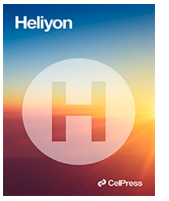
Heliyon 9 (2023) e21191

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Effects of different processing conditions on the carotenoid's composition, phenolic contents, and antioxidant activities of *Brassica campestris* leaves

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ARTICLE INFO

Keywords:
Mustard
Carotenoids
Boiling
Frying
Freezing
Microwaving
Sonication

ABSTRACT

Leafy vegetables are enriched with health-promoting compounds such as carotenoids and polyphenols. Different processing treatments have been shown to affect the amounts of these compounds. In this study, mustard (*Brassica campestris*) leaves were subjected to various processing treatments, boiling, frying, freezing, sonication, microwaving, and blanching. Carotenoid contents were determined using HPLC-DAD while the total phenolic, flavonoids, anthocyanin, and antioxidant activities were determined using established spectroscopic protocols. It has been found that different processing treatments concentrated the lutein, flavoxanthin, and β-carotene contents of mustard leaves, while frying has been found to have deleterious effects on these compounds. During boiling the concentration of violaxanthin, antheraxanthin, flavoxanthin, and lutein was significantly increased to 87.4, 29.9, 20.4, and 340.8 μg/g respectively versus control. The total anthocyanin and phenolic contents of mustard leaves were better preserved during frying having values of 6.2 mg/L and 1281.2 mg/100g, respectively, whereas the total flavonoid contents (TFC) in the control sample was 111.8 mg/100g. Among the studied treatments the highest TFC was reported in the blanched samples (108.7 mg/100g), followed by sonication (107.1 mg/100g). During microwave and sonication, the antioxidant potential of the treated samples had significantly increased while in other treatments, it was reduced.

1. Introduction

Brassica is the most important genus within the plant family *Brassicaceae*, which comprises crops and species with significant global economic importance. *Brassica* species can be used for multiple purposes depending on their form or kind. Oil seeds, condiments, forage, and vegetable crops are the different forms of Brassica spp. which are essential elements of the human diet, hence this family significantly contributes to the bioeconomy of a country. Vegetable leaves are enriched with several colored pigments; chlorophylls and carotenoids are the pigments not only associated with the color of leaves [1,2] but the carotenoids also help the chlorophyll during photosynthesis [3]. They also function as antioxidants, immunological boosters, and anti-tumor agents [4]. These carotenoids may have positive effects on health [5]. The conjugated double bonds seen in carotenoids have been mostly attributed to the quenching impact of singlet oxygen or the free radical scavenger effect [6]. Carotenoids have been reported to be more resistant to deterioration

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https://doi.org/10.1016/j.heliyon.2023.e21191
Received 27 May 2023; Received in revised form 20 September 2023; Accepted 18 October 2023
Available online 22 October 2023

when exposed to heat [7]. It has been found that processed foods tend to be higher in carotenoids than their raw counterparts [8–10]. Cooking and other processing techniques such as blanching, canning, sterilizing, and freezing are anticipated to have an impact on the yield, composition, and bioavailability of their essential elements [11–13]. The antioxidant activity of vegetables may be impacted by quality changes, antioxidant breakdown, and leaking of vital contents into nearby water during processing; particularly during boiling [14]. Domestic cooking is based on common techniques, which include boiling, microwaving, frying, and steaming [15]. During boiling, steaming, and microwaving, the contents of β-carotene were significantly increased in mustard leaves [16]. Conversely processing treatments like boiling, steaming, pressure cooking, microwaving, and sautéing had significantly decreased the total carotenoid contents in mustard leaves when compared to their raw counterpart [17]. Likewise, microwaving has the potential to concentrate the flavonoid contents in mustard leaves when compared with boiling or steaming [16]. Microwaving and steaming could concentrate the total phenolic contents and antioxidant activities of mustard leaves, whereas the chlorophyll content was significantly decreased during such treatments [18]. Mustards are commonly prepared and thoroughly devoured because they are now a significant part of our food menu. However, when they are made at home, the flavor is prioritized over maintaining their nutritional value and health-promoting ingredients [19,20]. However, there is a lack of literature regarding the effects of different processing techniques such as frying, freezing, boiling, microwaving, blanching, and sonication on the carotenoid's contents in mustard leaves. Keeping this point in mind, the present study was planned to determine the fate of carotenoids and total bioactive contents during various processing treatments of mustard leaves collected from Pakistan.

2. Materials and methods

2.1. Materials

Fresh leaves of *Brassica campestris* L. (field mustard) were collected from the vegetable market in Batkhela, District Malakand, Khyber Pakhtunkhwa, Pakistan. The sample (5 kg) was randomly taken from the commercial market (Latitude = 34.6138° N, longitude = 71.9283° E, and altitude = 648.4 m). The plant was identified by Dr. Gul Rahim, Department of Botany, University of Malakand, and a voucher specimen was deposited and recorded in the Herbarium of the University (code H/UOM.BG.864). The chemical used in this study were chlorophyll *a* (CAS # 479-61-8), chlorophyll *b* (CAS # 519-62-0), lutein (CAS # 127-40-2), β-carotene (CAS # 7235-40-7), gallic acid (CAS # 149-91-7), quercetin (CAS # 117-39-5), cyanidin-3-glucoside (CAS # 7084-24-4), DPPH (CAS # 1898-66-4), ABTS (CAS # 30931-67-0) were from Sigma-Aldrich (Germany).

2.2. Sample processing techniques

Different processing techniques were applied of which each technique was repeated in triplicates. Mustard leaves were boiled for 2, 4, 6, 8, and 10 min in a stainless-steel pot. During microwave processing, the leaves were microwaved for 3, 6, 9, 12, and 15 min at 850W using a Dawlance Microwave oven (Pakistan). The leaves were fried for 1, 2, 3, 4, and 5 min in sunflower oil at 180 °C. During sonication, the sample leaves were sonicated for 05, 10, 15, 20, and 25 min using a Power Sonic 405 (Hwashin Technology Co, South Korea). Blanching was done for 30, 60, 90, 120, and 150 s while freezing was performed in the Deep freezer (PEL Arctic Pro, Pakistan) for 24, 48, 72, 96, and 120 h. The sample size in all treatments was 200 g while the unprocessed fresh leaves were taken as control.

2.3. Extraction of carotenoids

Carotenoid extraction was performed from each processed sample using the method developed in our lab [21]. Briefly, the grinded paste (1 g) from each processed and control sample was mixed with 5.0 mL acetone (ice cold) containing BHT (0.1 %). The whole mixture was allowed to shake for 60 min using Orbital Shaker (Biobase, China). Acetone (5.0 mL) was added to the mixture and agitated for 30 min before filtration. The procedure was continued till the leaves were discolored. Under vacuum at 35 °C, the solvent was evaporated, and the remaining residue was dissolved in methanol (2.0 mL), followed by filtration using Agilent PFTE syringe filters (0.45 μm) and was shifted into an HPLC vial.

2.4. Chromatography

The separation of carotenoid pigments was performed using a reverse-phase HPLC-DAD system. The HPLC system (1260 infinity Better) was equipped with an auto-sampler, quaternary pump, degasser, DAD, and reversed-phase column (Agilent Zorbax C18, with specification 4.6 × 100 mm, 3.5 μm maintained at 25 °C) was used for the purpose.

As previously described in the method [21], a tertiary gradient system was used comprising methanol-deionized water (92:8 v/v) as solvent A, ammonium acetate (0.1 mM), deionized water as solvent B and MTBE (100 %) as solvent C. The flow rate was adjusted to 1 mL/min with an injection volume of 50 μL. According to the published protocol [21], the spectra were determined at 190–750 nm. Open Lab Chemstation software (Agilent Technologies, Germany) was used to obtain the chromatograms at 450 and 650 nm. The identification of compounds was determined using the retention time and absorption spectra of the available standards (chlorophyll *a*, & *b*, lutein, β-carotene). However, if the standards were not available, the identification was performed by comparing the absorption spectra of the unknown compounds with the ones reported in the literature. From the peak area and calibration curve, the compounds were quantified and represented as μg/g on a fresh weight basis.

2.5. Total phenolic contents

To determine the total phenolic compounds (TPC) in the mustard extract, a method previously reported was used [22]. Briefly, 0.5 mL of sample extract was mixed with 2.5 mL of Folin Ciocalteu reagent (0.2 N) and 1 mL Na_2CO_3 (7.5 %). The mixture was kept in the dark for 1 h as incubation time and the absorbance was taken at 765 nm against the blank using a spectrophotometer (Shimadzu-1700, Tokyo Japan). The TPC of each sample was measured in triplicate as mg/100g of gallic acid equivalents (GAE) against the gallic acid standard calibration curve.

2.6. Total flavonoid contents

The total flavonoid contents (TFC) in the mustard extract were determined using quercetin as a reference compound. An aliquot of 0.5 mL aluminum chloride (2.0 %) was added to 0.5 mL extract and incubated for 1 h. Through a spectrophotometer (Shimadzu-1700), the absorbance of the sample mixture was determined at 420 nm. The TFC of each sample was measured in triplicate as mg QE/g against the standard calibration curve of quercetin.

2.7. Total anthocyanin contents

Total anthocyanin contents (TAC) in mustard were determined using the pH differential protocol [23]. An aqueous solution of sodium acetate (0.4 M) and potassium chloride (0.025 M) was prepared and their pH was adjusted to 4.5 and 1.0, respectively. After this, 1.0 mL of sample extract was mixed with 4.0 mL of the sodium acetate solution. The initial absorbance at 520 nm and the final absorbance at 700 nm were recorded. Likewise, 1.0 mL of sample extract was added to 4.0 mL of potassium chloride solution read the initial absorbance at 520 nm and final absorbance at 700 nm. TAC of each sample was determined in triplicate as cyanidin-3-glucoside equivalents (mg/L).

$$\mathrm{TAC} = \frac{\mathrm{A} \times \mathrm{MW} \times \mathrm{DF} \times 1000}{\varepsilon \times 1}$$

Where A = (*Abs*520nm – *Abs*700nm) at pH 1.0 – (*Abs*520nm – *Abs*700nm) at pH 4.5 MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside, DF = dilution factor, l = pathlength in cm, ε = 26 900 M extinction coefficient, in $L \times mol^{-1} \times cm^{-1}$, for cyd-3-glu and 1000 = factor for conversion from g to mg.

