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Celery (*Apium graveolens*) as a Natural Nitrite Alternative in Minced Meat Preservation

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Abstract

NITRITE is a key meat additive used in curing to enhance color, flavor, and inhibit microbial spore growth, but it poses health risks to consumers, making it essential to find safer, eco-friendly alternatives. Meat products provide an ideal environment for *Staphylococcus aureus* growth and toxin production, leading to tissue damage, nutrient absorption, and bacterial spread. Thus, our main goal was to investigate the antibacterial properties of nitrite, celery (*Apium graveolens*) extract, and their potential synergistic effect against *S. aureus* in preserved minced meat. *S. aureus* was detected in 22 fresh meat samples (44 %) with a mean count of 3.54 ± 0.52 $\log_{10}$ CFU/g. All *S. aureus* isolates carried the *sea* gene, and 66.6% harbored *icaA* and *hla* genes. Celery extract showed notable antibacterial activity, producing an 18 mm inhibition zone in the well diffusion test. Celery extract showed a minimum inhibitory concentration (MIC) of 512 µg/mL, while nitrite required over 1024 µg/mL. In *S. aureus*-inoculated minced meat, celery extract reduced bacterial count by 12%, nitrite by 9%, and their combination achieved a 16% reduction. Our study concluded that celery extract is a promising and safe natural alternative to synthetic nitrites, and both nitrite and celery extracts demonstrated synergistic antibacterial activity against *S. aureus* in meat.

Keywords: *Staphylococcus aureus*, Antibacterial, chemical preservatives, plant-based, ground meat, virulence genes.

Introduction

Meat and meat products can provide humans with essential nutrients and energy. The long history of meat consumption demonstrates the significance of meat and meat products in human diets. The susceptibility of meat to microbial infection and spoiling is high because of its rich content of water, proteins, minerals, and nutrients, which create an ideal environment for bacterial growth [1]. *Staphylococcus aureus* is a Gram-positive bacterium that origins a wide range of infectious illnesses, including skin infections, bacteremia, endocarditis, pneumonia, and food poisoning. *S. aureus* is among the most economically significant foodborne pathogens globally. It produces heat-stable toxins that cause food poisoning and toxic shock-like syndrome and acts as a superantigen by stimulating T-cell proliferation. Its virulence is linked to factors such as hemolysin, thermonuclease, lipases, and hyaluronidase [2]. *S. aureus* is concerning due to its heat and acid resistance and frequent presence in ready-to-eat foods. Enterotoxins A, D, and B, especially type A, are linked to food poisoning, with type B also known as a bioweapon [3]**.** Virulence genes (*hla*, *hlb*, *icaA*) support tissue invasion, adhesion, and biofilm formation, enhancing *S. aureus* survival [4].

The rising demand for ready-to-eat foods has pushed the food industry to adopt new processing techniques, including chilled and semi-prepared meals. These often involve meat curing using salt, nitrate (NO_3^-), and nitrite (NO_2^-), which have long been used to preserve meat by lowering moisture and water activity. Although nitrites improve color and

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offer antimicrobial and antioxidant effects, health concerns arose in the 1950s–60s with the discovery of N-nitroso compounds (NOCs), potentially carcinogenic substances formed when nitrites react with amides in food or the stomach [5]. Studies show that nitrosamine formation can be reduced by the antioxidant and bacteriostatic effects of vitamins, plant extracts, herbs, spices, and fruits. Nitrite levels in plants are highest in leaves, followed by stems and roots, with the lowest in flowers. Nitrite is more reactive than nitrate in acidic environments such as the stomach. The effectiveness of plant extracts varies depending on raw material, extraction method, and growth stage; for instance, extracts from flowering herbs and leaves have stronger antioxidant activity than those from vegetative stages or stems [6].

