Impact of *Nigella sativa* and Clove Oils on Cell Wall Genes Expression in Multidrug Resistant *Staphylococcus aureus*

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Abstract

Antimicrobial resistance of methicillin resistant *Staphylococcus aureus* (MRSA) became on the top list of the serious problems that have a negative impact on public health. Therefore, this study aimed to determine the *in vitro* antibacterial activities of *Nigella sativa* (*N. sativa*) and clove essential oils against multidrug resistant (MDR) methicillin resistant *Staphylococcus aureus* (MRSA) isolates recovered from different sources with a special reference to their role on the expression of penicillin binding protein (*pbp*2) and muscle ring finger (*mur*F) genes. Antibiotic susceptibility testing of 51 staphylococcal isolates comprising 34 *Staphylococcus aureus* (*S. aureus*) and 17 coagulase negative staphylococci (CoNs) revealed a highest sensitivity against vancomycin (100%) and marked resistance patterns against β-lactam (beta-lactam) antibiotics. The results revealed that 97.1% of *S. aureus* isolates demonstrated a high level of MDR pattern, being resistant to more than 3 antibiotics of different classes. The *in vitro* antibacterial effects of clove and *Nigella sativa* essential oils against 11 MDR isolates using disc diffusion method indicated that both oils exhibited strong inhibitory efficiencies with inhibition zone diameters up to 45 and 20 mm, respectively. Besides, broth microdilution test of both essential oils revealed maximum activities against the tested strains with minimum inhibitory concentrations (MICs) up to 0.5 and 8 μg/mL for clove and *N. sativa* oils, respectively. Quantitative RT-PCR (real time polymerase chain reaction) analysis revealed the effective role of *N. sativa* and clove oils on the down-regulation of *S. aureus mur*F and *pbp*2 genes. In conclusion, the above findings highlight the promising antibacterial functions of *N. sativa* and clove essential oils in the treatment of emergent resistant *S. aureus* infections.

Keywords: *Staphylococcus aureus*, *N. sativa*, clove oil, microdilution, MDR.

Introduction

Staphylococcus aureus (*S. aureus*) is a common pathogen associated with serious community and nosocomial infections as well as being a major cause of food poisoning due to the production of enterotoxins [1].

The occurrence of *S. aureus* intoxication depends on its capability to survive and multiply under a variety of conditions in addition to the production of extracellular toxic compounds such as haemolysins, nuclease, coagulase, lipase and enterotoxins. In both human and animal medicine, methicillin resistant *Staphylococcus aureus* (MRSA) are among the most threatening bacteria involved in various infections [2]. Staphyloccal infections are considered public health problems worldwide. Therefore, the development of strategies to control the survival and growth of *S. aureus* is of a great interest.

Increased demand for safe and natural antimicrobials has motivated researchers to investigate the antimicrobial efficacies of many natural compounds against pathogenic microorganisms especially *S. aureus* [3]. Essential oils are complex mixtures of volatile molecules produced by the secondary metabolism of aromatic plants. Understanding the antibacterial actions of essential oils is of a great importance for their application in pharmaceutical industries. The antibacterial activities of essential oils' compounds appear

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to be due to their lipophilic nature, which allows their accumulations in biological membranes [4]. Consequently, they can disrupt the cytoplasmic membrane, increase its permeability to proteins and other cell contents and inactivate membrane-embedded enzymes [5,6].

One of the most important essential oils is *Nigella sativa,* which has several therapeutic effects such as prevention of cancer, anti-inflammatory, analgesic and antibacterial effects [7]. Most of these activities have already been attributed to thymoquinone, which is the major component of the essential oils of this seed.

Clove is another important agent which is used as a flavouring agent. The essential oils of clove have been known to posses various antibacterial and antioxidant properties due to the presence of 70-90% of their components as eugenol [8,9].

The expression of high level methicillin resistance in MRSA requires not only the presence and expression of the *mec*A gene, but the functioning of a surprisingly large number of additional auxiliary genes including *S. aureus* penicillin binding protein (*pbp*2) structural gene [10]. Moreover, the muscle ring finger (*mur*F) is a cell wall biosynthetic gene which is also essential for *S. aureus* in methicillin resistance [11].