2.8. DPPH antioxidant activity

To determine the free radical scavenging potential of the mustard extract, a DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used [24]. Briefly, 100 μL of the sample extract was added to 1900 μL of DPPH reagent (0.1 mM), freshly prepared in methanol. The reaction mixture was kept in the dark for 30 min as the incubation time and the absorbance of each sample was measured at 517 nm. For each test, the assay was repeated three times and the % RSA activity was determined.

2.9. $ABTS^{++}$ radical scavenging activity

$ABTS^{++}$(2,2′-azo-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical was used to determine TEAC (Trolox equivalent antioxidant capacity) of the mustard leaves as reported early by (Ayaz et al., 2015) [25] with some modification. Potassium per-sulfate (2.45 mM) and ABTS (7.0 mM) solutions were prepared, mixed, and incubated in the dark at room temperature for 12–16 h. A dark-colored ABTS radical solution was formed after the completion of the incubation period. Then, 200 μL of each sample was mixed with 2.0 mL of the ABTS solution in the cuvette. The activity of the sample to reduce the absorbance was determined through a spectrophotometer. The activity was expressed in % inhibition.

2.10. Iron chelation activity

Iron (Fe) chelation activity was carried out using a ferrozine solution, which forms a magenta-colored complex with ferrous ions [26]. The activity was conducted using the method of Mladěnka, Macáková [27] with some modifications. In brief, a 50 mL solution of ferrous chloride at a concentration of 2 mM was prepared. Ferrozine solution (50 mL) with a concentration of 5 mM was also developed. In a test tube, 10 μL of the sample's methanolic extract was added, followed by the addition of 50 μL ferrous chloride and vigorous mixing for 3 min. Following this, 200 μL of ferrozine solution was added to the reaction mixture, which was then shaken and incubated for 5 min. The absorbance of the mixture was read spectrophotometer at 562 nm through a spectrophotometer. The activity of the sample extract was calculated by the % inhibition.

2.11. Statistical analysis

Statistical analysis was performed using ordinary one-way ANOVA with multiple comparisons of variables using Dunnett's multiple comparison tests at $P < 0.05$ versus control samples.

3. Results

3.1. Identification of carotenoids in mustard

In the mustard sample, 17 carotenoids and chlorophylls were identified and quantified (Fig. 1). Chromatographic characteristics of the compounds such as absorption spectra and their retention times are listed in Supplementary materials. Peak 1 represents the first compound ε-Apo-13-luteinol eluted at 0.9 min with a maximum absorption (λ_{max}) of 276 nm. Similarly, peaks 2 and 3 represent the compounds divinyl chlorophyll *a* and violaxanthin, respectively. Divinyl chlorophyll *a* was eluted at 4.6 min with λ_{max} 666, 620, and 432 nm, while violaxanthin was eluted at 6.7 min at λ_{max} 470, 440, and 420 nm. Antheraxanthin was identified at peak 4, eluted at 8.9 min with λ_{max} 474, 446, and 422 nm. 13′-*Z*-violaxanthin and 13-*Z*-zeaxanthin were the next compounds identified by characteristic peaks with elution times 9.6 and 10.2 min, respectively. The absorption maxima of 13′-*Z*-violaxanthin were 466, 438, and 414 nm, while for 13-*Z*-zeaxanthin it was determined at 470, 440, 416, and 330 nm. Flavoxanthin was identified at λ_{max} 448, 422, and 400 nm and was eluted at 11.1 min, while lutein was the next compound identified by its characteristic peak at λ_{max} 474, 446, and 422 nm with an elution time of 14.7 min. Peak 9 represents 9-*Z*-β-carotene identified at 9 min with λ_{max} 474, 446, and 423 nm. Peaks 10 and 11 were identified for the compounds 9-*Z*-lutein and 9′-*Z*-lutein and were eluted at 17.4 and 21.3 min, respectively. 9-*Z*-Lutein was identified at λ_{max} 470, 440, 420, and 330 nm, while 9′-*Z*-lutein was identified at 468, 440, 420, and 330 nm. Hydroxy chlorophyll *b* and chlorophyll *b* were the next compounds identified at peaks 12 and 13 and were eluted at 22.5 and 23.2 min, respectively. The absorption spectra 648, 600, 464, and 342 nm were determined for hydroxy chlorophyll *b,* and chlorophyll *b* was identified at 650 and 464 nm. Similarly, chlorophyllide *a*' and hydroxy chlorophyll *a* were the next compounds eluted at 24.3 and 25.5, respectively. Chlorophyllide *a*' was identified at λ_{max} 664, 616, and 430 nm, while hydroxy chlorophyll *a* was identified at λ_{max} 664, 618, and 430 nm. Peaks 16 and 17 represent the last two identified compounds: chlorophyll *a* and β-carotene, respectively. Chlorophyll *a* was eluted at 26.2 min with λ_{max} 663 and 430 nm, while β-carotene was eluted at 32.3 min with absorption spectra of 476, 450, and 422 nm.

3.2. Effects of different processing conditions on carotenoid composition

3.2.1. Effect of boiling

Table 1 shows the effects of boiling time (2, 4, 6, 8, and 10 min) on the carotenoid amount versus control. The amount of ε-apo-13-luteinol (9.0 μg/g) and divinyl chlorophyll *a* (8.0 μg/g) was quantified in the sample boiled for 2 min which was significantly decreased ($p < 0.05$) as the boiling time increased. The highest amounts of violaxanthin (87.4 μg/g) were found in the sample boiled for 10 min. However, the highest amounts of antheraxanthin (105.2 μg/g) were quantified in the sample boiled for 2 min, which was significantly decreased as the boiling time increased. Similarly, the amount of 13′-*Z*-violaxanthin, 13-*Z*-zeaxanthin, and flavoxanthin was found to significantly increase (187.5, 251.6, and 113.9 μg/g, respectively), in the sample boiled for 2 min which was significantly decreased when boiling time increased. In the same way, the amounts of lutein, 9-*Z*-β-carotene, 9-*Z*-lutein, and 9′-*Z*-lutein were also found to increase (910.4, 95.5, 46.7, and 285.4 μg/g) respectively, in the group treated for 2 min. However, the highest amounts (417.3 μg/g) of hydroxy chlorophyll *b* were determined in the sample boiled for 4 min. The amount of chlorophyll *b* and chlorophyllide *a*' was 184.5 μg/g and 88.9 μg/g, respectively, during boiling for 2 min. Likewise, hydroxy chlorophyll *and* chlorophyll were quantified as 468.1 and 197.5 μg/g, respectively, in the sample treated for 2 min. The amounts of these compounds were significantly decreased as the boiling time increased. β-Carotene was the last compound identified in the boiled sample. The highest amounts of β-carotene were 198.3 μg/g

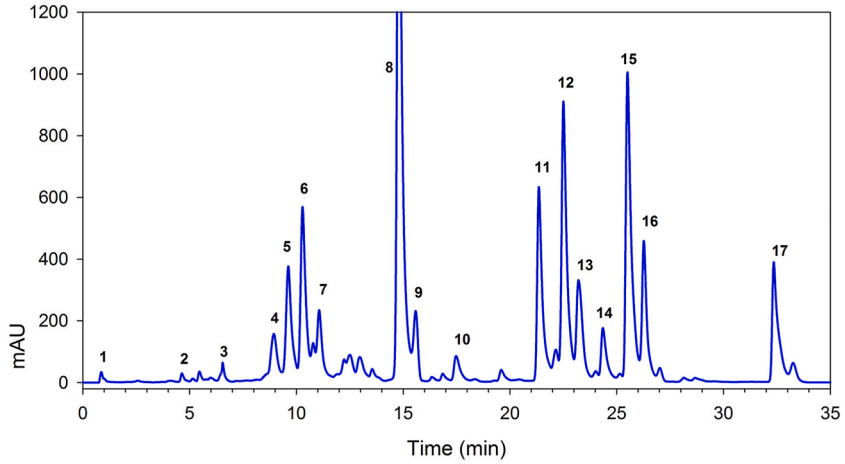

Fig. 1. HPLC-DAD chromatogram of mustard carotenoid (Boiled sample) at 450 nm. Each peak number represents individual compound with details as [1]; ε-Apo-13-luteinol [2], divinyl chlorophyll *a* [3], violaxanthin [4], antheraxanthin [5], 13′-*Z*-violaxanthin [6], 13-*Z*-zeaxanthin [7], flavoxanthin [8], lutein [9], 9-*Z*-β-carotene [10], 9-*Z*-lutein [11] 9′-*Z*-lutein [12], hydroxy chlorophyll *b* [13], chlorophyll *b* [14], chlorophyllide *a*' [15], hydroxy chlorophyll *a,* [16] chlorophyll *a* and [17] β-carotene.

Table 1
Effects of boiling on the carotenoid's concentration (μg/g) in mustard leaves.

Peak	Identity	Boiling Time (min)					
		Control	02	04	06	08	10
1	ε-Apo-13-luteinol	0.0 ± 0.0a	9.0 ± 0.2b	5.8 ± 0.1c	2.5 ± 0.1d	2.3 ± 0.1a	1.84 ± 0.1a
2	Divinyl chlorophyll *a*	11.7 ± 0.4a	8.0 ± 0.2b	4.1 ± 0.1c	4.5 ± 0.1d	11.7 ± 0.1a	4.93 ± 0.1e
3	Violaxanthin	0.0 ± 0.0a	19.4 ± 0.3b	2.9 ± 0.1c	26.9 ± 0.3d	80.9 ± 1.0e	87.4 ± 1.3f
4	Antheraxanthin	0.0 ± 0.0a	105.2 ± 2.2b	101.8 ± 1.1c	38.5 ± 0.4d	19.4 ± 0.5e	29.9 ± 1.6f
5	13′-*Z*-Violaxanthin	17.3 ± 0.4a	187.5 ± 2.1b	125.1 ± 1.6c	10.5 ± 0.4d	7.5 ± 0.3e	27.4 ± 1.1f
6	13-*Z*-Zeaxanthin	0.0 ± 0.0a	251.6 ± 0.4b	112.5 ± 1.0c	24.6 ± 0.3d	12.3 ± 0.3e	14.6 ± 0.6f
7	Flavoxanthin	1.6 ± 0.1a	113.9 ± 1.7b	84.3 ± 1.2c	11.5 ± 0.1d	13.5 ± 0.1e	20.4 ± 0.8f
8	Lutein	152.9 ± 3.5a	910.4 ± 1.5b	827.5 ± 1.7c	97.0 ± 0.6d	220.7 ± 1.1e	340.8 ± 0.3f
9	9-*Z*-β-Carotene	5.9 ± 0.1a	95.5 ± 0.8b	91.2 ± 1.5c	7.8 ± 0.2a	4.6 ± 0.5a	28.0 ± 0.5d
10	9-*Z*-Lutein	13.5 ± 0.5a	46.7 ± 0.8b	42.7 ± 1.3c	3.6 ± 0.1d	10.3 ± 0.6e	12.5 ± 0.6a
11	9′-*Z*-Lutein	15.1 ± 0.5a	285.4 ± 2.1b	167.4 ± 1.4c	17.7 ± 0.2d	4.3 ± 0.2e	22.8 ± 1.2f
12	Hydroxy chlorophyll *b*	0.0 ± 0.0a	411.2 ± 1.2b	417.3 ± 2.3c	2.3 ± 0.1d	50.6 ± 0.3e	0.4 ± 0.0a
13	Chlorophyll *b*	35.2 ± 0.4a	184.5 ± 2.3b	187.9 ± 1.4c	0.1 ± 0.0d	4.8 ± 0.3e	177.4 ± 1.6f
14	Chlorophyllide *a*′	0.0 ± 0.0a	88.9 ± 1.6b	49.4 ± 1.3c	1.5 ± 0.0a	13.8 ± 0.2d	18.9 ± 1.1e
15	Hydroxy chlorophyll *a*	0.0 ± 0.0a	468.1 ± 1.8b	296.5 ± 1.1c	1.2 ± 0.0a	4.8 ± 0.6d	34.8 ± 0.9e
16	Chlorophyll *a*	0.0 ± 0.0a	197.5 ± 1.3b	125.4 ± 1.6c	0.0 ± 0.0a	12.6 ± 0.3d	14.0 ± 1.3e
17	β-Carotene	0.4 ± 0.4a	198.3 ± 1.8b	55.1 ± 1.1c	0.0 ± 0.0a	0.0 ± 0.0a	1.4 ± 0.1a