Plant-based nitrite alternatives, including Celery (*Apium graveolens* var. *dulce*), have been used for centuries in meat curing, flavoring, traditional medicine, and pharmaceuticals, making it an important commercial medicinal plant due to its antimicrobial effects and high phenolic content, particularly in essential oils, which offer strong free radical scavenging and health benefits, and they also has long been used as a curing and flavoring agent in meat products [7]. Celery powders (CPs) serve as a natural nitrate and nitrite source in meat processing, valued for their mild flavor and low pigment content. The extraction of bioactive compounds, such as nitrites and nitrates, from medicinal plants is typically carried out through physical and chemical methods. However, chemical methods like solvent extraction often result in low extraction ability and the presence of toxic chemical residues [8]. In contrast, the milling technique is considered more environmentally friendly, while water extraction offers a relevant approach for extracting phenolic components. It also produces a safer and more convenient extract or product, which is more suitable for certification [9].

In this context, our study aimed to explore the prevalence of *S. aureus* in fresh meats and the percentage of *S. aureus icaA*, *hla*, and *sea* virulence genes utilizing the Polymerase Chain Reaction (PCR) method. Additionally, the study assessed the use of celery extract as a source of nitrite and nitrate in the preparation and preservation of meat products by demonstrating the antimicrobial effects of celery extract on meat and meat products, and identifying the minimum concentration needed to inhibit the growth of *S. aureus*.

Material and Methods

Sample collection

From August and November 2024, a total of fifty fresh bovine minced meat samples were randomly selected from local meat stores in Sharkia Governorate, Egypt. Every sample was carried in sterile bags under cold, aseptic conditions. The samples were preserved at a temperature of 4 °C during transport. Microbiological analyses were performed immediately upon arrival at the laboratory.

Isolation and Identification of S. aureus

The technique previously described by the International Commission of Microbiological Specification for Foods (ICMSF) was used to isolate *S. aureus*, with some modifications (10). Briefly, 25 g of meat samples were homogenized and combined with 225 mL of sterile-buffered peptone water (Oxoid, UK). The resulting homogenates were subsequently subjected to serial tenfold dilutions in sterile distilled water. For each dilution, 1 mL was applied to the surface of two distinct sterile Baird-Parker agar plates (Oxoid, UK). The dishes were incubated at 37°C for 24-48 h. Colonies were enumerated and reported as log colony-forming units per gram of food (CFU/g). For further confirmation of *S. aureus*, individual colonies of presumptive *S. aureus* were further examined using Gram staining and biochemical tests, and coagulase activity [11].

Calculating the total count of S. aureus and Staphylococci

Baird-Parker agar was inoculated with one milliliter of each serial dilution utilizing a sterile spreading tool. The plates were incubated for 48 hrs at 37°C. Black colonies were calculated to define the total Staphylococcal count/g. *S. aureus* colonies, identified by their distinctive appearance [11], were purified on nutrient agar and confirmed by Gram staining, biochemical tests, and coagulase activity. PCR was utilized to confirm *S. aureus* and detect its virulence genes.

Biofilm Formation Assay

The isolates were characterized phenotypically by culture on Congo red agar (CRA) plates, as described by Arciola et al. [12]. Briefly, agar plates were prepared by adding 37 g/L brain heart infusion broth (Oxoid, UK), 0.8 g/L Congo red stain, 50 g/L sucrose, and 10 g/L agar (Oxoid, UK), followed by incubation at 37 °C for 24 and 48 h. The macroscopic characteristics of the *S. aureus* isolates in the CRA were observed. Crusty black colonies, with a dry filamentous appearance, were recorded as biofilm producers, smooth pink colonies as nonproducers, and intermediate colony morphology (pink with dark centers resembling bull's eyes), as potential biofilm producers [13].

Identification of Virulence Genes

The *S. aureus* virulence genes, including *hla*, *sea*, and *icaA*, were assessed utilizing the conventional uniplex PCR method. DNA was extracted utilizing the QIAamp DNA Mini Kit (Qiagen, Germany), in accordance with the manufacturer's guidelines. The

oligonucleotide primers (Metabion, Germany) used are listed in Table 1. The EmeraldAmp Max PCR Master Mix (Takara, Japan) was used for PCR amplification, in accordance with the manufacturer's guidelines, in the Applied Biosystems 2720 heat cycle. PCR products were separated using a 5 V/cm gradient electrophoresis on a 1.5% agarose gel (Applichem, Germany) in 1× TBE buffer at room temperature. Twenty µl of uniplex and 30 µl of multiplex PCR products were loaded per well for gel analysis. Gels were photographed utilizing a documentation system (Biometra, Alpha Innotech), and computer software was used to analyze the data.