It has long been known that certain cell-wall targeting antibiotics can influence the expression of staphylococcal genes encoding cell wall repair enzymes, but a complete understanding of the influence of essential oils on staphylococcal cell wall genes expression is currently lacking. Therefore, owing to the prominent effects of essential oils, the present study focused on studying the antibacterial activities of *N. sativa* and clove oil against multidrug resistant MRSA isolated from different sources in addition to assessing the impact of sub-inhibitory concentrations of these oils on the expression of two cell wall biosynthesis-associated genes, *mur*F and *pbp*2.

Material and Methods

Clinical specimens

One hundred and fifty-five samples were collected from animals (n=115) and human

(n=40) sources from different areas at Sharkia Governorate, Egypt. The samples from animal origin included milk from cows showing clinical mastitis (n=100) and food specimens (n=15) [sausage (n=6), burger (n=4) and minced meat (n=5)], while those from human subjects were urine (n=7), pus (n=28) and sputum (n=5). Informed consents were obtained from all the participants in this study and it was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt. The samples were transported in an ice box and microbiological examination was performed within 24 h.

Phenotypic characterization of staphylococcal isolates

Primary isolation of staphylococcal isolates was carried out onto mannitol salt agar (Oxoid, Hampshire, England, UK). Single, well-isolated colony from overnight cultures was subcultured onto blood agar for testing their beta hemolysis and milk agar for pigment production [12]. The isolates were identified by standard bacteriological methods including cultural characteristics, Gram's stain and biochemical tests such as catalase and tube coagulase tests [13].

Antimicrobial resistance patterns

Resistance of all staphyloccal isolates to various antibiotics was evaluated by disc diffusion test according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [14]. Antibacterial discs (Oxoid, Hampshire, England, UK) containing lincomycin (2 µg), chloramphenicol (30 µg), gentamycin (10 µg), tobramycin (10 µg), erythromycin (15 µg), ampicillin (10 µg), ciprofloxacin (5 µg), amoxicillin/ clavulanic acid (20/10µg), trimethoprim/ sulphamethoxazole (1.25/23.75 µg), oxacillin (1 µg), amoxicillin (25 µg) and vancomycin (30 µg) were used. Identification of MRSA strains was performed by molecular detection of *mec*A gene in oxacillin-resistant *S. aureus* isolates using PCR assay [1].

Antibacterial activities of the essential oils

Preliminary screening of the antibacterial activities of *N. sativa* (Star chemical pharmaceutical) and clove oils (MEBACO)

was done on 11 multidrug resistant (MDR) *S. aureus* isolates with respect to their resistance patterns by the disc diffusion method as previously described [15]. It was performed using 18 h bacterial cultures adjusted approximately to 10^8 CFU/mL in 10 mL Müller Hinton broth. The bacterial suspension was spread over the plates of Müller-Hinton agar (Oxoid, Hampshire, England, UK) using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. The used essential oils were dissolved in 10% aqueous dimethyl-sulfoxide (DMSO, Oxoid, Hampshire, England, UK)) under aseptic conditions. Empty Whatman sterile discs no. 5, 6 mm diameter (Oxoid, Hampshire, England, UK) were impregnated with 10 μl of different concentrations (1:1, 1:2, 1:4, 1:8 and 1:16) of the respective essential oils prepared in DMSO and were then placed on the agar surface inoculated with the bacterial culture. A sterile disc moistened with aqueous DMSO was placed on the seeded Petri-plate as a vehicle negative control. The plates were left for 30 minutes at room temperature to allow the diffusion of oils and then were incubated at 37ºC for 24 h. After the incubation period, the zones of inhibition around the discs were measured and recorded.

