE

Optimized ultrasonic-assisted extraction factors			
Solid-to-solvent ratio	**Frequency**	**% ethanol concentration**	**Extraction time**
1:10	60 kHz	90 %	25 min

partitioned individually and sequentially with chloroform, ethyl acetate and finally n-butanol (250 mL) four times for each solvent, excluding n-butanol. All remaining fractions were dried with anhydrous sodium sulfate and filtered. A rotary evaporator was used to achieve complete dryness of the fractions. The ethyl acetate fraction was subjected to preliminary analysis using chemical tests for flavonoids, phenolic acids, cardioactive glycosides and terpenoids (1,15). This fraction was chosen to evaluate its cytotoxic effect.

Determination of the cytotoxic effect of ethyl acetate fraction against SKGT-4, AGS and A431 cell lines

SKGT-4

The esophageal carcinoma cell line was established in 1989 from a primary tumor from an 89-year-old Caucasian male who had dysphagia secondary to a well-differentiated adenocarcinoma arising in the Barrett epithelium of the distal esophagus (16).

AGS cell line

This cell was obtained from the stomach tissue of a white female patient (54 years) having gastric adenocarcinoma (17).

A431 cell line

This cell was isolated from the skin tissue of an 85-year-old female having epidermoid carcinoma by the research team supervised by D. bJ. Giard (18).

The *in vitro* experiments involving these cell lines were conducted following the institutional ethical protocols. Cell lines were authenticated and all procedures adhered to relevant biosafety and ethical guidelines for cell culture research.

Maintenance of cell cultures

The SKGT-4 cell line was cultured in minimum essential media MEM (US Biological, USA) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS), penicillin (100 IU) and streptomycin (100 IU). Incubation of the cells was optimized in a humidified atmosphere at 37 °C. Exponentially growing cells were employed to accomplish the experiment (19). The same procedure was applied to the other cell lines used in the study.

MTT procedure

A 96-well microplate was used to seed SKGT-4 cells at a density of 10000 cells and incubated for 72 hr at 37 °C until the development of a monolayer confluence. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to estimate cytotoxicity. The cells were exposed to serial concentrations of ethyl acetate fraction (31.2, 62.5, 125, 250, 500, 1000 µg/mL).

After 72 hr of incubation in each well, 28 µL (2 mg/mL) of MTT dye solution was added. The incubation continued for an additional 3 hr. 100 µL of dimethyl sulfoxide (DMSO) was added to each well and incubated for 15 min. A microplate reader measured the optical density at 492 nm (20). Cytotoxicity % was calculated using the following equation (21).

$$\text{Cytotoxicity \%} = (\text{OD control} - \text{OD sample})/\text{OD control} \times 100$$

OD control is the mean optical density of untreated wells and OD sample is the optical density of treated wells.

Statistical analysis

The collected data were statistically examined using GraphPad Prism 8, one-way ANOVA and multiple comparison Tukey's test with a level of significance $\alpha = 0.05$. The values were expressed as the triple measurements' mean ± standard deviation.

Results

Extraction by UAE and Soxhlet

Phytochemicals are extracted from plant raw materials via two popular methods: UAE and Soxhlet extraction. In the UAE, the optimized extraction factors were determined and summarized (Table 2) based on extraction yields (grams of the extracts) (Fig. 2) and the colour intensity and number of separated spots in TLC chromatograms of various extracts obtained according to the examined variables (Fig. 3).

Frequency and percent ethanol concentration showed a nonsignificant effect on the extraction yield as specified by p-values of 0.665, 0.162 for both factors, respectively. Meanwhile, solid-to-solvent ratio has a highly significant effect (p-value 0.0037 and time also shows a significant effect (p-value 0.0416) (Fig. 4). Upon optimizing the extraction factors, the percentage yield was 18.2 %. In Soxhlet extraction, the percentage yield of the crude hydroalcoholic extract was 19.11 %. Soxhlet and UAE have comparable yields, but better TLC chromatograms for the Soxhlet extraction method. In Fig. 5, a characteristic blue

