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Combined efficacy of thymol and silver nanoparticles against *Staphylococcus aureus*

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Due to the looming spread of resistance to classical antimicrobial agents, innovative therapeutic methods are in dire need to combat the onslaught of resistant bacterial pathogens. This study examines the antimicrobial efficacy of a phytochemical and a metallic nanoparticle against the top Gram positive resistant pathogen. The potential synergy of these two agents was also evaluated. The antibacterial activity of thymol and silver nanoparticles were tested individually using disc diffusion technique. The extent of synergy of their combination was evaluated using the checkerboard assay. Twenty clinical isolates of *Staphylococcus aureus* characterized as methicillin resistant or methicillin sensitive *Staphylococcus aureus* were utilized and the extent of synergism was calculated from fractional inhibitory concentration indices. Thymol exhibited an antistaphylococcal activity regardless of whether the isolates were phenotypically resistant or sensitive to methicillin. Combining thymol with silver nanoparticles resulted in at least additive or synergistic effect for all the examined strains and methicillin resistant strains were inhibited in the combinatorial assays to a greater extent comparative to when silver nanoparticles or thymol were used singly.

Key words: Silver nanoparticles, thymol, *Staphylococcus aureus*, synergy, fractional inhibitory concentration index.

INTRODUCTION

The everlasting battle between humans and infectious diseases causing pathogens continues. Emerging at the front line of challenges to human health is bacterial resistance and its alarming spread. This ongoing rise in resistance is critically threatening the immeasurable medical advancements made possible by antibiotics over the past 70 years (WHO, 2014). Multi-drug resistant-methicillin resistant *Staphylococcus aureus* (MDR-MRSA) is a constantly evolving paradigmatic pathogen. The silently vicious incarnations of *S. aureus* widespread in

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community and hospital environments have posed serious clinical imbroglio (Magiorakos et al., 2012). More than 95% MRSA worldwide do not respond to first line antibiotics and resistance has even evolved to the more recent antimicrobial agents like linezolid, vancomycin, teicoplanin and daptomycin (Kaur and Chate, 2015).

Aggravating the problem of accelerating bacterial resistance to currently approved antibiotics is the lack of investment in antibiotic discovery by the pharmaceutical industry (Spellberg et al., 2007). This situation is so dire that the World Health Organization has recognized multidrug resistant (MDR) bacteria as one of the top three threats to human health (Bassetti et al., 2011). Novel approaches to combating infections caused by these bacteria are sorely needed. An alternative hope resides in medicinal plants, since nature is the only resource to offer an assortment of chemical compounds that can be utilized for new drug discovery. Moreover, phytohemicals are privileged with their lower mammalian toxicity, lesser environmental effects and wider public acceptance (Paranagama et al., 2003). The array of phytochemicals that have been studied for their antimicrobial activity ranges from phenolic compounds, alkaloids, saponins, glucosinates to terpenoids. Research in this field was not halted by the discovery of antibacterially active phytochemicals but extended to their combination with antibiotics that provided the next approach to combat multidrug resistant bacteria (Nostro et al., 2004).

Nanosilver is a versatile antimicrobial agent with established efficacy against a broad range of microorganisms, including bacteria, yeast, fungi, algae and even viruses (Rai et al., 2009). Recently, silver nanoparticles (AgNPs) were considered predominantly attractive for the production of a novel class of antimicrobials opening up a totally new approach to combat a wide assortment of bacterial pathogens (Morones et al., 2005; Shrivastava et al., 2010). In fact, its broad spectrum of activity against morphologically and metabolically different microorganisms, its multifaceted mechanism, as well as, the rare incidence of resistance to elemental silver puts it forth as a potential candidate in the context of the continuing rise in drug resistant strains of bacteria (Panacek et al., 2006; Franci et al., 2015) .

While the approaches to combat antibiotic resistance encompass an array of perspectives from antibiotic stewardship programs, antibiotic alternatives to antivirulence drugs, combination therapy is the norm in treatment of many infections. One of the potential candidates in this respect is the combination between metallic nanoparticles and phytochemicals that may lead to new choices to overcome the onslaught of microbial resistance. Eventually, the aim of the present study was to assess the susceptibility of staphylococcal isolates, characterized as methicillin sensitive *Staphylococcus aureus* (MSSA) or methicillin resistant *S. aureus* (MRSA), to the phytochemical compound "thymol". Furthermore, the synergistic potential of the combination of both thymol and silver nanoparticles was investigated.