The minimum inhibitory concentrations (MIC) of *N. sativa* and clove oils were determined using the broth micro-dilution method [16]. The concentration of stock solution of the used essential oils was adjusted at 1024 μg/mL DMSO and the inoculum size was approximately prepared as 5×10^5 CFU/mL. A double fold serial dilution was made for each essential oil in custom-designed 96-well panels (Corning, New York, USA) starting from a concentration of 1024 μg/mL. Controls of broth medium with the microorganism or the essential oil alone were included as positive and negative controls, respectively. The microplates were incubated at 37ºC for 24 h. The first dilution with no microbial growth was recorded as MIC of the essential oil. Subsequently, aliquots from each well were plated onto Müller Hinton agar and incubated at 37ºC for 24 h to determine the MBC (minimum bactericidal concentration) of the oils. The plates were checked for growth of bacterial colonies and MBC was evaluated as the lowest oil concentration at which no growth was observed on the plates.

Impact of essential oils on the expression of *murF* and *pbp2* genes

Eleven MDR *S. aureus* isolates grown in Müller Hinton broth were supplemented with 0.5× MIC of each of *N. sativa* and clove oils. After 24 h incubation, aliquots of suspended cells containing approximately 10^8 CFU/mL were centrifuged and the supernatant was decanted and the pellet was used immediately for RNA extraction [17,18]. Total RNA extraction was accomplished according to the protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). Quantitative RT–PCR (real time polymerase chain reaction) was performed in triplicates using one-step RT-PCR kit with SYBR green (Qiagen, Germany, GmbH) in the Stratagene MX3005P real time PCR machine. The expression patterns of *murF* and *pbp2* genes were measured by qRT–PCR using primers and cycling conditions detailed previously [19]. The relative quantitation of mRNA expression in each sample was normalized to the constitutive expression of the 16S rDNA housekeeping gene. The transcription levels were analyzed using the comparative $\Delta\Delta C_t$ method [20].

Statistical analysis

In order to assess the influence of *N. sativa* and clove MIC and MBC on *S. aureus* isolates from both human and animal sources, independent sample t-test was used following $\log_2$ transformation. Analysis was done using Statistical Package for Social Sciences version 22.0 (IBM Corp., Armonk, NY, USA). Results were reported in means ± SEM (Standard Error of Mean), t-statistic and probability values. The value of $P < 0.05$ was used to indicate the statistical significance

Results

Isolation and phenotypic characterization of Staphylococcus species

Fifty-one staphylococci isolates (32.9%) were recovered from 155 samples collected from different sources. Staphylococci were identified by conventional methods; all the 51 strains were Gram-positive cocci, non-motile, non-spore forming, arranged in grape-like clusters, grown onto mannitol salt agar,

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fermentative and catalase test positive, therefore, being identified as staphylococci. Among them, 34 isolates (21.9%) were tube coagulase test positive, β-hemolytic and produced the characteristic golden yellow pigments, therefore, they were considered as coagulase positive *S. aureus*, while the other 17 isolates (10.9%) strains were identified as coagulase negative *S. aureus* (CoNs).

Table (1): Antimicrobial susceptibility patterns of staphylococcal isolates

Antibiotic	Percentage of staphylococcal isolates (%)			
	S. aureus (n = 34)		CoNs (n = 17)	
	S	R	S	R
Vancomycin	100	0	100	0
Sulphamethoxazol/trimethoprim	88.2	11.8	76.5	23.5
Chloramphenicol	76.5	23.5	76.5	23.5
Ciprofloxacin	67.6	32.4	100	0
Amocillin/clavulanic acid	50	50	82.4	17.6
Gentamicin	47.1	52.9	88.2	11.8
Tobramycin	26.5	73.5	88.2	11.8
Erythromycin	23.5	76.5	52.9	47.1
Oxacillin	2.9	97.1	5.9	94.1
Ampicillin	2.9	97.1	17.6	82.4
Amoxicillin	2.9	97.1	23.5	76.5
Lincomycin	2.9	97.1	0	100

CoNs: coagulase negative staphylococci , S: Sensitive, R: resistant

Antimicrobial resistance patterns

The *in vitro* antimicrobial resistance profiles of all staphylococci isolates are summarized in Table (1). Antibiogram analysis revealed that the highest sensitivities of *S. aureus* were reported against vancomycin (100%), followed by Sulphamethoxazol/trimethoprim (88.2%) and chloramphenicol (76.5%), while the highest rates of resistance were observed against β-lactam antibiotics and lincomycin (97.1%). All 33 isolates (97.1%) which appeared oxacillin resistant phenotypically with antimicrobial susceptibility test were positive for the presence of *mec*A gene and were therefore confirmed as MRSA strains.

