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Phytochemical And Physicochemical Properties Of Betalains Extracted From Red Beetroot (*Beta Vulgaris* L.)

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Abstract

The aim of this study was characterized the physiochemical and phytochemical properties of betalains extracted from red beetroot as antioxidant, antibacterial, and anticancer activity. Betalains were extracted from red beetroot using different single or mixed solvents in comparison with ultrasonic-assisted extraction (UAE) using different conditions from solid to solvent-ratio and duration of extraction. The content of total phenolics (TP) and flavonoids (TF), color characteristics (CIE L^* a^* b^*) and HPLC profile were analyzed in betalains extracted under optimized conditions. Among different tested media, 0.1% ascorbic acid at 1:30 (g/ml)/ 10min and 20% ethanol acidified with 0.1% ascorbic acid at 1:15 (g/ml)/2.5 min significantly ($p \leq 0.05$) exhibited higher betalains content of 1085.80 and 1760.00 mg/100gm, respectively, compared to 1662.50 mg/100g when ultrasonic assisted extraction was applied at 1:30 (g/ml)/ 10min at 40°C. High content of TP and TF as well as antioxidant activity (ABTS and DPPH) were found in the betalains recovered with optimal conditions (1:15(g/ml)/ 2.5 min). Betalains extracted was effective as antimicrobial against most of the tested microorganisms as well as a high anticancer activity against MCF-7 than Caco-2 and HepG2 cancer cells. Betalains can be recommended for the application as natural food colorants and bioactive ingredients in the food industry.

Keywords: Betalain, Beta vulgaris L; extraction; antioxidant; antimicrobial; anticancer; color parameters

1. Introduction

Choosing nutritional and safe foods is necessary for living a healthy life. The purpose of adding colorants to food is to improve quality, enhance attraction, adjust for color loss during processing, and persuade customers to purchase a safe product**[1, 2].** Many people prefer natural colorants to synthetic ones, which are seen to be safer or healthier. As there are dark sides to keep in mind: some of the artificial colorings are thought to trigger allergic reactions, and neurological conditions, and may be carcinogenic to humans **[2-4].** Nature produces a variety of compounds adequate for food coloring, such as water-soluble anthocyanins, betalains, carminic acid, and oil-soluble carotenoids and chlorophylls. Numerous studies have focused on the health-benefitting qualities of natural pigments, particularly the extensively researched antioxidant and antibacterial activities**[5-8].**Nevertheless, it was suggested that they might be applied as bioactive pigments**[9].**These results have encouraged the use of natural pigments as food colorants**[10].** The red betacyanins and the yellow betaxanthins of red beetroot (*Beta vulgaris*) are the two primary groups of the very important water-soluble betalains pigment. Because of this, betanins are permitted to be used as a natural food colorant, medications, and cosmetics. Betalains obtained from red beetroot are a substantial healthier alternative to artificial antioxidants for healthy foods because of their polyphenol content and antioxidant activity**[11, 12]**.The prevention or treatment of long-term diseases is another area where betalains are crucial **[13].**Hence, procedures that aim to save extraction time, energy, and solvent quantities require further consideration**[14-16].**Furthermore, an essential indication for assessing the effectiveness of betalains extraction is the assessment of the relationship between solvent ratios, temperature, and extraction time**.** Process variables affecting the amount of betaxanthin and betacyanin in beetroot peel juice

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extracted using conventional methods were examined **[17]**. The extraction procedure can be improved by optimizing the operating time and temperature, as well as using less expensive solvent. The optimum extraction conditions and the highest concentration of color components (952,5 and 1361 mg/L of betaxanthin and betacyanin, respectively) were achieved using aqueous ethanol (15%) at a ratio of 0.8 (w/v) at 20°C/h. Betacyanin (0.11–4.24 mg/g) more significant than betaxanthin(0.02–2.89 mg/g) in red beet extracts produced with ultrasonic-assisted extraction (UAE). While betacyanin is not eliminated at higher temperatures, betaxanthin extraction is affected by both 25% ethanol concentration and extraction efficiency. The optimal conditions for discarding red beet pigments were 89 w (UAE) at 53° C/35 min and 1:19 g/ml solid-liquid ratio **[18]**.**[19]**investigated bioactive components of beetroot pomace extracts, focusing on phenolics (218–376 mg GAE/g D.W. extract), flavonoids (201–570 mg RE/g D.W. extract), betacyanins (19–24 mg/g D.W. extract), and betaxanthins (11–23 mg/g D.W. They found that extracts increased the reducing power with concentration and showed a significant correlation between phytochemical components and scavenging activity(from 0.133 to 0.275 mg/mL).The characteristics of betalain and phenolic compounds in beets have been correlated to antimicrobial, anticancer, anti-inflammatory, cytotoxic, and hepatoprotective activities in earlier studies**[11, 20-22].**

New technologies and sources can use natural colorants to create a wider range of hues and enhance stability in food products. These materials could be able to give color and health-promoting qualities as well. To achieve diverse colorimetric qualities and stabilities, similar to synthetic food colorants, inspiration from natural color processes is essential [23, 24].The objectives of this study were to optimize the extraction process conditions of betalains from red beetroot. The total phenolic and flavonoid contents as well as antioxidant, antimicrobial, and anticancer activities were determined in betalains extract obtained under the optimum conditions.