MATERIALS AND METHODS

Bacterial strains isolation and identification

Twenty (20) clinical strains of *Staphylococci* from the routine laboratory of Damanhour Main Hospital were enrolled in the present study. The species level confirmation was done using Gram staining, colonial morphology, coagulase positivity and inoculation onto mannitol salt agar (Oxoid). The strains were categorized as MRSA or MSSA based on cefoxitin disc (30 µg) agar screening method (CLSI, 2015). Standard strains of *S. aureus*: *S. aureus* ATCC 43300 and *S. aureus* ATCC 13150 corresponding to methicillin resistant and sensitive strains, respectively, were also included in the current study.

Synthesis and characterization of citrate coated silver nanoparticles

Citrate coated silver nanoparticles were prepared using silver nitrate as the source material and trisodium citrate as the reducing and capping agent. All chemicals used were of analytical grade. 10 ml of 1mM $AgNO_3$ was heated in a water bath and to this solution, 1 ml of 1% trisodium citrate was added gradually and shaking was done till a pale yellow colour was formed. Heating was ceased and the prepared $AgNO_3$ were left to cool at room temperature. The suspension thus obtained was purified by centrifugation at 12000 rpm for 15 min and the supernatant was discarded. A dried powder of the nanosized silver was obtained by freeze-drying. For characterization and assessment of AgNPs antibacterial activity, the freeze dried powder was resuspended in deionized water (Munro et al., 1995).

The shape and nanodimension of the synthesized AgNPs were assessed using transmission electron microscope (Jeol, Tokyo, Japan) by placing drops of the silver nanoparticles solutions on carbon-coated TEM grids. The particle size distribution of the prepared AgNPs was determined by laser light scattering on a Beckman Coulter Particle Size Analyzer (NS submicron particle size analyzer, Japan). Zeta potential was assessed using Zetasizer Nano ZS (Malvern UK). UV-Vis spectroscopy of 10-fold diluted dispersion of AgNPs was recorded using UV-6800 UV/VIS spectrophotometer (Jenway, Germany).

Susceptibility testing of methicillin sensitive and methicillin resistant *S. aureus* isolates

The susceptibility of the MRSA and MSSA isolates to thymol (Sigma Chemical Co., UK) was evaluated using Mueller Hinton Agar (MHA, Oxoid) as described in the diffusion method commonly used for antibiotics (CLSI, 2015). Sterile paper discs (6 mm in diameter) impregnated with 10 µl of either AgNPs (at final content of 10 µg/disc) or thymol solutions were laid on the surface of MHA plates with inoculations of approximately 10^5 CFU/ml of the respective strain. Thymol was prepared as a 1:10 diluted stock in dimethyl sulphoxide (DMSO). Antimicrobial activity was expressed in terms of the inhibition zone (IZ) diameters obtained following incubation at 37°C for 18-24 h.

Test for synergism by disc diffusion method

The synergistic potential of nanosilver and thymol was evaluated using sterile discs impregnated with 5 µl of both thymol and AgNPs

