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INVESTIGATING NON-THERAPEUTIC PHARMACEUTICAL SUBSTANCES FOR IMPROVING *IN-VITRO* EFFICACY OF CLINDAMYCIN PHOSPHATE AGAINST MRSA AND *Staphylococcus epidermidis*

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The aim of present study was to investigate the effect of pharmaceutical excipients and other active substances on antimicrobial efficacy of standard antibiotic against resistant and susceptible microorganisms. Pharmaceutical excipients (sodium lauryl sulfate [SLS], Tween-80, citric acid, NaOH, NaCl) and active substances (fusidic acid, sorbic acid) were investigated to check in-vitro efficacy and their effect on the efficacy of standard antibiotic. Clindamycin was selected as standard antibiotic. Clindamycin was found to be ineffective against methicillin-resistant Staphylococcus aureus (MRSA). Fusidic acid and SLS showed concentration dependent effect against MRSA. Other tested substances were also ineffective against MRSA, and also failed to improve the susceptibility of MRSA towards clindamycin. The clindamycin + fusidic acid (0.05 µg, 0.1 µg), and clindamycin + SLS (0.5 mg, 1 mg) showed concentration dependent effect on Staphylococcus epidermidis (S. epidermidis). Clindamycin combinations with fusidic acid or SLS showed better inhibition of S. epidermidis, than individual substance. At lower concentration of clindamycin (2 µg), the sorbic acid (25 µg) improves its effectiveness. SLS (0.5 mg, 1 mg) and clindamycin (4 µg, 10 µg) showed almost equal zone of inhibition against S. epidermidis, respectively. Present findings showed that certain pharmaceutical excipients (e.g. SLS) are effective against resistant and susceptible microbes, and suggested that more excipients should be screened for their antimicrobial potential and their ability to improve the efficacy of standard antibiotics.

Keywords: Methicillin-resistant *Staphylococcus aureus*, Pharmaceutical excipients, Clindamycin, Sodium lauryl sulfate, *S. epidermidis*

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INTRODUCTION

The incidences of antimicrobial resistance (AMR) are serious global health threat (Theuretzbacher 2013). If pathogenic microorganism develops resistance against multiple antibiotics, then it will pose a great challenge to effectively treat the infection (Edwards *et al*. 2014). AMR is the development of ability in a microbial cell to survive the normal lethal doses of antimicrobial drug; or the development of insensitivity in a microbe to the antimicrobials, against which the microbe was susceptible earlier (Godsey, Zheleznova Heldwein and Brennan 2002; Tanwar *et al*. 2014). The mechanisms responsible for drug resistance includes: detoxification by enzymatic modification or cleavage of drug molecules, genetic alteration of the cellular targets, decreased permeability of cell membrane and drug efflux by multidrug transporters (Godsey, Zheleznova Heldwein and Brennan 2002; Tanwar *et al*. 2014; Thompson *et al*. 2013; Chovanová, Mikulášová and Vaverková 2013).

Overuse, misuse and use at sub-therapeutic level of antimicrobials often lead to the adaptation of microorganisms and development of resistance. Use of antimicrobials in the food producing animals (e.g. poultry) and agriculture sector exceeds their indirect use in humans and it may further augment the emergence of resistant pathogens (Centers for Disease Control and Prevention 2013). Antibiotics have largely contributed to enhance the production of food producing animals (e.g. poultry animals) by decreasing the incidences of fatal infectious diseases. Continuous non-therapeutic use of antibiotics (viz. growth promoter) in animal feed and improper use in the treatment of diseases may also leads to resistance (Diarra and Malouin 2014). The lost or impaired effectiveness of antimicrobials against pathogenic microbes will complicate the treatment of patient undergoing surgery and organ transplantation.