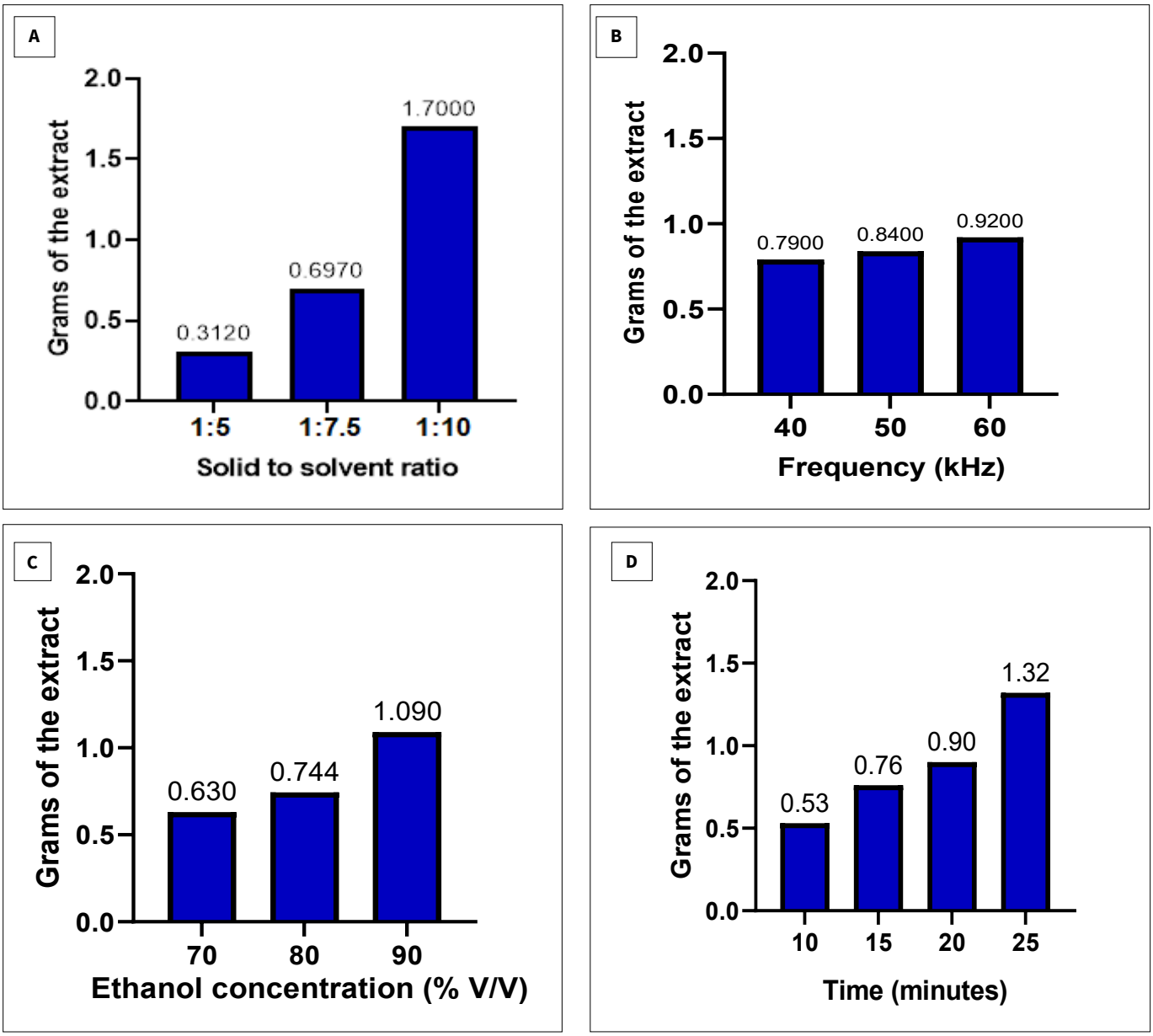
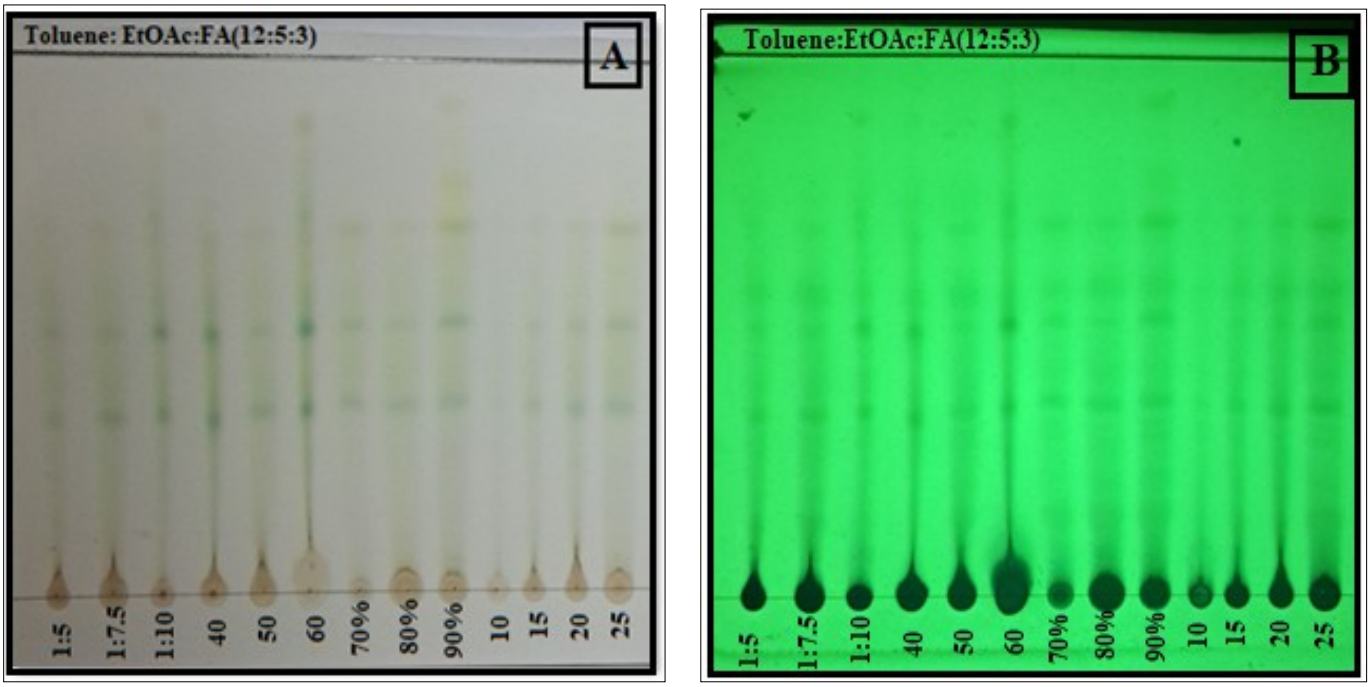


