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Efficiency of Some Essential Oils and Nano-Chitosan against Multidrug Resistant *Staphylococcus Aureus* That Isolated from Some Food

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Abstract

This study aimed to use natural antibacterial agents to prevent the growth of multidrug-resistant *Staphylococcus aureus* that was isolated from some food samples. Among 150 different food samples, only 50 samples were recorded to contain *Staphylococcus aureus* at different counts. Fresh minced meat and sausage samples exhibited the highest *Staphylococcus aureus* counts while, fresh meat and chicken showed the lowest counts. PCR using species-specific PCR primer from DNA mixture was done. Results indicated that PCR able to amplify the specific DNA fragment for *Staphylococcus aureus*. Antibiotic susceptibility of *Staph. aureus* strains revealed varying degrees of resistant patterns against the antibacterial agents. *Staph. aureus* was resistant to 75% of examined antibiotics. *Staph. aureus* was resistant to 12 antibiotics, On the other hand, *Staph. aureus* was sensitive to Levofloxacin and Vancomycin. Most essential oils gave antibacterial activity against tested bacterial strain. The minimum inhibition zone was recorded by Black bean (4 mm), while the maximum one was recorded by cinnamon (24 mm) followed by clove (20 mm).

Key words: Cinnamon, Clove, Ginger, Parsley, Marjoram, Garlic, Thyme, nano-chitosan and *Staphylococcus aureus.*

Introduction

Staphylococcus aureus is facultative anaerobic, non-motile, and mesophilic. In addition, they are characterized by producing enterotoxins. These bacteria have nowadays exhibited great antibiotic resistance which is developed as a result of horizontal gene transfer and mutation **(Kroning *et al.*, 2016).** *Staphylococcus aureus*, despite not producing spores, has the ability to contaminate food products during their preparation and processing. This is particularly concerning due to its ability to withstand a wide range of temperatures, ranging from 7-48°C, with an optimal temperature range of 30-37°C. Moreover, it exhibits resilience in environments with pH levels around 4-9 and demonstrates robust survival at pH levels of 7-7.5.

Additionally, it can tolerate high sodium chloride concentrations of up to 15%. *Staphylococcus aureus* also displays notable tolerance to conditions of dehydration-induced stress. **(Chaibenjawong and Foster, 2011).**

Resistance of bacteria to antibiotics is based on the ability of the bacterial cell to prevent any antibiotic or bactericidal effects, to certain levels, not to mention the extent to which excessive and/or improper use is likely to contribute to enhancing this resistance **(Kraemer *et al.*, 2019)**. The most common cases are episodes of bacterial food poisoning, caused by *Salmonella*, *Staphylococcus*, *Clostridium perfringens*, *Escherichia coli*, *Bacillus cereus*, and rarely *Clostridium botulinum* **(Gupta, 2017).**

The present trends in food processing are centred around the utilization of natural compounds, which are regarded as safe alternatives that meet regulatory requirements. These natural food additives are commonly referred to as "Generally Recognized As Safe" 'GRAS' **(Bondi *et al.*, 2017)**. Essential oils, herbs and spices, vinegar, bacteriocins, organic acids, chitosan, sugar, salt, and fermentation are commonly used as bio preservatives. These substances work collectively to inhibit the growth of bacterial pathogens, with their effectiveness depending on the concentration at which they are added **(Jandali *et al.*, 2019).**

Materials and Methods

Sampling

One hundred fifty different food samples i.e., fresh meat products, fatty tissue and cheese were obtained from a butcher shops, a frozen meat shops and a dairy shops in Cairo, Egypt, and were immediately transported in an ice chest to the laboratory examinations.

Media used

In this study, all used media were ready-made, which were purchased from HIMEDIA and Oxoid Companies. These media were, Nutrient Agar (NA) media, Tryptone Soy Agar (TSA), Mueller Hinton Agar (MHA), Mannitol-salt Agar (MSA) medium and Blood Agar medium.