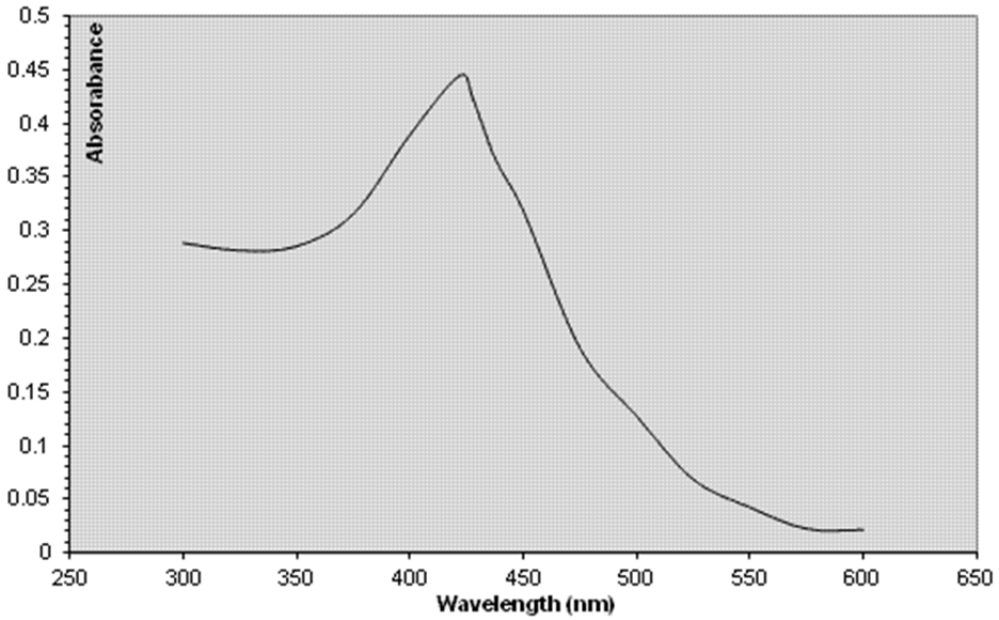

Figure 1. UV-Vis spectrum of citrate coated AgNPs with surface plasmon resonance bend at 422 nm.

and proceeding as aforementioned. The percentage fold increase in inhibition zone was calculated using the formula B-A/A x100 where A and B are the inhibition zones in mm obtained for thymol alone and in combination with AgNPs, respectively (Singh et al., 2013).

Test for synergism of thymol and silver nanoparticles by checker board assay

Minimum inhibitory concentrations (MIC) of thymol and silver nanoparticles were determined individually by the standard microdilution method (CLSI, 2015; Wiegand et al., 2008). Two fold serial dilutions of thymol in DMSO were used comprising 32, 64, 128, 256, 512 and 1024 µg/ml (Hamoud et al., 2014). Simultaneously, the MIC of AgNPs was assessed using gradient concentrations of AgNPs in Mueller Hinton broth (MHB) (5, 10, 20, 40, 80 and 160 µg/ml) (Paredes et al., 2014). A growth control (bacterial inoculum in MHB with 1% DMSO) and a sterility control (MHB with 1% DMSO) were also included in the assay. The MIC was taken as the lowest concentration of the agent that showed no visible turbidity matching with a negative control after incubation at 35°C for 24 h.

The checkerboard dilution method was used to evaluate the *in vitro* synergy of thymol and silver nanoparticles. From the stock solutions of thymol and silver nanoparticles, a two-fold serial dilution to at least double the MIC was distributed in a microtiter plate. The bacterial suspensions were then added to reach inoculums of $5x10^5$ CFU/ml. Thymol dilutions were placed along the abscissa of the microtiter plate in ascending concentrations starting at four dilutions below the MIC and ending at two times the MIC. Meanwhile, AgNPs dilutions were distributed along the ordinates of the plate. The microtiter plate was incubated overnight at 35°C and MIC was read as the lowest concentration of the agent at which no visible growth occurred. The fractional inhibitory concentration index (FICI) was used to evaluate synergy as per the Clinical Laboratory Standards Institute guidelines for broth microdilution (CLSI, 2015). FICI was calculated using the formula FICI=FIC A + FIC B, where FIC A is the MIC of thymol in combination/MIC of thymol alone, and FIC B is the MIC of AgNPs in combination/MIC of AgNPs alone. The combination was considered synergistic when the FICI ≤ 0.5. However, indifference was indicated by an FICI>0.5 and antagonism was depicted by an FICI > 4 (Hsieh et al., 1993; Petersen et al., 2006; Meletiadis et al., 2010).

Statistical analysis

All experiments were done in triplicates. The mean values were calculated and the standard deviation was determined. The IZ diameters of methicillin resistant and methicillin sensitive strains of *S. aureus* were compared using Fisher's exact test.

RESULTS

In the present study, characterization of the prepared AgNPs demonstrated a narrow absorption peak at wavelength of 422 nm (Figure 1). Moreover, spherical, non-aggregated nanoparticles were observed in the TEM micrograph with a diameter range of 21 to 41 nm with an average particle size of 26.9 nm and polydispersity index of 0.73 (Figure 2). A zeta potential of -33.9 mv ± 6.87 reveals high aggregation stability for the prepared AgNPs (Figure 3).