2. Materials and methods

Red beetroots (Beta vulgaris L.) as a natural source of betalains were obtained from the Agriculture Research Center (A.R.C.), Ministry of Agriculture Giza, Egypt. The antimicrobial activity of betalains was tested against four bacterial cultures: Salmonella typhimurium (ATCC 6539), Escherichia coli (ATCC 8739), Staphylococcus aureus (ATCC 6538), and Bacillus subtilis (ATCC 6633) and two fungal cultures: Aspergillus niger(ATCC 10535) and Candida albicans (ATCC 10221). The microbial cultures were obtained from The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The cell lines gained from the Egyptian Vaccination and Serum Vacsera Co. (vaccines and sera), Egypt were used to determine the in-vitro cytotoxicity of betalains. Human hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7), and human colon adenocarcinoma (Caco-2) are the positive groups, whereas the human oral cavity cell line is the negative group. All other chemicals reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA).

2.1. Preparation of red beetroots

The red beetroots were cleaned from impurities, washed in running tap water, and cut into small pieces. Beet samples were dried in an air circulation oven (Thermo scientific: Heraeus) at 50°C for 24 hours, pulverized to powder using a Braun grinder and stored in a brown glass vial at -20°C before the experiments.

2.2. Extraction of betalains

Betalains were extracted from red beetroot according to the method of [18, 25] with slight modifications. Distilled water, citric acid solution (0.5%), ascorbic acid solution (0.1%), and ethanol solution (20%) media were used to extract betalains from dried red beetroot at different solid to solvent-ratio(1:10, 1:15, and 1:30mg/ml) and times (2.5, 5 and 10 min) at 40°C[26]The effect of the extraction methods using ultrasonic assistant extraction (UAE; Elmasonic P 30 H; Schmidbauer GmbH Elma Co., Singenam Hohentwiel, Germany, 37/80 Hz, 100 W) was also evaluated with the most effective mixed solvent on betalains content. The liquid solution of betalains extract was separatory centrifuged at 10.000 rpm for 10min, filtered, and stored in brown glass vial at -20 °C until analysis.

2.3. Determination of total betalains content

Spectrophotometric measurements of the absorbance at 538 and 480 nm were used to quantify the amounts of betaxanthins and betacyanins in beetroot extracts, respectively, and it was calculated as betalains content (BC).

$$BC(mg/g) = (A \times DF \times MW \times 1000)/(\varepsilon \times l)$$

Where:

Ais the absorption at 538 and 480 nm for betacyanins and betaxanthins, respectively as well as DF is the dilution factor while, l is the path length of the cuvette (1cm). For quantification of betacyanins and betaxanthins, molecular weights (MW) and molar extinction coefficients (ε) of the representative compounds, i.e. betacyanins (MW = 550 gmol-1; ε = 60.000 l mol-1 cm in H2O) and betaxanthins (MW = 308 g mol-1; ε = 48.000 l mol-1 cm in H2O) according to [26, 27].

2.4. Color analysis

The color was measured for all samples by the Spectrometer (Momcolo) (Moomcolor, DALOO, MOM, Hungary). The CIE L*, a*, and b* color scheme was used to represent color according to the method described by [28]. Before each test run, the equipment was calibrated with a white plate supplied by the manufacturer. For each sample, three spectral measurements were estimated, including lightness (L*) value (from dark to light), redness (a*) value (from reddish to greenish), and yellowness (b*) value (from yellowish to bluish). Chroma (c) and hue0 (h) were calculated from the obtained a*and b* values as follows:

Chroma (c) = √ (a^2+b^2)

hue (H^0)= are tan b/a

A 360° grid is used to depict the hue angle, with 0° representing red, 90° yellow, 180° green, and 270° blue. [29].

