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Introduction

Health-promoting natural source elements, including bioactive peptides (BAPs) and phenolic compounds, have recently received increased attention. Bioactive peptides can be obtained from both plant and animal sources. These peptides are not biologically active in their native sequences, but enzymatic, chemical, and microbial hydrolysis can activate them and make them biologically active (1). Bioactive peptides typically consist of three to thirty amino acids (2). These products own various bioactivities, including antibacterial, immunomodulatory, anticancer, antioxidant, anti-inflammatory, and antihypertensive properties. The bioactivities of many peptides are determined by their

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The Antibacterial Activities of Bioactive Peptides Generated using Protein Extracted from Chia Seeds, and quinoa Seeds in Iraq

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Abstract

Foodborne pathogens are one of the significant causes of food deterioration and a public health concern worldwide. Antimicrobial peptides in plant protein sequences, such as Chia (*Salvia hispanica L.*) and quinoa (*Chenopodium quinoa Wild.*), might inhibit bacteria. Therefore, this study assesses the potential application of chia and quinoa peptides in food preservation by evaluating their antibacterial activity and stability. Bradford colorimetric assay was used to determine the crude protein content of the samples. Then, the peptide was extracted by organic acids. The antibacterial activity was assessed through a bacterial activity assay, and the extract's minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined against several bacterial strains. Additionally, the disc diffusion assay was used to assess the antibacterial efficacy of the seed extract. Two chia and quinoa peptides (2-5 and 5-10 kDa) were obtained by enzymatic hydrolysis of a protein-rich fraction and subsequent ultrafiltration. Gram-positive bacteria were susceptible to 3 kDa. These fractions showed a more significant inhibition effect against *B. cereus* with percentages of 90.93 % and 85.94% for Chia and Quinoa seeds, respectively, for both MIC and MBC values, indicating potential for application in antibacterial formulations. Clear inhibition zones were seen in the disc assay, which demonstrated the potential of the prepared sequence as a preservative for controlling the proliferation of Gram-positive and harmful bacteria in food products. In conclusion, this study approved the efficacy of peptide generated from Chia seeds, and quinoa seeds fraction 2-5 KDa as an antibacterial agent against *B. cereus*. Additionally, it reflects its antibacterial action in food products.

Keywords: Antibacterial activity, Chia seeds, Quinoa seeds, Peptide extract



structure, content, and sequence (3). Thousands of BAPs with diverse biological functions have been identified, particularly the secondary structure and amino acid makeup that affect a range of bioactivities (4). BAPs can produce localized effects on the gastrointestinal tract or circulatory system, while maintaining their physiological roles, like proteins, which are fully absorbed by the gut. However, in plants, one of the sources of bioactive peptides is the Quinoa (*Chenopodium Quinoa Wild*). It is the Andes' mother grain, which is an annual plant. For almost 5,000 years, Andean cultures in South America mainly relied on its seeds as their primary food supply (5). Quinoa seeds have exceptional nutritional properties; they have more fat, fiber, and protein than traditional seeds. Quinoa is regarded as a substitute crop in many parts of the world for food for humans and animals since it has a high nutritional content and adapts well to varying soil and climate conditions (6). One key criterion in the plant's evaluation as a good alternative source of nutrition is its high protein, balanced amino acid content, quality fiber content, and energy content in the seeds and vegetative sections (7). *Salvia hispanica*, often known as chia, is an annual herbaceous plant belonging to the *Lamiaceae* family. It is native to southern Mexico and northern Guatemala, the latter being the world's largest producer of chia (8). Chia seeds (*Salvia hispanica L.*) have historically been used worldwide as a source of omega-3 and polyunsaturated fatty acids (8). Chia seeds have been an essential part of human diets since 1500 BC, and research has indicated that they provide health benefits. Because of their nutritional benefits and potential medical uses, chia seeds have emerged as one of the most widely consumed foods worldwide (9). This study assessed the potential application of chia and quinoa peptides in food preservation by evaluating their antibacterial activity and stability.