Antibiotics

Sixteen common antibiotics used in medical practice belonging to different groups where the tablets were purchased from Turkish, Poland, Oxoid, Gentamicin (10µg/mL), Piperacillin/tazobactam (100µg/mL), Cefotaxime (30µg/mL), Imipenem (10µg/mL), Meropenem (10µg/mL), Ciprofloxacin (10µg/mL), Trimethoprim/sulphamethoxazole (1.25/23.75µg/mL), Ceftriaxone (30µg/mL), Levofloxacin (5µg/mL), Cefoxitin (30µg/mL), Polymixin B (300µg/mL), Erythromycin (15µg/mL), Clindomycin (2µg/mL), Vancomycin (30µg/mL), Amoxicillin/clavulanic acid (30µg/mL) and Amikacin (30µg/mL). These antibiotics were selected based on common antibiotics used in medical practice, health treatment, and the recommended dose from each of them **(Aween *et al.*,2014).**

Natural antibacterial agents

Essential oils (EOs)

The following nine E.Os (98% purity) were procured from the Medical and Aromatic Oils Unit at the National Research Center. Essential oils in this study were Cinnamon oil *(Cinnamomum zeylanicum)*, thyme oil *(Thymus vulgaris)*, Clove oil *(Syzygium aromaticum)*, Parsley oil *(Petroselinum crispum)*, Garlic oil *(Allium sativum L.)*, Ginger oil *(Zingiber officinale)*, Marjoram oil *(Origanum majorana)*, Black seed oil *(Nigella sativa)* and Mint oil *(Mentha)*.

Nano-chitosan characterization

Nano-chitosan (size: 50–100 nm) was purchased from Nano-Fab Technology, New Maadi, Cairo.

DNA analysis for identification

Bacteria	Sequences	Amplified product	Reference
***Staphylococcus* sp.**	F (5-GCGATTGATGGTGATACGGTT-3) R (5-AGCCAAGCCTTGAACGAACTAAAGC-3)	270 bp	**Hu *et al.*, (2011)**

Extraction of DNA, Oligonucleotide primers and agarose gel electrophoreses were done according to **Sambrook *et al.*, (1989)**.

Cycling conditions of the primer during PCR

Bacteria	Primary denaturation	Secondary Denaturation	Annealing	Extension	No. of cycles	Final extension
***Staphylococcus* sp.**	94˚C, 5 min.	94˚C, 30 sec	55˚C, 30 sec	72˚C, 30 sec	35	72˚C, 7 min.

Total count of bacteria

The aerobic bacterial count was determined by performing plate agar count method according to **FDA, (2002)**. After incubation at 35ºC for 24 hours, the number of colony forming units (CFUs) was counted and calculated per 100 grams of the sample.

Antibiotics sensitivity

A volume of 1 milliliter of each bacterial inoculum containing approximately 10^6 colony forming units (CFU) was streaked onto sterile Petri dishes containing Muller and Hinton Agar (MHA). Pre-existing antibiotic disks were then placed at the center of the inoculated plates, which were subsequently incubated at 37°C for 24 hours **(Bauer *et al.*, 1966)**. The results of the sensitivity test conducted on the tested pathogenic bacterial strains were categorized into three groups: sensitive, intermediate, and resistant, based on the size of the observed inhibition zones. This categorization was done in accordance with the guidelines set by the Clinical Laboratory Standards Institute **(CLSI,2015).**

Antibacterial activity

To perform the agar well diffusion method, one millilitre of the bacterial inoculum with the highest level of antibiotic resistance was spread evenly onto sterile Muller and Hinton Agar (MHA) supplemented with 0.01%

v/v tween 80%. Using a sterile cork-borer, wells with a diameter of 9 mm were created in the agar. Each well was then filled with 100 µl of either individual essential oils (EOs) or their combinations (v/v). The plates were incubated at room temperature for 1 hour, followed by incubation at 37°C for 24 hours. This method adhered to the described protocol by **(López *et al.*, 2005)**.

Statistical analysis

The obtained data were statically analyzed Using CoStat version 6.400 (CoHort software, Monterey, CA, 93940, USA). For comparison between means, standard deviation (SD) was used.