2.5. Separation and identification of bealains

Betalains profile analysis was performed as described by[30, 31] using an Agilent infinity 1260HPLC- system equipped with a binary pump, multicolumn thermostat, vial samples VL, and UV-Vis dual wave length detector (set at 470 nm and 538 nm). A mass spectrometer type Agilent G6470A series triple quad LC/MS (Agilent Technologies GmbH–Co.kG, Waldbronn, Germany) was connected to the HPLC system. The column used was of protosil 120-3-C18 ACE-EPS, 3 µm, 120A, 250×3 mm (Bischff Analytic Engineering and Equipment GmbH, Leonberg, Germany) with pressure limits of 400 bar, 0.5 ml/ min. at 100% A= 285 bar and column temperature of 30°C. Two eluents A (1% formic acid) and B (Acetonitrile) Merck, Darmstadt, Germany were utilized with a 0.5 ml/min flow rate. with a gradient concentration ratios during the run time as follows: 1: 0 min – 100 % A0-2 min; 100% A,1-60min; 100-75% A,60-65min,75-20%A; 65-66 min,20% A; 66-68 min,100% A; 68-72 min,100% A. (Total time. 72 min). Samples were treated for 5 min under ultrasonic conditions to remove dissolved gases and then vortexed and diluted 1:50 with distilled water and mixed again 5 µl at 10 °C were injected. Dilution with ethanol led to appear of precipitate after 2-10 min.

The applied MS/MS conditions were as follows: Collision Gas is nitrogen; Pressure 3.41E-5 Torr; detection is MRM and fragmentor voltage: 80 V, while cell accelerator voltage average was individually optimized. On the other side, the ESI conditions are as follows: Positive/Negative Ion-Mode; with nitrogen as Sheath gas at 275°C and flow rate of 11L/min. Capillary current was 4200 nA and the nebulizer pressure was 35.0 psi.; MRM as described by the aforementioned authors.

2.6. Total phenolic content

The total phenolic content of betalains extracts was determined with Folin Ciocalteu reagent as described by [32] with slight modification. A small vial containing 2.5 mL of ethanol (95%) was filled with approximately 50 µL of the extract. Following homogenization and centrifugation at 1000 rpm /10min, 5 mL of deionized water and 1 mL of ethanol (95%) were added. Folin Ciocalteu reagent (50%, 500µL) was added to each sample. The reaction mixture was left to stand for 60 min in the dark after the addition of 1mL of 5% Na2CO3 and stirring with a vortex mixer. After a second round of homogenization in a vortex mixer, samples were tested for absorbance at 725 nm using a UV-VIS Double Beam/UVD-3500 (Labonmed, Inc., California, USA). A standard curve made using Gallic acid in ethanol (95%) was used to determine the percentage amount of phenolics in the samples. Gallic acid equivalent (mg GAE/100g) was used to express the average value of the triplicate estimation (mg GAE/100g).

2.7. Total flavonoids content

According to [32] an aluminum chloride (AlCl3) colorimetric test was used to determine the total flavonoid content of betalains extract. Briefly, 100 µL of the extract was combined with 300 µL of 5% sodium nitrite (NaNO2). After 6 min of agitation, 300 µL of 10% AlCl3 solution was added and left for 7 min before 1.5 mL of 1 M NaOH was added. The mixture was diluted to a final volume of 2.5 mL with distilled water and mixed thoroughly and the absorbance was measured at 510 nm and Catechinwas usedto make the calibration curve. The content of total flavonoids was represented as mg Catechin equivalent/ 100g of sample (mg CE/100g).

2.8. Antioxidant activity

2.8.1. ABTS radical scavenging assay

According to [33] ABTS reagent stock solution was made by reacting 7 mM of ABTS with 2.45 mM of potassium persulfate in equal parts for 16 h at 25 °C in the dark. Then, 1 mL of ABTS + solution was diluted with 60 mL of ethanol: water (1:1) to produce the working solution, which had an absorbance of 1.0 0.02 units at 734 nm. A sample of 50 µL extract was combined with 4.95 mL of the ABTS+ solution and allowed to react for an hour in the dark. The absorbance was then determined at 734 nm. Trolox was used to prepare the standard curve. The outcomes were expressed as mg Trolox equivalent (TE)/100g extract. More dilution was performed when the measured ABTS value was higher than the linear range of the standard.

2.8.2. DPPH radical scavenging assay

The capacity of the betalains extract to scavenge2,2-diphenylpicrylhydrazyl(DPPH) was measured based on the method[33].The leftover DPPH radicals that exhibit maximal absorbance at 517 nm were measured after the reduction of the purple DPPH radical to the yellow DPPH radical, which is the basis for this procedure. Briefly, 3.95 ml of DPPH solution (0.2 mM DPPH in methanol) was added to 100µL of extracts and left to react. Using a Labomed, Inc. spectrophotometer and a blank of pure methanol, the reduction in absorbance was measured at 517 nm after 60 min of incubation in the dark. A calibration curve was made using Trolox. The antioxidant activity was calculated as mg Trolox equivalent (TE)/100g extract.