Materials & Methods

Plant preparation and extraction

Plant material was grounded in a blender or coffee mill. 10% CH₃COOH is the extractant employed, and a commercial cocktail of proteinase inhibitors (Sigma-Aldrich, USA) was used. It was applied at a ratio of 1:10 (w:v) to the ground material. The extraction process took place at room temperature for one hour while vigorous stirring was ongoing. The mixture was run through a sieve. Then, the fine particles were removed by centrifuging. A Whatman paper filter was used to filter the supernatant. The supernatant obtained from large volumes is further concentrated 1.5–2 times using a rotary evaporator (10). In the second step, Organic solvent precipitation was employed as a saturation technique. After carefully swirling the filter and adding cold acetone (– 70 °C) in a 1:7 ratio, the mixture was kept at +4 °C for 6–8 hours. The precipitated fraction was collected by centrifugation and dried at room temperature. Then, the dried precipitate was dissolved in trifluoroacetic acid (TFA), and the low molecular weight components were removed using pipetting. The freeze-drying activity obtained from ultrafiltration tubes with molecular weights of 10 and 2 kDa was used in agar disc diffusion and antibacterial tests (10).

Determination of Protein and Peptide Content

Protein concentration was determined using the method described previously by Bradford assay (11). Moreover, the peptide concentration was estimated by the O-Phthaldialdehyde (OPA) test (12).

Antibacterial Activity

Antibacterial Assay

The antimicrobial activity of Chia and Quinoa seeds was assessed against Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *pseudomonas aeruginosa*) bacteria. The antibacterial assay was ascertained using the methodology reported by (13). After being adjusted to pH 6, the antibacterial activity of the 2 KDa peptides was evaluated using a 96-well microtiter plate assay. The 96-well plates were inoculated with bacteria in nutrient broth and used as a control. 100 µL of fermented supernatant of 2 KDa peptides and 100 µL of new pathogen culture were added to the plates. The plates were then incubated at 37°C for 24 hours. Using an ELISA microplate reader system, the absorbance at 630 nm was measured to evaluate the antimicrobial activity (the test was carried out in triplicate). The inhibition has been computed using the following formula:

$$\text{Inhibition \%} = \frac{(\text{control 24h} - \text{control 0h}) - (\text{sample 24h} - \text{sample 0h})}{(\text{control 24h} - \text{control 0h})} \times 100$$

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

With a few modifications, the methodology was carried out by (14). To dilute the bacteria from the overnight culture, introduce 100 µL of the bacterial suspension into the suitable growth medium. 96-well microplates were plated with bacterial suspension. Serial dilutions ranging from 0.33 to 1.64 mg/mL were made using various concentrations of 5 kDa peptides in microdilution tubes. The concentration sample at which the growth of bacteria could be inhibited was found using the supernatant. Following a 24-hour incubation period for bacteria at 37°C, the turbidity of each well was measured and compared to the control to determine the degree of microbial growth inhibition. On the other hand, MBC was ascertained by inoculating 100 µL of bacterial suspension from wells containing supernatant at MIC of Chia and Quinoa peptides at a concentration greater than the MIC (6, 3, and 1.5 mg/ml). The minimum bacterial concentration (MBC) that prevents agar plate growth following the incubation period is a well-defined term.

Agar Disc-Diffusion Assay

The antibacterial activity has been measured using an Agar disc-diffusion experiment following the protocol outlined (15). Pathogenic bacteria were Streaked onto Muller-Hinton Agar (MHA) plates using a sterilized swab. Thirty microliters (µL) of peptides (mg/mL) were added on plain paper discs (6 mm). The positive control contains 10 mcg/disc of ciprofloxacin. After incubation for 24 hours at 37°C, the diameter of the inhibition zones was measured, and the antibacterial activity with ciprofloxacin was employed as a positive control.