Concerning the CoNs, vancomycin and ciprofloxacin showed the maximum overall sensitivity rates against the isolates (100%, each), followed by gentamycin and tobramycin (88.2%, each). On the other hand, high levels of resistance were recorded for lincomycin (100%), followed by oxacillin (94.1%).

As apparent from Table (2), the majority of *S. aureus* strains (n=33, 97.1%) demonstrated multidrug resistance (MDR) pattern to 3 or more antibiotics of different classes. Additionally, 88.2% of CoNs showed the MDR pattern.

Table (2): Resistance percentages of staphylococcal isolates to one or more antimicrobials

No. of antibiotics	Percentage of staphylococcal isolates (%)	
	S. aureus (n=34)	CoNs (n=17)
1	0	0
2	2.9	11.8
3	2.9	11.8
4	5.9	11.8
5	14.7	11.8
6	17.6	11.8
7	8.8	11.8
8	20.6	5.9
9	17.6	5.9
10	8.8	0

CoNs: coagulase negative staphylococci

Antimicrobial activities of N. sativa and clove essential oils

Preliminary screening of antibacterial activities of *N. sativa* and clove essential oils against 11 MDR isolates was carried out by disc diffusion method. The growth inhibition zone diameters of the tested MRSA isolates are presented in Table (3). It was indicated that the concentrations of the essential oils were directly proportional to their inhibitory activities. There was no inhibition of *S. aureus* growth with vehicle control (DMSO). Concerning the antibacterial activities of the investigated essential oils, it was demonstrated that clove oil, followed by *N. sativa* essential oils showed high antibacterial activities against the tested isolates with inhibition zones diameters ranged from 9 to 45 mm and 1 to 20 mm, respectively.

Table (3): Inhibition zone diameters (mm) of different concentrations of *N. sativa* and clove essential oils against 11 MDR *S. aureus*

S. aureus isolate code No.	Inhibition zone diameters (mm)						
	N. sativa		Clove				
	1:1	1:2	1:1	1:2	1:4	1:8	1:16
45 Sg	16	12	20	16	12	9	-
15 M	17	11	32	28	22	15	-
1 M	13	9	40	34	25	20	-
13 M	18	13	45	35	27	21	10
40 U	20	16	35	30	22	17	10
59 Ps	13	9	30	28	20	18	-
58 Ps	18	11	40	30	24	21	17
60 Ps	16	1	35	29	20	14	-
55 Ps	15	8	25	16	12	10	-
57 Ps	16	12	26	21	18	13	-
61 Ps	15	10	25	15	12	9	-

Sg: Sausage, M: Milk, U: Urine, Ps: Pus, -: Resistant

Referring to the large inhibition zones observed with disc diffusion assay for *N. sativa* and clove essential oils, the MIC and MBC values for these compounds were determined with broth microdilution assays and their results were reliable with the diameters of inhibition zones observed with disc diffusion method. The results confirmed the antimicrobial activities of *N. sativa* and clove oils with higher MIC values for clove (up to 0.5 µg/mL), followed by *N. sativa* oils (up to 8 µg/mL) (Table 4). Statistical results showed that both *N. sativa* and clove extracts had significant inhibitory and bactericidal effects ($P<0.05$) against all studied *S. aureus* isolates from both human and animal sources.

Table (4): Minimum inhibitory and bactericidal concentrations (µg/mL) of *N. sativa* and clove essential oils against *S. aureus* isolates

S. aureus isolate code No.	*N. sativa* (µg/mL)		Clove oil (µg/mL)	
	MIC	MBC	MIC	MBC
45 Sg	64	128	32	64
15 M	16	32	1	2
1 M	64	128	0.5	1
13 M	32	64	0.1	0.25
40 U	8	16	1	2
59 Ps	64	128	1	2
58 Ps	16	32	0.5	1
60 Ps	32	64	0.5	1
55 Ps	32	64	4	8
57 Ps	32	64	2	4
61 Ps	16	32	4	8

Sg: Sausage, M: Milk, U: Urine, Ps: Pus, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

Regulation of *mur*F and *pbp*2 genes expression

The effects of sub inhibitory concentrations of *N. sativa* and clove essential oils on the transcriptional modulation of cell wall biosynthesis genes (*pbp*2 and *mur*F) of *S. aureus* were assessed by quantitative RT-PCR. Both tested virulence genes were constitutively expressed in all isolates with different expression patterns indicating various responses. Relative expressions (fold-change or fold-difference of expression levels) of the representative genes were compared to those within the control untreated isolates, which are assigned a value of 1.