2.9. Antimicrobial activity of betalains extract

The agar-well diffusion method was used to examine the antimicrobial activity of betalains extract which dissolved in dimethylsulfoxide (DMSO) at 100µg/ml. Bacterial and fungal strains were grown at 37o C/ 24hr at pH 7.2-7.4 on MÜeller-Hinton agar slants for bacteria (MHA, Himedia, Mumbai, India) and at 25 o C/ 48h on fungal agar slants (FA, Himedia, Mumbai, India).

Overall, 10ml of MHA, FA nutrient agar melted at 50°C were inoculated with 100 µL of adequately diluted and thoroughly mixed active microbial inoculums. The inoculated mixture was put into a 15-cm sterile Petri dish and left to solidify. Using a sterile metal tube, 96 wells with a 6 mm diameter were created and the agar was removed, leaving wells filled with 100 µL betalains extract and left 2 h at room temperature. Test plates were incubated at 30–35 °C/24 h for bacteria or at 25 °C /48 h for yeasts and 72 h for molds. The resulting inhibition zones have been measured, and the average values have been calculated. In the same settings as the tested extract, the standard medications gentamycin and fluconazole (10 µg/well) for bacteria and fungi, respectively were employed as positive controls [22, 34].

2.10. Minimum inhibitory concentrations for bactericidal and fungicidal effects

The minimum inhibitory concentration (MIC)was determined by the broth micro dilution method to assess the least inhibitory concentration and the minimum bactericidal and fungicidal concentrations (MBC and MFC) of betalains extract according to[35].A loop was used to collect one colony of each microbial strain, which was subsequently inoculated into 25 ml of broth medium. After incubation at 37°C/18–24 h, 109 CFU/ml of bacterial suspension was obtained. Every stock solution was diluted with buffered peptone water (Oxoid) to yield suspended bacterial cultures at a concentration of 105 CFU/ml. In a test tube, 0.5–5 µL/ml dilutions of betalains extract in broth medium were combined with bacterial suspensions to yield a volume of 4 ml and a final concentration of around 5×104 CFU/ml. Earlier specified temperatures were used to incubate the final solutions. The MIC is defined as the lowest concentration of betalains extracts that inhibits observable microbial growth. The MBC and MFC were measured by sub-culturing 100 µL from each negative test tube onto PCA plates. MBC or MFC was defined as the lowest concentration yielding a negative subculture or a single colony following incubation. The experiments were conducted in duplicate four times.

2.11. In vitro cytotoxicity activity by MTT assay

Microculture Tetrazolium Inhibition Assay (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) MTT) was used to investigate the cytotoxicity of betalians extract on colon adenocarcinoma (Caco-2), liver cell line (HepG-2), Caucasian breast adenocarcinoma (MCF-7), and normal oral cavity cells OEC [36]. In 96-well plates, 1×105 cells were seeded in 0.2 ml of medium in each well. Following incubation, the media from the wells was removed carefully for the MTT experiment. The minimum essential medium (MEM) without foetal calf serum (FCS) was used to rinse each well two to three times, and then 200 µl of MTT (5 mg/ml) was added. In a 5% CO2 incubator, the plates were incubated at 37°C /6-7 h to test for cytotoxicity. After incubation, 1 ml of DMSO (a solubilizing agent) to each well was added, micropipette-mixed, and left for 45sec. Owing to forming of formazan crystals, the presence of live cells was shown by developing a purple hue. The suspension was placed in a spectrophotometer cuvette, and the OD (optical density) readings were measured at 595 nm using

DMSO as a blank. The concentration required for a50% inhibition of viability (IC50) was visually estimated after measurements were conducted. A Standard Graph was created using the extract concentration on the X-axis and relative cell viability on the Y-axis.

Cell viability (%) = Mean OD/Control OD × 100

Where mean OD is the mean of optical density for different concentrations of betalains on treated cells and control OD is the mean of optical density for different concentrations of betalains on control cells.

2.12. Statistical analyses

Analysis was done using the statistical analysis system software for Windows (Statistical Analysis System, Version 9.1.3, SAS Institute Inc. Cary, NC, USA) according to [37]. The data were given as mean± SD. Analysis of variance was used for statistical analysis, and Duncan's multiple range tests were used to compare the experimental findings (P≤0.05)[38].

3. Results and discussions

3.1. Optimal conditions for extraction of betalains

Different extraction conditions (i.e. type of solvents, solid-solvent ratios, times, and extraction techniques) were applied to yield a maximum betalains content from red beetroot. Results in Table (1) illustrate that among all tested solvents at 1:30 (g/ml)/10min, 0.1% ascorbic acid and 0.5% citric acid significantly (p≤0.05) extracted the highest content of betalains (1085.80 and 910.09 mg/100g respectively). [26, 39] reported that acidification enhances the extraction of betalains.

Table 1: Total betalains contents extracted with different solvents from red beetroot.

