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Research Article
Bacteriostatic and Bactericidal Activity of Deer Musk on Multidrug Resistance Bacteria

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Abstract

Background and Objective: Infecting agents (e.g., bacteria, fungi, virus and parasite) have comprised high levels of multidrug resistance (MDR) with increased morbidity and mortality; so the main aim of this study was to investigate and demonstrate the antimicrobial activity of deer musk on multidrug-resistance bacteria and to proof that musk had a bacteriostatic and bactericidal effects against MDR bacteria. **Materials and Methods:** Deer musk evaluated 11 multi-drug resistance (MDR) species were selected, namely, *Staphylococcus capitis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Enterococcus faecalis* the MDR strain were tested by means of disk diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by the time-kill method recommendation by CLSI. In addition, the antimicrobial susceptibility of 8 commonly used antimicrobials was examined on the same MDR bacterial strains. **Results:** The minimum inhibitory concentration MIC and MBC values were <2% (v/v) against all MDR strains except *Pseudomonas aeruginosa*, but the deer musk has bacteriostatic and bactericidal activity against *Pseudomonas aeruginosa* at >2% (v/v), in addition changes was observed in the morphological form of the bacterial colonies in of most of MDR bacteria such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa* and *Klebsiella baumanni* and indicating that the musk had an effect on bacterial cellular membranes. **Conclusion:** The findings of this study indicated that deer musk has a bacteriostatic and bactericidal effects on the growth of all tested MDR bacteria.

Key words: Musk, antimicrobial drug, multidrug resistance, antimicrobial susceptibility, infecting agent, minimum inhibitory concentration, minimum bactericidal concentration

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Musk has been used since ancient times as a medicine and fragrance. It is extracted from a small sac that is formed secondary to a fold in the genitalia of deer, the extracted substances are dried for use as a fragrance, which is usually brown to black in color. Muscone (3-methylcyclopentadecan-one-1) is the active component in musk, which also is the cause for the odor and has medicinal properties. Other major components are steroids and lipids. In addition, it could contain paraffin, triglycerides, waxes, mucopyridine and nitrogenous substances[1]. Natural musk has an inhibitory effect due to its composites and metabolic yields such as alkaloids, flavonoids, sterols and antibiotics. These composites may disrupt bacterial and fungal cells through raising the permeability of cell membranes causing a leak important substances that led to cell death. In addition, it may inhibit the microbes via preventing the synthesis of nucleic acids causing construction of abnormal proteins. Volatile oils in musk could be the reason for its inhibitory effect[2,3], the previous study has shown that musk has a fungistatic, fungicidal, parasitostatic, bactericidal and bacteriostatic effect. The previous study has shown a bactericidal and bacteriostatic effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa*[1,4-8]. Today, antibiotic resistance exists worldwide. Infections caused by antibiotic resistant bacteria increased risk and expend more health-care incomes than infections with non-resistant bacteria. According to World Health Organization (WHO) recently updated resistance in *Klebsiella pneumoniae* common intestinal bacteria to the last choice treatment (carbapenem antibiotics) has found in all regions of the world. As a result of the resistance, carbapenem antibiotics do not work in more than half of people treated for *Klebsiella pneumoniae* infections. Fluoroquinolone resistance in *Escherichia coli* is very widespread[9]. There are countries in many parts of the world where this treatment for urinary tract infections is now ineffective in more than half of patients. The rising frequency of extended spectrum beta-lactamase (ESBL) producing isolates from Saudi Arabia also recognized. Where particular establishments had 65% ESBL rates among *Klebsiella pneumoniae* and 29% ESBL rates among *Escherichia coli*. Consequently, these growth rates have been related to many reported mortality and outbreaks[10]. Resistance to colistin, which is last alternative treatment for infections caused by Enterobacteriaceae, has lately been identified in some countries and regions, making untreatable infections. A literature review of multidrug resistance in Gram-negative bacilli presented a considerable growth in the frequency of carbapenem-resistant in Saudi Arabia over the last decade. Carbapenem resistant *Acinetobacter baumanni* from Saudi Arabia have also increased dramatically over the years[9,11-15].