Fig. 2. Effect of four factors on crude extract weights using a one-factor experiment at a time.

A: Solid-to-solvent ratio; B: Frequency; C: % ethanol concentration; D: Extraction time



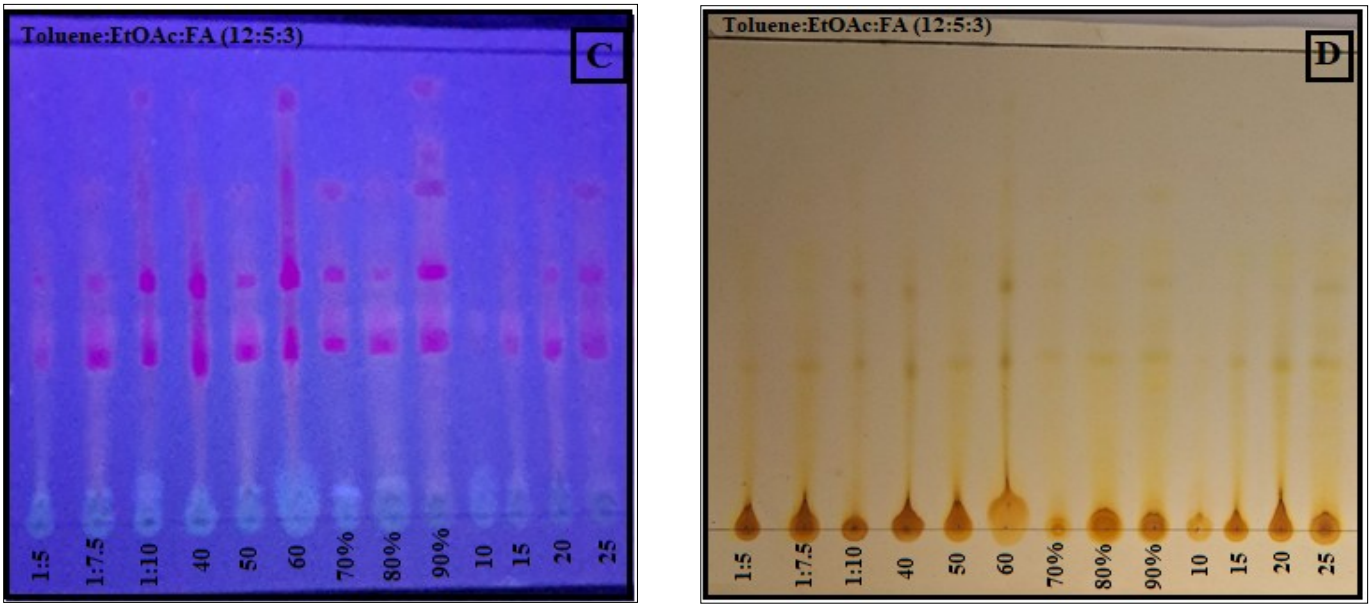


Fig. 3. Comparative TLC chromatograms for extracts obtained from a one-factor experiment at a time.

