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Antioxidants and Anticancer Activity of Beetroot (*Beta vulgaris* L.) Extract

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ABSTRACT

Synthetic drugs, originally developed to combat diseases, have been found to carry potential risks to both human health and the environment. This realization has sparked a renewed interest in medicinal plants, which contain secondary metabolites and antioxidant compounds capable of mitigating the effects of free radicals. Among these plants, *Beta vulgaris* L. subsp. vulgaris, commonly known as beetroot, stands out as a versatile crop with numerous health benefits and applications across various industries. This study aims to explore the chemical composition of beetroot and evaluate its antioxidant and anticancer properties, highlighting its potential as a natural alternative to synthetic drugs in promoting health and preventing chronic diseases. The research methodology involved several key steps: Beetroot composition was determined using AOAC (Association of Official Analytical Chemists) methods. Extractions were performed using various solvents: water, ethanol, citric acid, ascorbic acid, and a mixture of citric and ascorbic acids. Total phenolic content was measured using the Folin-Ciocalteu method. HPLC (High-Performance Liquid Chromatography) analysis was conducted to identify individual phenolic compounds and vitamins. Total antioxidant capacity was assessed using the Trolox equivalent method. Cytotoxicity was investigated in HCT (colon cancer) and MCF-7 (breast cancer) cell lines using the MTT assay protocol, with cell viability calculated using trypan blue staining and hemacytometer counting. The study's findings revealed several interesting aspects of beetroot: Chemical composition: The analysis showed variations in protein, carbohydrates, fiber, moisture, fat, and ash content compared to previous studies. Mineral composition: Significant levels of iron (Fe), sodium (Na), magnesium (Mg), manganese (Mn), zinc (Zn), and calcium (Ca) were detected. Vitamin content: Vitamins C, A, D, and E were quantified. Extraction efficiency: Different extraction methods yielded varying amounts of dry extracts, with the mixture of citric and ascorbic acids (EMCAA) showing the highest extraction ratio. Phenolic compounds: Concentrations were highest in ascorbic acid (EAA) and the mixture of citric and ascorbic acids (EMCAA) extracts. Antioxidant capacity: All extracts demonstrated total antioxidant capacity, with aqueous ethanol extract (AEE) showing the highest value. Anticancer effects: Beetroot extracts exhibited inhibitory effects on MCF-7 and HCT cancer cell lines, with increasing effectiveness over time. In conclusion, this study demonstrates that beetroot possesses a diverse nutritional composition, significant antioxidant properties, and potential anticancer effects. These findings underscore its value as a functional food and potential therapeutic agent, offering a promising natural alternative to synthetic drugs in promoting health and preventing chronic diseases.

Keywords: Chemical composition, Cytotoxicity, Natural alternative, Nutritional value, Phenolic compounds

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INTRODUCTION

Recent discoveries highlighting the potential risks of synthetic drugs to human health and the environment have sparked a renewed interest in medicinal plants, which contain valuable secondary metabolites and antioxidant compounds capable of neutralizing harmful free radicals, offering a safer and more sustainable approach to disease prevention and treatment [1-4].

Beta vulgaris L. subsp. *vulgaris*, part of the Chenopodiaceae family (Angiosperms), is commonly known as beetroot. This herbaceous plant can be classified as either annual or biennial and is cultivated for its edible roots and leaves. The color of the beet varies from yellow to red depending variety all over the world, beets are used human consumption [5].

Beetroot is a versatile vegetable with many uses. Its roots are commonly used in preparing salads, jams, soups, and juices. The leaves, often overlooked, are packed with antioxidants and vitamins, making them a nutritious alternative to spinach in cooking. One of the most distinctive features of beetroot is its vibrant color, which comes from compounds called betalains. These natural pigments have applications beyond the kitchen. They're used commercially and in the pharmaceutical industry as natural food colorings, in cosmetics, drug formulations, and even in paint production. In an interesting development, researcher Firas has explored the potential of beetroot as a natural alternative to nitrates in the production of pastrami. In Iraq, where pastrami is traditionally made as a type of sausage, this finding could have significant implications for the meat processing industry. By using

beetroot in traditional pastrami production, it may be possible to create a more natural product while maintaining its traditional qualities [6].