Analytical Statistics

The collected data were examined using SPSS. The statistical differences between the samples and the controls were evaluated using one-way analysis of variance (ANOVA). Tukey's post-test analysis evaluated significant differences between the obtained bioassay data at a 95% confidence interval. The data is presented using the triplicate experiment mean \pm standard deviation (SD).

Result

Yield of the Peptide after Ultrafiltration

The resulting peptides after the ultrafiltration of Chia and Quinoa seeds extract, supernatant with 10 and 5 kDa cutoff, respectively, as reported in Figure. (1), and centrifuged at 6000 for 60 minutes. All the extract filters go down in the tubes to the size of 10 kDa, while 50% of the filtrate is obtained by 5 kDa tubes. The ultra-filtration separated the low molecular peptides below 2 KDa from other non-active high molecular compounds and impurities and concentrated the peptides.

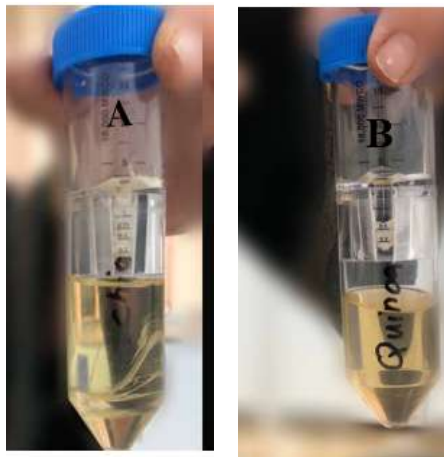


Figure. 1: A. Chia, and B. Quinoa Yield of the Peptide after Ultrafiltration

Antibacterial Assay

The antibacterial activity of 2 KDa peptides in Chia seeds shows the highest antibacterial activity, 90.93% in Chia seeds and 85.94% in Quinoa seeds against *B. cereus*. In contrast, the lowest activity in Chia was 64.58% against *Staph. Aureus*, 84.11%, and 65.24% (the values represent the growth inhibition percentage) towards *E. coli* and *P. aeruginosa*, respectively, presented in Table. (1) and Quinoa seeds show the lowest active 58.23% against *P. aeruginosa*.

Table.1: Shows the antibacterial activity of peptides extracted from Chia and quinoa seeds (μ l).

pathogens	Mean Concentration	
	Chia seed	Quinoa seeds
<i>E. coli</i>	84.11 ^a ± 0.10	59.20 ^c ± 0.05
<i>P. aeruginosa</i>	65.24 ^b ± 0.73	58.23 ^c ± 0.01
<i>Staph. aureus</i>	64.58 ^c ± 0.50	67.5 ^b ± 0.30
<i>B. cereus</i>	90.93 ^a ± 0.65	85.94 ^a ± 0.09

SD: standard deviation; **P**: One-way Anova; Different letters refer to a significance at $p \leq 0.001$

Minimum Inhibitory Concentration (MIC)

The MIC of peptides for Chia and Quinoa is required to inhibit 100% of all four pathogens' growth, as shown in Tables (2 and 3). Five distinct concentrations of Quinoa and Chia seeds (1.56, 3.125, 6.25, 12.5, 25, and 50) were examined in the current investigation. Gram-positive and Gram-negative bacteria strains were used (*B. cereus*, *Staph. Aureus*, *E. coli*, and *P. aeruginosa*). The *S. aureus* and *E. coli* examined yielded MIC values ranging from 0.58 to 4.4 mg/mL. Additionally, the strength of this antibacterial activity changed depending on the enzyme and the various protein extraction techniques employed during hydrolysis.