(1:5,1:7.5, 1:10 denote solid-to-solvent ratio), (40,50,60 denotes frequency in kHz), (70 %, 80 % and 90 % denote % ethanol concentration), 10,15,20,25 min denotes extraction time. A, B, C and D: evaluated at daylight, under UV light 254nm and 336 nm, after spraying with 5 % KOH, respectively

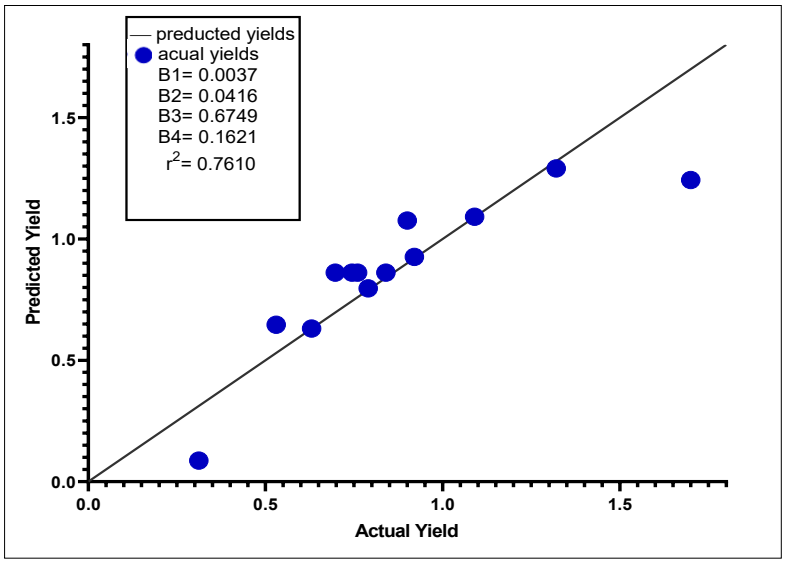


Fig. 4. Actual yield versus predicted yield using multiple linear regression.

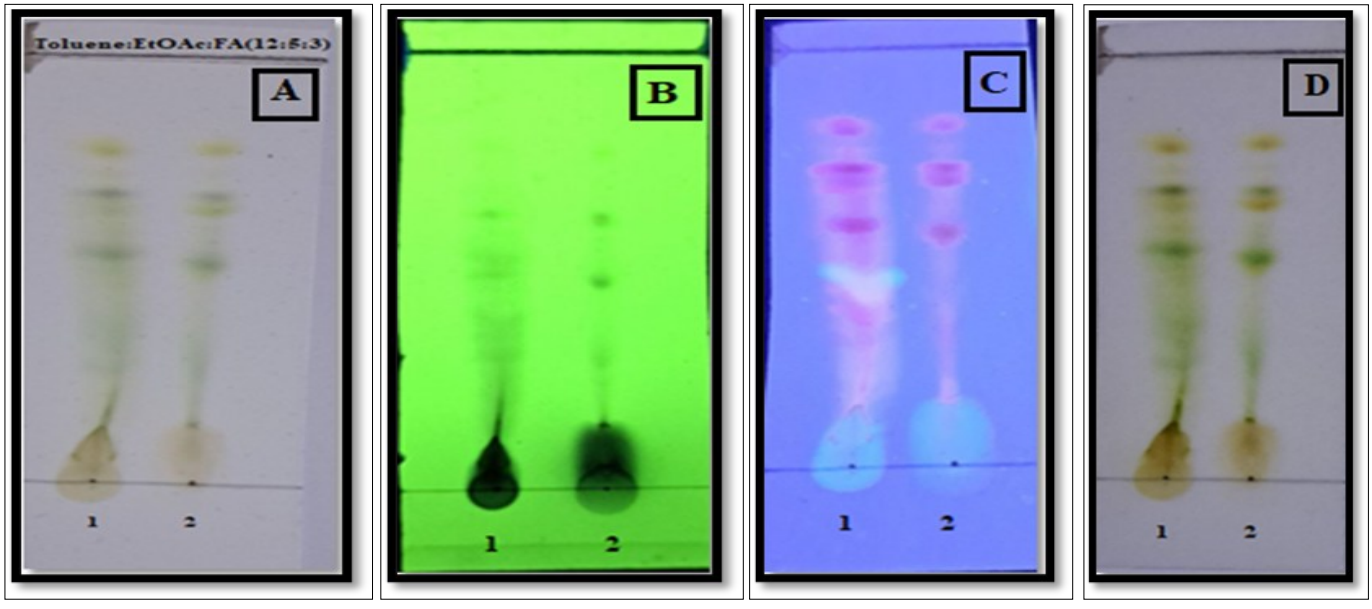


Fig. 5. Comparative TLC chromatogram of extracts obtained from Soxhlet extraction [1] and optimized ultrasonic-assisted extraction [2].