The organic beetroot dye extract demonstrates remarkable versatility through its exceptional homogeneity, stability at room temperature, and non-toxic properties, enabling its innovative application across diverse sectors including paint manufacturing, optical technologies, industrial processes, color-sensitive industries, and specialized fields like tinted optical contact lens production, while simultaneously offering an environmentally friendly alternative to synthetic colorants due to its natural origin and sustainable characteristics [7].

Beetroot ranks as the 10th most widely cultivated vegetable in the world and is known for its high antioxidant content. These antioxidants act as free radical scavengers, helping to prevent oxidative damage to proteins, DNA, and lipoproteins. Such oxidative damage to macromolecules can lead to chronic diseases including cancer, cataracts, cardiovascular disease, neurodegenerative disorders, and strokes. Fortunately, the antioxidant compounds found in beetroot can help mitigate these risks. Additionally, beetroot contains high concentrations of secondary metabolites, including phenolic acids, flavonoids, and ascorbic acid, further contributing to its health benefits [8].

Beet (*B. vulgaris* L.) is a globally consumed vegetable prized for its high content of biologically active substances. These include betalains, inorganic nitrates, polyphenols, folates, as well as various minerals and vitamins found in its tuberous root. Consumption methods vary by region, with beets being eaten whole, cooked, canned, or minimally processed. Beetroot serves multiple purposes beyond its use as a vegetable. Its juice and extracts are utilized in traditional medicine, as food coloring, and as additives in both food products and cosmetics. The plant is renowned for its potent antioxidant and anti-inflammatory properties, making it a potentially valuable aid in the treatment of numerous health conditions [9]. Beetroot is important for diabetics because it contains a percentage of amylase inhibitors and has the ability to prevent a rapid rise in blood glucose levels [10]. Beetroot is valued for its diverse range of health benefits. It is recognized for its antioxidant properties, which help protect cells from damage. Additionally, beetroot has antimicrobial effects, potentially aiding in fighting off harmful microorganisms. Its anti-inflammatory qualities may help reduce inflammation in the body, while its anti-allergic properties could assist in managing allergic reactions. Beetroot is also noted for its antithrombotic and antiatherogenic characteristics, which may contribute to cardiovascular health by preventing blood clots and the buildup of plaque in arteries. Furthermore, it offers cardioprotective benefits, supporting overall heart health. Lastly, beetroot has vasodilatory properties, meaning it can help relax and widen blood vessels, potentially improving circulation and lowering blood pressure [11]. The purpose of this work is to study the chemical composition of beet roots and study of their effect such as antioxidant and anticancer activity.

MATERIALS AND METHODS

beetroot approximate composition was determined using AOAC methods 17(1), using hexane solvent to extract the fat , Crude proteins were calculated according to the following formula: %N x 6.25 [12]. Total carbohydrate was calculated by difference. All Samples of *B. vulgaris* L. were collected in Baghdad area. Species identification of *B. vulgaris* L. was done by Dr. Ibtehaj Hakeem and it has been registered with herbarium Code, 3589 in Baghdad University

Extraction

Water Extraction (WE)

An aqueous beetroot extract was prepared by homogenizing 50 g of beetroot in 250 mL of distilled water. The mixture underwent maceration for 72 hours at room temperature. After this period, it was centrifuged at 8000 rpm for 30 minutes. The resulting crude extracts were then filtered to remove any remaining solid particles. Finally, the filtered extract was subjected to evaporation at 40°C under reduced pressure. This process continued until the extract was completely dry, yielding a concentrated beetroot extract. This method effectively extracts water-soluble compounds from beetroot, preserving their properties while removing excess water [13].

Alcoholic Extraction (Ethanol) (AEE)

Beetroot tubers were combined with ethanol in a 1:20 ratio and blended using an electric mixer for 30 minutes. The resulting mixture was then strained through a soft cloth, with this process repeated three times. The liquid extract was then separated by centrifugation at 7000 rpm for 30 minutes. Following this, the liquid was concentrated using a rotary evaporator set at 40°C. The concentrated solution was then dried in an oven, also at 40°C. The resulting powder was stored in a sealed, opaque container and frozen. Finally, the yield of the extracted powder was calculated [14].