Multidrug AMR limits the therapeutic options available for the treatment of infection and compel healthcare providers to use comparatively toxic and or expensive antimicrobials. Treatment of infections caused by resistant microbes is difficult, complicated and expensive; even some times it results in failure. In such cases patients suffers for prolonged duration and chances of mortality are also increased. According to the Centres for Disease Control and Prevention (2013), in United States each year at least 2 million people acquire serious bacterial infections that are resistant to one or more of the recommended antibiotics (Centres for Disease Control and Prevention 2013). The European Antimicrobial Resistance Surveillance Network reported that resistance to methicillin by *Staphylococcus aureus (S. aureus)* is close to 50% in some European Union Member states (World Health Organization 2011). The World Health Organization (WHO) report 'Antimicrobial Resistance Global Report on Surveillance' raises serious concerns and suggested harmonised regional and global surveillance of the AMR (World Health Organization 2014).

Development and marketing authorisation of new antibiotics take almost a decade. Developing countries have infrastructure and capacity constraints to develop new antimicrobial drugs, so in such situations alternate approaches can be adopted to combat the challenges of AMR. The use of pharmaceutical excipient to enhance the anti-microbial efficacy of substance or to increase the susceptibility of resistant microbe towards the existing antimicrobial substances is another gray area which can be explored. Several pharmaceutical excipients have been screened against numerous microbes and there are reports revealing that some excipients have anti-microbial activity. No *et al*. (2002) reported antibacterial activity of chitosan and found that the activity differed with molecular weight of chitosan and the type of microorganism tested.

The pH 4.5–5.9 of the medium also influences the chitosan's anti-microbial activity. Tsai and Su (1999) observed that higher temperature (25˚C and 37˚C) and acidic

pH increased the bactericidal effects of chitosan, while sodium ions (100 mM Na^+) reduce chitosan's activity against *Escherichia coli* (*E. coli)*, probably by forming complex with chitosan. Divalent cations (Ba^{2+} > Ca^{2+} > Mg^{2+}) at concentrations of 10 mM and 25 mM reduced the antibacterial activity of chitosan.

Hoque *et al*. (2016) investigated the efficacies of N-(2-hydroxypropyl)-3-trimethylammonium chitosan chlorides (chitosan derivatives) polymers against multidrug resistant (MDR) bacteria and pathogenic fungi; and observed that polymers disrupted cell membrane and hinder resistance development in bacterial cell. Pharmaceutical excipient propylene glycol exhibited bactericidal activity against *Staphylococcus mutan (S. mutans), Escherichia faecalis (E. faecalis)* and *E. coli* and polyethylene glycol 1000 exhibited bactericidal activity against *S. mutans and E. coli* (Nalawade, Bhat and Sogi 2015). Sater, Ojcius and Meyer (2008) reported that hydroxyethyl cellulose at pH 5 in a concentration-dependent manner inhibits the *Chlamydia trachomatis* (*C. trachomatis*) infection of cervical epithelial cells.

Lampe *et al*. (2004) investigated antichlamydial activity of several commonly used excipients. Benzalkonium chloride and PEG 400 showed significant activity, while citric acid, EDTA, potassium benzoate and sorbic acid showed moderate activity against *C. trachomatis* (Lampe *et al*. 2004).

On the basis of current available literature, it can be suggested that pharmaceutical excipients should be tested for their antimicrobial potential and to provide an alternate way to counter the microbial resistance by enhancing efficacy of antimicrobial drug substances. Hypothesis of present investigation is based on the assumptions that combined use of potential excipient with standard antibiotics may improve efficacy of antimicrobial agent or the susceptibility of resistant microbe, and provide an alternate improved composition against resistant microbes.

METHODS

Sodium lauryl sulfate (SLS) and sodium chloride were purchased from Winlab, Leicestershire, U.K. Clindamycin phosphate, manufactured by Saniver Ltd. Hong Kong, China. Fusidic acid, manufactured by Ercros, Aranjuez, Spain. Sodium hydroxide, manufactured by Merck, E. Merck D-6100 Darmstadt F. R. Germany. Sorbic acid, manufactured by Merck. Citric acid, manufactured by Avonchem Ltd. Waterloo St. West, Macclesfield, Cheshire, U.K. Tween-80, manufactured by Alpha Chemika, Mumbai, India. MilliQ water was prepared by using MilliQ Direct8. The microbial strains were methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 35591 and *Staphylococcus epidermidis* (*S. epidermidis*) ATCC 35984.