A– Evaluated at daylight; B- Under UV light 254nm; C– Under UV light 336 nm; D– After spraying with 5 % alcoholic KOH

fluorescence spot was observed only in the extract obtained from Soxhlet extraction (R_f value of 0.44), which left for further investigation. Thus, Soxhlet was more efficient and accordingly, large-scale extraction and fractionation were performed. The phytochemical analysis was done for each fraction. In the ethyl acetate fraction, the presence of flavonoids, phenolic acids, terpenoids and cardioactive glycosides was specified (22).

The cytotoxic effect of the ethyl acetate fraction

The cytotoxic potential of the ethyl acetate fraction of the *Amaranthus viridis* aerial parts 80 % ethanolic extract demonstrated cytotoxic activity against SKGT-4, AGS and A431 following concentration-dependent patterns with IC_{50} values of 170.1, 119.8 and 122.7 µg/mL, respectively.

After 72 hr, an increase in cytotoxic potential was observed. In SKGT-4, the percentage of cell cytotoxic effect increased from 18.6 % to 77.16 % when the concentration was raised from 31.2 µg/mL to 1000 µg/mL. In the AGS cell line, the percentage cytotoxic effect rose from 9.3 % to 65.55 % when the concentration was scaled up from 31.2 µg/mL to 1000 µg/mL.

In A431, a maximum cytotoxic effect of 74.44 % was shown when the concentration was 1000 µg/ml, while a weak cytotoxic action of 9.09 % was observed when the concentration was 31.2 µg/ml (Fig. 6).

Morphological alterations reflect malignant cells' sensitivity to cytotoxic therapy. Cancerous cell structural changes, viability and behavior are influenced by anticancer agent-specific mechanisms of action and microenvironment within the cancer cells. The morphological changes associated with the cytotoxic effect are verified in Fig. 7.