Extraction using Multiple Concentrations of Citric Acid (EMCC)

Citric acid solutions at 1%, 0.5%, and 0.2% concentrations were prepared, labeled as EMCC 1%, EMCC 0.5%, and EMCC 0.2% respectively. For each concentration, the same extraction process used for the aqueous method was applied separately. This approach allowed for a comparison of extraction efficiency across different citric acid concentrations.

Extraction using 1% Ascorbic acid Solution (EAA 1%)

A 1% ascorbic acid solution was prepared, and the extraction process followed the same steps as those used for the aqueous extraction method.

Extraction using a Mixture of Citric acid (0.5%) and Ascorbic Acid (0.1%)(EMCAA)

A solution was prepared by combining 0.5% citric acid with 0.1% ascorbic acid. The extraction process adhered to the same steps used in the aqueous extraction method. This approach ensured consistency in the extraction procedure while allowing for the evaluation of the combined effects of citric and ascorbic acids on the extraction efficiency of the desired compounds.

Colorimetric Assurance of Absolute Phenols

The quantity of phenolic compounds was determined using the standard Folin-Ciocalteu method (AOAC, 1984). A 0.10 mL sample of the concentrate was mixed with 0.50 mL of Folin-Ciocalteu reagent (diluted 1:10), 1.00 mL of distilled water, and 1.50 mL of 20% sodium carbonate solution. The mixture was then left to react for 1 hour at room temperature (25°C) in darkness. Following this, the absorbance of the solution was measured at 760 nm using a Jenway 6505 UV/Vis Spectrophotometer. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. The calibration curve range was set between 5-50 mg mL−1. This method provides a reliable measure of antioxidant capacity in plant extracts.

HPLC condition of Phenolic Compounds

Quantification of individual phenolic compounds was conducted using reversed-phase HPLC analysis. The analysis employed a SYKAMN HPLC chromatographic system equipped with a UV detector. Separation was achieved using a C18-OSD column (25 cm x 4.6 mm) maintained at 30 °C. A gradient elution method was utilized, with eluent A (methanol) and eluent B (1% formic acid in water, v/v). The elution profile was as follows: 0-6 min, 40% B; 7-20 min, 50% B, with a flow rate of 0.9 mL/min. Sample and standard injection volumes of 100 µL were automatically introduced using an autosampler. Spectral data were acquired at 280 nm. This method ensures accurate identification and quantification of phenolic compounds [13].

Sample Preparation for HPLC Vitamin Assay

5 gm of each freeze-dried plant materials were absorbed 20 ml water. Then 1 ml 0.1 M NaOH and 25 ml phosphate support (1M, pH 5.5) were added to it and saved in dull for 24 hours. The arrangement was first separated through a Whatman No. 1 channel paper and the subsequent filtrate was taken in a 25 ml volumetric jar and arrangement was finished off sufficient with HPLC grade water. The example arrangement was separated through 0.45 µm film channel before infusion into HPLC framework the stock arrangements of test were saved in a cooler for additional utilization. Then, HPLC technology was used to diagnose and estimate vitamins, according to previous research that is has been explained by Seal and Chaudhuri [15].

Total Antioxidants Capacity (TCA) Assay

Total antioxidant capacity was estimated according to the method described by Pascu et al. [16]. TCA was calculated by plotting the absorbance as a function of Trolox concentration (0 – 400 nmol/ml) and interpolating the net absorbance value obtained for the samples (Fig. 1). This method provides a standardized measure of antioxidant activity, allowing for comparison across different samples and studies. The use of Trolox, a water-soluble vitamin E analog, as a reference standard ensures reliable and reproducible results. The assay's sensitivity enables accurate quantification of antioxidant capacity in various plant extracts and food samples.