Solvents		Betalains contents (mg/100 g)		
	time/ min	g. red beet root /mL solvent		
		1:10	1:15	1:30
Distilled water	2.5	434.82^{Cc}	573.61^{Cb}	850.14^{Ca}
	5	356.46^{Gc}	583.86^{Cb}	755.99^{Ea}
	10	419.10^{Ec}	469.52^{Eb}	654.09^{Fa}
0.1% Ascorbic acid	2.5	453.10^{Ac}	661.10^{Ab}	1072.56^{Aa}
	5	453.82^{Ac}	666.44^{Ab}	1110.84^{Aa}
	10	443.89^{Bc}	672.15^{Ab}	1085.80^{Aa}
20% Ethanol	2.5	291.74^{Hc}	446.89^{Fb}	646.71^{Fa}
	5	365.49^{Fc}	445.45^{Fb}	747.68^{Ea}
	10	428.67^{Dc}	491.87^{Db}	806.36^{Da}
0.5% citric acid	2.5	435.90^{Cc}	672.03^{Ab}	880.73^{BCa}
	5	436.83^{Cc}	669.09^{Ab}	879.40^{BCa}
	10	445.92^{Bc}	613.84^{Bb}	910.09^{Ba}

Mean values (n=3) followed by the same small letters within the same row are not significantly different (P≥0.05)). Means with the same capital letters within the same column are not significantly different (P≥0.05)

In similar studies, it was found that extraction with ascorbic acid at concentrations of 0.1% and 0.25% (w/v) enhanced the red color stability of beet red pigment [40].However, acidified conditions at higher ratios and longer extraction times were preferred for betalains extraction. In previous research, the highest pigment yields (0.303 mg/g to 0.377 mg/g) were obtained by modifying extraction parameters such as time and the solid to solvent ratio in an acidic solution [41]. Even though betalains are hydrophilic pigments, the extraction yield is increased when organic solvents combined with water rather than they were used alone, like ethanol.

Table (2) displays the efficiency of extraction of betalains with mixed solvents on betalains content.

Table 2
Total betalains contents extracted with different mixed solvents from red beetroot.

Solvents	time/min	Betalains contents (mg/100 g) g. red beet root /mL solvent 1:10	1:15	1:30
20% Ethanol (0.1% ascorbic acid)	2.5	622.20^{Fc}	1760.00^{Aa}	1416.14^{Bb}
	5	667.69^{Ec}	1670.85^{Ba}	1334.58^{Cb}
	10	759.51^{Cc}	1463.06^{Cb}	1533.60^{Aa}
20% Ethanol(0.5% citric acid)	2.5	826.89^{Bc}	894.97^{Fb}	1148.13^{Da}
	5	696.39^{Dc}	874.21^{Fb}	1194.60^{Da}
	10	882.98^{Ab}	873.80^{Fb}	1044.59^{Ea}
0.1% Ascorbic(0.5% citric acid)	2.5	747.32^{Cc}	1254.62^{Da}	788.66^{FGb}
	5	667.61^{Ec}	1192.90^{Ea}	831.89^{Fb}
	10	665.54^{Ec}	893.09^{Fa}	761.90^{Gb}

Mean values (n=3) followed by the same small letters within the same row are not significantly different (P≥0.05)).
Means with the same capital letters within the same column are not significantly different (P≥0.05)

The extraction yield of betalains progressively increased with using different mixed solvents instead of a single solvent (Tables 1 and 2). Acidified 20% ethanol with 0.1% ascorbic acid showed the highest significant favorable impact on the efficiency of betalains extraction at 1:15/ 2.5min. (1760.00 mg/100g) decreased to 1533.60 mg/100 g when the extraction ratio and time increases to 1: 30 (g/ml)/ 10min. Meanwhile, 20% ethanol acidified with 0.5% citric acid recorded a significant loss of betalains content in the same conditions. This may be due to the solute being diluted with a more potent solvent. These results agree with those reported by [42-44], who reported that acidified ethanol increased the amount of total betalains extracted from red beetroot. According to [44, 45], One particularly famous co-solvent that is employed with water is ethanol, the primary goal of adding a co-solvent to water is to lessen its polarity so that it behaves more like a medium- or low-polarity solvent, which can improve extraction efficiency.

The effect of ultrasound assistance extraction (UAE/ 40 °C, 100 W) on the extraction of betlains displayed in Table (3).

Table 3: Total betalains contents extracted under different conditions with ultrasound assistance (UA: 80F/100Pw).