In addition, third generation cephalosporin antibiotics were a failure for the treatment of gonorrhea. A Saudi national surveillance on Gram-positive cocci established that 33% of *Streptococcus pneumoniae* are resistant penicillin G, 26% are resistant to erythromycin and 32% of *Staphylococcus aureus* are methicillin-resistant[11-13,15-17]. This study was carried out to evaluate the antibacterial effect of natural Musk on MDR bacteria and expand by evaluating the effect on the above-mentioned widespread pathogenic bacteria.

MATERIALS AND METHODS

Total time required to conduct this study about 4 months from September-December, 2017. Acquisition and analysis of data, drafting of article and revision January-April, 2018.

Musk: Black musk were provided as an oily solution commercially available at Abd-El Samad El Korashy Official stores treated in natural environmental conditions at temperature 25-28°C.

Microbial strain: Overnight cultures of the following 11 clinical MDR bacteria were used throughout the study: *Staphylococcus capitis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Actinobacter baumanni*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Enterococcus faecalis* were obtained from Bio House Medical Lab. The inoculum prepared by making a direct NB (Oxoid, UK) suspension of isolated colonies selected from an 18-24 h NA (Oxoid, UK) agar plate. Adjusted the suspension turbidity spectrophotometrically 0.1 at 625 nm equivalent to a 0.5 McFarland standard, this results in suspension containing approximately 1.5×10^8 CFU mL^{-1}.

Antibiotics: The antibiotics-standard Doxycycline (DO, 30 µg), Cefotaxime (CXM, 30 µg), Amoxicillin/clavulanic acid (AMC, 30 µg), sulfamethoxazole trimethoprim (SXT, 25 µg), Azithromycin (AZM, 15 µg), Penicillin G (PG, 10 units), Norfloxacin (NOR, 10 µg) and Clindamycin (DA, 10 µg) were used as a reference antibiotics in the disk diffusion method.

Antimicrobial activity of musk by agar disk diffusion method: Antimicrobial activity of deer musk at different concentrations on the growth MDR bacterial strains was carried out and determined by disk diffusion method protocol suggested in the CLSI[18]. Diffusion in solid media method was used for 11 MDR species with musk and it used with the antibiotics-standard as a reference antibiotics against a giving MDR strain. Commercially available antimicrobial disks and the filter paper discs (about 4 mm in diameter) impregnated with 50 µL of musk at the concentrations of (100% (v/v) and 10% (v/v)) stock solution of the musk to be used was freshly dissolved in 10% dimethyl sulfoxide (DMSO; BDH, UK) to obtain a stock concentration of 10% (v/v) applied on the surface of MHA (Oxoid, UK) agar plates were inoculated with a standardized inoculum of the test MDR bacteria using a sterile cotton swab by pressing slightly. The plates were incubated for overnight at 37°C and the diameters of inhibition zone were measured.

Minimum inhibitory concentrations: The MIC of musk was determined using a broth microdilution method on 11 MDR strains. In general, the protocol used followed that recommended by CLSI. Stock solution of the musk to be used was freshly dissolved in 10% dimethyl sulfoxide (DMSO; BDH, UK) in sterile MHB broth to obtain a stock concentration of 10% (v/v). Serial two-fold dilutions were prepared in 96-well microplates (Falcon, corning-life sciences, USA). This was serially diluted two fold to obtain concentration ranges of 10% (v/v) to 0.01% (v/v). An overnight culture of the 11 MDR microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale which each bacterial suspension was standardized at a cell density of 1.5×10^8 (CFU mL^{-1}). After well-mixing, the 96-well microtitration plates have incubated for 18 h at 37°C. Two columns were left with the purpose to be used as a positive control (without antibiotic and inoculated with MDR) and a negative control (without antibiotic and not inoculated with the bacterium). Each MIC assay was performed thrice and the MIC value was considered as the lowest concentration of the musk causing an absence of bacterial growth and preventing visible turbidity and the MIC assay included ampicillin as a reference antibiotic.