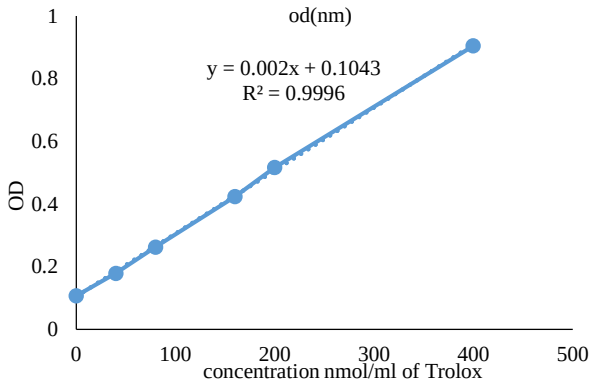


Fig. 1 The standard curve for determination of total antioxidant capacity using trolox equivalent method

The standard procedure that should be applied to complete all the steps during the development of the final color is summarized below. In a test tube, 1 mL of CuCl2 solution (1.0 × 10^-2 M), 1 mL of alcoholic neocuproine solution (7.5 × 10^-3 M), and 1 mL of NH4Ac buffer solution at pH 7.0 are added and mixed thoroughly. Afterwards, (X) mL of extracted beetroot is combined with (1.1 - X) mL of water, resulting in a total volume of 4.1 mL, which should be mixed well to ensure homogeneity. Absorbance against a reagent blank is measured at 490 nm after a 30-minute incubation period. The equivalent antioxidant activity of Trolox can be traced back to the original extract by considering all dilutions and proportions related to the initial mass of beetroot extracted. This process allows for the determination of total antioxidant capacity (TCA) in nmol/mL Trolox units, providing valuable insights into the antioxidant potential of the beetroot extract and its possible health benefits. Accurate measurements and careful adherence to this protocol are essential for reliable results in antioxidant analysis.

Investigation of Cytotoxicity in Cell Lines (*HCT, MCF-7*) The Steps of the MTT Assay Protocol were Followed

MCF-7 and HCT cancer cells, along with normal human HdFn fibroblast cells, were cultured in RBMI1640 medium supplemented with 10% bovine serum and 1% penicillin-streptomycin antibiotics. The cultures were incubated in a 5% CO2 gas incubator at 37 °C to maintain optimal growth conditions. Cells were examined using an inverted microscope to ensure their vitality, check for contamination, and confirm that they had grown to the required density of approximately 10,000 cells/mL. Once the desired cell density was reached, the cells were transferred to a growth chamber. The culture medium was removed, and the cells were washed with a phosphate-buffered saline (PBS) solution to eliminate any residual serum and antibiotics. Trypsin-EDTA enzyme was then added to detach the adherent cells from the culture vessel. The cells were incubated at 37°C for one-minute while being monitored closely until they transitioned from a monolayer to a single-cell suspension. Following this detachment process, fresh growth medium containing serum was added to neutralize the trypsin activity. The cell suspension was collected and placed into centrifuge tubes for further processing. Centrifugation allowed for the pelleting of the cells while removing the trypsin and used culture medium. After centrifugation, the supernatant was discarded, and the sedimented cells were resuspended in fresh culture medium containing 10% serum. To determine cell viability and count, a specific volume of the cell suspension was mixed with an equal volume of trypan blue dye. This dye exclusion method is commonly used in cell biology to assess cell viability; viable cells remain unstained while non-viable cells take up the dye and appear blue under a microscope. Cell counting was performed using a hemocytometer slide, allowing for accurate quantification of both total cell numbers and percentage viability based on the equation used in this analysis. This meticulous process ensures that both cancerous and normal fibroblast cells are prepared appropriately for subsequent experiments, enabling researchers to study cellular responses under controlled conditions effectively. The careful handling of these cells is crucial for obtaining reliable experimental data in cancer research.

$C = N \times 10^4 \times F/ml$

When C: Number of cells in one ml of solution.

N: Dilution factor of the solution.

F: Dilution factor of the solution and 10^4 is Slide dimensions.

The percentage of cell viability in the sample was calculated according to the equation:

Vitality of living cells % = [(Living cells)/(Dead cells)] ×100 .

RESULTS AND DISCUSSION

Chemical Compositions of Beetroot and the Amount of some Vitamins and Elements

The chemical composition of beetroot is illustrated in Table(1), The percentages of protein (1.6%),carbohydrates (9.6%), fiber were (1.6 ,9.6 ,3.2 %) respectively which were higher than that obtained by Kale *et al*., [17], the percentages of moisture ,fat and ash were(84.3,0.2,1.1%) respectively which were lower as compared to that reported by Kale *et al*., [17], which were (1.32,7.59,1.9,0.3,87.4,1.4%) respectively. This difference can be attributed to storage and transportation conditions, crop service during the cultivation stages, light, fertilizer and the influence of environmental factors.