Results and discussion

This study aimed to use natural antibacterial agents to prevent the growth of multidrug-resistant *Staphylococcus aureus* that was isolated from some food samples.

Results in **Table (1)** showed that, out of 150 different food samples, only 50 samples were recorded to contain *Staphylococcus aureus* at different counts. Fresh minced meat and sausage samples exhibited the highest *Staphylococcus aureus* counts while, fresh meat and chicken showed the lowest counts. Obtained results are in harmony with findings that mentioned by **(EFSA, 2016)**.

Pathogens usually multiply within the gastrointestinal tract. In some cases, they may also produce harmful toxins when food is stored incorrectly, which can then be transferred to the human digestive tract and ingested later. Therefore, foodborne illnesses can result from either direct infection by foodborne pathogens, indirect poisoning from toxins, or a combination of both.

Table 1. *Staphylococcus aureus* count (100g sample) in deferent food samples.

No.	Sample	Count *10^3	No.	Sample	Count *10^3
1.	Sausage	15	26.	Quraish cheese	25
2.	Fresh meat	20	27.	Quraish cheese	15
3.	Quraish cheese	17	28.	Sausage	10
4.	Sausage	30	29.	Liver	21
5.	Fresh minced meat	40	30.	Quraish cheese	25
6.	Fresh meat	26	31.	Fresh meat	16
7.	Fresh minced meat	25	32.	Fresh meat	20
8.	Fresh minced meat	35	33.	Sausage	20
9.	Sausage	40	34.	Kiri cheese	17
10.	Kiri cheese	15	25.	Fresh meat	19
11.	Fresh meat	18	36.	Liver	17
12.	Sausage	22	37.	Kiri cheese	20
13.	Liver	15	38.	Quraish cheese	14
14.	Sausage	36	39.	Kiri cheese	12
15.	Kiri cheese	12	40.	Liver	13
16.	Fresh meat	9	41.	Quraish cheese	18
17.	Liver	12	42.	Liver	16
18.	Fresh meat	15	43.	Fresh meat	20
19.	Chicken	18	44.	Kiri cheese	24
20.	Liver	11	45.	Quraish cheese	17
21.	Quraish cheese	14	46.	Chicken	25
22.	Fresh meat	22	47.	Fresh meat	18
23.	Fresh meat	26	48.	Quraish cheese	23
24.	Chicken	24	49.	Quraish cheese	27
25.	Quraish cheese	32	50.	Kiri cheese	24

DNA analysis of *Staphylococcus aureus*

To monitor food mediated contamination by *Staphylococcus aureus*. DNA extraction was used to isolate the DNA from fresh bacterial growth followed by species-specific PCR with standard amplification conditions to perform the specific DNA fragment **(Fig. 1)** using specific primers. Furthermore, the adopted PCR using species-specific PCR primer from DNA mixture was done. Results indicated that PCR able to amplify the specific DNA fragment for *Staphylococcus aureus*. DNA electrophoresis using agaros (1.5%) in the presence of GeneRulerTM DNA ladder Mix (100-1000-bp) was done.