Bactericidal activity of musk by kill-time test: Bactericidal component of musk effect determined by kill-time test. The geometric dilution method that has been standardized and described in CLSI[18]. In the presence of a selected concentration of the musk and determined by measuring the number of viable bacteria at various time intervals. The resulting graphic depiction is known as the time-kill curve. In this study, the killing curve method was prepared in duplicate, the 96-well microplates were used to determine the MBC. Two Gram-negative bacteria were selected one of them had the highest MIC value and two Gram-positive bacteria that had the lowest MIC values from all 11 MDR bacteria. About 10 µL of musk dilution added to 90 µL MHB in the first well containing an inoculum of around 10^6 CFU mL^{-1} of bacteria. In order carryover of musk dilution, 10-fold dilution series were prepared in sterile MHB and 10 µL of these dilutions from and the initial broth well were then removed and spread onto musk-free MH agar and incubated for 24 h at 35°C. The colony-forming units (CFU) were counted at fixed incubation time's interval (30 min, 1, 2, 4 and 24 h). The minimal bactericidal concentration (MBC) was defined as that yielding growth of fewer than five colonies (>99% killing), time-killing curves was prepared at 37°C in duplicate at musk concentration 10 and 0.16% (v/v) and it used another row without musk and inoculated with the bacterium as a growth control.

RESULTS

Antimicrobial effect of musk by disk diffusion method: The antimicrobial effects of musk and reference antibiotics (RA) by using disc-diffusion method against the tested microorganism are presented in Table 1 and 2, respectively. According to the results a similar pattern of antimicrobial activity was observed in all of the bacteria and presented strong antimicrobial effect in wild types and MDR bacteria and the best result of antimicrobial activity was shown in all tested microorganism at 100% (v/v) and the bacteria reduced susceptibility to musk with the lowest concentration 10% (v/v). The maximum inhibition zone was observed in *Streptococcus agalactiae*. According to the antimicrobial susceptibility patterns shown in Table 1, *Pseudomonas aeruginosa* exhibited reduced susceptibility to musk. The inhibition zones of musk effect at 100 and 10% (v/v) against *Streptococcus agalactiae* and *Staphylococcus aureus* illustrated in Fig. 1.

Table 1: *In vitro* antimicrobial activity of musk to several micro-organisms determined by diameter (mm) zone of inhibition

Microbial strains	Musk 10% v/v	Musk 100% v/v
Staphylococcus aureus	14	23
Klebsiella pneumoniae	11	18
Escherichia coli	9	17
Enterococcus faecalis	12	18
Actinobacter baumanni	9	14
Pseudomonas aeruginosa	8	12
Serratia marcescens	11	13
Staphylococcus capitis	13	21
Proteus mirabilis	10	16
Streptococcus agalactiae	21	43
Streptococcus pyogenes	11	23