The mineral composition of beetroot were analyzed and results revealed that Fe ,Na, Mg,Mn,Zn,and Ca were 297.5, 9384.6 , 890.4, 19.79, 21.43 and 347.9 (ppm) respectively , While the Kale *et al*., [17] recorded that the percentage of Fe, Ca,Mn , Na and Zn were 7.5 ,122,79,725.8 and 2.1%. This difference in the presence of elements can be attributed to the beet variety, crop service during the cultivation stages, the influence of environmental factors and soil type [18].

The same table shows the amounts of vitamins in beetroot (C, A, D, E) which were 50.89, 250.8, 0.48 and 1.0 ppm.

The percentage of these vitamins were differs to what was found by Mudgal *et al* [5], as the percentage of vitamins C, A, and E were 49, 360, 3.0 ppm.

Table1 The percentage of chemical composition of beetroot and the amount of some vitamins and elements (ppm)

chemical composition	V%%%g
Moisture (g)	84.3
Protein (g)	1.6
Carbohydrate (g)	9.6
Fiber, total dietary g	3.2
Total lipid (fat) (g)	0.2
Ash (g)	1.1
Vitamins and minerals composition	ppm
Vit C	50.89
Vit A	250.8
Vit D	0.48
Vit E	1.00
Na	9384.6
Mg	890.4
Mn	19.79
Ca	347.9
Fe	297.5
Zn	21.43

Figure 2 shows the quantities of extracted in dried materials (the moisture content of the extracts was 8.5-9 %) from beets (100 g). The figure shows that the extracted quantities of WE, AEE, EMCC1%, EMCC0.5%, EMCC0.2%, EAA1%, and EMCAA were 6.92, 6. 75, 8.62, 8.26, 8.04, 7.59 and 9.39 g/ 100 g from beetroot tubers.

The figure shows that EMCAA had the highest extraction ratio (9.39) compared to the other types of extraction solutions. In general, the alcohol extraction had the lowest extraction rate, and this is due to the effect of acids and water in the extraction processes. The results were largely consistent with the previous mentioned research [19], that the use of ethanol alcohol reduces the amounts of extracted materials.

The Percentage of Phenolic Compounds in the Extracts

Figure 3 shows the percentage of total phenolic compounds in different beetroot extracts. It is noted from the figure that the highest percentages were in EAA and EMCAA which were 172, 173 % respectively.

The incorporation of citric and ascorbic acids significantly enhanced the extraction of phenolic compounds compared to water and alcohol-based methods. The data reveals an inverse relationship between EMCC concentration and phenolic compound extraction efficiency. As citric acid concentrations decreased from 1% to 0.5% and 0.2%, the percentage of extracted phenolic compounds correspondingly dropped from 45.9% to 42.99% and 33.48%. Notably, the WE (water extract) and AEE (alcohol extract) yielded lower percentages of phenolic compounds compared to the EMCAA (extract mixture of citric and ascorbic acid) extracts. This disparity underscores the pronounced effect of ascorbic acid in maximizing phenolic compound extraction. These findings highlight the superiority of citric and ascorbic acid-based extraction methods in isolating phenolic compounds from beetroot. The results suggest that optimizing acid concentrations could further enhance extraction efficiency, potentially leading to more potent antioxidant-rich extracts for various applications in food and nutraceutical industries. These results are consistent with the previos research [20] that it has been shown regarding the ability of ascorbic acid to extract high amounts of phenolic compounds from grapes. It has been explained [21] that using ethanol at a concentration of 50% and a mixing ratio of 1:50 gave the best extraction rate of phenolic compounds compared to other concentrations and mixing ratios.