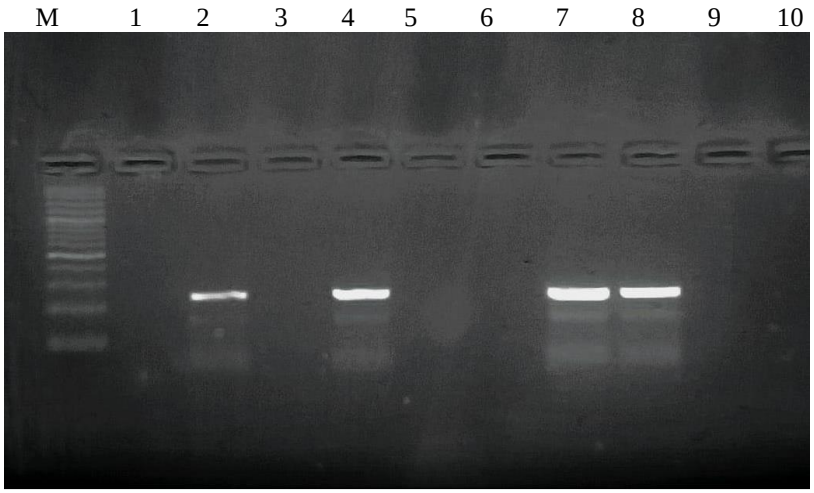

Fig.1. PCR-DNA fragments of *Staphylococcus aureus* at 270-bp, four positive samples (2,4,7,8) and four negative samples (1,3,5,6).

Sensitivity of *Staphylococcus aureus* strains to different antibiotics

Antibiotic susceptibility of *Staph. aureus* strains in **(Table 2)** revealed varying degrees of resistant patterns against the antibacterial agents. *Staph. aureus* was resistant to 75% of examined antibiotics. According to **(Xu *et al*., 2020)** who confirmed that any strain resistant to three or more antibiotics classified to multidrug resistant. This confirms that the tested strain was multidrug resistant.

Staph. aureus was resistant to 12 antibiotics, Gentamicin, Piperacillin/tazobactam, Cefotaxime, Imipenem, Meropenem, Ceftriaxone, Cefoxitin, Polymixin B, Erythromycin, Clindomycin, Amoxicillin/clavulanic Acid, and Amikacin.

These results are in agreement with those obtained by **Akanbi *et al*., (2017)** who found that the highest resistance of *S. aureus* recorded to ampicillin and penicillin 96.7% followed by rifampicin 80%. On the other hand, *Staph. aureus* was sensitive to Levofloxacin and Vancomycin.

Table 2. Sensitivity of *Staphylococcus aureus* to different antibiotics.

Antibiotics	Disc content µg/mL	*Staphylococcus aureus*	
		Inhibition zone (mm)	Interpretive standard of (I.Z)
Gentamicin	10	10.0±0.6	R
Piperacillin/tazobactam	100	12.0±0.5	R
Cefotaxime	30	10.0±0.3	R
Imipenem	10	12.5±0.2	R
Meropenem	10	10.5±0.4	R
Ciprofloxacin	10	14.0±0.2	I
Trimethoprim/sulphamethoxazole	23.75/1.25	15.0±0.1	I
Ceftriaxone	30	7.0±0.1	R
Levofloxacin	5	16.5±0.6	S
Cefoxitin	30	10.0±0.5	R
Polymixin B	300	11.4±0.4	R
Erythromycin	15	10.2±0.4	R
Clindomycin	2	13.0±0.1	R
Vancomycin	30	16.0±0.1	S
Amoxicillin/clavulanic Acid	30	10.5±0.7	R
Amikacin	30	16.0±0.5	R
Percentage of resistant			75.0%

R, Resistant; I, Intermediate; S, Sensitive; CLSI, Clinical Laboratory Standards Institute; N.I, No Inhibition; I.Z, Inhibition zone; I.S, Interpretive standard

Also, results are compatible with **Ali *et al*. (2007)** confirmed that *Staph. aureus* is resistant to a large group of antibiotics called the β-lactams, which include the penicillin and the cephalosporins. Methicillin resistant *Staph. aureus* (MRSA) isolates are often multiple-resistant to commonly used

antimicrobial agents including chloramphenicol, ciprofloxacin and tetracycline. Summing up, excessive use of antibiotics has accelerated the development of methicillin resistance, and resistance in *Staph. aureus* can be explained by mutation or modification of antibiotic targets, inactivation of β-lactam antibiotics by β-lactamase, a reduction in membrane permeability, or increased activity of efflux pumps **(Lade and Kim, 2021).**

Antibacterial activity of essential oils and N-chitosan against *S. aureus*

The antimicrobial efficacy of essential oils and N-chitosan was assessed against *Staphylococcus aureus*. The data obtained from (Table3) demonstrated that most essential oils exhibited antibacterial activity against the tested bacterial strain. Particularly, cinnamon oil displayed a significant level of antibacterial activity against *Staphylococcus aureus*, as evidenced by an inhibition zone of 24 mm. These results align with the findings of **Behbahani *et al.*, (2020)**, who similarly reported that *Cinnamomum zeylanicum* oil effectively inhibited the growth of pathogenic and spoilage bacteria, primarily Gram-positive strains such as (*Listeria innocua, Staphylococcus aureus, and Bacillus cereus*), compared to Gram-negative strains like (*Escherichia coli, Pseudomonas aeruginosa, and Salmonella typhi*).