Table 2: The Minimal inhibitory concentration of peptide extract of Chia seeds against pathogenic bacteria

Bacterial Species	Conc. of Chia seeds (mg/ml)						P value	Mean of Total MIC
	1.56 μ l	3.125 μ l	6.25 μ l	12.5 μ l	25 μ l	50 μ l		
<i>Escherichia coli</i>								
Mean \pm SD of MIC	34.71 ^c \pm 0.05	39.71 ^c \pm 0.09	42.86 ^d \pm 0.20	47.66 ^c \pm 0.15	54.29 ^b \pm 0.50	59.63 ^a \pm 0.10	p \leq 0.001	80.13 \pm 8.131
<i>P. aeruginosa</i>								
Mean \pm SD of MIC	35.88 ^c \pm 0.1	37.98 ^{ac} \pm 0.20	43.44 ^d \pm 0.70	48.91 ^c \pm 0.20	59.21 ^b \pm 0.050	66.02 ^a \pm 0.90	p \leq 0.001	80.00 \pm 10.00
<i>Stap. aureus</i>								
Mean \pm SD of MIC	33.59 ^e \pm 0.5	36.99 ^d \pm 0.080	37.46 ^d \pm 0.32	42.58 ^c \pm 0.22	47.17 ^b \pm 0.40	52.76 ^a \pm 0.60	p \leq 0.001	82.07 \pm 6.36
<i>B.cereus</i>								
Mean \pm SD of MIC	34.22 ^d \pm 0.03	36.04 ^d \pm 0.80	38.09 ^d \pm 0.54	45.85 ^c \pm 0.10	50.08 ^b \pm 0.30	57.33 ^a \pm 0.50	p \leq 0.001	78.60 \pm 8.87

SD: standard deviation; **P**: ANOVA; Different letters refer to a significant at $p \leq 0.001$

Table3: The Minimal inhibitory concentration of peptide extract of Quinoa seeds against pathogenic bacteria

Bacterial Species	Conc. of Quinoa seeds (mg/ml)						P value	Mean of Total MIC
	1.56 μ l	3.125 μ l	6.25 μ l	12.5 μ l	25 μ l	50 μ l		
<i>Escherichia coli</i>								
Mean \pm SD of MIC	18.24 ^e \pm 0.01	23.23 ^d \pm 0.05	28.27 ^c \pm 0.09	35.86 ^b \pm 0.08	38.99 ^b \pm 0.15	45.21 ^a \pm 0.20	p \leq 0.001	31.63 \pm 0.09
<i>P. aeruginosa</i>								
Mean \pm SD of MIC	14.62 ^f \pm 0.05	19.03 ^e \pm 0.21	24.92 ^d \pm 0.30	32.06 ^c \pm 0.20	46.68 ^b \pm 0.05	55.09 ^a \pm 0.23	p \leq 0.001	32.06 \pm 0.12
<i>Stap. aureus</i>								
Mean \pm SD of MIC	12.59 ^f \pm 0.02	14.59 ^e \pm 0.03	25.56 ^d \pm 0.13	29.98 ^c \pm 0.22	34.98 ^b \pm 0.50	54.16 ^a \pm 0.60	p \leq 0.001	28.64 \pm 0.24
<i>B.cereus</i>								
Mean \pm SD of MIC	10.94 ^e \pm 0.01	14.07 ^d \pm 0.09	17.43 ^d \pm 0.01	20.04 ^c \pm 0.11	29.47 ^b \pm 0.46	32.72 ^a \pm 0.51	p \leq 0.001	20.77 \pm 0.19

SD: standard deviation; P: ANOVA; Different letters referees to a significant at $p \leq 0.001$

Minimum Bactericidal Concentration (MBC)

The MBC of 2 KDa peptides for Chia and Guinoa is required to inhibit 100% of all four pathogens' growth as shown in Tables (4, and 5). In this study, the MBC values ranged from 625–721.33 mg/mL Concentration of peptide extract of Chia seeds against pathogenic bacteria and 236.77 - 583.78 mg/mL for peptide extract of Quinoa seeds.