Total Antioxidants of Beetroot Extracts by TAC Method

Figure 4 illustrates the total antioxidant percentages of various beetroot extracts, comparing them to a control model. The Total Antioxidant Capacity (TAC) assay is employed to measure the free radical scavenging ability of test solutions or suspensions. This method evaluates how effectively antioxidants in a sample neutralize reactive oxygen species, offering insights into the sample's overall antioxidant potential. The TAC assay is crucial for understanding a substance's capacity to combat oxidative stress. By quantifying this ability, researchers can assess the potential health benefits of different extracts or compounds. This information is valuable for developing nutritional supplements, functional foods, or even pharmaceutical products aimed at reducing oxidative damage in the body. The comparison with a control model allows for a standardized evaluation, helping to identify which beetroot extracts demonstrate superior antioxidant properties. This data can guide further research into optimizing extraction methods or selecting the most potent beetroot varieties for antioxidant-rich products [22]. The figure shows that the total antioxidants appeared in all extracts and they appeared at close values.

The highest and lowest results for AEE were found in EMCC 1% and EMCC 0.5% which were 45.18 , 38 ,37.85% respectively ,while the results of TAC for WE ,EMCC 0.2%, EAA and EMCAA were 42.85 , 44, 43.35 and 42.51 % respectively. These results are consistent with previous research that it hs brrn shown [23], about beets containing antioxidants as a result of them containing a large group of phenolic compounds. The difference in the percentage of total antioxidants in the extracts may be due to the effect of the solutions in extracting different quantities and types of beets. It has been explained [24], the possibility of increasing antioxidants in sugar beet plants by using vermicompost and glutathion as fertilizer.