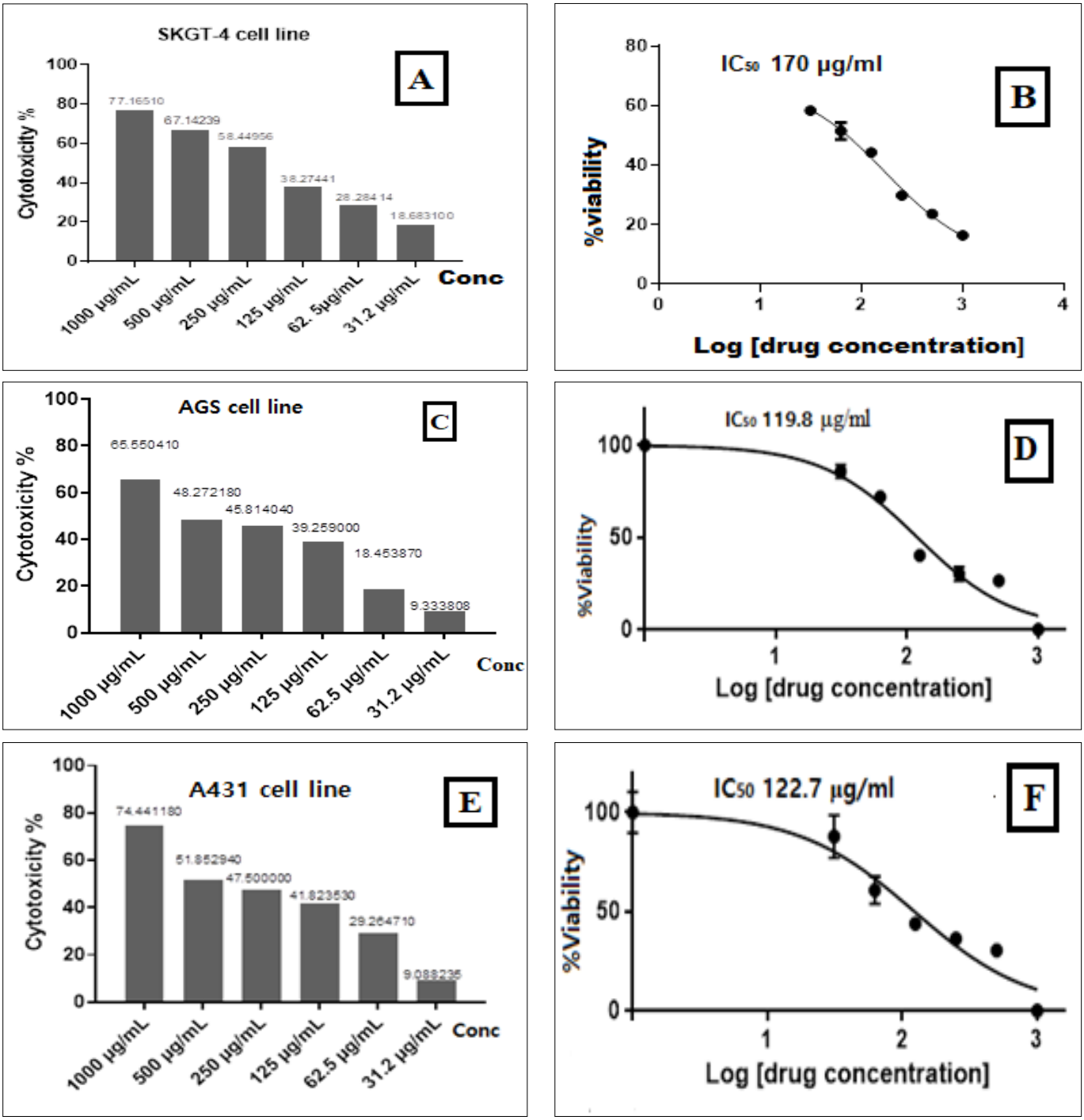


Fig. 6. Ethyl acetate fraction mediated cytotoxicity and IC_{50} values in selected cancer cell lines. (A, C and E): Cytotoxic percentage of ethyl acetate fraction on SKGT-4, AGS and A431 cells; (B, D and F) IC_{50} values of ethyl acetate fraction on the SKGT-4, AGS and A431 cell lines