The results showed that both tested genes were found to be down regulated after exposure to 0.5x MIC of *N. sativa* and clove essential oils (Table 5). The lowest mRNA expression levels were ubiquitously detected in the isolates exposed to clove oil, where the transcriptional levels of *pbp*2 and *mur*F genes were remarkably decreased up to 0.2952-and 0.3186-fold, respectively. Likewise, the use of *N. sativa* oil also repressed the expression levels of the *pbp*2 and *mur*F genes up to 0.4293- and 0.5987-fold, respectively.

Table (5): Relative expression of *pbp*2 and *mur*F of *S. aureus* after treatment with sub-MIC of *N. sativa* and clove essential oils

S. aureus isolate code No.	Relative expression[a]			
	*pbp*2		*mur*F	
	N. sativa sub-MIC	clove sub-MIC	*N. sativa* sub-MIC	clove sub-MIC
45 Sg	ND	0.9539	ND	0.5664
15 M	0.4293	0.9659	0.8526	0.9596
1 M	ND	0.7900	ND	0.9138
13 M	ND	0.7320	ND	0.8526
40 U	0.4475	0.3686	0.5987	0.3186
59 Ps	ND	0.7371	ND	0.8586
58 Ps	0.9931	0.5285	0.9931	0.5285
60 Ps	ND	0.9593	ND	0.8706
55 Ps	ND	0.2952	ND	0.7900
57 Ps	ND	0.7371	ND	0.9302
61 Ps	0.6417	0.7792	0.8527	0.9202

Sg: Sausage, M: Milk, U: Urine, Ps: Pus, *pbp*2: Penicillin binding protein, *mur*F: Muscle ring finger, ND: Not done
[a] Values represent the fold change in comparison with the transcription level of the control untreated isolates, which is assigned a value of 1.0

Discussion

There is a growing concern about the rapid rise in the resistance of *S. aureus* to antimicrobial agents [21]. Therefore, the present study aimed to detect the antibacterial activities of *N. sativa* and clove oils against a group of MDR *S. aureus* isolates from different sources.

Methicillin resistant *S. aureus* are prevalent worldwide and are an important cause of nosocomial infections, resulting in increased morbidity and mortality in the hospital settings [22]. Knowledge of the pattern of antibiotic resistance among *S. aureus* isolates is very important both chemically and epidemiologically [23]. Analyzing the antibiogram results of *S. aureus* herein revealed that vancomycin showed a maximum overall sensitivity against all the tested isolates (100%) as was recorded in a previous report in India [24]. These findings confirm that vancomycin remains the drug of choice in the treatment of infections caused by organisms resistant to β-lactame antibiotics [25]. Another observation in the present study was the maximum resistance rates of all the tested isolates against β-lactam antibiotics. A similar trend in the level of β-lactam resistance was reported in Ethiopia [26]. The majority of *S. aureus* isolates in the present study were MDR (97.1%). This high prevalence corroborated an earlier study in India [27].

Molecular confirmation of oxacillin resistance showed the amplification of *mec*A gene in 33 (97.1%) of the MDR isolates confirming them as MRSA. This was however higher than the studies conducted in Ilorin, Nigeria (34.7%) [28], France (6%), Ireland (5%) and United Kingdom (2%) [29]. However, a high prevalence rate of MRSA (83%) was reported in Pakistan [30]. This confirms the high regional variations in the findings from different countries and cities.

Medicinal plants are considered rich sources of antimicrobial agents [31]. *N. sativa* has gained a special interest as a medicinal plant and there has been several reports dealing with the antimicrobial activities of its crude extract and some of its constituents [32]. In this study, *N. sativa* essential oil showed a marked inhibitory activity against MRSA tested as was previously observed [33].