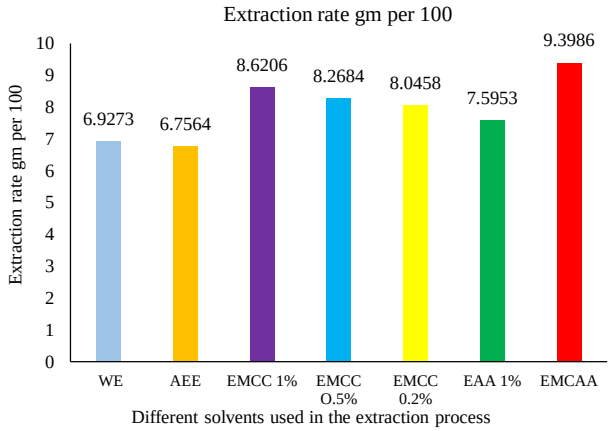


Fig. 2 Amounts of dry extracts from 100 g of beetroot

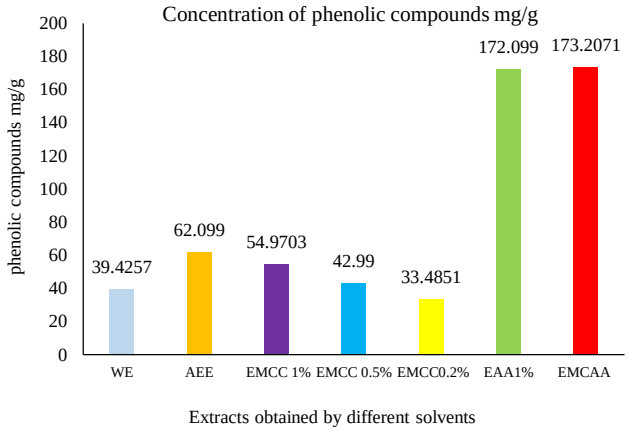
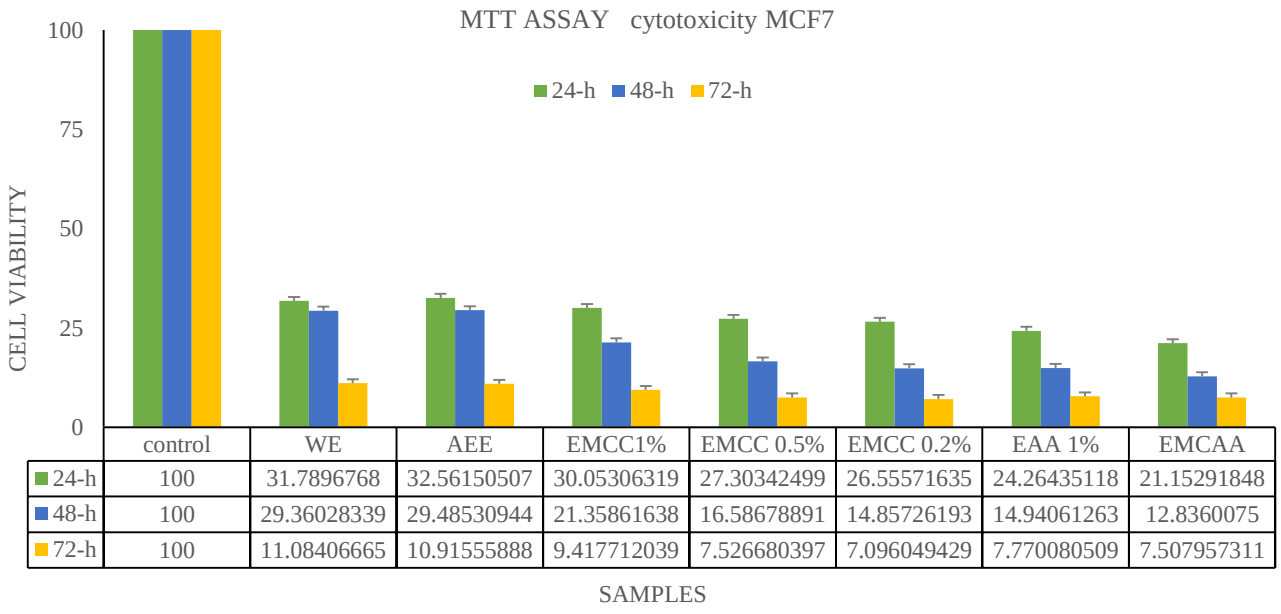
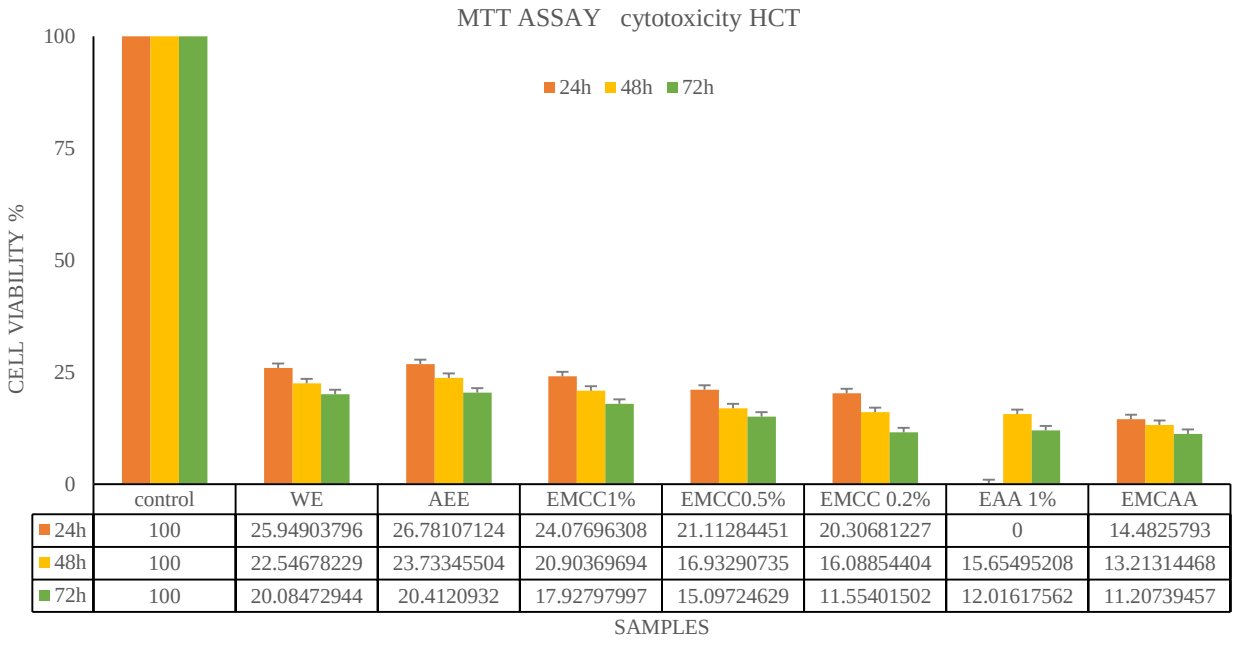