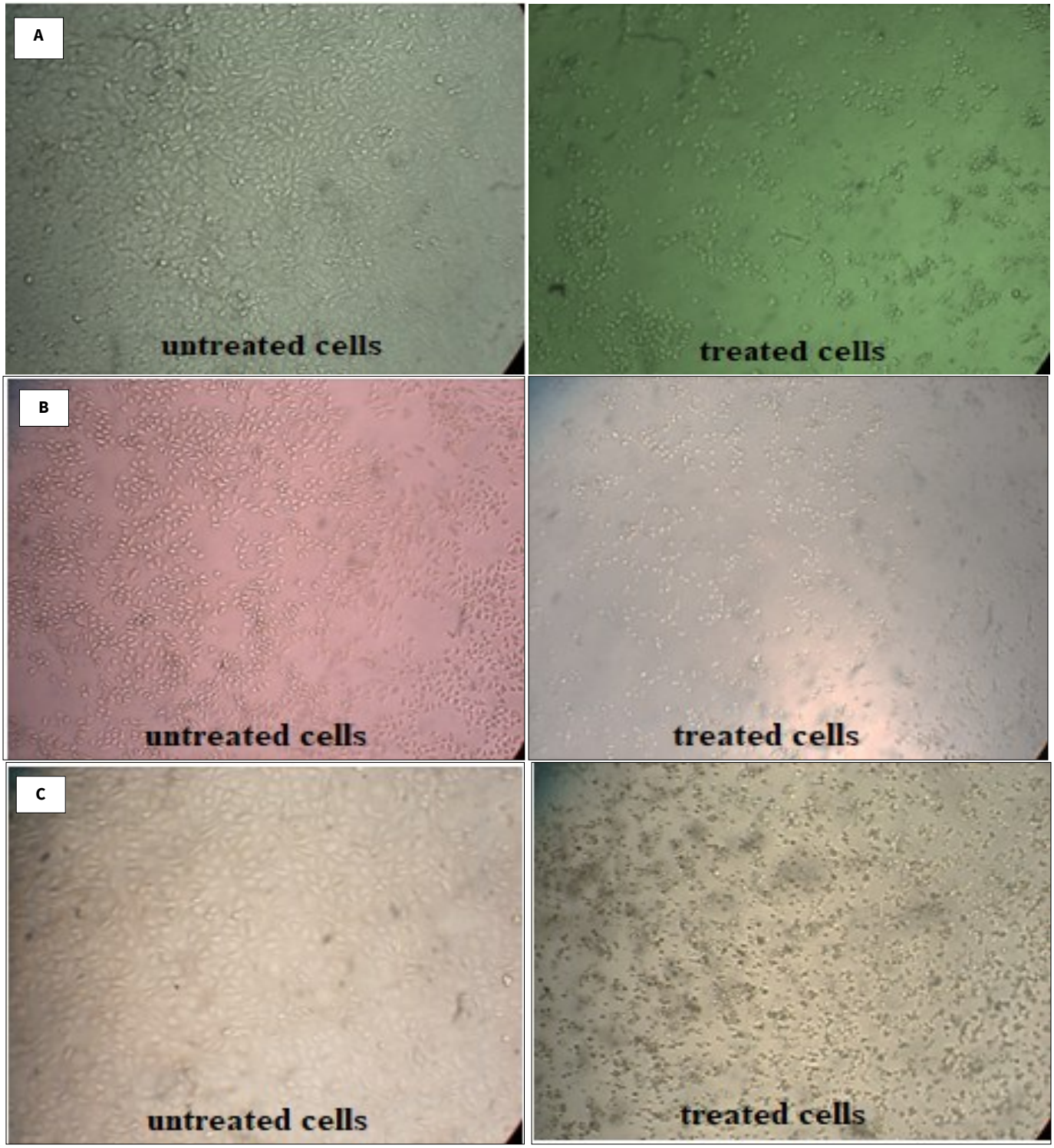


Fig. 7. Morphological changes in the tested cell lines pre– and post– treatment with ethyl acetate fraction. A: SKGT-4, B: AGS and C: A431 cell lines

Discussion

UAE is an advanced non-conventional extraction that reduces the solvent and time required for extraction (9). Based on the results in Table 2, the optimum solid-to-solvent ratio was 1:10. Usually, as solvent volume increases, extraction efficiency increases through dissolution process enhancement. Insufficient solvent volume prevents components from the plant matrix from being effectively transferred to the solvent at low solvent-to-solid ratios (23). The solution's high viscosity at a low solvent-to-solid ratio makes the cavitation effect more challenging (24). Increasing the solvent-to-solid proportion decreases the density of the blended mixture, reducing the attenuation of ultrasonic power and propagation of ultrasonic waves. This aids in the transfer of influential energy while improving the cavitation effect, thus increasing the extraction efficiency and yield (25). As part of the ultrasonic waves is released in the form of heat, solvent loss by evaporation can disrupt the solid-to-solvent ratio and hence, solubilization is reduced; this is most noticeable when the ratio is small. This explains why a solvent-solid ratio had the most noticeable effect (26).

Optimized extraction time is a crucial parameter as it saves time and power. The extraction was executed at different sonication times (10, 15, 20 and 25 min); the highest yield was achieved at 25 min. During extraction, the solute is in contact with the extraction solvent, so increasing the extraction time will improve the extraction for a specific time range. Sonication

extraction comprises two phases; the washing phase is carried out during the first 20 min and accounts for 90 % of the extraction. The second phase is slow extraction, taking about 60 min (27). Thus, the optimal time should be determined; beyond this time, the extraction yield will reach a plateau or may decrease. The longer the extraction time, the higher the probability of solvent loss by evaporation, which reduces the solvent-to-solid ratio and thus adversely affects yield (28). Prolonged extraction time also triggered the destruction and oxidation of phytoconstituents (27).

The selected frequencies were 40,50 and 60 kHz, considered low frequencies that are not proposed to affect the extracted compounds' integrity (29). Cavitation is primarily affected by the frequency (30). For efficient extraction, mechanical force and strong shear generated by low-frequency, high-intensity ultrasound are required; meanwhile, high-frequency, low-power density produces many reactive radicals that are ultimately terminated by phytochemical degradation. A previous study demonstrated increased anthocyanin levels with growing frequency, achieving a maximum level of 62-64 kHz (31). Researchers commonly operate a prob device in a range between 20 and 60 kHz to extract bioactive compounds from natural sources (32). Based on the extraction yield, non-significant differences were observed among the tested frequencies. Nevertheless, higher numbers and distinguishable spots were obtained at 60 kHz; therefore, this frequency was chosen.

In Fig. 4, there is a statistically insignificant increase in the extraction yield with increasing percent ethanol concentration. Ethanol is the most widely used solvent in the UAE. Aqueous ethanol enhances the extraction of methoxylated and hydroxylated compounds, affecting extract yield and quality. Cellular material swelling and increased plant matrix permeability are triggered by water incorporation, thus enhancing the extraction (32, 33).

As a result, setting optimized UAE parameters is essential as it enhances extraction efficiency, maintains the integrity of the phytochemicals and improves the extract quality while achieving a reduction in the environmental impact and resources used (34).

Soxhlet is a traditional extraction process that consumes time and solvent and needs specialized glassware (35). Here, Soxhlet was more efficient than UAE based on the TLC profile. Certain constituents were present in the Soxhlet but not in the UAE extract. This can be explained by the fact that these constituents might require heat during their extraction to improve their solubility in the extracting solvent (36,37). Also, in the UAE compound's integrity might be compromised (structural changes, thermal degradation and free radical formation) due to the high frequency used in the extraction process (38).

