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Sustainable recovery of phenolics from mustard seeds and study of their antioxidant and antimicrobial properties

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Abstract

This study aimed to investigate the effects of pH and temperature on the recovery of phenolic compounds from germinated and non-germinated mustard seeds. For this purpose, a Central Composite Rotatable Design (CCRD) was applied to mustard from two species (*Brassica nigra* and *Sinapis alba*). The evaluated responses included total phenolic content (TPC), antioxidant (ABTS, DPPH, FRAP) and antimicrobial activities, as well as phenolic identification and quantification by HPLC-DAD. The most suitable pH and temperature conditions for obtaining extracts with higher TPC content and antioxidant activity, varied according to sample type: for non-germinated white mustard (pH 5 at 37.5 °C); for germinated white mustard (pH 7.1 at 46.4 °C); for non-germinated black mustard (pH 5 at 25 °C) and for germinated black mustard (pH 8 at 37.5 °C). Under optimized conditions, sinapic acid was identified as the major phenolic compound in all samples, ranging from 700 µg g⁻¹ (non-germinated white mustard) to 1270 µg g⁻¹ (germinated black mustard). Furthermore, all mustard extracts inhibited microbial growth to varying degrees depending on the bacterial strain and sample evaluated. Overall, the results highlight the potential of green extraction using pH adjustment and mild temperatures for the recovery of phenolic antioxidants with antimicrobial activities.

Keywords: Mustard; Germination; Green extraction; Optimization; Phenolic compounds; Bioactive properties.

Highlights

- Different pH and temperature combinations were defined to recover the maximum bioactive compounds from mustard
- Optimal pH and temperature combinations enhanced the recovery of bioactive compounds from mustard
- Mustard extracts showed bacteriostatic activity against some of the most common pathogens



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1 Introduction

Since their first appearance in the scientific literature in 1983 (Kozłowska et al., 1983), mustard seeds have received increasing attention due to their phenolic compounds and antioxidant properties. In 1994, Shahidi et al. (1994) obtained the results of mustard grain antioxidant potential that encouraged further investigations. The defatted fraction – as a result of lipid extraction for biodiesel feedstock, for example – is considered a promising source of phenolic compounds, especially sinapic acid and its derivatives (Martinović et al., 2020).

As a result, in recent years, different extraction methods or solvents have been employed to achieve the same objective: mustard phenolic compound recovery and characterization. Methanol, water, and acetone are commonly used (Sharma et al., 2017; Martinović et al., 2020) either individually or in aqueous mixtures as aqueous methanol (Thiyam-Holländer et al., 2014) and aqueous ethanol (Reungoat et al., 2021), and methanol, acetone, and formic acid (Engels et al., 2012). Enzymatic (Reungoat et al., 2021; Rasera et al., 2023a), acid, and alkaline hydrolysis (Martinović et al., 2020) were also applied to mustard phenolic extraction. Additionally, the Quick, Easy, Cheap, Effective, Rugged, and Safe method (QuEChERS method) has emerged recently as an alternative extraction method (Niciácio et al., 2021).

Our research group has performed an optimization study to choose the most adequate solvent combination to obtain defatted mustard extracts with greater antioxidant potential, which was defined to be water and acetone in equal proportions (Rasera et al., 2019). Additionally, germination was explored as a process to improve the antioxidant potential and to understand the changes in soluble and insoluble mustard phenolic compounds (Rasera et al., 2020). Enzymatic hydrolysis (Rasera et al., 2023a) was also applied to the same defatted material. All results observed encouraged our research group to carry out more investigations with mustard seeds.

In parallel, the concept of green extraction has emerged to address the challenges of the 21st century. Chemat et al. (2019) developed practice guidelines for the green extraction of natural products and their principles. The aim is to reduce or eliminate energy consumption and petroleum solvents, as well as to ensure safety and quality in the extract. Therefore, one of the six principles addressed by Chemat et al. (2019) was followed in this work as a green strategy for phenolic recovery: “use of alternative solvents and principally water or agro-solvents”.

pH and temperature are two of the strategic conditions for the release and maintenance of the stability of phenolic compounds (Gil-Martín et al., 2022). Therefore, we explored the possibility of extracting phenolics with antioxidant potential from defatted mustard seeds by altering the pH and temperature conditions.

Moreover, mustard essential oil has been widely investigated for its antimicrobial potential. Allyl isothiocyanate, a non-phenolic compound derived from sinigrin hydrolysis by myrosinase, is considered the major compound responsible for this activity (Peng et al., 2014; Reyes-Jurado et al., 2019; Bahmid et al., 2021a). However, no data were found on defatted mustard extracts and their antimicrobial activity, even with the remarkable presence of phenolic compounds, known as antimicrobial molecules, in such material (Zamuz et al., 2021).

Therefore, the main objective of this work was to evaluate the recovery of phenolic compounds from non-germinated and germinated mustard seeds under different pH and temperatures. To achieve the results, a Central Composite Rotatable Design (CCRD) was employed as a statistical tool to verify the effects of the variables (pH and temperature) on TPC content and antioxidant activities. In addition, phenolic identification and quantification, as well as antimicrobial activity, were evaluated using extracts obtained under optimized conditions.