Table 4: The Minimum Bactericidal Concentration of peptide extract of Chia seeds against pathogenic bacteria

pathogens	MBC of Chia seed	
	50 μ l	25 μ l
<i>E. coli</i>	578.78 ^c \pm 0.3	625 ^b \pm 0.6
<i>P. aeruginosa</i>	704.65 ^a \pm 0.01	774 ^a \pm 0.09
<i>Stap. aureus</i>	432.43 ^d \pm 0.07	579.51 ^c \pm 0.56
<i>B.cereus</i>	614.77 ^b \pm 0.62	721.33 ^a \pm 0.39

SD: standard deviation; P: One-way ANOVA; Different letters referee to a significance at $p \leq 0.001$

Table 5: The Minimum Bactericidal Concentration of peptide extract of Quinoa seeds against pathogenic bacteria

pathogens	MBC of Quinoa seeds.	
	50 µl	25 µl
<i>E. coli</i>	380.73 ^b ± 0.01	236.77 ^c ± 0.06
<i>P. aeruginosa</i>	536.64 ^a ± 0.3	583.78 ^a ± 0.01
<i>Staph. aureus</i>	359.73 ^b ± 0.05	531.37 ^b ± 0.21
<i>B.cereus</i>	177.37 ^c ± 0.02	263.90 ^c ± 0.08

SD: standard deviation; P: One-way ANOVA; Different letters refer to a significance at p ≤ 0.001

Agar Disc-Diffusion Assay

Peptides 2-5 KDa and 5-10 KDa from Chia seeds were found to display the largest zone of inhibition against *B. cereus* (94.2a, 138.16a), while *P. aeruginosa* 2-5 KDa (100.48a) and *B. cereus* 5-10 KDa (153.86a) were found to exhibit the largest zone of inhibition from Quinoa seeds. The clean zone for the positive control CIP is higher (94.20a) with *B. cereus* and lower (56.52b, 47.01c, and 37.68d) for *E. coli*, *P. aeruginosa*, and *Staph. aureus* as shown in Tables (6, and 7) and Figure. (2, 3).

Table 6: The disc diffusion assay of peptide extract of Chia seeds against pathogenic bacteria

Compounds	Growth inhibition % of Chia seed		Ciprofloxacin	P value
	Mean KDa 2-5	Mean KDa 5-10		
Bacterial Species				
<i>E. coli</i>	94.2 ^a ± 0.03	75.36 ^d ± 0.09	56.52 ^b ± 0.01	p ≤ 0.001
<i>P. aeruginosa</i>	87.92 ^b ± 0.07	84.78 ^c ± 0.057	47.01 ^c ± 0.03	
<i>Staph. aureus</i>	87.92 ^b ± 0.10	94.2 ^b ± 0.3	37.68 ^d ± 0.01	
<i>B.cereus</i>	94.2 ^a ± 0.06	138.16 ^a ± 0.01	94.20 ^a ± 0.02	

SD: standard deviation; P: One-way ANOVA; Different letters refer to a significance at p ≤ 0.001

Table 7: The disc diffusion assay of peptide extract of Quinoa seeds against pathogenic bacteria

Compounds	Growth inhibition % of Quinoa seeds.		Ciprofloxacin	P value
	Mean KDa 2-5	Mean KDa 5-10		
Bacterial Species				
<i>E. coli</i>	94.2 ^b ± 0.01	78.5 ^c ± 0.4	56.52 ± 0.01	p ≤ 0.001
<i>P. aeruginosa</i>	100.48 ^a ± 0.32	94.2 ^b ± 0.063	47.01 ± 0.03	
<i>Staph. aureus</i>	87.92 ^c ± 0.04	78.5 ^c ± 0.01	37.68 ± 0.01	

<i>B. cereus</i>	94.2 ^b ± 0.081	153.86 ^a ± 0.9	94.20 ± 0.02	
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SD: standard deviation; P: One-way ANOVA; Different letters refer to a significance at $p \leq 0.001$