The susceptibility of the MRSA and MSSA isolates to thymol, individually and combined with nanosilver was evaluated using disc diffusion method. The IZ diameter (mm) around the different discs with and without AgNPs against the test strains is shown in Table 1. Silver nanoparticles displayed negligible activity against both resistant and sensitive strains of *S. aureus.* Thymol exhibited an inhibitory effect where relatively larger IZ diameters were observed for MSSA isolates (30-35

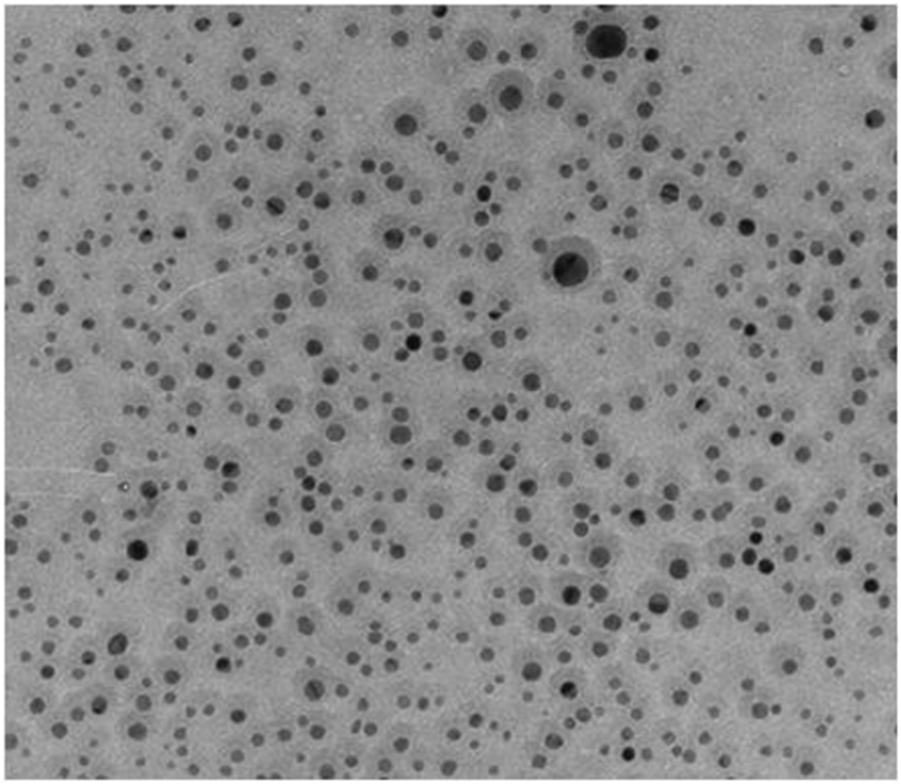

Figure 2. Transmission electron micrograph of prepared AgNPs (Mag. 35000X).