Celery Extract Preparation

Celery extract was prepared following the procedures of Martins et al. [14]. Briefly, 100 ml of distilled water and 10 grams of dry celery powder were mixed, and the mixture was agitated at 150 rpm for one hour at room temperature. Whatman No. 1 filter paper was then utilized to filter the mixture, and the supernatant was freeze-dried.

In Vitro Antibacterial Activity of Celery Extract

The well diffusion method was used to evaluate the antibacterial activity of the crude extracts as described by Modzelewska et al. [15]. Sterile Petri dishes were filled with 15 mL of Mueller-Hinton Agar (MHA, Oxoid, UK) to form the base plates. A bacterial suspension (10 ml, 1.0×10^8 CFU/ml, McFarland standard 0.5) was spread evenly utilizing an aseptic cotton swab. Six mm wells were aseptically created utilizing a sterile cork borer that was filled with 50 µl of the extract. Plates were incubated at 37 ± 2 °C for 24 h, and then the inhibition zones were measured.

Determining the Minimum Inhibitory Concentration (MICs)

To evaluate the MIC values, the broth dilution method was utilized as described by Khotimah et al. [16]. Briefly, one colony was suspended in 5 mL of nutritional broth. The suspension was adjusted to the 0.5 McFarland standard (1.0×10^8 CFU/ml). Equal volumes of bacterial inoculum were mixed with either nitrite or celery extract. Each tube was then incubated for 24 hours at 37 ± 2 °C. The lowest concentration that prevented bacterial growth was defined as the minimum inhibitory concentration (MIC) [16].

Determining the effect of Celery extract, Nitrite, and their mix on S.aureus artificially inoculated in minced meat.

The most virulent *S. aureus* strain was prepared in accordance with the previously described method by Kantachote and Charernjiratrakul [17]. The *S. aureus* strain was added to a 0.1% sterile peptone water tube and was incubated for 24 h at 37 °C. Using tube dilution techniques, the cell count for *S. aureus* was adjusted to 6 $\log_{10}$ CFU/ml. A total of 1000 g of minced meat was kept in the refrigerator at 4°C for two hours before use. After this time, *S. aureus* was aseptically added to the minced beef samples at a concentration of approximately 6 $\log_{10}$ CFU/g [18]. The samples were maintained at room temperature for 15 minutes [19]. The initial load of the inoculated samples was assessed by counting the number of *S. aureus* in each of the four groups of minced beef samples, with each group weighing 250 g. The four groups included one control group, untreated and uninfected, and three inoculated minced meat and treated with aqueous plant extracts of celery, 150 ppm nitrate, and a combination of both (nitrate with celery). To achieve uniform mixing, the plant extracts were combined with the minced beef samples for one hour. The levels of *S. aureus* were counted, converted to logarithmic form, and expressed as $\log_{10}$ CFU/g of the sample.

Statistical analysis

One-way analysis of variance (ANOVA) was utilized to analyze the data**.** Duncan's Multiple Range Test was utilized to compare means using SPSS version 14 (2006). The results were reported as mean ± standard error (SE), and the microbial counts were expressed as $\log_{10}$ CFU/g. *p*-values less than 0.05 were regarded as statistically significant.

Results

Incidence and count of Staphylococcus aureus

The overall prevalence of pathogenic coagulase-positive *S. aureus* among the 50 tested fresh bovine meat samples was 44% (22/50). On mannitol salt agar, *S.aureus* appeared as smooth, circular, yellow colonies after 24 h at 37°C due to mannitol fermentation. On Baird-Parker agar, colonies were convex, smooth, gray to black, 2–3 mm in diameter, with clear halos. Microscopically, *S. aureus* appeared as grape-like Gram-positive clusters, and β-hemolysis was also observed on blood agar. The mean *S. aureus* counts were log 3.54±0.52 CFU/ g

Detection of Biofilm-Productive S. aureus

The Congo Red test showed that 13 out of 22 (59%) *S. aureus* isolates produced biofilms (Fig. 1). Among them, 6 isolates (27.2%) showed strong biofilm formation, and 7 (31.8%) showed moderate formation. The remaining 9 isolates did not produce biofilms.