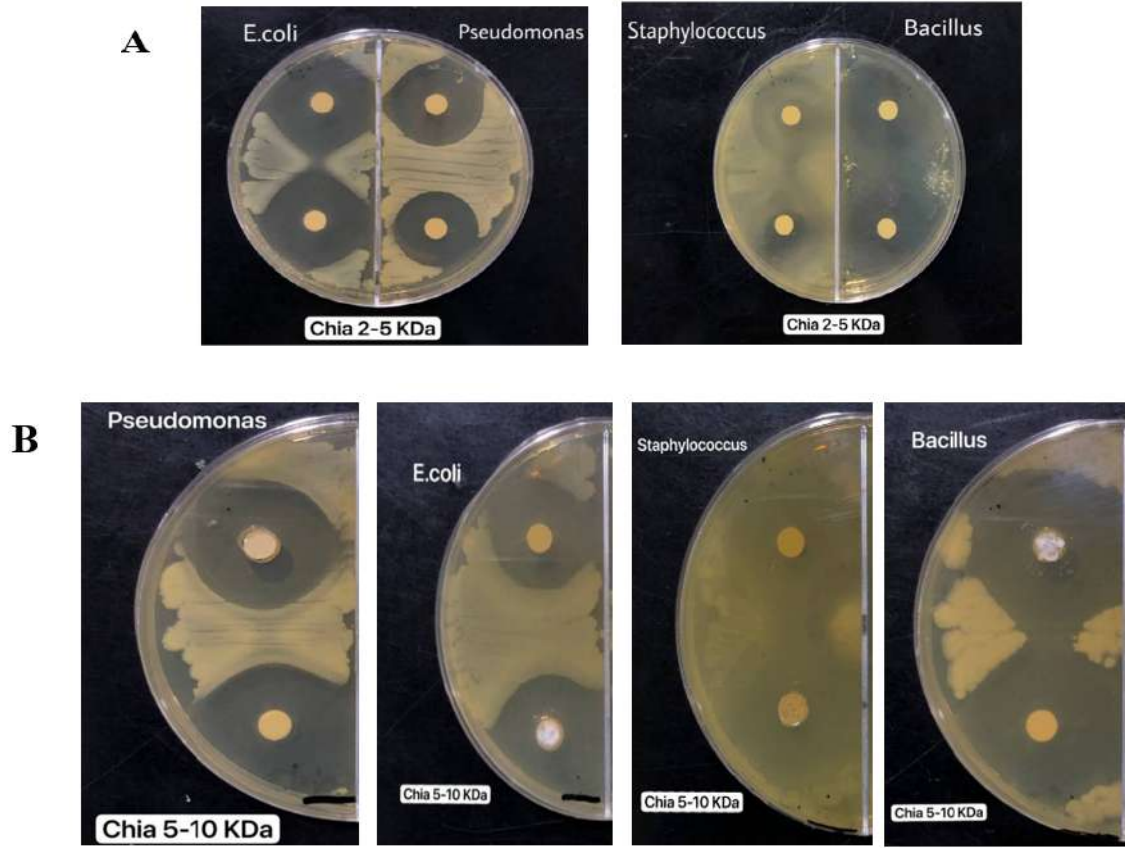
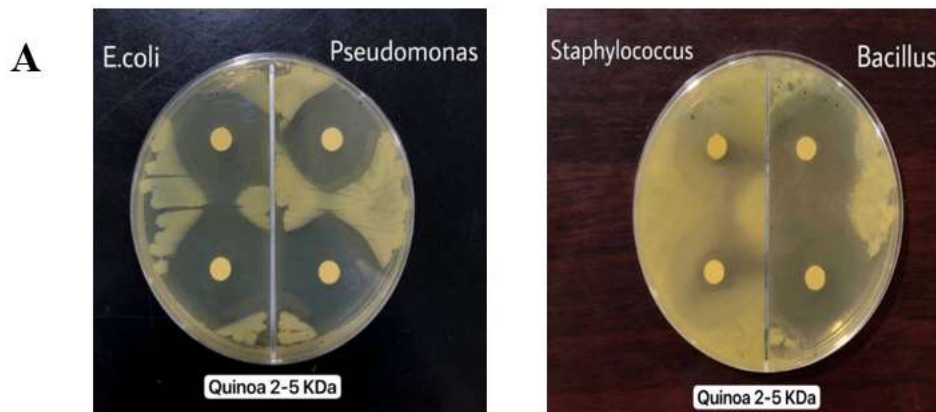