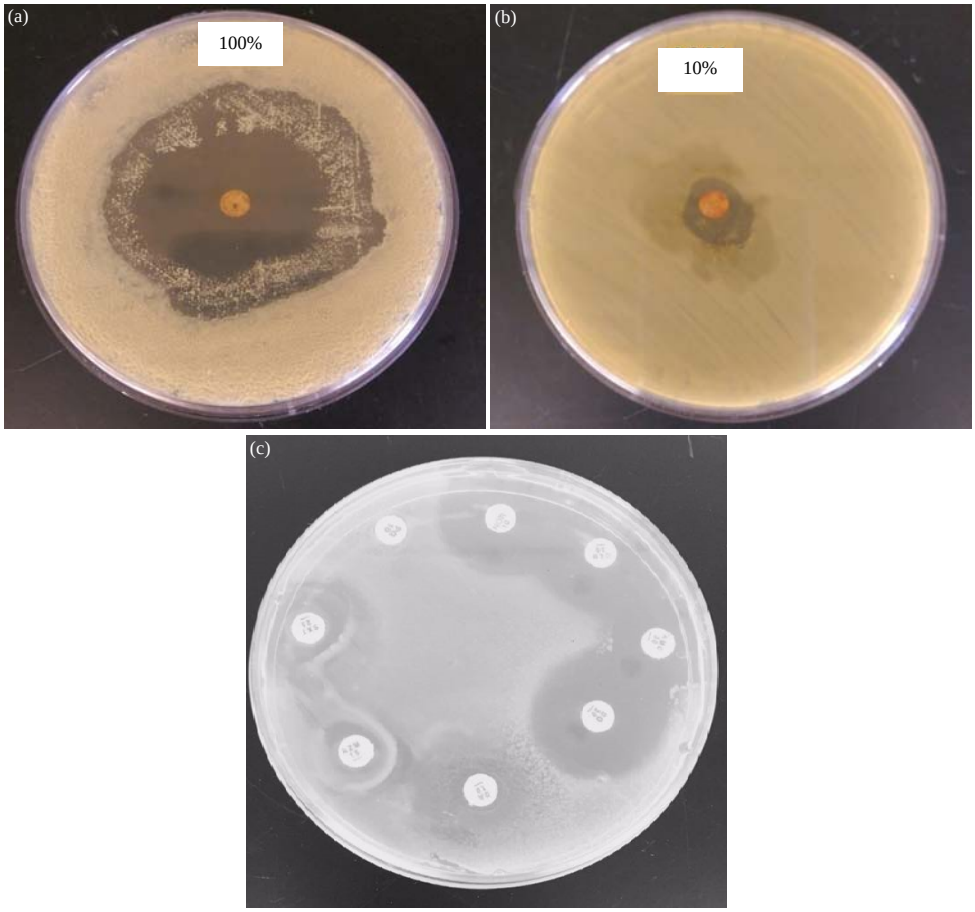

Fig. 1(a-c): Effect of musk treatment by disk diffusion method at (a) Concentration 100% (v/v), (b) Concentration 10% against *Streptococcus agalactiae* and (c) References antibiotic treatment against *Staphylococcus aureus*

Table 2: Antimicrobial effect of standard antibiotics comparing to deer musk against MDR bacteria by disk-diffusion method

	Antimicrobial*								
Microorganism	DO	CXM	AMC	SXT	AZM	PG	NOR	DA	Musk
Escherichia coli	R	R	S	R	S	R	R	R	S
Enterococcus faecalis	S	R	R	R	R	S	S	R	S
Klebsiella pneumoniae	S	S	S	S	S	R	S	R	S
Actinobacter baumanni	S	R	S	R	S	S	S	S	S
Staphylococcus aureus	R	S	R	S	S	R	S	S	S
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	S
Serratia marcescens	S	R	R	S	S	R	S	R	S
Staphylococcus capitis	S	R	R	R	R	S	S	R	S
Proteus mirabilis	R	S	R	R	S	S	S	S	S
Streptococcus agalactiae	R	S	R	S	S	R	S	S	S
Streptococcus pyogenes	S	R	S	R	S	S	S	R	S

DO: Doxycycline (30 µg), CXM: Cefotaxime (30 µg), AMC: Amoxicillin/clavulanic acid (30 µg), SXT: Sulfamethoxazole trimethoprim (25 µg), AZM: Azithromycin (15 µg), PG: Penicillin G (10 units), NOR: Norfloxacin (10 µg) and DA: Clindamycin (10 µg), Musk 100% v/v applied in the tested microorganisms, Susceptibility patterns: R: Resistant and S: Susceptible

Minimum inhibitory concentration (MIC): The mean MIC (v/v%) of the musk against the bacterial strains are shown in Table 3. The musk showed an inhibitory effect on the growth of the tested MDR which the MIC values of musk treatment getting between 10% v/v and 0.1 (v/v) for all MDR bacteria. According to MIC values, the Gram-positive bacteria showed MIC values <2% (v/v), whereas the best activity was noted towards musk with *Streptococcus agalactiae* with the lowest