Solvents	time/min	Betalains contents(mg/100 g) g red beet/mL solvent 1:10	1:15	1:30
20% Ethanol(0.1% ascorbic acid)	2.5	867.18^{Fc}	932.06^{Eb}	1114.29^{Ia}
	5	1013.59^{Ac}	1152.14^{Ab}	1613.80^{Ba}
	10	932.96^{Dc}	1162.63^{Ab}	1662.50^{Aa}
	30	1002.41^{Bc}	1080.46^{Bb}	1565.19^{Ca}
20% Ethanol(0.5% citric acid)	2.5	750.87^{Hb}	953.30^{DEa}	965.22^{Ka}
	5	924.95^{Dc}	1027.98^{Cb}	1396.60^{Fa}
	10	864.35^{Fc}	938.30^{Eb}	1351.68^{Ga}
	30	890.47^{Eb}	888.81^{Gb}	1286.59^{Ha}
0.1% Ascorbic(0.5% citric acid)	2.5	976.09^{Cb}	910.67^{Fc}	1071.24^{Ja}
	5	821.34^{Gc}	963.09^{Db}	1438.06^{DEa}
	10	624.71^{Jc}	937.50^{Eb}	1453.51^{Da}
	30	676.31^{Ic}	968.25^{Db}	1419.50^{Ea}

Mean values (n=3) followed by the same small letters within the same row are not significantly different (P≥0.05)).
Means with the same capital letters within the same column are not significantly different (P≥0.05)

The maximum content of betalains yield (1662.50 mg/100 g) extracted with UAE by 20% ethanol acidified with 0.1% ascorbic acid at 1:30 (g/mL) / 10 min. The effect of ultrasound assistance extraction (UAE/ 40 °C, 100 W) on the extraction of betlainsis displayed in Table (3). The maximum content of betalains yield (1662.50 mg/100 g) extracted with UAE by 20% ethanol acidified with 0.1% ascorbic acid at 1:30 (g/mL) / 10 min was greater than those obtained under the traditional extraction procedure (1533.60 mg/100 g) at the same conditions. Ultrasound-assisted extraction is a useful method of extraction that works better than some traditional extraction methods. On the other hand, UAE showed improvement in extraction yields through acidified ethanol (0.1% ascorbic and 0.5% citric acid) or through 0.1% ascorbic and 0.5% citric acid combined. The underlying idea of ultrasound-assisted extraction is sonic cavitation, which leads to microjetting **[46].** The impacts of microjetting, such as surface peeling and particle breakup, can help to increase extraction yield**[47].**

3.2. Betalains profile analysis by HPLC

Table (4) displays the HPLC analysis of betaxanthins (at 480 nm) and betacyanins (at 540 nm) of betalains extracted from red beetroot under optimal conditions. Only five significant betalains were identified and isolated from the extracted betalains, including betaxanthins (vulgaxanthin I, Miraxanthin V, and miraxanthin IV) and betacyanins (betanin and isobetanin). The two main components of Betanin and Vulgaxanthin I, which were identified from betalains extracted by 20% ethanol acidified (0.1% ascorbic acid) under optimal conventional method at 1:15 (g/mL)/2.5 min. (84.91 and 57.15%, respectively) followed by 1:30(g/mL)/10 min. (80.41 and 50.67%, respectively). Vulgaxanthin IV* and isobetanin were enhanced more by optimal UA conditions at 1:30(g/mL)/10 min than by the conventional approach (18.09 and 22.54%, respectively).

Table 4: HPLC profile of betaxanthins and betacyanins in red beetroot extracted under optimized conditions at 470 nm and 538 nm.

Major compounds of betalains	RT (min.)	Peak area (%)		
		g: mL 20% ethanol acidified (0.1% ascorbic acid)		
		1:15 /2.5 min	1:30 /10 min	1:30 UA/10min
Betaxanthins (470 nm)				
Vulgaxanthin I	12.25	57.15	50.67	46.66
Miraxanthin V	31.48	11.07	19.66	4.94
Vulgaxanthin IV*	39.05	14.70	17.68	18.09
Betacyanins (538 nm)				
Betanin	27.78	84.91	80.41	75.19
Isobetanin	29.8	12.46	12.72	22.54

RT, retention time; UA: ultrasound assistant

These results are consistent with those of [48], who reported that the two primary pigment-related compounds present in red beet are betalain and isobetalain. According to the extraction efficiency, the conventional method (1:15/2.5min.) has demonstrated compatibility in the extraction and recovery of betalains from red beetroot. Acid extracts likewise provided the maximum quantity of betalains. In a while, betaxanthin concentration reduced considerably in UA betalains extract. This occurrence was explained by the betacyanin's stability, diffusivity, and conventional extraction technique applied to the extracts, which held the molecule [49].

3.3. The bioactive compounds content and antioxidant activity of betalains extracts

Table (5) shows the total contents of betalains, phenolic (TP), and flavonoids (TF), as well as antioxidant activity (ABTS and DPPH) of betalains extracted from red beetroot under optimized conditions. High contents of TP and TF (962.00 mg GAE/100 g and 402.40 mg CE/100 g, respectively) as well as high antioxidant activity (755.60 and 311.20 mg TE/100 g for ABTS and DPPH, respectively) were significantly ($p \leq 0.05$) found for the betalains extracted with the conventional method at 1:15 (g/mL)/2.5min.