Different letters (a-f) represent significant differences with respect to control using Dunnett's multiple comparisons test at a <0.05.

during boiling for 2 min and were significantly decreased as the boiling time increased further.

3.2.2. Effects of frying

Table 2 shows the effects of frying time (1, 2, 3, 4, and 5 min) on the carotenoid contents versus control. ε-Apo-13-luteinol and chrysanthemaxanthin were the compounds quantified as 7.4 and 38.8 μg/g, respectively, in the sample fried for 1 min. However, it has been observed that by increasing the frying time, the amount of these compounds was significantly decreased ($P < 0.05$). During 1 min of frying, the amount of flavoxanthin was 50.6 μg/g, and this amount significantly decreased as the frying time elapsed further. Dihydro-lutein was 13.3 μg/g after frying for 1 min, which was significantly increased to 19.2 μg/g, after 3 min of frying. The amount of lutein and 9-*Z*-β-carotene was 228.9 and 15.1 μg/g, respectively, in the sample fried for 1 min, which was significantly higher than the control sample (152.9 and 5.9 μg/g) respectively. However, by increasing the frying time, the amount of these compounds was significantly affected. 9-*Z*-lutein and 9′-*Z*-lutein were quantified as 13.5 and 15.1 μg/g in the control samples. However, the amounts of these compounds were significantly decreased while frying except for 9-Z-lutein's concentration (20.8 μg/g) during frying for 3 min. During frying for 1 min, the amount of chlorophyll *b,* 13′-hydroxy-lactone, chlorophyll *b,* and hydroxy chlorophyll *a* was 52.3, 16.6, and 16.3 μg/g, respectively. Pyropheophytin *a* became more concentrated (3.2 μg/g) during frying for 2 min, while the amount of chlorophyll *a* was 1.6 μg/g and was significantly increased in the sample fried for 3 min. The amounts of β-carotene were 2.0 μg/g during frying for 3 min, which was significantly higher among the other treatments.

3.2.3. Effects of microwave

Table 3 shows the effect of microwave heating (3, 6, 9, 12, and 15 min) on the amounts of the identified compounds. The amounts of divinyl chlorophyll *B* and divinyl chlorophyll *a* in the control samples were 33.2 and 11.7 μg/g, which were significantly increased

Table 2
Effects of frying on the carotenoid's concentration (μg/g) in mustard leaves.

Peak	Identity	Frying Time (min)					
		Control	01	02	03	04	05
1	ε-Apo-13-luteinol	0.0 ± 0.0a	7.4 ± 0.3b	7.3 ± 0.3c	3.7 ± 0.1d	6.7 ± 0.2e	5.2 ± 0.1f
2	Chrysanthemaxanthin	0.0 ± 0.0a	38.8 ± 0.7b	38.8 ± 0.7c	11.7 ± 0.3d	2.4 ± 0.1e	0.9 ± 0.1a
3	Flavoxanthin	1.6 ± 0.1a	50.6 ± 1.0b	50.5 ± 1.0c	9.7 ± 0.2d	1.7 ± 0.1a	0.0 ± 0.0e
4	Dihydro-lutein	0.0 ± 0.0a	13.3 ± 0.2b	13.3 ± 0.2c	19.2 ± 0.5d	10.4 ± 0.1e	0.0 ± 0.0a
5	Lutein	152.9 ± 3.5a	228.9 ± 0.7b	228.9 ± 0.7c	93.9 ± 0.8d	30.3 ± 0.2e	1.6 ± 0.0f
6	9-*Z*-β-Carotene	5.9 ± 0.1a	15.1 ± 0.2b	15.1 ± 0.2c	12.6 ± 0.3d	1.7 ± 0.1e	0.0 ± 0.0f
7	9-*Z*-Lutein	13.5 ± 0.5a	10.2 ± 0.1b	10.2 ± 0.1c	20.8 ± 0.3d	4.9 ± 0.2e	0.0 ± 0.0f
8	9′-*Z*-Lutein	15.1 ± 0.5a	13.7 ± 0.7b	13.7 ± 0.7c	5.3 ± 0.1d	2.1 ± 0.2e	0.0 ± 0.0f
9	Chlorophyll *b*	35.2 ± 0.4a	52.3 ± 2.2b	52.3 ± 2.2c	11.5 ± 0.2d	29.5 ± 0.7e	0.0 ± 0.0f
10	13′-Hydroxy-lactone chlorophyll *b*	0.0 ± 0.0a	16.6 ± 0.4b	16.6 ± 0.4c	6.9 ± 0.2d	4.6 ± 0.4e	0.0 ± 0.0a
11	Hydroxy chlorophyll *a*	0.0 ± 0.0a	16.3 ± 0.6b	16.4 ± 0.6c	1.7 ± 0.1d	0.5 ± 0.1a	0.0 ± 0.0a
12	Pyropheophytin a	0.0 ± 0.0a	3.1 ± 0.1b	3.2 ± 0.1c	0.8 ± 0.1a	0.0 ± 0.0a	0.0 ± 0.0a
13	Chlorophyll *a*	0.0 ± 0.0a	0.30 ± 0.01a	0.0 ± 0.0a	1.6 ± 0.1b	0.0 ± 0.0a	0.0 ± 0.0a
14	β-Carotene	0.43 ± 0.40a	0.10 ± 0.03a	0.0 ± 0.0a	2.0 ± 0.1b	0.5 ± 0.1a	0.0 ± 0.0a

Different letters (a-f) represent significant differences with respect to control using Dunnett's multiple comparisons test at a <0.05.

($P < 0.05$) to 96.9 and 118.9 μg/g respectively while microwaving for 3 min. The highest amount of 13′-*Z*-violaxanthin was 347.5 μg/g when microwaved for 3 min. The amounts increased significantly from 6 to 12 min of treatments and significantly decreased at 15 min. Except for 6 min, the amounts of antheraxanthin increased significantly with treatments. Similarly, the amounts of lutein increased significantly (1203.3 μg/g) at 3 min and then decreased significantly to 1031.0 μg/g at 6 min. Further treatments significantly increased the amounts of lutein reaching 1725.6 μg/g when microwaved for 15 min. The amounts of 9-*Z*-β-carotene were significantly increased to 120.0 μg/g during microwaving for 15 min. Similarly, the amounts of 9-*Z*-lutein and 9′-*Z*-lutein significantly increased during microwaving for 15 min and reached 75.9 and 107.2 μg/g, respectively. The amounts of chlorophyll *b* and 13′-hydroxy-lactone chlorophyll *b* significantly increased to 878.8 and 203.9 μg/g, respectively, in the samples microwaved for 15 min. The highest amounts of 13′-hydroxy-lactone chlorophyll *a* were determined to be 251.8 μg/g in samples treated for 6 min. The amount of pyropheophytin *A* was 42.6 μg/g in the sample microwaved for 3 min, which was significantly reduced by increasing the microwave heating time. Among the treated samples, the highest amounts of β-carotene and 9-*Z*-β-carotene were 553.1 and 75.9 μg/g in the group microwaved for 12 min and was significantly decreased while microwaving for 15 min.

3.2.4. Effects of sonication

Table 4 shows the effects of sonication (5, 10, 15, 20, and 25 min) on the amounts of the identified compounds. The amounts of divinyl chlorophyll *b* and divinyl chlorophyll *a* were significantly increased ($P < 0.05$) to 40.1 and 24.6 μg/g respectively, in the sample sonicated for 25 min, versus the control group having 33.2 and 11.7 μg/g, respectively. The highest amounts of 13′-*Z*-violaxanthin were 105.8 μg/g in the sample treated for 20 min. The amount (8′R)-neochrome and flavoxanthin was increased to 29.9 and 15.3 μg/g, respectively, in the group treated for 20 min. Similarly, among the treated groups, the amount of lutein was significantly increased to 392.2 μg/g in the group sonicated for 20 min. The amount of 9-*Z*-β-carotene was 28.9 μg/g in the samples sonicated for 20 min and was significantly decreased to 8.7 μg/g when treated for 25 min. Likewise; the amount of 9-*Z*-lutein and 9′-*Z*-lutein was significantly increased to 19.8 and 30.7 μg/g, respectively, in the group sonicated for 20 min. The amount of chlorophyll *b* 13′-hydroxy-lactone, chlorophyll *b,* and chlorophyll *b'* was significantly increased to 278.2, 82.7, and 180.2 μg/g respectively, in the group treated for 20 min and was further declined as the sonication time proceeded. β-Carotene and 9-*Z*-β-carotene also became more concentrated specifically in the group treated for 20 min and were quantified as 77.2 and 10.9 μg/g, respectively.