2 Material and methods

2.1 Material and microorganisms

Black (*Brassica nigra*) and white mustard (*Sinapis alba*) seeds were purchased from a local market in Piracicaba (São Paulo, Brazil). Folin and Ciocalteu's phenol reagent, sodium carbonate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), (TPTZ), and RP-HPLC standards were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in commercially available grade.

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella* Typhimurium (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus cereus* (ATCC 10876), and *Listeria monocytogenes* (ATCC 7644) used for antimicrobial tests were from the Food Microbiology Laboratory II, School of Food Engineering, University of Campinas (Campinas, Brazil).

2.2 Mustard grain germination and flour preparation

First, mustard seeds were soaked for 12 h in abundant water. Afterward, the seeds were placed in a paper filter that was curled up and placed in the following adequate germination conditions: for black mustard, germination proceeded at 25 °C for 48 h with alternating periods of darkness and light; for white mustard, germination occurred at 25 °C for 72 h in the dark (Rasera et al., 2020).

Non-germinated and germinated seeds were lyophilized, ground, and defatted using hexane in five cycles (20 min each). Non-germinated mustard flours were vacuum-packed and stored at -18 °C.

2.3 Effects of pH and temperature on the extraction of phenolic compounds from mustard

The effects of two variables (pH and temperature) on the recovery of phenolic compounds and the antioxidant potential of extracts from white and black mustards (non-germinated and germinated) were evaluated using a Central Composite Rotatable Design (CCRD) (Table 1). One gram of defatted and lyophilized samples was mixed with 10 mL of each corresponding buffered solution: pH 2 (100 mmol L⁻¹ HCl-potassium chloride buffer), pH 2.9 (100 mmol L⁻¹ citrate buffer), pH 5 (100 mmol L⁻¹ acetate buffer), pH 7.1, and pH 8 (100 mmol L⁻¹ phosphate buffer).

Phenolics were extracted for 1 h under continuous stirring (100 rpm) at different temperatures (Table 1). After that, the dispersions were centrifuged (17 000 g) at 25 °C for 10 min, and the supernatants were collected for further analysis.

Table 1. Central composite rotatable design (CCRD) matrix used to study the effects of different pH and temperature combinations on the recovery of phenolic compounds from non-germinated and germinated mustard.

Assays	Independent variables (coded and real values)	
	x ₁ (pH)	x ₂ (Temperature (°C))
1	-1 (2.9)	-1 (25)
2	1 (7.1)	-1 (25)
3	-1 (2.9)	1 (46.4)
4	1 (7.1)	1 (46.4)
5	-1.41 (2.0)	0 (37.5)
6	1.41 (8.0)	0 (37.5)
7	0 (5.0)	-1.41 (25)
8	0 (5.0)	1.41 (50)
9	0 (5.0)	0 (37.5)
10	0 (5.0)	0 (37.5)
11	0 (5.0)	0 (37.5)

For the statistical analysis from CCRD, mathematical models were generated as described by Equation 1 below:

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} X_i X_j \quad (1)$$

where Y is the estimated response, i and j equal values from 1 to the number of variables (n), β_0 is the intercept term, β_i and β_{ij} are the linear and quadratic coefficients, respectively, and X_i and X_j are the coded independent variables.

The coefficient of determination R^2 and the F test (analysis of variance – ANOVA) were used to verify the statistical adequacy of the proposed models generated from the CCRD. The estimated effects of the independent variables obtained from the CCRD were considered statistically significant when p-values ≤ 0.05 . Statistica® 13 software from TIBCO Software Inc. (Palo Alto, California, USA) was used for the CCRD, data analysis, and model building.

2.4 Total phenolic content and antioxidant properties

The total phenolic content (TPC) was estimated according to Swain & Hillis (1959) with the modifications proposed by Pereira et al. (2017). The results were expressed as mg of gallic acid equivalents per g of lyophilized and defatted sample (mg GAE g⁻¹).

To support the antioxidant activity of mustard extracts, different methodologies were used, as follows: ABTS and DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) assays (Rasera et al., 2023b). All results were expressed as μmol of Trolox equivalents per g of dry and defatted sample ($\mu\text{mol TE g}^{-1}$).

2.5 Phenolic compounds identification and quantification by HPLC-DAD

Phenolic compounds identification and quantification were performed following Rasera et al. (2023b) using a Shimadzu ODS-A column (4.6 mm 250 mm, 5 μm) in a reversed-phase and photodiode array detector (SPD-M10AVp, Shimadzu Co., Kyoto, Japan). Twenty microliters of mustard extracts were injected after filtration (0.22 μm), and the run was conducted at a flow rate of 1.0 mL min^{-1} . Mobile phase A consisted of water/formic acid (99.75/0.25, v/v), and mobile phase B consisted of acetonitrile/formic acid/water (80/0.25/19.75, v/v). A gradient was conducted starting with 10% B and increasing to 20% B at 10 min, 30% B at 20 min, 50% B at 30 min, 100% B at 35 min, and 10% B at 40 min, finishing at 45 min. The chromatograms were analyzed using Class-VP® software.