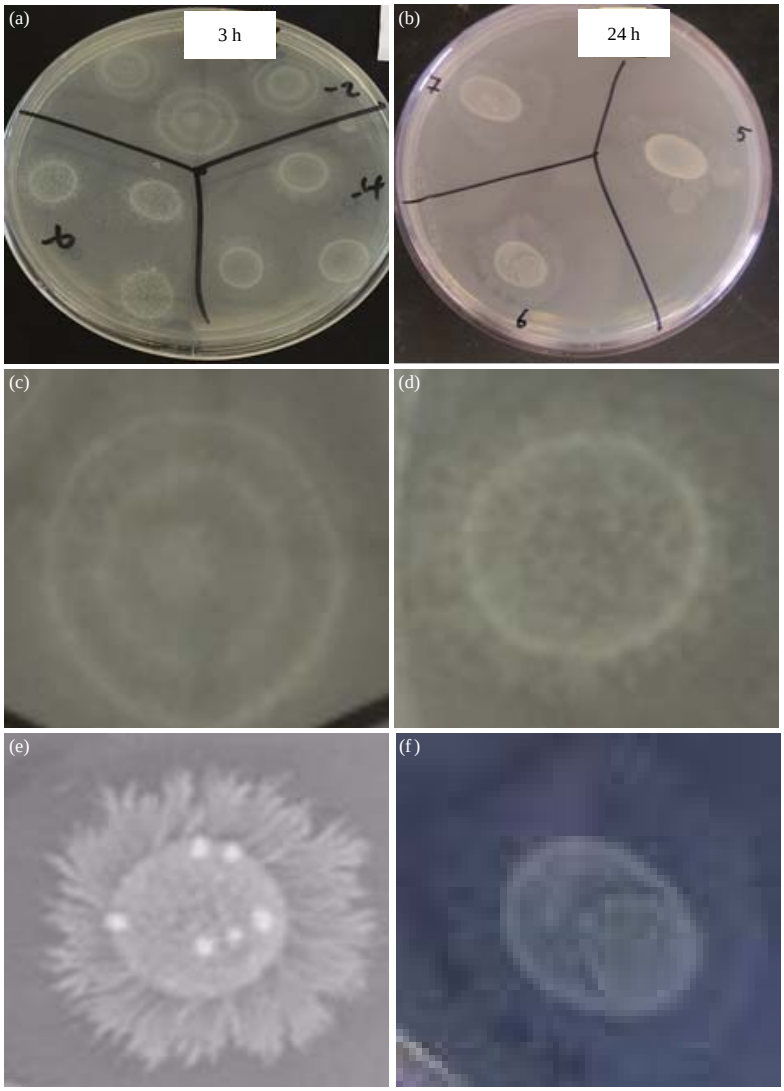

Fig. 2(a-f): Alteration in the morphological form of the *Streptococcus agalactiae* colonies with time interval (a) 3 h, (b) 24, (c-e) Magnification of A (f) Magnification of B

Table 3: Minimum inhibitory concentration values (%, v/v) of musk at 37°C against MDR microbial strains

Microbial strains	MIC % (v/v)
Staphylococcus aureus	0.62
Klebsiella pneumoniae	1.25
Escherichia coli	0.62
Enterococcus faecalis	0.31
Actinobacter baumanni	1.25
Serratia marcescens	1.25
Staphylococcus capitis	0.62
Pseudomonas aeruginosa	2.50
Proteus mirabilis	1.25
Streptococcus agalactiae	0.16
Streptococcus pyogenes	0.62

MDR: Multidrug resistance bacteria

MIC value $\leq$1% (v/v) and the MIC values of some strain of Gram-negative bacteria was <2% (v/v) and the *Pseudomonas aeruginosa* showed some resistance effect to musk at lowest concentration but interestingly it found to be active with musk by increased the incubation time and concentration of musk.

Cell morphology: Changes were observed in the morphological form of the bacterial colonies treated with musk in the most of MDR bacteria such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Pseudomonas aeruginosa* and *Klebsiella baumanni* indicating that the musk had an effect on bacterial cellular membranes Fig. 2. Figure 2a and b presented the alteration in the morphological form of the *Streptococcus agalactiae* colonies after musk treatment at different concentration with different time's interval 3 and 24 h, Fig. 2c-f represented magnification of Fig. 2a and b for morphological changes resulting from musk treatment

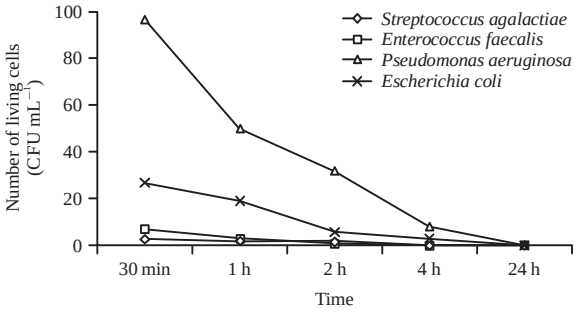

Fig. 3: Time killing curve of musk against MDR bacteria musk concentration at 10% (v/v), a viable bacteria count CFU mL^{-1} was performed after 30 min, 1, 2, 4 and 24 h

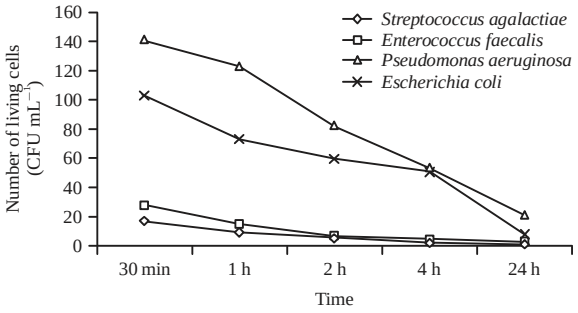

Fig. 4: Time killing curve of musk against MDR bacteria (MBC) at musk concentration 0.16% (v/v), a viable bacteria count CFU mL^{-1} was performed after 30 min, 1, 2, 4 and 24 h

showed the alteration in the morphological form of the *Streptococcus agalactiae* colonies after musk treatment at different concentration with different time's interval 3 and 24 h.