The cytotoxic tendency of ethyl acetate was evaluated against SKGT-4, AGS and A431. This fraction demonstrates an IC_{50} in the 119-170 µg/mL range on the examined cell lines. The cytotoxic effects of plant extracts depend on several factors, including the extract's contents, the evaluated concentrations, duration and specific inhibition mechanisms. Also, the cytotoxic effect of the same extract varies across different cell lines (39). On SKGT-4, the examined fraction showed the highest IC_{50} among the other cell lines, meanwhile demonstrating the lowest IC_{50} on the AGS cell line; thus, it exerts a more potent cytotoxic action on this cell line. Consequently, a less concentration is required to inhibit AGS cell proliferation.

The variation in cytotoxic effects among cell lines is likely due to differences in cellular targets and constituent sensitivity, triggering different interactions of ethyl acetate fraction constituents with malignant cell contents. According to preliminary phytochemical investigations, phenolics (flavonoids and phenolic acids), terpenoids and cardioactive glycosides were recognized in the ethyl acetate fraction and the cytotoxic effect is correlated with the existence of these compounds. Phenolics have cytotoxic potential on these cell lines through diverse mechanisms; In SKGT-4, extract containing phenolic compounds enhances cell death through mitochondrial release of cytochrome C and triggers a variety of caspases, such as caspase 9, 8 and 3, simultaneously mitigating the expression of BCL-2 and BclXL (40). In AGS, the phenolics triggered programmed cell death by modifying BAX and *Bcl-2* gene expression (increased BAX/BCL-2 ratio inducing apoptosis in cancer cells) (41). In A431 cells, the cytotoxic effect of phenolics was observed through modifying the level of the expression of certain proteins: ERK, p38 and JNK (c-Jun NH2-terminal kinase) through stimulatory changes in MAPK (Mitogen-Activated Protein Kinase) signaling pathway (42). For cardioactive glycosides, cytotoxic effects can be clarified by cell cycle arrest at G_2/M phase and enhancement of apoptosis (43). Inhibition of topoisomerase I, P53 and HIF-1 alpha synthesis, in addition to increased ROS production and calcium intake, are among the mechanisms associated with the cytotoxic potential of cardioactive glycosides (44). For terpenoids, the cytotoxic effect against A431 is produced by cell cycle arrest at G_0/G_1 phase, modifying molecular targets, which are involved in tumor initiation and progression; tubulin in the initiation stage and HYAL, ODC in the progressive stages (45). Certain terpenoids trigger autophagy in the SKGT-4 cell (46).

Flavonoids, phenolic acids and other types of phenolic compounds induce cytotoxic actions on cancerous cells; however, not all phenolics exhibit the same action, i.e., selectivity toward cancer cells. Differences in the chemical structure of phenolics are another factor affecting cancer cell sensitivity to these compounds. The number and position of hydroxyl groups, the aromatic ring and the unsaturated chain are key structural features required for phenolic compounds to have a cytotoxic effect.

Malignant cell sensitivities to phenolics can greatly vary depending on the tissues from which these cells originated. This suggests that the cytotoxic potential may relate to the type of cancer cell. Even among flavonoids with high structural similarities, compound-specific actions modulate precise biochemical reactions; therefore, the growth of certain tumors could be differentially influential, indicating tissue-specific cytotoxic action (47,48).

Conclusion

The choice of a specific extraction method depends on the target compounds and their applications. In the UAE, the solid-to-solvent ratio and time have the most significant impact on yield, while varying the aqueous ethanol concentration and frequency does not influence the yield. However, an improved qualitative representation of phytochemicals was achieved at maximum concentration and frequency.

UAE is particularly effective when time is a critical factor in the extraction process. Soxhlet is the preferred method for compounds that may require heat for extraction or perhaps

degrade under the frequencies used. The ethyl acetate fraction demonstrated a cytotoxic effect on various cell lines associated with cell-type components and their interactions with the phytochemicals present in this fraction. This cytotoxic effect was concentration-dependent, with the AGS cell line being more sensitive to this fraction. Further research is needed to identify the target phytoconstituent(s) and clarify the cytotoxicity mechanism. To the best of our knowledge this is the first study concerning the extraction and optimization of UAE of Iraqi *Amaranthus viridis* and determining its cytotoxic effect on these cell lines.

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Authors' contributions

NSJ and EJK equally contributed to the manuscript regarding the concept, design, analysis and interpretation of data. All authors read and approved the final manuscript.

Compliance with ethical standards

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

Ethical issues: None

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