2.6 Minimum inhibitory concentration (MIC)

The minimal inhibitory concentration was determined according to Budri et al. (2015) in 96-well flat-bottomed cell culture plates. Inocula of six bacterial strains -*Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella* Typhimurium (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus cereus* (ATCC 10876), and *Listeria monocytogenes* (ATCC 7644) - were prepared at a density of 0.5 McFarland using a densitometer (Densichek, BioMérieux, Durham, NC) in 0.85% saline solution. Extracts were diluted (50 mg mL^{-1}) in tryptone soy broth (TSB) with yeast extract, and 400 μL was added to the first well. After that, a serial dilution (1:2) was performed, transferring from the first well to the second well, and so on until the last column, to reach final volumes of 200 μL . Subsequently, 2 μL of bacterial suspension was inoculated with a final concentration of 105 UFC mL^{-1} . Positive growth controls (bacteria in TSB without extracts), sterility controls (extracts in TSB without bacteria), and negative controls (only TSB) were also included. Plates were incubated at 35 °C for 24 h (except *Listeria* and *Pseudomonas*, which were incubated at 32 °C), and then 50 μL of 0.01% resazurin was added to each well. Five minutes later, the blue color indicated no bacterial growth, while the pink color indicated the opposite. The minimal inhibitory concentration was obtained in the well with the lowest concentration of extract, which remained blue, while the well whose color turned pink indicated bacterial growth. The results were expressed as the TPC content of each sample per mL of culture medium (mg GAE mL^{-1}).

2.7 Minimum bactericidal concentration (MBC)

To determine the MBC, 10 μL from the MIC wells and the previous wells without growth were inserted into nutrient agar plates using the droplet technique (Knezevic et al., 2016). The plates were incubated at 37 °C for 24 h (except *Listeria* and *Pseudomonas*, which were incubated at 32 °C). The sections without growth indicated bactericidal activity, while the sections with positive growth indicated bacteriostatic activity of the extract analyzed.

2.8 Calculations and statistics

Statistical analysis was performed using analysis of variance (ANOVA), and mean comparisons were conducted using Tukey's test in Minitab® 19 software from Minitab Inc. (State College, Pennsylvania, USA). The values were expressed as the arithmetic mean ($n = 3$) and were considered significantly different when the p value ≤ 0.05 .

3 Results and discussion

3.1 Effects of pH and temperature on phenolic extraction and antioxidant properties of mustard

CCRD results for white and black mustard are presented in Tables 2 and 3, respectively. Significant effects ($p \leq 0.05$) of pH and temperature were observed, although their magnitude varied depending on the sample and response (Table S1, Supplementary Material).

Table 2. Total phenolic content (TPC) and antioxidant potential of extracts from non-germinated and germinated white mustard obtained under different pH and temperature conditions.

Assay	Non-germinated white mustard grains			
	TPC	ABTS	DPPH	FRAP
	mg GAE g^{-1}	$\mu\text{mol TE g}^{-1}$	$\mu\text{mol TE g}^{-1}$	$\mu\text{mol TE g}^{-1}$
1	8.50 ± 0.55^f	34.44 ± 1.11^c	31.82 ± 1.07^c	46.36 ± 1.44^c

Each value is expressed as the mean ($n = 3$) $\pm$ standard deviation (SD). The results that showed the same letters in the same column are not significantly different at the 5% level of significance according to Tukey's test.

Table 2. Continued...

Assay	Non-germinated white mustard grains			
	TPC	ABTS	DPPH	FRAP
	mg GAE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹
2	10.27 ± 0.22 ^{def}	103.53 ± 0.50 ^b	16.91 ± 0.80 ^c	56.14 ± 1.86 ^{bc}
3	11.26 ± 0.03 ^{cde}	36.17 ± 1.24 ^c	38.37 ± 0.76 ^{ab}	51.17 ± 1.65 ^{de}
4	13.74 ± 1.19 ^a	106.98 ± 5.59 ^{ab}	20.53 ± 1.89 ^d	60.89 ± 3.29 ^{ab}
5	11.42 ± 1.18 ^{bcd}	82.58 ± 2.16 ^c	33.74 ± 0.99 ^c	64.35 ± 2.50 ^a
6	11.50 ± 0.37 ^{bcd}	110.96 ± 1.84 ^a	13.05 ± 0.70 ^f	59.82 ± 0.04 ^{ab}
7	9.41 ± 0.51 ^{ef}	67.88 ± 2.60 ^d	34.36 ± 0.54 ^c	60.66 ± 0.27 ^{ab}
8	12.99 ± 0.45 ^{abc}	78.89 ± 0.60 ^c	33.96 ± 1.89 ^c	60.48 ± 2.26 ^{ab}
9	10.56 ± 0.37 ^{de}	67.08 ± 3.58 ^d	34.85 ± 1.56 ^c	54.23 ± 0.15 ^{cd}
10	13.26 ± 0.66 ^{ab}	70.72 ± 2.12 ^d	34.94 ± 1.15 ^{bc}	64.11 ± 0.55 ^a
11	13.47 ± 0.38 ^a	71.06 ± 1.70 ^d	39.13 ± 0.59 ^a	63.88 ± 0.20 ^a
Assay	Germinated white mustard grains			
	TPC	ABTS	DPPH	FRAP
	mg GAE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹
1	15.92 ± 0.71 ^d	47.50 ± 4.15 ^c	40.66 ± 0.58 ^{cd}	71.05 ± 1.16 ^d
2	18.61 ± 1.09 ^b	116.52 ± 7.39 ^b	37.90 ± 0.47 ^d	86.94 ± 2.80 ^c
3	16.4 ± 0.27 ^{cd}	38.47 ± 1.39 ^c	40.22 ± 1.16 ^{cd}	61.37 ± 1.02 ^e
4	19.19 ± 0.13 ^{ab}	124.32 ± 7.05 ^{ab}	37.82 ± 0.28 ^d	102.34 ± 6.16 ^a
5	18.14 ± 0.29 ^{bc}	103.33 ± 3.25 ^c	41.93 ± 2.65 ^{bc}	97.55 ± 2.87 ^{ab}
6	17.72 ± 1.00 ^{bcd}	130.41 ± 7.66 ^a	27.64 ± 2.54 ^c	92.76 ± 1.40 ^{bc}
7	17.32 ± 0.36 ^{bcd}	85.93 ± 2.65 ^d	46.77 ± 0.76 ^a	98.18 ± 1.73 ^{ab}
8	20.73 ± 0.29 ^a	81.04 ± 1.06 ^d	41.54 ± 1.27 ^{bcd}	91.06 ± 1.29 ^{bc}
9	18.50 ± 0.90 ^b	83.13 ± 2.37 ^d	43.34 ± 0.39 ^{abd}	97.10 ± 2.38 ^{ab}
1	18.75 ± 0.47 ^b	85.20 ± 2.13 ^d	45.33 ± 0.59 ^{ab}	102.20 ± 2.51 ^a
11	13.51 ± 0.79 ^c	76.61 ± 0.75 ^d	40.10 ± 1.65 ^{cd}	70.46 ± 1.61 ^d