Fig. 3 Concentration of phenolic compounds in beetroot extracts



	control	WE	AEE	EMCC1%	EMCC 0.5%	EMCC 0.2%	EAA 1%	EMCAA
24-h	100	31.7896768	32.56150507	30.05306319	27.30342499	26.55571635	24.26435118	21.15291848
48-h	100	29.36028339	29.48530944	21.35861638	16.58678891	14.85726193	14.94061263	12.8360075
72-h	100	11.08406665	10.91555888	9.417712039	7.526680397	7.096049429	7.770080509	7.507957311

Fig. 5 Effect of different types of beetroot extracts against cancer cells MCF7



	control	WE	AEE	EMCC1%	EMCC0.5%	EMCC 0.2%	EAA 1%	EMCAA
24h	100	25.94903796	26.78107124	24.07696308	21.11284451	20.30681227	0	14.4825793
48h	100	22.54678229	23.73345504	20.90369694	16.93290735	16.08854404	15.65495208	13.21314468
72h	100	20.08472944	20.4120932	17.92797997	15.09724629	11.55401502	12.01617562	11.20739457

Fig. 6 Effect of different types of beetroot extracts against cancer cells HCT

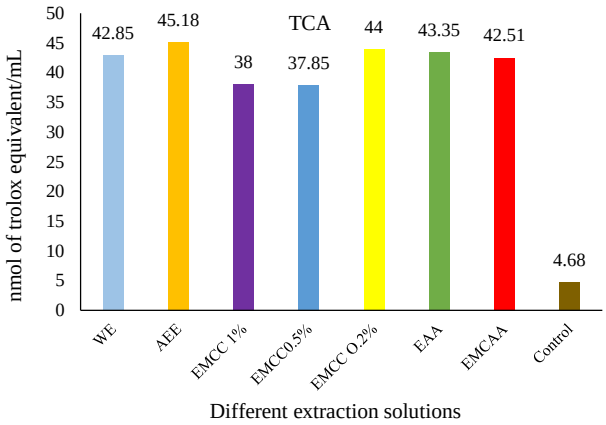


Fig. 4 TAC for different beetroot extracts

Effect of Different Beetroot Extracts on Cell Lines (HCT, MCF-7)

The graph illustrates the percentage of viable cells compared to the untreated control group. Cells from the third passage were seeded in 96-well plates at a density of 10,000 cells per well. After a 24-hour growth period, the desired treatments were applied. Cell viability was assessed using the MTT assay at 24, 48, and 72 hours post-treatment to evaluate the effectiveness of the treatments on cellular health. The results provide valuable insights into how different conditions influence cell survival and proliferation over time. The samples exhibited less than 50% survival rate. Figure 5 demonstrates the impact of beetroot extracts on MCF7 cancer cells. The graph shows an increasing effect of the extracts over time for all samples. The AEE extract exhibited the highest anti-cancer activity at 72 hours, while the lowest inhibition rate of 32.56% was also observed for AEE extract. Similarly, Figure 6 presents the MTT assay results for HCT cancer cells. This figure also indicates an increasing effect of all extracts on cancer cells over time. The most potent anti-cancer activity was observed after 72 hours, while the least inhibitory effect was seen after 24 hours of treatment. These results highlight the effective role of compounds found in beetroot against various cancer cell lines. These results are consistent with what Saber et al., [25] and Romero *et al*., [26] showed regarding the ability of alcoholic beetroot extract to effectively inhibit Colorectal cancer (CRC) and Cervical cancer cells after using MTT assay.

CONCLUSION

This study has revealed that beetroot possesses a diverse nutritional profile, with variations in macronutrients, minerals, and vitamins compared to previous research findings. Different extraction methods yielded varying amounts of bioactive compounds, with EMCAA demonstrating the highest extraction ratio. Citric and ascorbic acid extractions proved most effective in obtaining phenolic compounds. All extracts exhibited significant antioxidant capacity, highlighting beetroot's potential as a source of natural antioxidants. Furthermore, beetroot extracts showed promising anticancer effects on MCF-7 and HCT cell lines, with increasing effectiveness over time. These findings underscore beetroot's potential as a functional food and its possible applications in developing natural therapeutic agents for cancer treatment.

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