3.2.5. Effects of freezing

Table 5 shows the effect of freezing treatments (24, 48, 72, 96, and 120 h) on the amounts of the identified compounds. The amounts of divinyl chlorophyll *b* and divinyl chlorophyll *A* were significantly increased in the sample thawed for 72 h and were quantified as 204.1 and 146.5 μg/g, respectively. However, further freezing significantly reduced the amounts of these compounds. During freezing for 96 h, the amounts of 13′-*Z*-violaxanthin and (8′R)-neochrome were significantly increased to 30.2 and 186.2 μg/g, respectively. 13/13′*Z*-Antheraxanthin became more concentrated (201.7 μg/g), particularly during freezing for 72 h. Among the freezing treatments, the highest amounts of 13/13′*Z*-neoxanthin and flavoxanthin were observed in a group thawed for 120 h and significantly increased to 82.2 and 111.4 μg/g, respectively. However, during freezing for 96 h, lutein, and 9-*Z*-β-carotene became more concentrated (633.0 and 50.7 μg/g), respectively. Similarly, the amount of 9-*Z*-lutein was significantly increased to 31.5 μg/g during freezing for 72 h. While the amount of 9′-*Z*-Lutein was significantly increased to 41.8 μg/g during freezing for 96 h. The amounts of chlorophyll *b* and 13′-hydroxy-lactone chlorophyll *b* were 322.0 and 81.9 μg/g respectively, in the samples thawed for 96 h while during freezing for 120 h, chlorophyll *b'* and β-carotene became more concentrated (167.4 and 31.7 μg/g), versus control (6.0 and 0.43 μg/g) respectively.

3.2.6. Effects of blanching

Table 6 shows the effects of blanching for 30, 60, 90, 120, and 150 s on the amounts of the identified compounds. It has been

Table 3
Effects of microwave heating on the carotenoid's concentration (μg/g) in mustard leaves.

Peak	Identity	Microwave Time (min)					
		Control	03	06	09	12	15
1	Divinyl chlorophyll *b*	33.2 ± 0.6a	96.9 ± 0.8b	24.8 ± 0.3c	11.5 ± 0.6d	5.3 ± 0.3e	22.1 ± 0.5f
2	Divinyl chlorophyll *a*	11.7 ± 0.4a	118.9 ± 1.3b	25.5 ± 0.4c	11.6 ± 0.2a	11.8 ± 0.2a	31.4 ± 0.6d
3	13′-*Z*-Violaxanthin	17.3 ± 0.4a	347.5 ± 2.7b	180.0 ± 0.5c	196.4± 0.3d	233.2 ± 0.5e	141.3 ± 0.3f
4	Antheraxanthin	0.0 ± 0.0a	80.8 ± 0.4b	58.3 ± 0.8c	95.7 ± 1.8d	104.7 ± 2.1e	146.3 ± 1.1f
5	Lutein	152.9 ± 3.5a	1203.3 ± 6.2b	1031.0 ± 1.9c	1069.0 ± 8.6d	1242.1 ± 5.7e	1725.6 ± 7.1f
6	9-*Z*-β-Carotene	5.9 ± 0.1a	72.6 ± 1.2b	51.1 ± 0.4c	65.0 ± 0.3d	95.5 ± 0.5e	120.0 ± 1.4f
7	9-*Z*-Lutein	13.5 ± 0.5a	41.0 ± 1.1b	38.2 ± 0.3c	35.3 ± 0.6d	47.6 ± 0.7e	75.9 ± 1.4f
8	9′-*Z*-Lutein	15.1 ± 0.5a	64.5 ± 1.3b	54.5 ± 0.3c	60.0 ± 0.6d	72.4 ± 1.4e	107.2 ± 0.5f
9	Chlorophyll *b*	35.2 ± 0.4a	664.2 ± 4.0b	511.6 ± 0.6c	465.5 ± 4.4d	645.5 ± 2.2e	878.8 ± 0.9f
10	13′-Hydroxy-lactone chlorophyll *b*	5.6 ± 0.3a	167.6 ± 0.4b	182.6 ± 0.6c	177.4 ± 1.7d	125.6 ± 1.6e	203.9 ± 1.4f
11	13′-Hydroxy-lactone chlorophyll *a*	0.0 ± 0.0a	125.9 ± 1.3b	251.8 ± 1.0c	217.1 ± 2.1d	167.3 ± 1.4e	147.7 ± 1.0f
12	Pyropheophytin *a*	0.0 ± 0.0a	42.6 ± 1.1b	0.6 ± 0.1a	4.1 ± 0.1a	27.9 ± 1.4c	28.0 ± 0.8d
13	β-Carotene	0.4 ± 0.0a	430.9 ± 1.1b	24.4 ± 0.2c	28.1 ± 0.4d	553.1 ± 4.9e	398.4 ± 3.8f
14	9-*Z*-β-Carotene	0.0 ± 0.0a	59.4 ± 1.6b	0.0 ± 0.0a	0.0 ± 0.0a	75.9 ± 1.1c	45.7 ± 1.7d

Different letters (a-f) represent significant differences with respect to control using Dunnett's multiple comparisons test at a <0.05.

Table 4
Effects of sonication on the carotenoid's concentration (μg/g) in mustard leaves.

Peak	Identity	Sonication Time (min)					
		Control	05	10	15	20	25
1	Divinyl chlorophyll *b*	33.2 ± 0.6a	27.2 ± 0.7b	13.4 ± 0.4c	47.9 ± 0.4d	53.5 ± 0.7e	40.0 ± 1.0f
2	Divinyl chlorophyll *a*	11.7 ± 0.4a	16.1 ± 0.9b	8.4 ± 0.2c	27.3 ± 1.1d	72.5 ± 1.5e	24.6 ± 0.3f
3	13′-*Z*-Violaxanthin	17.3 ± 0.4a	55.0 ± 0.6a	17.7 ± 0.4b	66.8 ± 1.6c	105.8 ± 2.3d	29.9 ± 0.3e
4	(8′R)-Neochrome	24.6 ± 0.3a	28.0 ± 0.4b	1.6 ± 0.1c	17.3 ± 0.3d	29.9 ± 0.6e	4.6 ± 0.4f
5	Flavoxanthin	1.6 ± 0.1a	13.5 ± 0.5a	1.7 ± 0.1b	7.6 ± 0.3c	15.3 ± 0.3d	2.3 ± 0.1a
6	Lutein	152.9 ± 3.5a	269.7 ± 0.9b	103.7 ± 0.8c	310.7 ± 2.6d	392.2 ± 1.2e	143.8 ± 1.0f
7	9-*Z*-β-Carotene	5.9 ± 0.1a	16.5 ± 0.5a	6.6 ± 0.5b	17.7 ± 0.4c	28.9 ± 0.4d	8.7 ± 0.4e
8	9-*Z*-Lutein	13.5 ± 0.5a	13.5 ± 0.3a	5.7 ± 0.2b	15.0 ± 0.3a	19.8 ± 0.6c	8.7 ± 0.2d
9	9′-*Z*-Lutein	15.1 ± 0.5a	21.6 ± 0.4b	10.0 ± 0.3c	24.9 ± 0.3d	30.7 ± 0.7e	12.9 ± 0.4f
10	Chlorophyll *b*	35.2 ± 0.4a	216.5 ± 0.3b	23.4 ± 0.6c	161.9 ± 1.1d	278.2 ± 2.0e	37.9 ± 0.4f
11	13′-Hydroxy-lactone chlorophyll *b*	5.6 ± 0.3a	59.9 ± 0.7b	3.6 ± 0.2c	37.1 ± 0.8d	82.7 ± 0.8e	11.4 ± 0.6f
12	Chlorophyll *b'*	6.0 ± 0.5a	79.6 ± 1.3b	2.5 ± 0.1c	50.4 ± 0.6d	180.2 ± 0.3e	5.8 ± 0.1a
13	β-Carotene	0.4 ± 0.4a	26.4 ± 0.8b	0.4 ± 0.0a	3.2 ± 0.1c	77.2 ± 1.1d	0.2 ± 0.0a
14	9-*Z*-β-Carotene	0.0 ± 0.0a	6.5 ± 0.3b	0.0 ± 0.0a	0.0 ± 0.0a	10.9 ± 0.2c	0.0 ± 0.0a

Different letters (a-f) represent significant differences with respect to control using Dunnett's multiple comparisons test at a <0.05.

Table 5
Effects of freezing on the carotenoid's concentration (μg/g) in mustard leaves.

Peak	Identity	Freezing Time (h)					
		Control	24	48	72	96	120
1	Divinyl chlorophyll *b*	33.2 ± 0.6a	55.1 ± 0.4b	94.2 ± 0.6c	204.1 ± 0.7d	128.8 ± 0.9e	133.1 ± 0.9f
2	Divinyl chlorophyll *a*	11.7 ± 0.4a	20.5 ± 0.7b	45.1 ± 1.1c	146.5 ± 2.1d	159.7 ± 1.2e	149.1 ± 1.9f
3	13′-*Z*-Violaxanthin	17.3 ± 0.4a	13.0 ± 0.5b	20.4 ± 0.5c	5.6 ± 0.3d	30.2 ± 1.1e	27.4 ± 0.4f
4	(8′R)-Neochrome	24.6 ± 0.3a	31.7 ± 0.2b	121.5 ± 1.1c	18.5 ± 0.5d	186.2 ± 1.2e	30.8 ± 0.3f
5	(13/13′Z)-Antheraxanthin	12.3 ± 0.3a	47.1 ± 0.6b	40.8 ± 0.6c	201.7 ± 1.0d	93.8 ± 2.5e	121.3 ± 1.2f
6	(13/13′Z)-Neoxanthin	1.2 ± 0.1a	31.9 ± 0.3b	27.2 ± 0.7c	24.2 ± 1.0d	37.0 ± 1.1e	82.2 ± 1.2f
7	Flavoxanthin	1.6 ± 0.1a	11.5 ± 0.3b	56.2 ± 0.8c	99.3 ± 0.8d	93.7 ± 1.0e	111.4 ± 1.2f
8	Lutein	152.9 ± 3.5a	80.8 ± 0.4b	308.7 ± 2.7c	459.7 ± 1.3d	633.0 ± 1.3e	486.9 ± 3.0f
9	9-*Z*-β-Carotene	5.9 ± 0.1a	7.8 ± 0.7a	25.2 ± 0.1b	11.4 ± 0.6c	50.7 ± 0.5d	39.2 ± 0.6e
10	9-*Z*-Lutein	13.5 ± 0.5a	1.6 ± 0.1b	8.6 ± 0.4c	31.5 ± 0.8d	16.7 ± 0.3e	16.7 ± 0.6f
11	9′-*Z*-Lutein	15.1 ± 0.5a	4.8 ± 0.1b	21.5 ± 0.5c	3.5 ± 0.1d	41.8 ± 0.6e	34.4 ± 0.5f
12	Chlorophyll *b*	35.2 ± 0.4a	7.9 ± 0.2b	94.9 ± 1.0c	146.5 ± 2.1d	322.0 ± 1.8e	8.3 ± 0.3f
13	13′-Hydroxy-lactone chlorophyll *b*	5.6 ± 0.3a	1.6 ± 0.1b	21.2 ± 0.5c	31.3 ± 0.9d	81.9 ± 0.9e	68.9 ± 0.7f
14	Chlorophyll *b'*	6.0 ± 0.5a	0.4 ± 0.0b	19.5 ± 0.3c	42.2 ± 0.8d	147.9 ± 0.7e	167.4 ± 1.3f
15	β-Carotene	0.43 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	3.4 ± 0.1b	31.7 ± 0.8c