Each value is expressed as the mean (n = 3) ± standard deviation (SD). The results that showed the same letters in the same column are not significantly different at the 5% level of significance according to Tukey's test.

Table 3. Total phenolic content (TPC) and antioxidant potential of extracts obtained from non-germinated and germinated black mustard under different pH and temperature conditions.

Assay	Non-germinated black mustard grains			
	TPC	ABTS	DPPH	FRAP
	mg GAE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹
1	13.02 ± 0.97 ^b	52.35 ± 1.70 ^c	25.85 ± 0.61 ^b	37.51 ± 0.34 ^{de}
2	9.44 ± 0.84 ^c	70.91 ± 3.42 ^{bc}	11.30 ± 0.74 ^c	21.26 ± 1.34 ^c
3	12.72 ± 0.54 ^b	58.36 ± 3.86 ^{de}	27.43 ± 0.38 ^b	29.95 ± 2.12 ^{cd}
4	11.26 ± 0.70 ^{bc}	75.44 ± 0.32 ^b	8.38 ± 0.75 ^d	41.87 ± 3.46 ^{cd}
5	12.70 ± 1.97 ^b	72.25 ± 3.13 ^{bc}	26.71 ± 0.43 ^b	40.93 ± 3.73 ^{cd}
6	10.51 ± 1.68 ^{bc}	85.70 ± 2.70 ^a	7.34 ± 0.48 ^d	44.26 ± 1.55 ^{cd}
7	19.93 ± 1.70 ^a	90.46 ± 0.84 ^a	30.54 ± 0.34 ^a	76.70 ± 2.78 ^a
8	12.48 ± 0.03 ^{bc}	70.18 ± 2.11 ^{bc}	27.35 ± 1.79 ^b	53.44 ± 4.85 ^b
9	11.44 ± 0.17 ^{bc}	66.23 ± 2.06 ^{cd}	26.01 ± 0.95 ^b	47.57 ± 1.66 ^{bc}

Each value is expressed as the mean (n = 3) ± standard deviation (SD). The results that showed the same letters in the same column are not significantly different at the 5% level of significance according to Tukey's test.

Table 3. Continued...

Assay	Non-germinated black mustard grains			
	TPC	ABTS	DPPH	FRAP
	mg GAE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹
10	11.78 ± 0.29 ^{bc}	65.36 ± 3.13 ^{cd}	26.77 ± 1.13 ^b	47.06 ± 1.82 ^{bc}
11	12.02 ± 0.85 ^{bc}	66.37 ± 5.03 ^{cd}	27.57 ± 1.12 ^b	46.22 ± 1.82 ^{bc}
Assay	Germinated black mustard grains			
	TPC	ABTS	DPPH	FRAP
	mg GAE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹	μmol TE g ⁻¹
1	16.40 ± 0.72 ^c	63.69 ± 1.39 ^f	31.30 ± 1.32 ^{de}	61.05 ± 3.53 ^c
2	23.25 ± 0.50 ^a	126.47 ± 1.82 ^b	23.66 ± 1.00 ^f	98.30 ± 5.63 ^b
3	18.53 ± 0.53 ^{bc}	62.64 ± 4.55 ^f	29.43 ± 1.13 ^c	67.62 ± 2.77 ^{de}
4	18.40 ± 1.84 ^{bc}	104.59 ± 10.86 ^{cd}	24.81 ± 0.75 ^f	74.72 ± 6.99 ^{cde}
5	20.88 ± 2.10 ^{ab}	108.49 ± 6.11 ^c	39.08 ± 2.31 ^a	88.26 ± 7.99 ^{bc}
6	23.01 ± 2.25 ^a	140.29 ± 1.18 ^a	15.06 ± 1.41 ^g	115.59 ± 10.01 ^a
7	9.60 ± 0.22 ^d	55.92 ± 0.18 ^f	22.11 ± 1.09 ^f	41.18 ± 1.36 ^f
8	22.10 ± 0.97 ^{ab}	93.77 ± 0.94 ^{de}	37.57 ± 0.72 ^{ab}	89.34 ± 3.33 ^{bc}
9	19.86 ± 1.58 ^{abc}	91.42 ± 2.22 ^e	35.41 ± 1.32 ^{bc}	77.95 ± 3.84 ^{cd}
10	19.98 ± 0.60 ^{abc}	90.84 ± 3.23 ^e	32.58 ± 1.42 ^{cde}	74.95 ± 7.37 ^{cde}
11	19.57 ± 0.56 ^{abc}	87.81 ± 0.16 ^e	33.19 ± 1.35 ^{cd}	78.55 ± 0.88 ^{cd}