Standard Solutions

The stock solutions of clindamycin phosphate (equivalent to clindamycin 1.0 mg/mL), fusidic acid (1.0 mg/mL) and sorbic acid (1.0 mg/mL) were prepared in MilliQ water. Solutions of sodium chloride (5% w/v), sodium hydroxide (0.5% w/v), citric acid (0.5%), Tween-80 (1%), and SLS (0.5% and 1% w/v) were also prepared in MilliQ water. The standard solutions were diluted to achieve the required quantity in fixed volume.

In-vitro Antimicrobial Study

The *in-vitro* efficacy of clindamycin phosphate and its combinations with other therapeutic or non-therapeutic pharmaceutical substances such as fusidic acid, sorbic acid, sodium chloride, sodium hydroxide, citric acid, Tween-80 and SLS were investigated against MRSA and *S. epidermidis*. The cup plate diffusion method was used for *in-vitro* investigations. The amounts of clindamycin (µg/cup) and the combination substances (µg/cup) are presented in Table 1. *In-vitro* activity of clindamycin against MRSA and *S. epidermidis* was compared with its combinations with other pharmaceutical substances. Sterilised Mueller-Hinton agar media was poured into sterilised Falcon® petri dish (1029TM Petri Dish 100 × 15 mm style, Becton Dickinson, USA) under laminar air flow and allowed to solidify under aseptic condition. MRSA and *S. epidermidis* suspensions ($1x10^8$ colony forming unit/mL) was applied on solidified Mueller-Hinton agar media by streaking technique. Cups of uniform size were cut by using sterile cork borer. The 50 µL of the diluted standard solution of clindamycin phosphate and 100 µL standard solution of other selected pharmaceutical substance (e.g. fusidic acid, sorbic acid, sodium chloride, sodium hydroxide, citric acid, Tween-80 and SLS) were filled into the corresponding cups under laminar air flow. Plates were covered with lids and incubated (Memmert, Schwabach, Germany) at 32°C for 20 h. The zones of inhibition were measured in millimeter by aid of ruler after 20 h.

RESULTS

The effect of therapeutic and non-therapeutic pharmaceutical substances on *in-vitro* antimicrobial efficacy of clindamycin phosphate against MRSA and *S. epidermidis* was investigated. The compositions of clindamycin with other therapeutic and non-therapeutic substance and their respective quantities are presented in Table 1. The amount and set of composition can be interpreted with the help of illustrations given below Table 1. MRSA and *S. epidermidis* were used as model micro-organisms. Zone of inhibition of the compositions given in Table 1 against MRSA are presented in Table 2. The efficacy of clindamycin was investigated at the concentrations of 2 µg, 4 µg, 5 µg and 10 µg per cup. The clindamycin did not show any sign of inhibition against MRSA at the tested concentrations (A, AA, A*, AA*; see Table 2). It indicates that selected MRSA for this study was resistant to the clindamycin or may show the susceptibility at much higher concentration. Present findings support the previous observations which suggest MDR isolates of methicillin-resistant *S. aureus* were resistant to clindamycin (Yang *et al*. 2017; Mesbah Elkammoshi *et al.* 2016). The clindamycin combinations with sorbic acid (A + C, AA + C), citric acid (A + D, AA + D), sodium chloride (A + E, AA + E), sodium hydroxide (A + F, AA + F) and Tween-80 (A* + H, AA* + H) did not showed any zone of inhibition against MRSA (Table 2). Though fusidic acid (0.1 µg) (B*) produced a zone of inhibition of about 17.6 ± 0.5 mm against MRSA, but there was no improvement in activity when combined with clindamycin (A* + B*, AA* + B*). The effect of fusidic acid against MRSA was concentration dependent (see Table 2, A + B, AA + B, A* + B*, AA* + B*). SLS individually inhibited the growth of MRSA. At lower concentration (0.5 mg, G*) the inhibitory effect of SLS was slightly weaker than higher concentration (1 mg, G). Results of present investigation suggested that any of these tested composition do not improve the susceptibility of MRSA to the clindamycin.

Table 1: Testing compositions of clindamycin phosphate and other pharmaceutical substances.