Bactericidal activity: The bactericidal activity results were registered 99.9% killing ($\geq$3 log10 CFU mL^{-1}) of four selected MDR bacterial strains obtained in the time-killing curves within five-time intervals are summarized in Table 4 and highest MBC values were observed in *Pseudomonas aeruginosa*. The Killing curves were prepared in duplicate and the results were identical to one dilution. Colony-forming unit (CFU) were calculated at five different time points (30 min, 1, 2, 4 and 24 h) to determine the number of living cells (CFU mL^{-1}) of each well using the agar plate count method. Starts with the concentration 10% (v/v) of musk after 24 h incubation ending with concentration 0.16% (v/v), yielding a log CFU mL^{-1} was a value of zero of selected MDR bacteria. According to time-killing curves that are shown in Fig. 3 and 4 remarked a perfectly bactericidal effect at both concentrations with Gram-positive *Streptococcus agalactiae*

Table 4: Minimum bactericidal concentration (MBC(%, v/v)) values of musk against a selected MDR bacteria at 37°C at different time periods (30 min, 1, 2 and 24 h), MBC values needed to achieve a reduction in numbers of 5 log CFU mL^{-1}

Microbial strains	MBC values % (v/v)
Escherichia coli	
30 min	5.00
1 h	5.00
2 h	2.50
4 h	2.50
24 h	1.25
Pseudomonas aeruginosa	
30 min	>10.00
1 h	>10.00
2 h	>10.00
4 h	10.00
24 h	5.00
Streptococcus agalactiae	
30 min	5.00
1 h	0.62
2 h	0.62
4 h	0.62
24 h	0.32
Enterococcus faecalis	
30 min	5.00
1 h	2.50
2 h	0.62
4 h	0.62
24 h	0.62

within 30 min at concentration $\leq$2% (v/v) and the *Enterococcus faecalis* showed exhibited good bactericidal activity within 30 min. However, the musk has demonstrated a perfectly bactericidal effect in Gram-negative bacteria *Pseudomonas aeruginosa* after 24 h incubation >2% (v/v) and present exhibited time-dependent bactericidal kinetics, whereas the bactericidal activity of musk increased by time against *Pseudomonas aeruginosa* and the *Escherichia coli* exhibited bactericidal activity <2% (v/v) after 24 h.

DISCUSSION

The main objective of the present study was to evaluate the ability of deer musk to inhibit the growth of pathogenic bacteria that are resistant to the different class of antibiotics and known as multidrug-resistant bacteria. Furthermore to provethe antibacterial effect of different concentrations of natural musk on the above-mentioned MDR bacteria. Additionally, herbal medicine based on oil products has shown the effect in literature with good safety profile[19]. Moreover, musk bacteriostatic and bactericidal effect has been previously discussed by Saddiq[7] and many studies were carried out to investigate the use of musk to inhibit the growth of many pathogenic micro-organisms for human, animals and plants, so it is important to examine newer drugs