Each value is expressed as the mean (n = 3) ± standard deviation (SD). The results that showed the same letters in the same column are not significantly different at the 5% level of significance according to Tukey's test.

For non-germinated white mustard, TPC and antioxidant activities varied within relatively narrow ranges, with the most favorable responses generally observed at the central extraction conditions (pH 5 and 37.5 °C). In contrast, germinated white mustard extracts showed higher overall values, with the best performance detected in assay 4 (pH 7.1 and 46.4 °C), indicating an enhanced extraction efficiency after germination (Table 2).

A similar trend was observed for black mustard. For non-germinated samples, the most suitable extraction conditions were pH 5 and 25 °C (assay 7), whereas germinated black mustard extracts exhibited higher antioxidant activities, particularly at pH 8 and 37.5 °C (assay 6) (Table 3).

ANOVA indicated that not all CCRD-derived models were statistically valid due to low coefficients of determination ($R^2 < 0.80$) and non-significant regression parameters ($p > 0.05$) (Table S1). This lack of significance reflects limited response variation under certain experimental conditions and does not invalidate the extraction process; rather, it restricts the detection of independent variable effects.

Conversely, statistically valid models were obtained for specific responses (Table S1). In these cases, pH was the main factor influencing phenolic extraction and antioxidant activity ($p \leq 0.05$), whereas temperature showed no significant effect ($p > 0.05$) within the evaluated range. These models presented R^2 values above 0.80 and significant F-tests ($p \leq 0.01$), confirming their adequacy for describing the system.

To harmonize the definition of the most appropriate extraction conditions for each type of material, an isolated evaluation of each result was performed. Thus, assays 10 (pH 5 and 37.5 °C), 4 (pH 7.1 and 46.4 °C), 7 (pH 5 at 25 °C), and 6 (pH 8 and 37.5 °C) were chosen as the most adequate extraction conditions for non-germinated and germinated white mustard and non-germinated and germinated black mustard, respectively.

For both white and black mustard extracts, germination increased the phenolic content and antioxidant potential, corroborating our previous findings (Rasera et al., 2020). Therefore, although the extraction solvent was changed and the phenolic profile differed accordingly, positive responses were observed for both non-germinated and germinated samples. This highlights the potential of aqueous extraction using different pH and temperature conditions.

The best results for TPC content and antioxidant activities were obtained with extraction temperatures lower than 50 °C in the studied range (from 25 °C to 50 °C). It is important to note that, in addition to temperature not being statistically significant in our results, it has a positive impact on extraction efficiency, especially at this studied range of temperatures. This behavior can be explained by several mechanisms, including improved solute solubility and diffusion coefficients, enhanced solvent penetration into plant cells, and desorption of phenolics; decrease in solvent viscosity, improving matrix particle penetration;

degradation of cell wall structure and permeability improvement; and attenuation of phenolics linkage to macromolecules and promotion of their release and diffusion rate (Benchikh & Louailèche, 2014; Böhmer-Maas et al., 2020; Čučuz et al., 2022).

However, temperatures higher than 50 °C may promote the degradation of phenolic compounds, leading to a reduction in their bioactivities, including antioxidant potential. In addition, it is important to consider that those molecules are vulnerable to oxidation, complexation, conjugation, hydrolysis, and polymerization, which can also change their bioactivities (Benchikh & Louailèche, 2014; Čučuz et al., 2022).

It is crucial to highlight that extraction conditions can be different for each material. Additionally, high extraction temperatures demand high energy costs, which were avoided in the present work to reduce energy intake, as proposed previously.

Furthermore, for all samples, the extraction pH was lower or close to 7, except for the selected condition for germinated black mustard (pH 8). In general, phenolic compounds are more stable at acidic pH values (Quatrin et al., 2020) and thus can be better extracted by adding acids to solvent extraction (Čučuz et al., 2022). However, pH effects depend on the chemical class and molecular structure of phenolic compounds, since variations in pH influence the ionization of phenolic hydroxyl groups, electron distribution, and molecular stability, thereby affecting solubility, reactivity, and antioxidant properties (Eran Nagar et al., 2021; Saarniit et al., 2023; Xu & Wang, 2025).