In addition, obtained results revealed that the most of tested essential oils inhibited *Staph. aureus* with various effects and gave different values of inhibition zone. The minimum inhibition zone was recorded by Black bean (4 mm), while the maximum one was recorded by cinnamon (24mm) followed by clove (20 mm). These results are in agreement with **Adesiji *et al*. (2015)** who found that clove essential oil showed the highest level of antibacterial activity against *Salmonella* spp.

Regarding the effect of N-chitosan either individually used or combined with the essential oils, it was investigated that N-chitosan/Cinnamon, N-chitosan/clove mixture were the most effective (28, 26mm, respectively) against *Staph. aureus.*

Table 3. Antibacterial activity of some essential oils and N-chitosan against *Staphylococcus aureus*

Essential oils/N-chitosan	Inhibition zone (mm)	Essential oils/N-chitosan	Inhibition zone (mm)
Cinnamon	**20**	**Parsley/clove**	**7**
Clove	**24**	**Ginger/clove**	**6**
Thyme	**5**	**Garlic/clove**	**5**
Parsley	**6**	**black bean/clove**	**5**
Ginger	**5**	**Marjoram/clove**	**5**
Garlic	**5**	**Mint/clove**	**20**
Black bean	**5**	**N-chitosan/clove**	**26**
Marjoram	**5**	**Parsley/Zaatar**	**5**
Mint	**7**	**Ginger/Zaatar**	**6**
Nano chitosan	**10**	**Garlic/Zaatar**	**5**
Clove/Cinnamon	**16**	**Black bean/Zaatar**	**5**
Zaatar/Cinnamon	**7**	**Marjoram/Zaatar**	**6**
Parsley/Cinnamon	**6**	**Mint/Zaatar**	**5**
Ginger/Cinnamon	**8**	**N-chitosan/Zaatar**	**16**
Garlic/Cinnamon	**6**	**Ginger/parsley**	**5**
Black bean/Cinnamon	**7**	**Garlic/parsley**	**6**
Marjoram/Cinnamon	**6**	**Black bean/parsley**	**5**
Mint/Cinnamon	**18**	**Marjoram/parsley**	**5**
N-chitosan/Cinnamon	**28**	**Mint/parsley**	**5**
Zaatar/clove	**5**	**Mint/ginger**	**5**
N-chitosan/parsley	**5**	**N-chitosan/ginger**	**7**
Garlic/ginger	**5**	**Black bean/Garlic**	**6**
black bean/ginger	**6**	**Marjoram/Garlic**	**6**
Marjoram/ginger	**7**	**Mint/Garlic**	**5**
Mint/black bean	**5**	**N-chitosan/Garlic**	**7**
N-chitosan/black bean	**20**	**Marjoram/black bean**	**8**
Mint/Marjoram	**8**	**N-chitosan/Marjoram**	**5**

15:1833–1838.
15:1833–1838.

Conclusion and recommendations

The development of multidrug pathogenic bacteria resistance has been partly enhanced by the improper application of a certain drug for treating a disease. Resistance of bacteria to antibiotics is based on the ability of the bacterial cell to prevent any antibiotic or bactericidal effects. Treatment of some disease infections may be of minimal effect due to the development of bacterial resistance which is gradually developed over time. Antibiotic

susceptibility of *Staph. aureus* strains revealed varying degrees of resistant patterns against the antibacterial agents. Essential oils can be used in the food preservation industry because they are involved in plant growth and development. Because it contains natural antimicrobial ingredients.

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