Different letters (a-f) represent significant differences with respect to control using Dunnett's multiple comparisons test at a <0.05.

observed that during blanching for 30 s, the amounts of ε-apo-13-luteinol were 15.9 μg/g and significantly reduced when blanched further. During blanching for 120 s, 9-*Z*-neoxanthin and flavoxanthin became more concentrated (329.3 and 137.7 μg/g), respectively. While freezing for 120 s, the highest amounts of lutein, 9-*Z*-lutein, 9′-*Z*-lutein and were **890.9**, **56.7**, and **20.5** μg/g, respectively. The

Table 6
Effects of blanching on the carotenoid's concentration (μg/g) in mustard leaves.

Peak	Identity	Blanching Time (Sec)					
		Control	30	60	90	120	150
1	ε-Apo-13-luteinol	0.0 ± 0.0a	15.9 ± 0.5b	6.9 ± 0.3c	0.6 ± 0.1a	9.3 ± 0.5d	9.9 ± 0.3e
2	9-Z-Neoxanthin	0.0 ± 0.0a	212.7 ± 1.1b	181.7 ± 4.6c	58.8 ± 0.1d	329.3 ± 2.6e	107.3 ± 1.0f
3	Flavoxanthin	1.6 ± 0.1a	101.4 ± 1.2b	106.9 ± 0.6c	21.5 ± 0.1d	137.7 ± 1.6e	98.7 ± 1.4f
4	Lutein	152.9 ± 3.5a	680.6 ± 5.5b	407.2 ± 0.8c	116.5 ± 1.2d	890.9 ± 1.4e	458.1 ± 2.8f
5	9-*Z*-Lutein	13.5 ± 0.5a	38.2 ± 0.5b	24.3 ± 0.8c	7.7 ± 0.3d	56.7 ± 1.2e	24.2 ± 0.2f
6	9′-*Z*-Lutein	15.1 ± 0.5a	15.7 ± 0.1a	9.4 ± 0.3b	3.1 ± 0.1c	20.5 ± 0.6d	13.1 ± 0.6a
7	(13/13′Z)-antheraxanthin	0.0 ± 0.0a	33.8 ± 1.2b	20.7 ± 0.6c	4.9 ± 0.2d	39.5 ± 0.6e	22.4 ± 0.1f
8	15-Hydroxy-lactone chlorophyll *a*	0.0 ± 0.0a	11.6 ± 0.4b	2.6± 0.1a	0.4 ± 0.1a	39.8 ± 0.1c	6.1 ± 0.1d
9	Hydroxy chlorophyll *b*	0.0 ± 0.0a	291.3 ± 2.3b	71.8 ± 1.1c	0.0 ± 0.0a	71.8 ± 0.8d	110.0 ± 0.9e
10	Chlorophyll *b*	35.2 ± 0.4a	92.0 ± 0.8b	20.2 ± 0.9c	10.7 ± 0.1d	555.2 ± 1.9e	34.9 ± 0.3a
11	Hydroxy chlorophyll *a*	0.0 ± 0.0a	158.8 ± 3.6b	27.5 ± 0.9c	0.0 ± 0.0a	530.9 ± 0.9d	51.8 ± 0.8e
12	13′-Hydroxy-lactone chlorophyll *a*	0.0 ± 0.0a	29.2 ± 1.0b	4.8 ± 0.2c	2.4 ± 0.0a	190.2 ± 1.1d	12.4 ± 0.7e
13	Chlorophyll *a*	0.0 ± 0.0a	2.8 ± 0.1b	0.2 ± 0.1a	0.0 ± 0.0a	9.5 ± 0.2c	0.0 ± 0.0a
14	β-Carotene	0.43 ± 0.04a	6.0 ± 0.1b	0.5 ± 0.0a	0.0 ± 0.0a	158.4 ± 1.7c	1.0 ± 0.1a

Different letters (a-f) represent significant differences with respect to control using Dunnett's multiple comparisons test at a <0.05.

contents of these carotenoids significantly increased upon 30 s treatments, then significantly decreased in dose dependent manner till 90 s and decreased again from its highest values during treatment for 150 s. Similarly, the highest amount of (13/13′*Z*)-antheraxanthin and 15-hydroxy-lactone chlorophyll was 39.5 and 39.8 μg/g respectively, in the group treated for 120 s. During blanching for 30 s, the amount of hydroxy chlorophyll *b* was 291.3 μg/g. This was significantly reduced when blanched further. However, the highest amounts of chlorophyll *b* and hydroxy chlorophyll *a* were 555.2 and 530.9 μg/g, respectively, during blanching for 120 s. The amounts of 13′-hydroxy-lactone chlorophyll and chlorophyll *a* were significantly increased to 190.2 and 9.5 μg/g, respectively, during blanching for 120 s. The amounts of β-carotene were also increased to 158.4 μg/g significantly in the group blanched for 120 s.

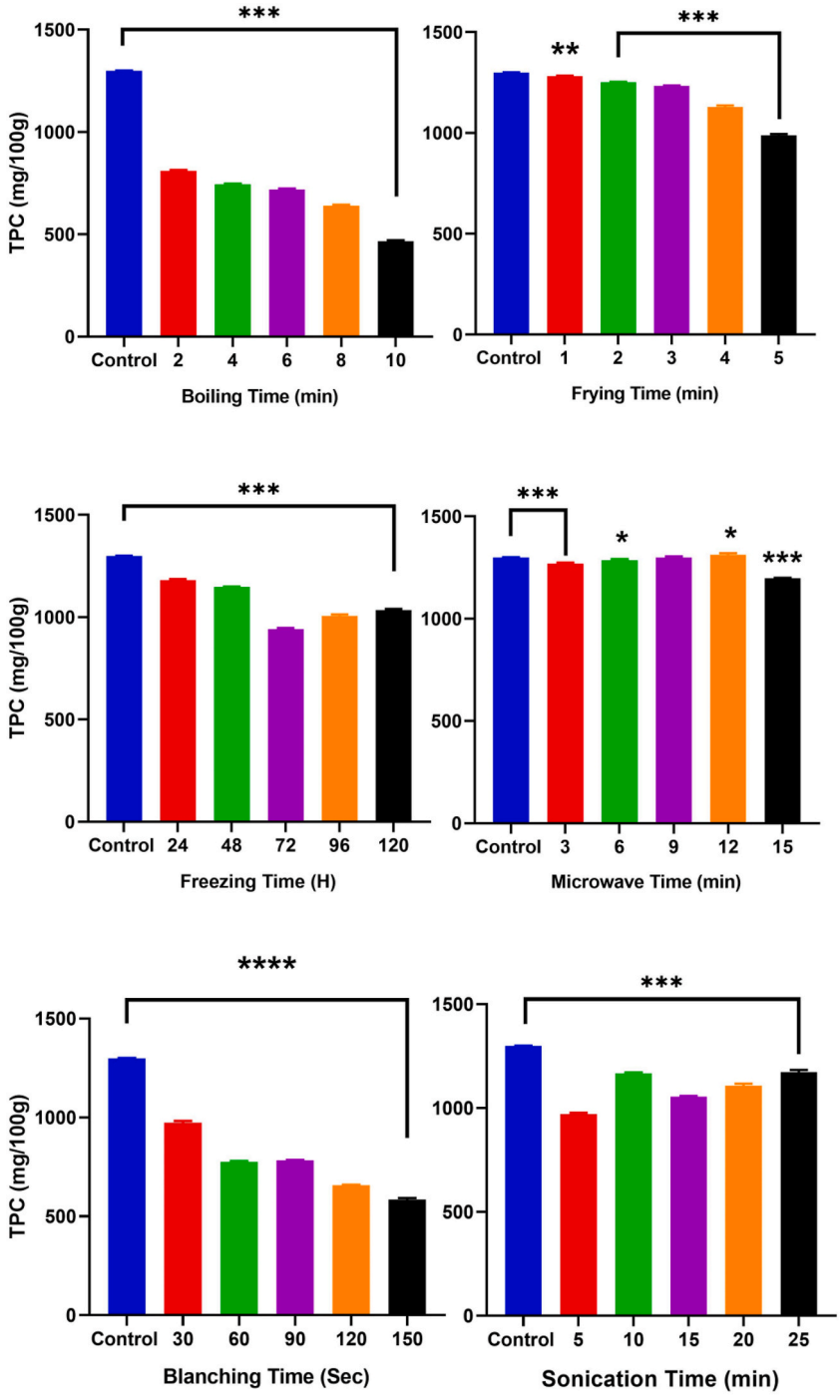

Fig. 2. Effects of different processing conditions on the total phenolic contents of mustard leaves. Data are the mean of triplicates with standard deviation. * = 0.01, ** = 0.003, *** <0.001, **** <0.0001 versus control in each treatment using Dunnett's multiple comparison tests.