Hydroxycinnamic, caffeic, syringic, and protocatechuic acids, for example, have their structure changed and/or are degraded under acidic conditions (Shahidi & Yeo, 2016). Friedman & Jürgens (2000) indicated that caffeic, chlorogenic, and gallic acids were unstable at high pH and that the pH- and time-dependent spectral transformations were not reversible. Chlorogenic acid was also stable under acidic pH, and catechin, epigallocatechin, ferulic acid, rutin, and trans-cinnamic acid resisted major pH-induced degradation (Friedman & Jürgens, 2000). Rutin and quercetin were better extracted at higher pH values, while gallic acid and kaempferol were better extracted at lower pH values (Čučuz et al., 2022). Additionally, anthocyanins can be degraded at alkaline pH values, such as pH 8, which explains their low bioaccessibility after digestion, since the intestinal phase is under alkaline pH (Zepeda-Ruiz et al., 2020).

Our findings are also consistent with recent studies demonstrating that pH is a critical factor governing the extraction efficiency and antioxidant capacity of mustard phenolics. Nguyen et al. (2023a) reported that pH significantly modulates both phenolic yield and antioxidant activity in different mustard extracts, while temperature plays a secondary role within moderate ranges. Similarly, optimization studies using green extraction approaches have shown that adjusting pH is an effective strategy to enhance the recovery of sinapates and related antioxidants from mustard matrices under mild processing conditions (Nguyen et al., 2023b).

3.2 Phenolic compound identification and quantification by HPLC-DAD

After determining the most suitable extraction conditions for each sample, phenolic compounds were identified and quantified by HPLC-DAD (Table 4).

Table 4. Identification and quantification of phenolic compounds of extracts from non-germinated and germinated black mustard obtained under the most adequate conditions defined in CCRD using high-performance liquid chromatography (HPLC).

Phenolic compound	Samples and phenolics quantification (µg g ⁻¹)				Validation parameters		
	Non-germinated white mustard	Germinated white mustard	Non-germinated black mustard	Germinated black mustard	LOD (µg) ²	LOQ (µg) ³	Linearity (R ²)
Gallic acid	85.59 ± 0.17	220.51 ± 0.85	53.27 ± 1.08	79.28 ± 0.61	0.0084	0.0256	0.9908
3,4-dihydroxybenzoic acid	nd ¹	nd	93.21 ± 7.61	nd	0.0096	0.0290	0.9991
Vanillic acid	nd	nd	49.19 ± 0.40	55.11 ± 1.65	0.0293	0.0890	0.9974
Caffeic acid	60.49 ± 0.70	114.33 ± 2.57	16.27 ± 0.31	27.95 ± 0.31	0.0147	0.0445	0.9980
p-Coumaric acid	83.00 ± 0.18	252.51 ± 1.55	77.83 ± 0.62	79.28 ± 0.11	0.0191	0.0581	0.9991
Ferulic acid	nd	nd	nd	nd	0.0082	0.0251	0.9968
Sinapic acid	698.47 ± 14.02	1667.51 ± 18.8	298.97 ± 28.26	1268.02 ± 108.35	0.0028	0.0086	0.9953
Rutin	nd	nd	88.11 ± 6.55	214.30 ± 3.89	0.0111	0.0336	0.9995
Quercetin	nd	nd	nd	nd	0.0043	0.0132	0.9870

Each value is expressed as the mean (triplicate) ± standard deviation (SD). ¹nd: non-detected. ²LOD: limit of detection. ³LOQ: limit of quantification.

Germination increased all phenolic compounds for both mustard species, except 3,4-dihydroxybenzoic acid, which appeared only for non-germinated black mustard. This germination-induced increase in phenolic compounds is consistent with the results presented in Tables 2 and 3.

Interestingly, despite the use of different pH and temperature conditions for each mustard sample, the phenolic profile remained consistent between non-germinated and germinated samples in both mustard species, with differences observed mainly in compound concentrations (Table 4).

Caffeic acid was the minor compound present in all samples analyzed. Additionally, rutin was only present in black mustard extracts (not germinated and germinated). Sinapic acid was the major phenolic compound extracted from both mustard seeds. The same result was observed for different studies, even with different mustard species or extraction with different solvents (Sharma et al., 2017; Nicácio et al., 2021; Achinivu et al., 2021). Sinapine, a derivative compound from sinapic acid, was also found to be a major compound in mustard seeds (Engels et al., 2012; Reungoat et al., 2021), despite having less radical-scavenging activity than sinapic acid (Thiyam et al., 2009).

The predominance of sinapic acid observed in the present study is in agreement with recent literature describing sinapic acid and its derivatives as the major phenolic constituents of mustard seeds. A recent comprehensive review highlighted sinapic acid as the most abundant phenolic compound across different mustard species, regardless of processing conditions (Nguyen et al., 2024). More recently, Polat Köse (2025) confirmed the dominance of sinapic acid in both *Sinapis alba* and *Brassica nigra* extracts using advanced chromatographic techniques, supporting the robustness of this phenolic marker across extraction strategies.