Clove oil is known to be used as an alternative to conventional antibiotic therapy due to its antibacterial activity [34]. Cloves are strongly pungent due to their high content of euganol, which is known to inhibit growth of Gram-positive and negative bacteria [30]. Herein, clove oil showed higher antibacterial activities against tested MRSA. These results are in harmony with those of another study conducted in Australia, where clove essential oil exhibited a great antibacterial effect against a large number of MRSA isolates [35].

The present results indicated that *mur*F and *pbp2* transcriptions were strongly suppressed with a down regulation when *S. aureus* exposed to 0.5x MICs of both *N. sativa* and clove oils. The results presented here verified other observations indicating that other plant extracts at sub-inhibitory concentrations induced a cell wall stress response in *S. aureus* that is modulated by the two-component VraSR system, which in turn enhances the transcription of genes encoding cell wall repair enzymes [19]. Nevertheless, the mechanisms by which *S. aureus* regulates virulence gene expression are extremely complicated and need further molecular investigations.

Conclusion

In conclusion, *N. sativa* and clove essential oils showed maximum antibacterial activities against MDR MRSA isolates with effective roles on the down-regulation of *mur*F and *pbp*2 genes. The above property could be exploited in developing these oils as anti-pathogenic agents against drug resistant and pathogenic *S. aureus*.

Conflict of interest: All the authors have no conflict of interest to declare.

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الملخص العربي

تأثير زيوت حبة البركة والقرنفل على تعبير جينات الجدار الخلوى للمكورات العنقودية الذهبية المتعددة المقاومة للمضادات الحيوية

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لقد أصبحت المقاومة الميكروبية للمكور العنقودى الذهبى المقاوم للميثيسيلين على قمة المشاكل الخطيرة المهددة للصحة العامة، لذلك تهدف هذه الدراسة الى تحديد الأنشطة المضادة للبكتريا لزيوت حبة البركة والقرنفل ضد المكورات العنقودية الذهبية المتعددة المقاومة للمضادات الحيوية والمعزولة من مصادر مختلفة معمليا وبإشارة خاصة الى دورهم على تعبير جينات murF و pbp2 . لقد أثبت اختبار القابلية للمضادات الحيوية أن ٥١ عزلة من عزلات الميكروب العنقودى (٣٤ من الميكروب العنقودى الذهبى و ١٧ من الميكروب العنقودى سالب التخثر) أعلى حساسية للفانكومايسين وانماط مقاومة واضحة ضد مضادات البيتا لاكتام. ومن الجدير بالذكر ان ٩٧.١ % من عزلات المكور العنقودى الذهبى اظهرت مستوى عالى من المقاومة المتعددة للمضادات الحيوية بكونها مقاومة لاكثر من ٣ مضادات حيوية من مجموعات مختلفة. وقد أوضحت نتائج التأثيرات المعملية المضادة للبكتريا لكلاً من زيت القرنفل وزيت حبة البركة ضد ١١ عزلة مقاومة للعديد من المضادات الحيوية باستخدام طريقة انتشار القرص أن كلاهما ذو تأثير مثبط قوى وأن منطقة التثبيط وصلت الى ٤٥ و ٢٠ مملليميتر على التوالى. وإلى جانب ذلك فقد أثبت اختبار التخفيف الدقيق للسوائل لكلاً من الزيتين أقصى نشاط ضد العترات المختبرة بحد أدنى مثبط يصل الى ٠.٥ و ٨ ميكروجرام/ملليليتر لزيت القرنفل و زيت حية البركة على التوالى. وقد اثبتت نتائج تحليل تفاعل انزيم البلمرة الناسخ العكسى والكمى دور زيوت حبة البركة والقرنفل الفعال فى تثبيط جينات murF و pbp2 فى المكور العنقودى الذهبى. وفى النهاية فقد ألقت النتائج السابقة الضوء على دور الزيوت الأساسية لحبة البركة والقرنفل الواعد كمضاد للبكتريا فى علاج العدوى الناشئة عن الميكروب العنقودى الذهبى المقاوم.