3.2.7. Effect on total phenolic contents

Fig. 2 shows the effect of various processing treatments on the total phenolic compounds (TPC) of the mustard sample. The TPC of the control sample was 1299 mg/100 g, which was significantly higher among the TPCs of various processing treatments. During boiling (B2 to B10), the highest TPC was 809 mg/100 g in the samples boiled for 2 min which was significantly decreased ($P < 0.05$) to 466.3 mg/100 g, when boiled for 10 min. During frying (1–5 min), The TPC of the frying treatment was observed as 1281.2 mg/100 g in 1 min, which was significantly decreased to 987.4 mg/100 g in the samples fried for 5 min. The TPC of freezing treatment (24–120 h) was 1180.4 mg/100 g in the samples treated for 24 h and significantly decreased to 1034.2 mg/100 g when thawed for 120 h. Likewise, the TPC was 1269.8 mg/100 g, during microwaving for 3 min, which was significantly increased to 1312.0 mg/100 g when microwaved for 12 min. Unlike other treatments, the TPC of the sonicated samples significantly increased from 971.1 to 1173.8 mg/100 g by increasing the sonication time. While in blanching (30–150 s), the TPC of the sample blanched for 30 s was observed as 974.1 mg/100

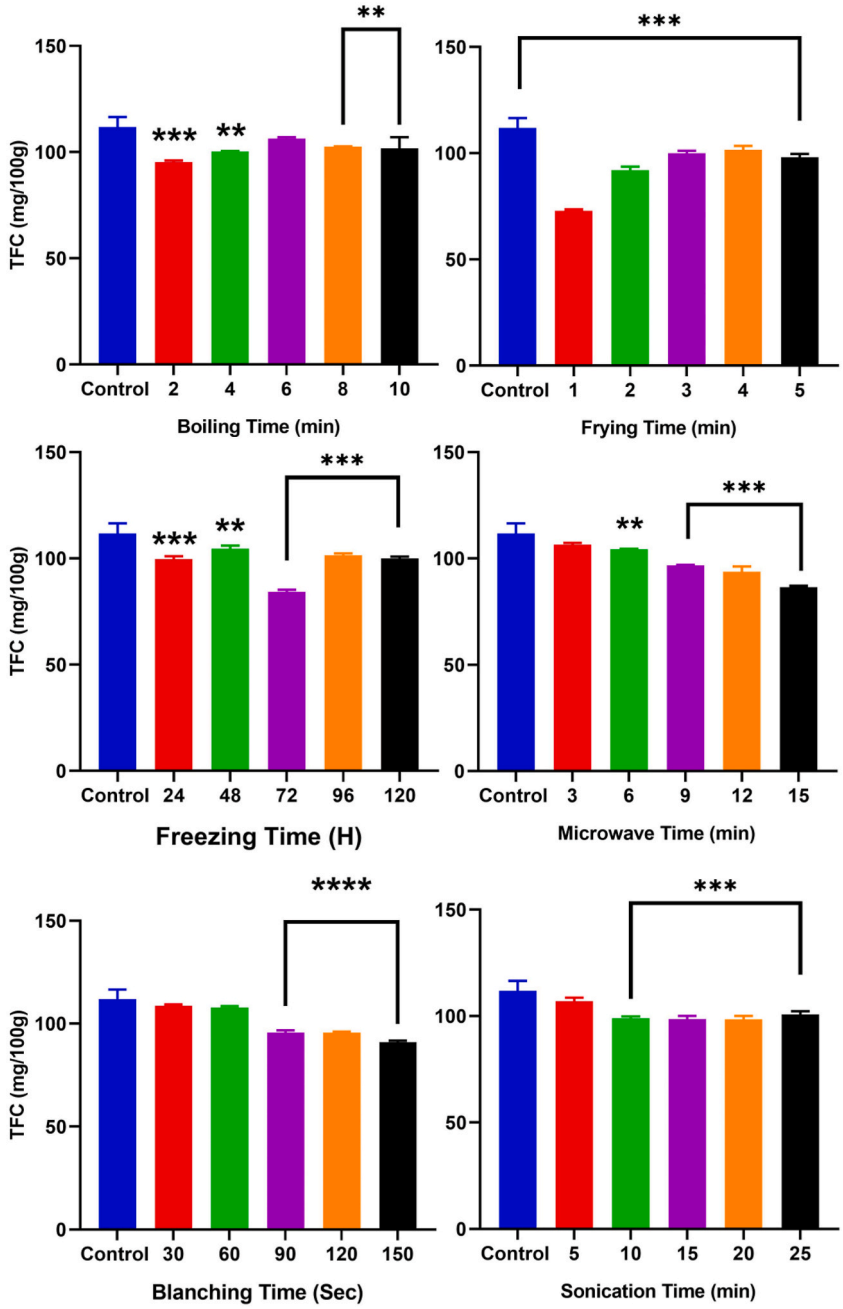

Fig. 3. Effects of different processing conditions on the total flavonoid contents of spinach. Data are the mean of triplicates with standard deviation. ** = 0.003, *** <0.001, **** <0.0001 versus control in each treatment using Dunnett's multiple comparison tests.

g, which was significantly decreased to 584.1 mg/100 g during blanching for 150 s.

3.2.8. *Effect on total flavonoid contents*

Fig. 3 shows the effect of processing treatment on the total flavonoid contents (TFC) of the treated sample. The TFC of the treated samples revealed that the processing treatments affect the amount of treated samples. For example, in the control samples, the TFC was

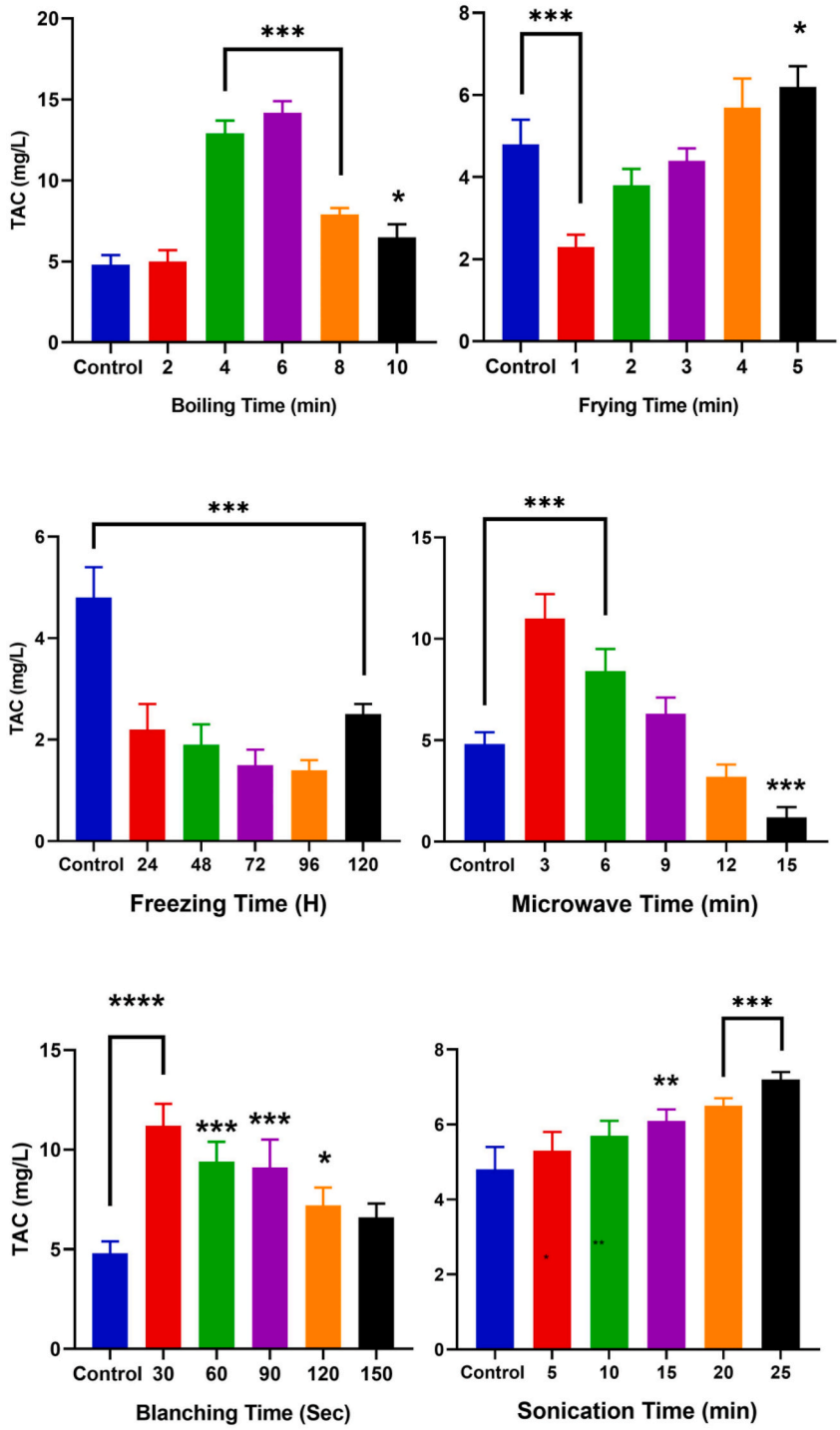

Fig. 4. Effects of different processing conditions on the total anthocyanin contents of mustard leaves. Data are the mean of triplicates with standard deviation. * = 0.01, ** = 0.003, *** <0.001, **** <0.0001 versus control in each treatment using Dunnett's multiple comparison tests.

111.8 mg/100 g, which was significantly higher among the observed TFCs of all treatments. During boiling, it has been observed that by increasing the boiling time from 2 to 10 min, the TFC has also significantly increased from 95.3 to 101.7 mg/100 g, respectively. By increasing the frying time (1–5 min), the TFC was significantly increased from 72.9 to 98.1 mg/100 g, respectively. During freezing, the highest TFC was 104.5 mg/100 g in the sample treated for 48 h. During microwaving, the TFC was significantly decreased from 106.6 to 86.6 mg/100 g in 3–15 min, respectively. The samples sonicated for 5 min revealed a TFC of 107.1 mg/100 g, which was