Figure2: Inhibition zone for Chia seed Peptides A (2-5 KDa) and B (5-10 KDa) for *E. coli*, *P. aeruginosa*, *Staph. aureus*, and *B. cereus*



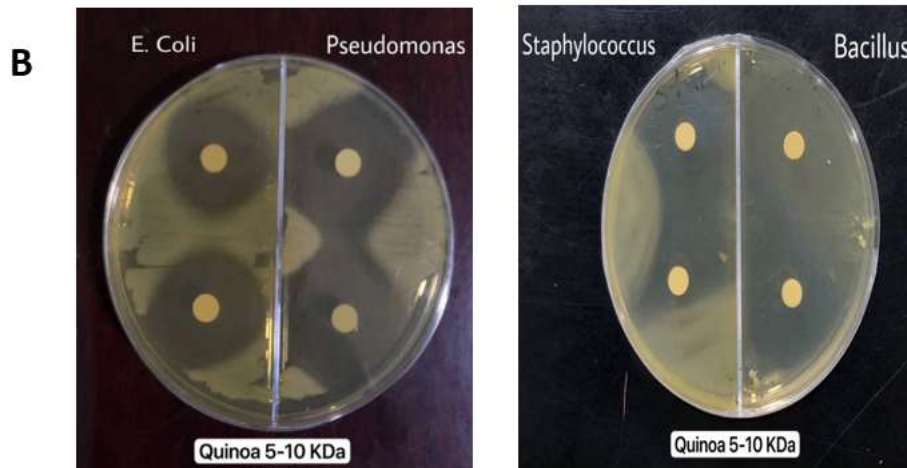


Figure 3: Inhibition zone for Quinoa seed Peptides A (2-5 KDa) and B (5-10 KDa) for *E. coli*, *P. aeruginosa*, *Staph. aureus*, and *B. cereus*

Discussion

In recent years, there has been a dramatic increase in the number of reported poisoning outbreaks caused by food-borne pathogenic bacteria. Consequently, there has been considerable interest in preventing food contamination by food-borne pathogens. Traditionally, various methods, such as heating, reducing water activity, smoking, fermentation, UV radiation and adding antimicrobial agents, have been used to prevent spoilage of foods. The addition of antimicrobial agents has been a particularly effective method for controlling microbial contamination (17). Protein hydrolysis is a useful technique to provide high-value-added products with antimicrobial activities known as bioactive peptides (18-20). Bioactive peptides are defined as inactive protein components of the primary protein structure and exhibit various physicochemical functions after their release by enzymatic hydrolysis (21). Antimicrobial peptides in protein sequences from plants, such as Chia (*Salvia hispanica L.*) and quinoa (*Chenopodium quinoa Wild.*), might have a crucial role in the inhibition of bacteria (22).

The yield of the peptide after ultrafiltration increased over time for chia and quinoa seed extracts, yielding a higher peptide concentration than camel milk fermented with *Lactobacillus plantarum*, which was 0.8 mg/ml (23). Protein decreased over time because of proteolysis into small peptides, as determined by the OPA and Bradford methods at 340 and 595 nm, respectively. The antibacterial activity of peptides in Chia seeds shows the highest compared with Quinoa seeds. The amphiphilic nature of peptides contributes to their antibacterial action. These peptides are folded in three dimensions, giving them a hydrophilic side comprising a polar and highly concentrated density of positively charged residues (amphiphatic) and a hydrophobic side comprising non-polar amino acid side chains. Therefore, by enabling peptide attachment and insertion into the bacterial membrane to produce transmembrane holes, this kind of arrangement causes membrane permeabilization (24). Similar research has been published (25), demonstrating the antibacterial activity of Chia against *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, and *Listeria innocua*. The values were less than in our investigation, it was <3kDa (61.93%, 65.05%, 60.97%, 51.73%), which involved hydrolyzing the chia seed protein sequentially catalase enzyme under microwave assistance. The cationic peptides' electrostatic attraction to negatively charged molecules, like teichoic acid