with lesser resistance, wherefore the natural products may present a new source of antimicrobial agents with probably novel mechanisms of action, drugs originated from natural sources show a considerable role in the inhibition and treatment of human diseases. In many developing countries, old-style medicine is one of the main healthcare systems[20], around 61% of new drugs established between 1981 and 2002 were formed from natural products and they have been effective, particularly in the infectious disease and cancer[21]. Many studies cite the inhibitory activity of musk against Gram-negative and Gram-positive organisms as well as fungi. The deer musk showed a great activity in inhibiting the growth of MDR bacteria, probably due to the presence of active ingredients that inhibit bacterial growth. Among the tested micro-organisms, these results are similar to that obtained from a recent study[1,4-6,8]. In the present study, it focused on the use of antimicrobial testing methods for the *in vitro* investigation of musk as a potential antimicrobial agent against MDR bacteria. The most known and basic method used in this study for antimicrobial susceptibility testing is the agar disk-diffusion method to see if there is any effect to musk on the bacteria and how effective it is and a microdilution method used to assess the MIC result for a musk. The MIC here is the lowest concentration of musk that inhibits more than 99% of the bacterial population. In addition, the time-killing test is used for determining the bactericidal effect of musk on the selected MDR microbial strain. The time-killing test reveals a time-dependent or a concentration-dependent antimicrobial effect, in this study, followed the time-dependent kill test to achieve the musk effect, also the musk showed effective against all tested micro-organisms especially against Gram-positive bacteria even in the low concentrations as *Streptococcus agalactiae, Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus capitis* and *Streptococcus pyogenes* which also has a significant effective against all tested Gram-negative microorganisms *Klebsiella pneumoniae*, *Escherichia coli*, *Actinobacter baumanni*, *Proteus mirabilis*, *Serratia marcescens*, *Pseudomonas aeruginosa*. This differentiation in antimicrobial effects between tested bacteria may be due to bacterial strains differences used in this study. This result is in agreement with another study which showed that the musk had an antibacterial effect against, *Lactobacillus acidophilus*, *Lactococcus lactis*, *Streptococcus thermophiles*, *Staphylococcus aureus* and *Penicillium puberulum* fungus[3]. According to the MIC and MBC results showed that musk had a bacteriostatic effect against all MDR bacterial strains that used in this study at the lowest concentration especially against *Streptococcus agalactiae* registered the lowest MIC value was <0.5% (v/v) and the antimicrobial sensitivity by disk diffusion method values registered >40 mm. The *Pseudomonas aeruginosa* is notorious for its involvement in nosocomial infections and frequent resistance to antibiotics and there are several studies showed that the *Pseudomonas aeruginosa* the most highly resistant to EO (essential oil)[19,22,23], although the musk comes as an oily nature *Pseudomonas aeruginosa* showed some resistance against the lowest concentration of musk which the MIC value was >2% (v/v) and it has reduced susceptibility in 1:10 of musk dilution but the musk can effect and inhibit the growth the *Pseudomonas aeruginosa* with a low concentration and can kill it at the highest concentration depending on increasing the time. Another observation in this study musk and its oil form is a component extracted from genitalia of deer that confirm the role of essential oils in change the morphology of the bacterial colonies by increasing membrane permeability, after 24 h of treatment with musk, the breakdown of rod bacterial cells has been observed and it may perform as a lubricant, which caused branching patterns of the bacteria. Several bacterial strains show colonial branching patterns through growth on poor semisolid substrates, these patterns reflect the bacterial supportive self-organization[24]. Other explanations, essential oils (EO) and their components have activity against a range of targets, mainly the membrane and cytoplasm and in certain cases, they totally change the morphology of the cells[22]. Moreover, the hydrophobicity of EO is reliable for the disruption of bacterial structures that leads to increased permeability of cell membrane because of a failure to discrete the EO from the bacterial cell membrane[25]. The antimicrobial activity of EO comes from its toxic effects on membrane structure and function, damaging the cytoplasmic membrane, cytoplasm coagulation, destructive the membrane proteins, the degradation of the cell wall and raised permeability and causing leakage of the cell contents[26-30].

CONCLUSION

It could be concluded that musk presented antibacterial activity against different bacterial strains, but at different levels and all the tested multidrug-resistant bacteria were more or less sensitive to musk has a significant role in human medication, displaying antimicrobial activities. So the main aim to study of the synergistic effects of musk and their components could be applied both to produce the best use of its antibacterial activity and to decrease its concentrations compulsory to accomplish a specific antibacterial effect for food safety and for health purposes and to prove the potential future use of musk as alternatives to common antibiotics and to regulate their capability to improve the activity of

antibiotics, according to some studies suggested that musk has a wide spectrum of antimicrobial activity including fungistatic, fungicidal, parasitostatic, bactericidal and bacteriostatic effects.

SIGNIFICANCE STATEMENT

Discovery of natural products such as musk with effective activity against pathogenic micro-organisms and some MDR strains in the current study as supported by earlier reports from Saudi Arabia, Iraq, Egypt and developed countries such as Germany, Switzerland and Korea further support the viability of observing for an alternative approach to manage drug resistant microbes.

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