Table 5: The bioactive compounds content, and antioxidant activity of betalains extracted from red beetroot under optimal conditions

20% Ethanol (0.1% ascorbic acid) ratios /time (min.)	Betalains(mg/100 g)	Total Phenols(mgGAE/100g)	Flavonoids (mg CE/100g)	Antioxidant activity (mg TE/100g)	
				ABTS	DPPH
1:15 /2.5 min	1760.00^{A}	962.00^{A}	402.40^{A}	755.60^{A}	311.20^{A}
1:30 /10 min	1533.60^{C}	801.60^{B}	325.20^{B}	615.20^{B}	246.80^{C}
1:30 UA/10min	1662.49^{B}	779.60^{C}	298.40^{C}	614.40^{B}	274.40^{B}

.
Mean values (n=3) followed by the same capital letters within the same column are not significantly different (P≥0.05); UA: ultrasound assistant; GAE 100 g-1, Gallic acid equivalent per 100 g of sample; CE 100g-1, Catechin equivalent per100 g of sample, Antioxidant activity (ABTS and DPPH radical scavenging capacities) of samples; TE100g-1, Trolox equivalent antioxidant capacity (TE100g-1).

Nevertheless, antioxidant activity values for betalains extracted using the conventional process (1:15) expressed a 32% increase in comparison to UA (1:30/10 min.), whereas values expressed a 40% increase in comparison with the conventional technique at 1:30/10 min. Since mixing ratios (solvent to beetroot) have a significant influence on extraction efficiency, as it decreased with the efficiency extraction increased. On the other hand, **[50-53]**investigated the relationship between the structure-activity of betalains concerning their ability to scavenge free radicals and increased antioxidant activity, and mentioned that betalain is characterized by its very high activity in scavenging free radicals.

3.4. Color parameters of betalains extracts

The primary aspect of food that draws consumers in and serves as a sign of its acceptance and quality is its color [54]. The L*, a*, b*, c*, and h* measures were used to depict the color characteristics of betalain extracts as displayed in Table 6. The extract obtained by conventional techniques (1:15 for 2.5 min) was determined to be the lightest color (L*), followed by 1:30 for 10 min, while the extract obtained by UA (1:30/10min.) was the darkest.

Table 6: Color parameters of betalains extracted from red beetroot under optimal conditions.

20% Ethanol (0.1% ascorbic acid)ratios /time (min.)	Color parameters				
	L*	a*	b*	h*	c*
1:15 /2.5 min	22.30^{A}	43.61^{A}	18.04^{A}	23.54^{A}	46.94^{A}
1:30 /10 min	20.56^{B}	43.50^{A}	17.37^{B}	21.71^{B}	46.75^{A}
1:30 UA/10min	20.28^{C}	41.41^{B}	17.14^{C}	21.51^{C}	45.17^{B}

Mean values (n=3) followed by the same capital letters within the same column are not significantly different (P≥ 0.05). L*, lightness; a*, yellowness; b*, redness; h*,hue; c*, chroma; UA: ultrasound assistance.

Nevertheless, compared to conventional methods (1:15 and 1:30), the extraction process has very little influence and is significant (P ≤ 0.05) at yellowness (a*). The levels of redness (b*) were frequently the highest in betalains extracted using conventional methods (1:15 for 2.5 min.). On the other hand, the UA (1:30 for 10 min) method showed the lowest a*, b*, and L* values compared to alternative treatments. In general, relative betaxanthin concentration increased with greater lightness levels but declined when betacyanins predominated. The relative ratios of betaxanthins and betacyanins also had an impact on the chroma, C*, which tended to rise with larger overall betalain contents (1:15 / 2.5 min). Depending on the content of betalains, the hue angle (h*) represents the overall color of the sample. h* varied from 23.54° for conventional 1:15/2.5 min. to 21.51° for UA (1:30/10 min.). All values were found to be significant (P ≤ 0.05) [55].

3.5. Antimicrobial activity of betalains extracts

The antimicrobial activity (i.e. inhibition zone (IZ), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC)) of betalains extracted from red beetroots under optimized conditions was tested against four bacterial strains and two fungal strains using the agar well diffusion method, (Table 7). The results suggest that the extract

displayed activity against all tested microorganisms with an inhibition zone ranging from 22.00 to 28.33 mm.

Table 7: Antimicrobial activity of of betalains extract based on microorganisms inhibition zone (IZ), minimum inhibitory concentration (MIC), and minimum bactericidal and fungicidal concentration (MBC/ MFC).