Identification of Virulence Genes

The PCR assay results showed that all six *S. aureus* isolates recovered from fresh meat samples (100%) carried *sea*, while four isolates (66.6%) carried *icaA* and *hla*. All *S. aureus* isolates had at least two virulence genes (Fig. 2).

Antibacterial Activity with the Well Diffusion Method

Celery extract showed antibacterial activity against *S. aureus*, producing an 18 mm inhibition zone. In contrast, no inhibition was observed with 150 ppm nitrite (Fig. 3), indicating the stronger antibacterial effect of celery.

Minimum inhibitory concentrations of the celery extract and nitrite

The minimum inhibitory concentrations (MICs) of nitrite and celery extract were determined using the dilution method to evaluate their bacteriostatic effects. The MIC of celery extract against *S. aureus* was 512 µg/mL, while the MIC of nitrite (150 ppm) exceeded 1024 µg /mL, indicating lower antibacterial activity compared to celery extract.

Effect of celery, nitrite, and their mix on S. aureus count in minced meat samples

The combination of 150 ppm nitrite and 10% celery extract showed enhanced antibacterial activity, resulting in the greatest inhibition of *S. aureus* growth and a drop in counts of *S. aureus* with a mean count of 5.12 ± 0.09 log CFU/g as seen in Table 2**.** This suggests that celery extract, when used with nitrite, can serve as an effective meat preservative against *S. aureus*, helping to maintain meat quality, extend shelf life, and reduce economic losses.

Discussion

Staphylococcus is commonly found in the environment and on humans and animals, especially in the nose, throat, skin, and hair [20]. Among the various species, *S. aureus* is the primary cause of infections and the third extreme common source of food poisoning worldwide [21]. Moreover, *S. aureus* was found in 22 out of 50 meat samples (44%), according to our results. This is similar to the results of Shawish and Al-Humam [22], who reported *S. aureus* among 38% of minced meat. Although Tarabees et al. [23] stated a higher rate of *S. aureus* (70%), Nadim [24] reported a lower rate of 28%.

In this study, the mean *S. aureus* counts were log 3.54±0.52 CFU/ g; the current results align with the results of Lela-Radwa (2.63×10^3 CFU/g) [25]. On the other hand, higher counts were observed by Elmali and Yaman [26] (1.0×10^5 CFU/g in kofta). Moreover, Ibrahim-Shimaa [27] found that minced meat had 5.83×10^5 CFU/g, whereas lower counts were noted by the same authors (2.2×10^2 and 6.61×10^2 CFU/g, respectively). The high *Staphylococcus* count in meat samples indicates cross-contamination, typically linked to human skin contact, handling, or secretions.

Staphylococcus aureus is a highly virulent bacterium whose pathogenicity is mainly driven by various virulence factors, including enzymes, adhesins, toxins, and surface proteins [4]. These factors support colonization, survival, and immune evasion and differ by infection type. The bacterium produces heat-stable enterotoxins, particularly enterotoxin A (SEA), which is strongly linked to food poisoning outbreaks [3] as well as membrane-damaging exotoxins [28]. In the present study, PCR results showed that all six *S. aureus* isolates from fresh meat samples carried the *sea* gene (100%),while *icaA* and *hla* were found in 66.6% of isolates. The predominance of *sea* in our results was in agreement with Rasmi et al. [4]**,** who reported *sea* as the most frequent gene (72.9%) among 59 isolates, followed by *icaA* (49.2%), and *hla* (37.3%). *Sea* was the dominant gene in both methicillin-resistant *S. aureus* (MRSA) (72.2%) and methicillin-sensitive *S. aureus* (MSSA) (79.9%). On the other hand Abdulmanea et al.[29] found a link between MRSA and the presence of the *icaA* and *hla* genes, with isolates carrying these genes showing high antibiotic resistance. The *icaA* gene, responsible for producing PIA (polysaccharide intercellular adhesin), plays a key role in biofilm formation and cell adhesion. *S. aureus* forms multilayered biofilms within a slime or glycocalyx matrix involving various proteins [30]. Hemolysins, encoded by the *hla* gene, help in iron acquisition and often have leucolytic effects. The anti-virulence drugs targeting hemolysins offer a promising strategy to reduce infection severity and, through quorum quenching, delay biofilm formation and drug resistance [31].