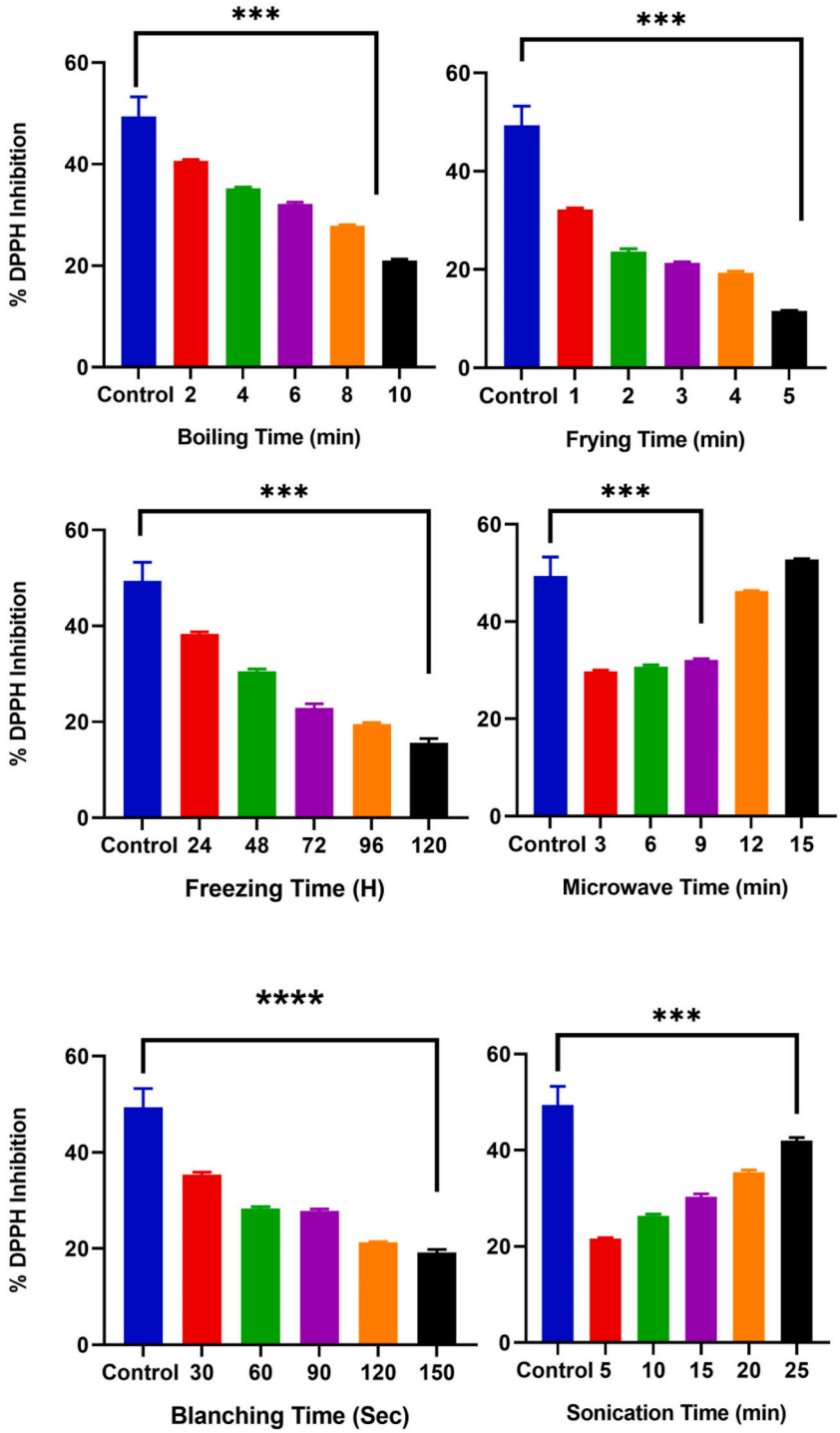

Fig. 5. Effects of different processing conditions on % DPPH radical scavenging activity of mustard leaves. Data are the mean of triplicates with standard deviation. *** <0.001 and **** <0.0001 versus control in each treatment using Dunnett's multiple comparison tests.

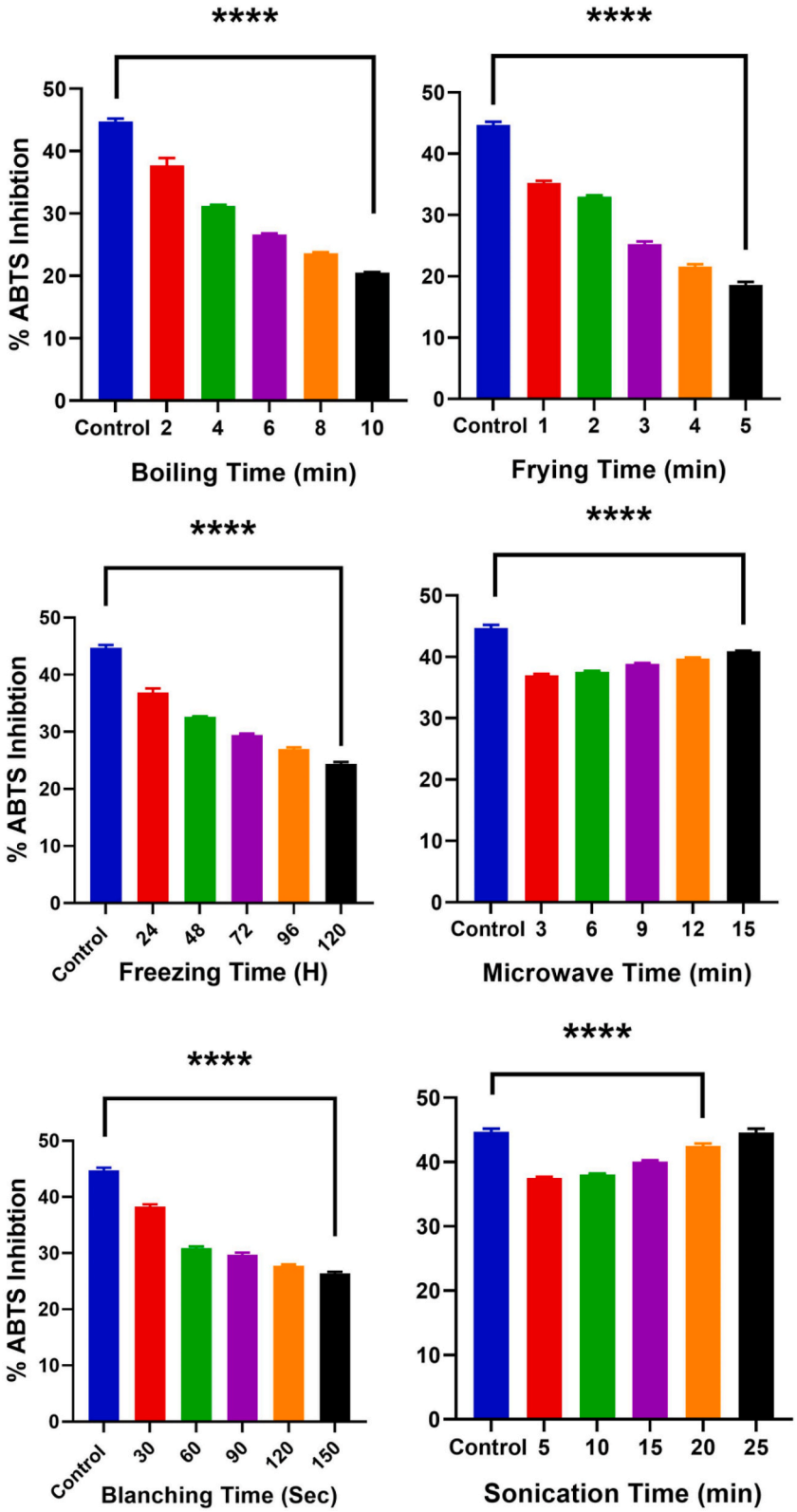

Fig. 6. Effects of different processing conditions on % ABTS^{++}radical scavenging activity of mustard leaves. Data are the mean of triplicates with standard deviation. **** <0.0001 versus control in each treatment using Dunnett's multiple comparison tests.

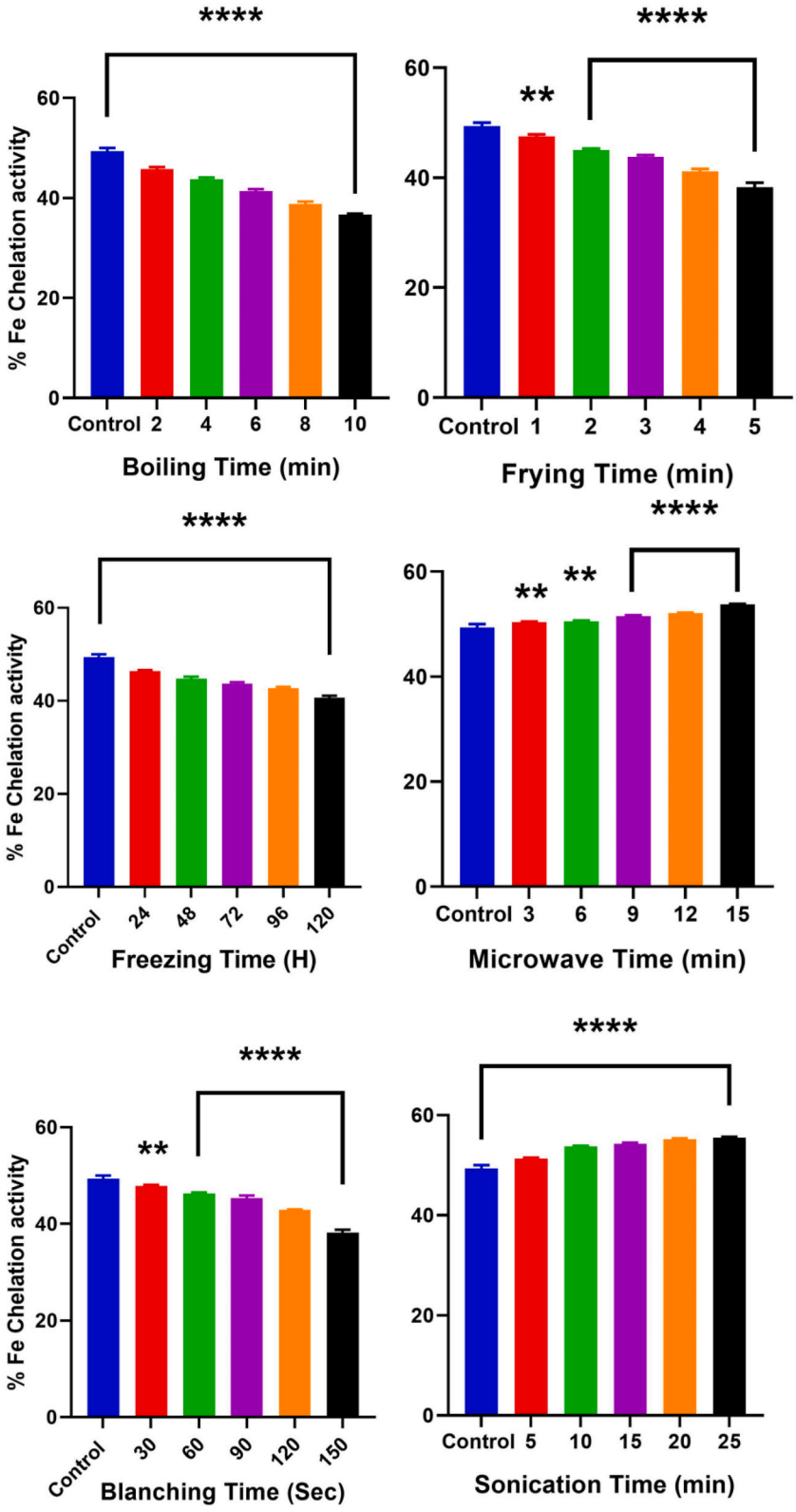

Fig. 7. Effects of different processing conditions on % Fe chelation activity of mustard leaves. Data are the mean of triplicates with standard deviation. ** = 0.003, **** <0.0001 versus control in each treatment using Dunnett's multiple comparison tests.

significantly reduced as the sonication time proceeded further. The TFC of the blanched sample has also significantly reduced from 108.7 to 91.1 mg/100 g in 30–150 s, respectively, during blanching.