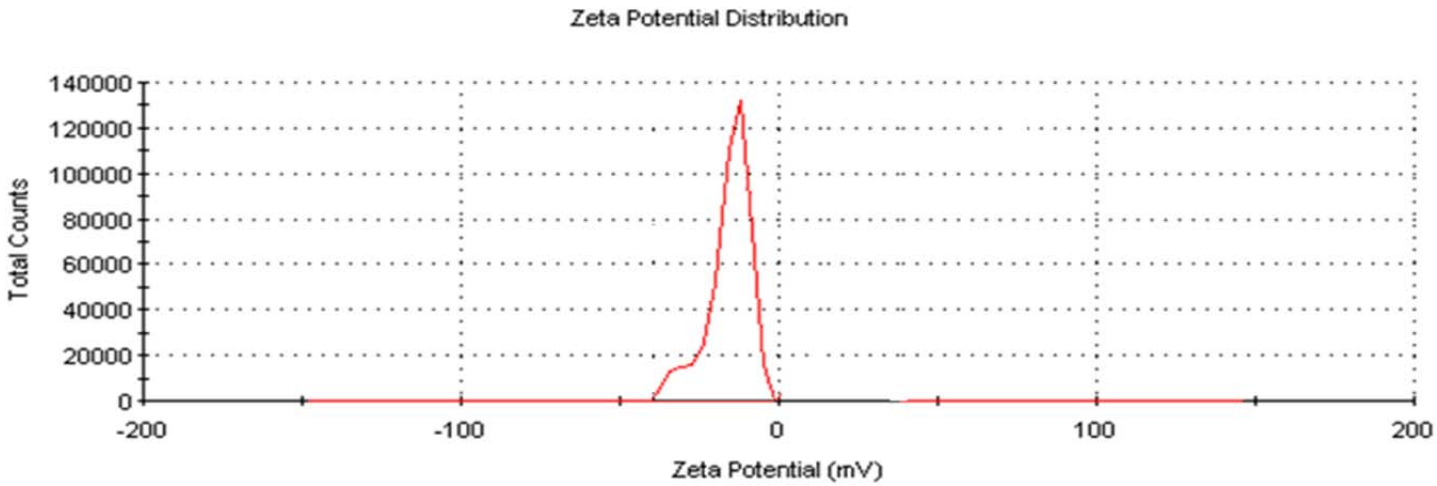

Figure 3. Zeta potential curve of citrate coated AgNPs (Zeta potential = -33.9 mV).

versus 29-34 mm for MRSA). The antibacterial activity of thymol was increased in combination with Ag-NPs, as shown by the fold increase percentage (Table 1).

The minimum inhibitory concentrations of thymol and AgNPs for both MRSA and MSSA were determined by broth microdilution technique. The observed MIC values of thymol and AgNPs for MRSA ranged from 128 to 256 and 40 to 160 µg/mL respectively, while those for MSSA ranged from 128 to 256 and from 20 to 80 µg/mL, respectively (Table 2). The table also shows the FICI results using the checkerboard technique, which demonstrated the mostly synergistic effect of the combination of both thymol and AgNPs, with few indifferent but no antagonistic combinations.

Table 1. Staphylococcal susceptibility to thymol and silver nanoparticles in terms of IZ diameter.

Test microorganism	IZ diameter (mm)		Fold increase (%)
	Thymol	Thymol + AgNPs	
Staphylococcus aureus ATCC 43300	28	33	17.9
MR1	31	36	16.1
MR2	31	34	9.7
MR 3	33	37	12.1
MR 4	34	35	2.9
MR 5	30	35	16.7
MR 6	32	33	3.1
MR 7	30	34	13.3
MR 8	29	35	20.7
MR 9	32	35	9.4
MR 10	30	31	3.3
Staphylococcus aureus ATCC 13150	34	38	11.8
MS 1	32	35	9.4
MS 2	32	38	18.8
MS 3	34	36	5.9
MS 4	35	39	11.4
MS 5	33	35	6.1
MS 6	30	34	13.3
MS 7	31	34	9.7
MS 8	33	37	12.1
MS 9	34	38	11.8
MS 10	32	34	6.3

MR: Methicillin resistant *S. aureus;* MS: Methicillin sensitive *S. aureus;* IZ: inhibition zone. Fold increase was calculated using the formula B-A/A*100, where A and B are the zone of inhibition (mm) obtained for thymol alone and in combination with AgNPs, respectively. In case of no inhibition zone, diameter of the disc (6 mm) was taken for the calculation. All experiments were repeated thrice and standard deviations were negligible.

Table 2. MIC of thymol and silver nanoparticles against methicillin-resistant and sensitive *S. aureus* and FICI results using checkerboard technique.

Test microorganism	MIC		Conditions at best synergy point		FICI
	Thymol (µg/ml)	AgNPs (µg /ml)	Thymol (µg/ml)	AgNPs (µg /ml)	
Staphylococcus aureus ATCC 43300	256	80	32	10	0.25 (S)
MR1	128	80	32	20	0.5 (S)
MR2	256	80	32	10	0.25 (S)
MR 3	128	40	32	10	0.5 (S)
MR 4	128	40	64	20	1 (I)
MR 5	256	80	32	10	0.25 (S)
MR 6	128	40	64	20	1 (I)
MR 7	128	80	32	20	0.5 (S)
MR 8	256	160	64	40	0.5 (S)
MR 9	128	80	64	40	1 (I)
MR 10	256	40	128	20	1 (I)
Staphylococcus aureus ATCC 13150	128	40	32	10	0.5 (S)
MS 1	128	20	128	20	2 (I)
MS 2	256	40	64	10	0.5 (S)

Table 2. Contd.