Nitrites are commonly used in meat products to prevent the growth of harmful microorganisms**.** They are typically added at levels below 150 ppm to ensure safety [32]. However, concerns arose in the 1950s–1960s when nitrites were linked to the formation of potentially harmful N-nitroso compounds [5]. Regulations generally limit nitrite levels in meat to a maximum of 200 ppm, whether as sodium or potassium nitrite. Celery inhibits *S. aureus* infection mainly due to its antioxidant compounds. Bioactive components like alkaloids and saponins in celery extract reduce oxidative stress and block redox pathways, helping to lessen infection severity [33]. The current result, obtained using the diffusion method, demonstrates celery's antibacterial activity against the isolated *S. aureus*; this is indicated by clear zones surrounding the wells. The extract's suppression of the test bacteria was seen in the clear zone. The antibacterial activity of celery extract is attributed to its phytochemicals [34**].** Antimicrobial effects of phytochemicals are closely linked to their phenolic compounds, including flavonoids, alkaloids, tannins, and saponins [35]**.** Flavonoids exert their antibacterial effects through various mechanisms, including inhibiting energy metabolism, nucleic acid production, and cytoplasmic membrane function**.** Saponins affect the permeability of bacterial cell membranes**.** Celery extract exhibits significant antibacterial and antioxidant properties and may promote wound healing by enhancing fibroblast proliferation and re-epithelialization [36]**.** In this

study, celery was selected as an alternative treatment for Staphylococci infections instead of nitrites. Because of its biochemical components, celery displays a number of possible advantages against *S. aureus*. Celery contains alkaloids, flavonoids, phenolic chemicals, saponins, and tannins, according to a previous study by Prakoso and associates [36], as celery extract's highest biochemical composition, phenolic chemicals, inhibits *S. aureus* from growing in solid and broth medium. Misic et al**.** [37] showed that celery extract can potentially be utilized as an antimicrobial agent against *Staphylococcus* infections in meat samples and was chosen as an alternative therapy to antimicrobial agents. Future research is advised to address the influence of nitrite and celery extract on the meat's sensory qualities and *S. aureus*'s virulence gene expression.

Conclusion

According to our data, meat products are a significant source of pathogenic *S. aureus*. The *sea* gene was present in all six *S. aureus* isolates, while *icaA* and *hla* genes were present in 66.6% of them. Celery (*Apium graveolens*) extract proved significant antibacterial activity, generating an 18 mm inhibition zone in the well diffusion test. The MIC was found to be 512 µg/mL, while nitrite required over 1024 µg/mL. The effects of this study concluded that celery extract reduced bacterial counts by 12%, nitrite levels by 9%, and the combination of both achieved a 16% reduction. It is advised that in the future, different dosages of celery extract be used as a herbal substitute for antibiotics for Gram-positive bacteria. When combined with nitrite, the celery extract will be a potent antibacterial agent against *Staphylococcus* species.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

TABLE 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
icaA	CCT AAC TAA CGA AAG GTA G AAG ATA TAG CGA TAA GTG C	1315	94˚C 5 min.	94˚C 30 sec.	49˚C 1 min.	72˚C 1.2 min.	72˚C 12 min.	[38]
Sea	TTGGAAACGGTTAAAACGAA GAACCTTCCCATCAAAAACA	120	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	[39]
hla	GAAGTCTGGTGAAAACCCTGA TGAATCCTGTCGCTAATGCC	704	94˚C 5 min.	94˚C 30 sec.	53˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	[40]

TABLE 2. Effect of celery, nitrite, and their mix on *S. aureus* count in minced meat samples after one hour.