(Gram-positive) and anionic phospholipids (LPS) (Gram-negative), which are asymmetrically arranged in the membrane architecture (26). Results in the first association of peptides with the bacterial membrane; consequently, these interactions remove the Mg²⁺ and Ca²⁺ ions or replace them with cationic antimicrobial peptides. The findings of the MIC and MBC values are necessary since they serve as a standard for choosing the right and efficient concentrations of substances (27). To inhibit pathogens from growing visibly, a minimum concentration of Quinoa seeds and Chia seeds is required. A previous study reported by Sun *et al.*, (2016) on antibacterial activity against *S. aureus* and *E. coli*, with MIC values for protein hydrolysates from Spirulina plates of 8 and 16 mg/mL, respectively numbers that were lower than those discovered in (28). The results of the current study on the antibacterial activity of quinoa extracts showed that the MIC values were much higher than those reported by Pereira *et al.*, (2020) (29). Nevertheless, for each protein extraction method, the least bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were found in most of the samples, in chia seed the hydrolysates produced by catalase enzyme exhibited antibacterial activity (16). Whereas, the MIC values were ranged between 0.107–0.867 mg/mL, 0.074–0.739 mg/mL and 0.111–0.592 mg/mL, for black, red and white varieties, respectively. All extracts showed better inhibitory efficacy against *B. cereus* with MIC values of 0.107 (black), 0.074 (red), and 0.111 mg/mL (white). On the other hand, the results revealed lower potential against *S. aureus*, with values of 0.867, 0.739, and 0.592 mg/mL for black, red, and white varieties, respectively. Peptides extracted from chia and quinoa seeds demonstrated a significantly higher antibacterial activity, lower MIC values and higher MBC towards the pathogenic bacteria compared to previous studies (28,30). Another study by (31) mentioned peptides from Chia Present Antibacterial Activity (31). Peptides from Chia and Quinoa seeds were found to display the largest zone of inhibition against *B. cereus*. In a different study, the antimicrobial activity of chia seeds was tested against three types of Pathogenic bacteria: *Salmonella spp.*, *Klebsiella*, and *E. coli*. It revealed the sensitivity of *Klebsiella* and *E. coli* with 0.5 mm and 1 mm, respectively for diameters of "zone of inhibition". According to a study by Yadav *et al.*, (2021), *Salmonella*, however, exhibited resistance and failed to generate a "zone of the growth inhibition of pathogens by various sensitivity assays, the disc diffusion assay is determined to have the least amount of inhibition in this study (32). This can be attributed to many factors, including solubility, diffusion through agar, and concentration saturation.

Conclusions

In conclusion, this study approved the efficacy of peptide generated from Chia seeds and quinoa seeds fraction 2-5 KDa as an antibacterial agent against *B.cereus*. Additionally, it reflects its antibacterial action in food products, suggesting its potential as an antibacterial agent in food products. Moreover, the results of this study showed that these seeds could be promising natural alternatives for combating bacterial infections. Further research is needed to explore their mechanisms of action and potential therapeutic uses fully.

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Competing interests statement

The authors approved that there is no conflict of interest from any parties upon publication of this manuscript.

Ethics statement

All authors approved that this research follows the journal's ethical guidelines as appeared on the journal's author guidelines page.

Author contributions

All the authors contributed equally to designing research, data collection, data acquisition, data analysis, reporting, and manuscript preparation.

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Data availability

Datasets related to this article will be available upon request to the corresponding author.

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