MS 3	128	20	256	40	4 (I)
MS 4	128	40	32	10	0.5 (S)
MS 5	256	80	64	20	0.5 (S)
MS 6	256	40	64	10	0.5 (S)
MS 7	256	40	512	80	4 (I)
MS 8	256	40	64	10	0.5 (S)
MS 9	128	20	32	5	0.5 (S)
MS 10	256	40	64	10	0.5 (S)

MR: Methicillin resistant *S. aureus*; S: synergism; MS: methicillin sensitive *S. aureus;* I: indifference; MIC: minimum inhibitory concentration; FICI: fractional inhibitory concentration index.

DISCUSSION

In 1960, the first strain of MRSA was isolated just one year after the use of methicillin as an alternative to penicillin. Recently, as per the WHO estimates, the incidence of MRSA has reached 70 to 80% of all the *S. aureus* isolates (Jevons, 1961; WHO, 2014). As the battle against super bugs including MRSA rages on, phytochemicals and metallic nanoparticles rise as promising candidates in the combat against MDR pathogens.

In the present study, the phytochemical "thymol" exhibited an inhibitory activity towards both methicillin resistant and sensitive strains of *S. aureus*, whereas silver exhibited negligible activity against either phenotypes when tested by agar diffusion technique. Thyme essential oil and its major phenolic component "thymol" were reported to exhibit a wide spectrum of antimicrobial activity (Dorman and Deans, 2000; Lambert et al., 2001; Friedman et al., 2002; Yap et al., 2014). Investigation of their putative inhibitory efficacy against MSSA and MRSA demonstrated that the susceptibility of the tested strains to thyme oil and thymol was insignificantly different (Nostro et al., 2004). Another study also reported that both methicillin resistant and sensitive strains of *S. aureus* were equally susceptible to thymol and its isomer carvacrol (Nostro et al., 2007). In line with the present results, it has been shown that all *S. aureus* strains were susceptible to thymol whether phenotypically sensitive or resistant to methicillin (Table 1).

While the mechanism of action of essential oil components has not been fully elucidated, most of the components investigated share commonality in their antibacterial mode of action (Nazzaro et al., 2013). Thymol's main mode of action is believed to involve outer and inner membrane disruptions, as well as, binding to intracellular targets disrupting a variety of cellular functions (Walsh et al., 2003; Turina et al., 2006; Xu et al., 2008). Relating the resistance phenotype of microorganisms with the activity of phyto-constituents is conflict ridden. In this regard, limited research has been done for the exploration of the capability of plant extracts in modulating bacterial resistance. Some phytocomponents have been reported to possess resistance modifying activity *in vitro.* Polyphenols in particular have been reported to reverse betalactam resistance in MRSA (Stapleton et al., 2004). Intriguingly, in the present study, there was an insignificant difference in the activity of thymol against both methicillin sensitive and resistant strains of *S. aureus* (Fisher's exact test, $P>0.05$) denoting that thymol activity was not affected by those resistance mechanisms that differentiate these strains (Table 1).

It is documented that a wide range of synthetic components exert antibacterial effects, but the primary impediment for their use is their toxicity. Among these, silver compounds raise as potent bactericidal agents whose medical antimicrobial application has been hindered by their potential toxic effects. Nevertheless, silver at nanoscale shows lower toxicity comparative to conventional silver preparations (Murphy et al., 2015). The evidence supporting AgNPs as effective antimicrobial agents is abundant with reported activity against various bacterial strains including *S. aureus, Staphylococcus epidermidis, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and even vancomycin-resistant strains (Ovington, 2004; Panacek et al., 2006; Lok et al., 2007). Disparities in literature, however, occur regarding the antistaphylococcal efficacy of AgNPs. In a study investigating the anti-MRSA effects of different concentrations of biosynthesized silver nanoparticles, it has been reported that the activity was variable with an inhibition zone ranging from 23 to 11 mm and the exerted effect inversely related to the concentrations investigated (Manzoor-ul-Haq et al., 2015). Where silver microbial susceptibility studies have been performed, the authors have assigned their own breakpoints to delineate susceptible and resistant strains. Most of these studies have produced different MIC data for silver, and this demonstrates the extent of variation that currently exists with regard to the pharmacological parameters of silver. For instance, results from the two studies that explored MIC values for *S. aureus* (around 100