Sample	Control -ve	Control+ve	nitrite	Celery	Celery and nitrite
***S. aureus* count**	3.8±0.01	6.06±0.03[a]	5.54±0.03[b]	5.33±0.02[c]	5.12±0.09[d]
Reduction %	-	-	9%	12%	16%

Fig. 1. Biofilm production of *S. aureus* on Congo red media.

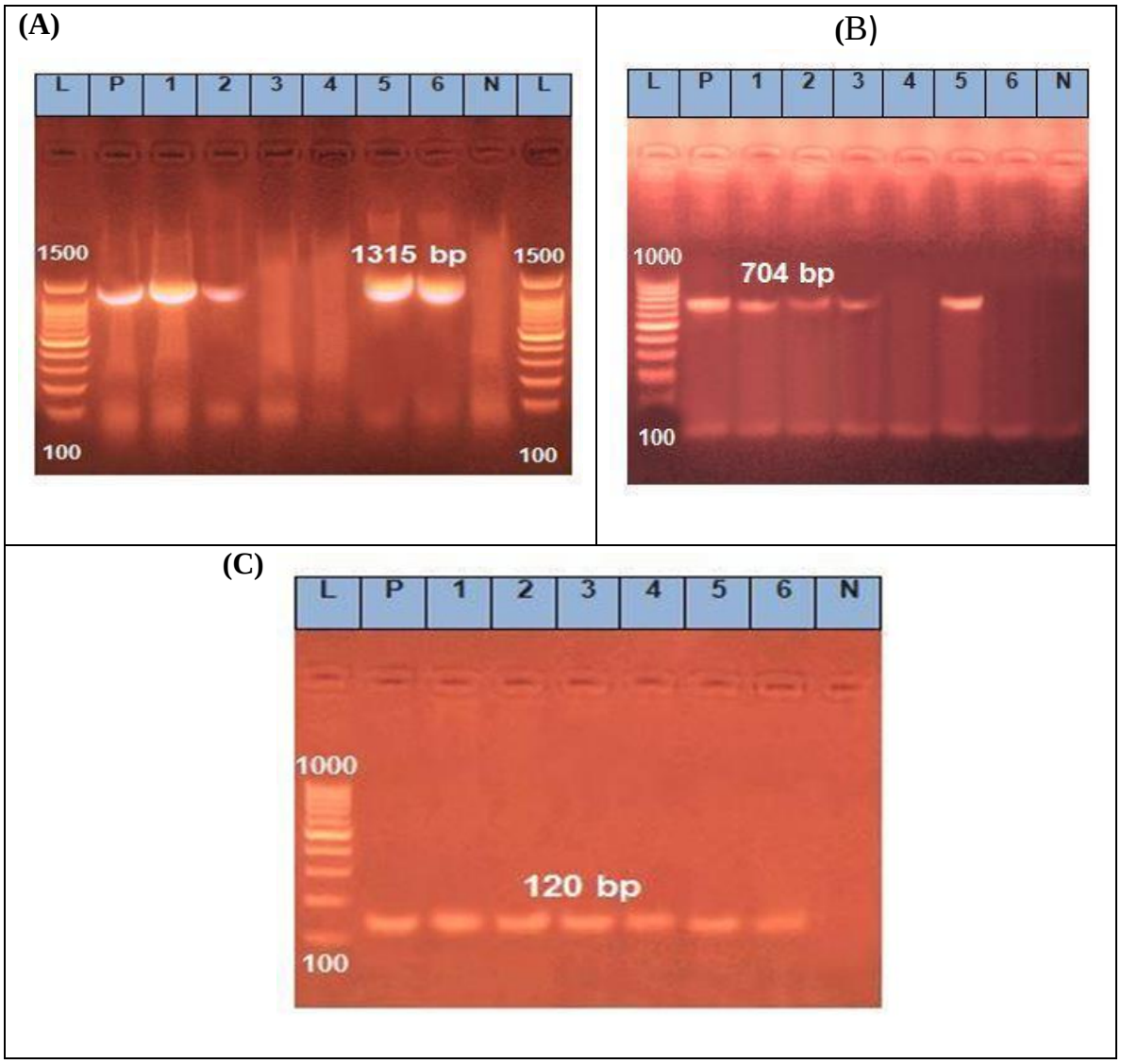

Fig. 2. Uniplex PCR identification of *S. aureus icaA* (A), *hla* (B), and *sea* (C) virulence genes on a 1.5% agarose gel using agarose gel electrophoresis. L: 100 bp ladder, P: positive control, 1-6: *S. aureus* isolates from fresh bovine meat samples

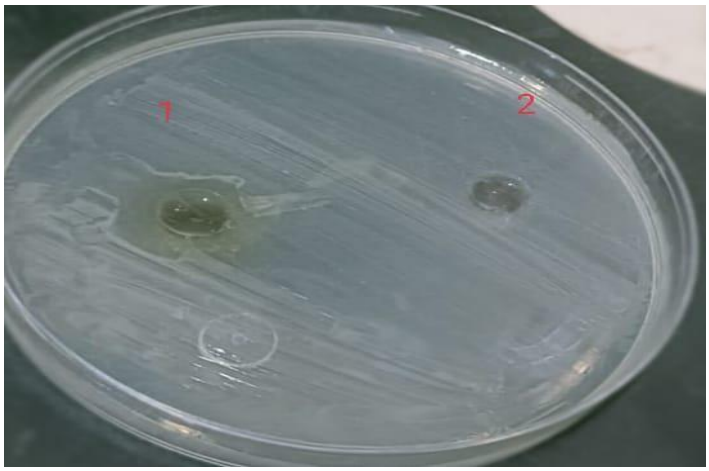

Fig. 3. Antibacterial effect of celery extract and nitrite against *Staphylococcus aureus.* 1: *S. aureus* was inhibited by celery extract, and 2: *S. aureus* was not inhibited by nitrite.

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الكرفس (أبيام كرافيولينس) كبديل طبيعي للنيتريت في حفظ اللحوم المفرومة

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الملخص