Sinapic acid is a 3,5-dimethoxy-4-hydroxycinnamic acid and a member of the phenylpropanoid family (Nićiforović & Abramović, 2014). Hydroxycinnamic acids are a subclass of phenolic acids derived from cinnamic acid and are characterized by a phenolic ring conjugated to a propenoic acid side chain, contributing to their biological activity. Sinapic acid occurs both in free form and predominantly as esterified derivatives, mainly sinapoyl esters such as sinapine (sinapoylcholine) and sinapoyl malate. The literature reveals that sinapic acid has many biological properties, such as antioxidant activity, anti-inflammatory and anticarcinogenic properties, anxiolytic properties, neuroprotective properties, antimicrobial activity, antihyperglycemic activity and antilipidemic activity (Tesaki et al., 1998; Nguyen et al., 2024; Polat Köse, 2025). Additionally, its derivatives also showed potential biological applications (Nićiforović & Abramović, 2014; Nguyen et al., 2024; Polat Köse, 2025). In addition, this phenolic acid has other applications, such as the synthesis of anti-UV ingredients (Rioux et al., 2020), non-endocrine disruptive antiradical additives (Jaufurally et al., 2016), bisphenol A as a substitute for polymer/resin synthesis (such as syringaresinol) (Janvier et al., 2017), and as a substrate for prepolymers (Diot-Néant et al., 2017).

Previous studies have reported considerable variability in the phenolic compound content of mustard, as summarized below: sinapic acids vary from 170 (Nicácio et al., 2021) to 15 000 $\mu\text{g g}^{-1}$; gallic acid ranges from 0.018 (Martinović et al., 2020) to 8 000 $\mu\text{g g}^{-1}$ (Sharma et al., 2017); 3,4-dihydroxybenzoic acids vary from 80 (Martinović et al., 2020) to 500 $\mu\text{g g}^{-1}$ (Sharma et al., 2017); vanilic acid ranges from 0.037 (Martinović et al., 2020) to 0.64 $\mu\text{g g}^{-1}$ (Nicácio et al., 2021); caffeic acid varies from 0.045 (Nicácio et al., 2021) to 1500 $\mu\text{g g}^{-1}$ (Sharma et al., 2017) ferulic acid ranges from 0.636 (Martinović et al., 2020) to 3000 $\mu\text{g g}^{-1}$ (Nicácio et al., 2020); rutin was quantified as 0.076 $\mu\text{g g}^{-1}$ (Martinović et al., 2020) and quercetin as 0.652 (Martinović et al., 2020).

It is clear that extraction conditions – solvents used, temperature, time, stirring – directly interfere with phenolic extraction and consequently its amount (Souza Silva et al., 2021). Additionally, mustard species or sample treatments, such as fat removal or the use of mustard seeds, mustard bran, or mustard flour, also impact the phenolic content (Nićiforović & Abramović, 2014). Hence, our results indicate that aqueous extraction with water and mild temperatures represents a promising green alternative for phenolic recovery, as high levels of individual phenolic compounds were obtained compared with those reported in other studies.

3.3 Antimicrobial activity of mustard extracts (MIC and MBC)

The aqueous extracts obtained in the present work inhibited the bacterial growth of some microorganisms that represent a major concern for the safety of food products. The results of the minimal inhibitory concentration (mg GAE mL^{-1}) are available in Table 5, and no extract presented bactericidal activity (MBC) against the strains and concentrations analyzed. Among the extracts, the best minimal inhibitory concentration was obtained with the non-germinated white mustard extract for *Pseudomonas*, which showed an average MIC of 0.77 mg GAE mL^{-1} .

Table 5. Minimal inhibitory concentration (MIC) of extracts obtained from non-germinated and germinated white and black mustard obtained under the most adequate conditions defined in CCRD.

Samples	MIC (mg GAE mL ⁻¹) ¹					
	<i>Listeria monocytogenes</i>	<i>Salmonella Typhimurium</i>	<i>Escherichia coli</i>	<i>Bacillus cereus</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
Non-germinated white mustard	3.11 ± 0.00 ^{Bb}	6.22 ± 0.00 ^A	6.22 ± 0.00 ^{Ab}	2.07 ± 0.90 ^{BCb}	0.77 ± 0.00 ^{Cb}	2.59 ± 0.90 ^{Bb}
Germinated white mustard	8.00 ± 2.77 ^{ABa}	> 9.60	9.60 ± 0.00 ^{Aa}	9.60 ± 0.00 ^{Aa}	4.80 ± 0.00 ^{Ba}	9.60 ± 0.00 ^{Aa}
Non-germinated black mustard	9.97 ± 0.00 ^{Ab}	9.97 ± 0.00 ^{Ab}	9.97 ± 0.00	9.97 ± 0.00 ^{Ab}	8.30 ± 2.88 ^{Aa}	> 9.97
Germinated black mustard	11.51 ± 0.00 ^{Aa}	11.51 ± 0.00 ^{Aa}	> 11.51	11.51 ± 0.00 ^{Aa}	9.60 ± 3.32 ^{Aa}	11.51 ± 0.00 ^A

¹MIC results are expressed as a function of TPC content expressed in mg of gallic acid equivalent per mL (mg GAE mL⁻¹). Each value is expressed as the mean (n = 3) ± standard deviation (SD). The results that showed the same letters in the same column are not significantly different at the 5% level of significance according to Tukey's test (lower case represents comparisons performed between the non-germinated and germinated mustard samples). The results that showed the same letters at the same line are not significantly different at the 5% level of significance according to Tukey's test (upper case represents comparisons performed between different strains).