Tested strains	IZ(mm) Betalains extract	Gentamycin/Fluconazole* (10µg/disc as a control)	MIC (µg/ml).	MBC/ MFC (µg/ml).
Staphylococcus aureus	24.00^{D}	22.00	156.25	312.50
Bacillus subtilis	25.00^{C}	20.00	156.25	156.25
Escherichia coli	28.33^{A}	20.00	78.125	312.50
Salmonella typhymurium	22.00^{E}	25.00	625.00	625.00
Candida albicans	26.33^{B}	21.00	156.25	312.50
A. niger	n.a.	19.00	———	————

Mean values (n=3) followed by the same capital letters within the same column are not significantly different ($P \geq 0.05$); IZ: inhibition zone (mm) including disc diameter of 4 mm. *: Inhibition zones (mm) observed by Gentamycin and Fluconazole as a positive control (10µg/disc) for bacteria and fungi respectively. n.a. Not active; DMSO: Dimethyl sulfoxide [(CH3)2SO] as a negative control, MIC: Minimum inhibitory concentration (µg/ml), MBC: minimum bactericidal concentration (µg/ml).

Gram-negative bacteria of *Escherichia coli* followed by yeast of *Candida albicans* showed more susceptibility inhibition zone than Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. It's significant to note that the extract showed high antibacterial activity against Escherichia coli (MIC = 78.125 µg/ml; MBC = 312.50 g/ml). On the other hand, no effect against *A. niger* and a low antibacterial activity against *Salmonella typhymurium* (MIC; MBC= 625.00 µg /ml) were found. Furthermore, *Salmonella typhymurium* showed the lowest sensitivity to the antibiotic Gentamycin and Fluconazole (10 µg /disc).High levels of antibacterial activity are attributed to the presence of phenolic compounds. The majority of studies on the action of phenolic chemicals on cellular membranes focused on how these changes in function and, in some cases, structure, inflammation, and increased permeability affected these membranes. Increases in cytoplasmic membrane permeability appear to be caused by a decrease in the cellular pH gradient, a decline in ATP levels, and the loss of the proton motive force, which results in cell death [19, 56, 57].

3.6. Anticancer activity of betalains extracts

The anticancer activity of betalains extracts was tested using four different human cancer cell lines were used: hepatic (HepG2), colon (Caco2), and mammary breast (MCF-7), in comparison with normal human oral cavity cell (OEC) as a negative group. The IC50 values of betalains extracted from red beetroots at a ratio of 1:15 (g/mL)/ 2.5 min are shown in Table 8.

Table 8: IC50 values of betalains extracted from red beetroot (Beta vulgaris L.) at a ratio of 1:15 (g ml)/ 2.5 min.

Extract	$IC_{50}$of the negative group(µg/well)	$IC_{50}$of positive group(µg/well)		
	OEC	HepG-2	Caco-2	MCF-7
Betalains	445.64 ± 6.13	451.22± 6.96	342.94 ± 1.32	333.23 ± 1.57

Data are mean ± SD from three separate experiments in the oral cavity (OEC) as a normal cell line, hepatic (HepG-2), colon (Caco-2), and mammary breast (MCF-7) cancer cell lines. The difference between IC_{50}(µg/well), and mean values (n=3) of betalains extract in all cell lines were significant at ($P \leq 0.05$).

The IC_{50} of extracted betalains on normal human oral cavity OEC was 445.64 µg/well. On the other hand, it was shown that extracted betalains had significant differences in their impact on the inhibition of human cancer cells. Betalains showed a high effect on mammary gland breast MCF-7 (333.23 g/well) than colon Caco-2 cancer cells (342.92 g/well). On the other hand, liver HepG-2 cells showed a lower

response to betalains (451.22 g/well).These results are consistent with the finding of a previous study [58]. Many studies have shown that betalains have strong in vitro antiradical scavenging abilities and antioxidant capacity [50, 59-64]. The ability of betalains to induce quinonereductase, a strong detoxifying enzyme related to cancer chemoprevention [65]. On the other hand, betalains might prevent endothelial cells in vitro models of inflammation from oxidizing[66]. Additionally, it was found that betanin effectively prevented skin and liver tumors brought on by some chemical carcinogens over the long term [58].

4. Conclusions

A conventional technique has been shown to effectively extract betalains and polyphenols from dried red beet powder. Low ethanol concentrations (20% ethanol) with 0.1% ascorbic acid have been established as the most suitable solvent combination in comparison to an ultrasound-assisted extraction method. Moreover, in vitro studies on the bioavailability of betanins are included. It seems that betalains therapy and betalains-rich food not only have the potential to replace supplemental treatments for disorders such as cancer, oxidative stress, and inflammation but are also non-toxic. Due to their excellent biological benefits, accessibility, cost, bioactivity, and toxicological safety, betalains can be recommended for application as natural food colorants and bioactive ingredients in the food industry.

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