3.2.9. Effects on total anthocyanin contents

Fig. 4 shows the effects of processing treatment on the total anthocyanin contents (TAC) of the treated sample versus control (4.8 mg/L). During boiling for 6 min, the TAC was significantly ($P < 0.05$) increased to 14.2 mg/L, whereas further treatment reduced its concentration. In frying, the TAC significantly increased from 2.3 to 6.2 mg/L of 1–5 min, respectively. Among the sub-treatments, the highest TAC in freezing was determined in the sample treated for 120 h (2.5 mg/L). Microwave heating affected the TAC of the samples. It has been observed that by increasing the microwave heating time (3–15 min), the TAC has significantly reduced (11.0–1.2) mg/L, respectively. The TAC of sonicated (5–25 min) samples was significantly increased from 5.3 to 7.2 mg/L. During blanching, the TAC of the treated samples has also significantly reduced from (30–150 s) 11.2 to 6.6 mg/L, respectively.

3.2.10. Effects of processing treatments on antioxidant activity

- ***DPPH radical scavenging activity***

Fig. 5 shows the effects of processing treatments on the antioxidant potential of the treated sample. It has been observed that the % RSA of the treated samples has significantly ($P < 0.05$) reduced versus control (49.4 %). During boiling, the %RSA of the treated samples was significantly reduced (from 2 to 10 min) from 40.7 to 21.0 %, respectively. Similarly, in frying, the %RSA of the treated samples has significantly decreased from (1–5 min) 32.2 to 11.6 %, respectively. It has been observed that microwave heating has significantly increased the antioxidant potential of the treated sample from (3–15 min) 29.7–52.7 %, respectively. However, freezing has significantly decreased the %RSA of the samples from 24 to 120 38.4 to 15.6 %, respectively. Sonication has significantly increased the %RSA of the treated samples from (S5 to S25) 21.6–42.0 %. However, during blanching, the %RSA of the treated samples has significantly reduced from (Bl30 to Bl150) 35.4 to 19.2 %, respectively.

- ***$ABTS^{++}$ radical scavenging activity***

Fig. 6 shows the antioxidant potential of mustard leaves extract to scavenge the $ABTS^{++}$ radicals. It has been observed that the activity of the treated sample was significantly ($P < 0.05$) decreased versus control (44.7 %). The activity was significantly reduced during boiling (2–10 min) from 37.3 to 20.5 %, respectively. Similarly, the activity was also affected while frying (1–5 min) from 35.2 to 18.6 %, respectively. Conversely, it has been observed that during microwaving (3–15 min), the activity significantly increased from 37.0 to 40.9 %, respectively. Sonication (5–25 min) has also shown some better potential to enhance the antioxidant potential of the treated samples from 37.5 to 44.6 %, respectively. During freezing (24–120 min), the activity was significantly reduced by 36.9 to 24.4 %, respectively. Likewise, the activity was also significantly reduced during blanching (30–150 s) from 38.3 to 26.4 %, respectively.

- ***Iron chelation activity***

Fig. 7 shows the iron chelation activity of the treated samples versus the control (49.4 %). Fe-chelation activity has significantly ($P < 0.05$) decreased during boiling (2–10 min) from 45.8 to 36.7 %, respectively. The activity was also significantly reduced while frying (1–5 min) from 47.5 to 38.3 %, respectively. Microwave (3–15 min) treated samples have shown a significant increase in the % inhibition activity from 50.4 to 53.8 %, respectively. Likewise, during sonication (5–25 min), the activity significantly increased from 51.3 to 55.5 %, respectively. During freezing (24–120 h) the activity was significantly reduced from 46.4 to 40.7 %, respectively. In the same way during blanching (30–150 s) the iron chelation activity of the samples was reduced from 47.9 to 38.2 %, respectively.

4. Discussion

Carotenoids are important bioactive compounds identified and quantified in this study including violaxanthin, antheraxanthin, lutein, and β-carotene. Paula Filho, Barreira [28] reported β-carotene in wild mustard. Violaxanthin, lutein, and β-carotene were reported previously [29,30]. The present study thus confirms the presence of these important carotenoids in mustard leaves. However, chlorophylls were not reported previously. This study further investigated the effects of different processing conditions on the carotenoid composition of mustard leaves.

It has been observed that different processing conditions can significantly affect ($P < 0.05$) the carotenoid contents of mustard leaves. When compared to other processing treatments, boiling has been found to retain and preserve the carotenoid contents effectively. For example, during boiling, we have identified 17 compounds in mustard leaves extract, while in the other treatments, only 14 compounds have been identified. According to Kao, Chiu [31] and Moyo, Serem [32] boiling is an effective approach for retaining fat-soluble chemicals and carotenoids that might be due to lesser diffusion and leaching into the surrounding water. According to de Sá and Rodriguez-Amaya [33], violaxanthin is a heat-sensitive carotenoid and cannot tolerate prolonged heating. In this study only the boiling treatment was observed to retain violaxanthin, therefore indicating the beneficial aspect of the treatment. Carotenoid contents in raw vegetables are more difficult to extract. therefore cooking may soften the food matrix in a way to releases the vital compounds [34]. This could be the reason that a significantly reduced amount of most of the compounds in the control (raw) sample was observed.

When compared to boiling, frying may provoke a detrimental effect on the concentration of most of the compounds; Flavoxanthin, lutein, β-Carotene, and chlorophyll *b* were significantly reduced during the process. According to Kao, Chiu [35], shallow contact frying is a better choice than deep fat frying because, during deep fat frying, carotenoids may be released into the surrounding oil or may also lead to degradation or isomerization of carotenoids. Lutein, β-carotene, 13′-*Z*-violaxanthin, and antheraxanthin contents were significantly increased during microwave heating. Conversely, it has been reported in different studies that during microwaving, the carotenoid contents of Chinese mustard [17] and broccoli [36] were significantly reduced. Similarly, it was also determined that carotenoid contents were significantly improved during blanching, this might be due to the release of compounds from cell matrices during the process [37,38]. In this study, freezing has been observed to preserve most of the carotenoid contents in mustard leaves. The findings of Dias, Camões [39] and Bouzari, Holstege [40] are parallel to this study as they found in different studies, that the freezing process did not cause carotenoid degradation of broccoli. Heating can improve the extractability of carotenoids by breaking carotene-proteins complexes in the food matrix and the carotenoid amounts may be triggered [41]. However, extreme heating or long-term exposure to heat treatment may cause oxidative deterioration of carotenoids [41]. This might be the reason that a significant improvement in the amount of the compounds in all treatments was observed in this study. Moreover, this could also be concluded that as the length of the treatment increased, the amounts of the compounds were significantly reduced particularly during frying, sonication, and blanching.

Phenolic compounds in mustard leaves were significantly decreased during various treatments versus control. In the previous study, it was reported that a significant increase in the phenolic profile of spinach was determined using similar processing treatments [42]. This indicates that different plants contain different compounds of which some are heat labile whereas some are not. According to Bernhardt and Schlich [43], the same cooking condition may have a different effect on the phenolic profile of different plants. During boiling or microwaving, the total phenolic contents may also be depleted due to the phenolic breakdown during the treatments [44]. Similarly, a study conducted by Price, Casuscelli [45] reported that only 18 % of the phenolic contents in broccoli were retained during boiling while the rest were leached into the surrounding water. Such findings can be matched to this study as a significant decline in the phenolic profile of mustard leaves was observed. Flavonoids usually exist in both free and conjugated forms. Conjugated flavonoids are found in fresh vegetables, while the formation of aglycones may be due to food processing [46]. Flavonoids are stable at high temperatures and over a long period of storage [47]. During various treatments such as frying, sonication, and blanching, the TACs of mustard leaves were significantly increased while prolonged exposure to microwave heating eventually declined the TAC contents of the treated samples. Such findings could not be compared to any other study because no relevant data regarding heat effects on anthocyanin contents in mustard leaves was found, and the findings of this study, therefore, contribute to its novelty. In this study, a significant decline was observed in the antioxidant potential of treated samples versus control. When compared to other treatments microwave and sonication have shown a significant increase in the antioxidant activities of mustard leaves. According to Oh, Kim [48] such an increase could be linearly correlated to the total phenolic or flavonoid contents. The antioxidant activity of vegetables may be influenced by qualitative changes, antioxidant breakdown, and their leaching into the surrounding water while cooking [49]. Antioxidant compounds in vegetables have been found sensitive to heat and storage and could be decreased during different processing steps [50]. These findings are in agreement with this study as a significant decrease was observed in the antioxidant activities during various processing treatments except microwave and sonication.

Different processing treatments affect the carotenoids as well as the poly-phenolic contents of the mustard leaves and such effects depend on the type and duration of the treatment. Among the studied treatments, we cannot point out a single treatment that has all the benefits of retention of carotenoids and phenolic compounds collectively at the same time. However, the amounts of lutein, flavoxanthin, and β-carotene were significantly enhanced during each treatment except frying, which has been observed to have a deleterious effect on these compounds. Sonication, microwave, and frying treatments would be a better choice to retain mustard leaves' phenolic and antioxidant properties.

5. Conclusion

Both positive and negative effects are associated with cooking regarding the retention of carotenoids or polyphenol contents. The bioactive composition of vegetable varieties might be varied even if similar cooking conditions are applied. Chromatography revealed seventeen carotenoids and chlorophylls in mustard leaves. The results of the treatments showed that different processing treatments concentrated the amounts of lutein, flavoxanthin, and β-carotene contents of mustard leaves, while frying has been found to have deleterious effects on these compounds. The mustard leaves' total anthocyanin and phenolic contents were better preserved during frying. In terms of the treated samples' antioxidant potential, microwave and sonication were found to be the best techniques.

Funding

This research received no external funding.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to the Department of Biochemistry, University of Malakand for providing the facilities to conduct this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21191.

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