strains) ranged from 8 to 80 mg/L (Ug and Ceylan, 2003; Hamilton-Miller et al., 1993). Notwithstanding, the many conflicts in literature regarding the antibacterial activities of AgNPs, it can be conceived that the effect of AgNPs is not solely dependent on nanoparticles characteristics but is also affected by method of assessment used, as well as, the bacterial class tested. In the current study, albeit, the negligible antistaphylococcal effect exerted by silver when tested by agar diffusion technique, AgNPs displayed MIC values ranging from 40 to 160 µg/ml for MRSA and 20 to 80 µg/ml for MSSA strains (Table 2). This may be related to the limited diffusion of AgNPs after disc impregnation and adsorption of certain amounts of AgNPs on the paper disc consequently resulting in lower or non-existent effect. Moreover, it is reported that broth microdilution technique is more reliable and reproducible than disc diffusion technique especially with respect to specific properties of nanoscale materials such as diffusion and aggregation stability in dispersion media (Panacek et al., 2015).

The antibacterial activity of AgNPs is reported to be multifaceted and underlies a decreased probability of resistance development (Franci et al., 2015). Silver nanoparticles were shown to inactivate bacterial enzymes, disrupt bacterial metabolic processes, increase the cytoplasmic membrane permeability, interact with DNA and generate reactive oxygen species that damage biomacromolecules (Lara et al., 2010; Franci et al., 2015). The studies reporting the different mechanisms by which AgNPs interact with microbes paralleled with several reports that investigated the synergistic potential of AgNPs with classical antibiotics (Fayaz et al., 2010; Hwang et al., 2012; Naqvi et al., 2013; Singh et al., 2013). In these studies, quantifying the synergistic effects of antibiotics in combination with AgNPs against an assortment of microorganisms has been investigated, employing the microdilution method. Albeit, the evidenced synergistic capacity of silver nanoparticles in these studies, no trends were observed for the synergistic effects of antibiotics with different modes of action and different chemical structures suggesting that these effects are non-specific.

Recently, several studies have indicated that AgNPs may strengthen the antibacterial effects of antibiotics against both susceptible and resistant bacteria, either additively or synergistically (Fayaz et al., 2010; Lara et al., 2010; Hwang et al., 2012). Consistent with these findings, combining two agents with reported multimodal antibacterial action, in the present study, has shown at least additive or synergistic effect for all tested strains (Table 2). Synchronically, the antibacterial activity of thymol increased when combined with AgNPs as was evident by the fold increase in inhibition zone diameters by disc diffusion method (Table 1). Notably, the resistant strains were inhibited in the combinatorial assays to a greater extent in comparison with when AgNPs and thymol were used in isolation. As per the FICI results, silver concentrations as low as 5 to 20 µg/ml were synergistic and the MIC of thymol was mostly four and eight orders of magnitude lower than those of non-combined thymol (Table 2).

The mechanism by which AgNPs enhanced *S. aureus* sensitivity towards thymol is probably complex taking into account the multiple-level mechanisms reported for both agents. It can be proposed that AgNPs promotes the disturbance of cell wall facilitating thymol's transport into the cell, resulting in disruptions and inactivation of biomacromolecules that has been previously shown to exert. Another interesting finding in the present study is that the synergistic effect of AgNPs in combination with thymol against both resistant and sensitive strains was mostly similar. Influencing resistance mechanisms that differentiate these strains cannot be ruled out but such combination is potentially significant for preventing the development of bacterial resistance. Detailed toxicological studies are needed to establish the safety of the combination regimen of these two agents in the light of decreased effectiveness of classical antibiotics against resistant organisms.

CONFLICT OF INTERESTS

The author(s) have not declared any conflict of interests.

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