The higher resistance of Gram-negative bacteria compared to Gram-positive bacteria observed for non-germinated white mustard, except for *Pseudomonas*, can be attributed to fundamental differences in cell envelope architecture (Sousa et al., 2012). Gram-negative bacteria possess an outer membrane rich in lipopolysaccharides, which acts as an additional permeability barrier and restricts the diffusion of antimicrobial compounds, including phenolic acids. In contrast, Gram-positive bacteria lack an outer membrane and are characterized by a thick but more permeable peptidoglycan layer, which may facilitate the interaction of bioactive compounds with the cytoplasmic membrane, resulting in increased susceptibility (Maher, 2023). Such composition influences MIC, since the destabilization of the double phospholipid layer, the disruption of the plasma membrane, and consequently the loss of vital intracellular components, enzymes, and energy production inactivation are the major action mechanisms of antimicrobial agents (Sousa et al., 2012; Li et al., 2022; Hou et al., 2022; Maher, 2023). For germinated white mustard and black mustard, no significant differences in the results were observed among the bacterial strains evaluated.

Mustard essential oil has already been extensively studied due to its antimicrobial activity, and allyl isothiocyanate (AITC) was reported to be the main compound responsible for this activity (Peng et al., 2014; Bahmid et al., 2021b; Reyes-Jurado et al., 2019). However, AITC preferentially partitions into the lipid fraction of the mustard matrix; consequently, defatted mustard may contain lower amounts of this compound (Bahmid et al., 2021b). Nevertheless, the present work shows defatted mustard potential for bacterial growth inhibition.

As previously reported, all extracts analyzed have phenolic compounds in their composition, such as gallic acid, 3,4-dihydroxybenzoic acid, ferulic acid, vanillic acid, caffeic acid, sinapic acid, rutin, and quercetin, which have already been shown to exhibit antimicrobial activity (Shao et al., 2011; Serafim et al., 2015; Gupta et al., 2021).

In fact, phenolic compounds can alter cell membrane permeability through interactions with membrane components, leading to leakage of cellular contents, bacterial depolarization, and disruption of the transmembrane pH gradient. In addition, they may interfere with intracellular bacterial functions through the formation of hydrogen bonds with enzymes (Zamuz et al., 2021).

However, such positive effects depend on multiple factors, including the position of hydroxyl groups on the aromatic ring and the length of the saturated side chain of phenolic acids (Añibarro-Ortega et al., 2020), bacterial susceptibility, plant cultivar, phenolic extraction procedures and solvents, synergistic inhibitory interactions among different phenolic compounds (Lima et al., 2019), bacterial strain and dose, compound structure (Zamuz et al., 2021), and exposure time. For example, flavonoids can inhibit nucleic acid synthesis and the function of the cytoplasmic membrane, whereas ellagitannins may induce cell adhesion, enzyme inhibition, form complexes with cell wall components, deprive substrates, disrupt membranes, and chelate metal ions (Reddy et al., 2020).

Interestingly, germination of white and black mustard did not enhance the inhibitory activity against all bacterial strains. The only exception was *Pseudomonas aeruginosa* for the extract from germinated black mustard, which inhibited bacterial growth at the same amount of phenolics as the non-germinated extract (9.60 and 8.30 mg GAE mL⁻¹, respectively). *Brassica oleracea* sprouts inhibited some bacterial growth, and the authors correlated it to the presence of organic acids, despite the presence of phenolics in sprouts (Vale et al., 2015). Therefore, it is important to see that phenolics are not the only compounds responsible for this activity.

Additionally, the extract pH did not inhibit bacterial growth. The same behavior was reported by Shen et al. (2014), who indicated a low influence of the acidic medium pH on the inhibitory effects exerted by blueberry ethanol extract.

Recent studies have also reinforced the antimicrobial potential of mustard-derived bioactive compounds. Salah et al. (2024) demonstrated that phenolic-rich extracts from germinated mustard sprouts exhibited enhanced biological activities, including antibacterial effects, supporting the role of phenolics in microbial growth inhibition. In addition, a recent comprehensive review emphasized that mustard phenolics contribute to antimicrobial activity through multiple mechanisms and highlighted their relevance for sustainable food-related applications (Hu & Yan, 2025), which is consistent with the bacteriostatic effects observed in the present study.

4 Conclusions

Mustard seeds are rich sources of bioactive compounds, particularly phenolic compounds. The evaluation of different pH and temperature conditions demonstrated that optimal extraction parameters depend on the mustard type and germination status, with pH emerging as the main factor influencing phenolic recovery within the evaluated ranges. Germination enhanced both phenolic content and antioxidant activity, and HPLC analysis identified sinapic acid as the predominant phenolic compound in all samples. In addition, the aqueous mustard extracts exhibited bacteriostatic activity against relevant foodborne pathogens, including *Listeria monocytogenes*, *Salmonella Typhimurium*, *Escherichia coli*, and *Staphylococcus aureus*. Overall, the use of pH–temperature-controlled aqueous extraction represents a green and effective alternative to conventional solvent-based methods and supports further studies on bioaccessibility and additional bioactive compounds in mustard.

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Data Availability Statement

The data supporting the findings of this study are available in the REDU – Repositório de Dados de Pesquisa da Unicamp under the DOI <https://doi.org/10.25824/redu/ISGQNI>.

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Conflict of interests

The authors declare that this study was conducted without any commercial or financial conflicts of interest.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. Analysis of variance (ANOVA) including regression models, R², F test, and probability values for the final reduced models generated for antioxidant potential of the extracts obtained from white and black defatted mustard under different pH and temperature conditions.

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