يُعد النيتريت من أهم الإضافات المستخدمة في حفظ منتجات اللحوم لما له من دور في تحسين اللون والنكهة ومنع نمو الأحياء الدقيقة. ومع ذلك، ارتبط استخدامه بمشكلات صحية خطيرة مثل تكوين مركبات النيتروزوأمين المسرطنة، مما دفع الباحثين للبحث عن بدائل طبيعية أكثر أمانًا.استهدفت هذه الدراسة تقييم فعالية مستخلص الكرفس (Apium graveolens) كبديل طبيعي للنيتريت في اللحوم المفرومة، خاصة ضد بكتيريا Staphylococcus aureus المسببة للتسمم الغذائي.تم فحص 50 عينة من اللحوم الطازجة، حيث وُجدت البكتيريا في 44% من العينات، وكل العزلات حملت جين sea، بينما وُجدت جينات icaA و hla في 66.6% منها.أظهر مستخلص الكرفس، الغني بالفلافونويدات والصابونينات والتانينات، نشاطًا مضادًا للبكتيريا بقطر تثبيط 18 مم، مع قيمة MIC بلغت 512 µg/mL، مقارنة بالنيتريت الذي احتاج تركيزًا أكبر من 1024 µg/mL.عند اختبار المستخلص في اللحم المفروم الملوث صناعيًا، انخفضت أعداد البكتيريا بنسبة 12% باستخدام الكرفس، 9% باستخدام النيتريت، بينما حقق المزيج بينهما أفضل نتيجة بانخفاض 16%.تشير النتائج إلى أن مستخلص الكرفس يمثل بديلاً طبيعيًا واعدًا للنيتريت الصناعي، مع إمكانية دمجه معه لتعزيز الفعالية المضادة للبكتيريا وتحسين سلامة وجودة اللحوم.

الكلمات الدالة : المكورات العنقودية الذهبية، مضاد للبكتيريا، مواد حافظة كيميائية، نباتي، لحم مفروم، جينات الضراوة.