	Composition codes (clindamycin + pharmaceutical substance)						
S No. 1	A	A + B	A + C	A + D	A + E	A + F	A + G
	Composition codes (clindamycin + pharmaceutical substance)						
S No. 2	AA	AA + B	AA + C	AA + D	AA + E	AA + F	AA + G
	Composition codes (clindamycin + pharmaceutical substance)						
S No. 3	A*	A* + B*	A* + G	A* + G*	G	A* + H	H
	Composition codes (clindamycin + pharmaceutical substance)						
S No. 4	AA*	AA* + B*	AA* + G	AA* + G*	G*	AA* + H	B*

Notes: Quantity of testing composition in their corresponding cup: A = clindamycin phosphate (2 µg); AA = clindamycin phosphate (5 µg); A* = clindamycin phosphate (4 µg); AA* clindamycin phosphate (10 µg); B = fusidic acid (0.05 µg), B* = fusidic acid (0.1 µg); C = sorbic acid (25 µg); D = citric acid (0.5 mg), E = sodium chloride (5 mg); F = sodium hydroxide (0.5 mg); G = SLS (1 mg), G* = SLS (0.5 mg), H = Tween-80 (1 mg).

Table 2: Average zone of inhibition (ZI in mm, *n* = 3) produce by testing compositions of Table 1 against MRSA.

	Composition codes (Clindamycin + Pharmaceutical substance)						
S No. 1	**A**	**A + B**	**A + C**	**A + D**	**A + E**	**A + F**	**A + G**
Average ZI	0 ± 0	13.6 ± 1.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	20 ± 0
	Composition codes (Clindamycin + Pharmaceutical substance)						
S No. 2	**AA**	**AA + B**	**AA + C**	**AA + D**	**AA + E**	**AA + F**	**AA + G**
Average ZI	0 ± 0	13.6 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	21.3 ± 2.3
	Composition codes (Clindamycin + Pharmaceutical substance)						
S No. 3	**A***	**A* + B***	**A* + G**	**A* + G***	**G**	**A* + H**	**H**
Average ZI	0 ± 0	18.3 ± 0.5	20 ± 0	17.6 ± 0.5	19.6 ± 0.5	0 ± 0	0 ± 0
	Composition codes (Clindamycin + Pharmaceutical substance)						
S No. 4	**AA***	**AA* + B***	**AA* + G**	**AA* + G***	**G***	**AA* + H**	**B***
Average ZI	0 ± 0	18 ± 0	20 ± 1	17.6 ± 0.5	17.3 ± 0.5	0 ± 0	17.6 ± 0.5

Notes: Quantity of testing composition in their corresponding cup: A = clindamycin phosphate (2 µg); AA = clindamycin phosphate (5 µg); A* = clindamycin phosphate (4 µg); AA* clindamycin phosphate (10 µg); B = fusidic acid (0.05 µg); B* = fusidic acid (0.1 µg); C = sorbic acid (25 µg); D = citric acid (0.5 mg); E = sodium chloride (5 mg); F = sodium hydroxide (0.5 mg); G = SLS (1 mg); G* = SLS (0.5 mg); H = Tween-80 (1 mg).

Table 3: Average zone of inhibition (ZI in mm, *n* = 3) produce by testing compositions of Table 1, against *Staphylococcus epidermidis*.

Composition codes (Clindamycin + Pharmaceutical substance)							
S No. 1	**A**	**A + B**	**A + C**	**D**	**A + E**	**A + F**	**A + G**
Average ZI	10 ± 0	18.6 ± 1.5	12 ± 0	-	10.3 ± 0.5	17 ± 0	20.6 ± 0.5
Composition codes (Clindamycin + Pharmaceutical substance)							
S No. 2	**AA**	**AA + B**	**AA + C**	**D**	**AA + E**	**AA + F**	**AA + G**
Average ZI	16.3 ± 0.5	21.3 ± 1.1	16.3 ± 0.5	-	14.6 ± 0.5	20.3 ± 1.5	22.6 ± 3.7
Composition codes (Clindamycin + Pharmaceutical substance)							
S No. 3	**A***	**A* + B***	**A* + G**	**A* + G***	**G**	**A* + H**	**H**
Average ZI	15.3 ± 0.5	23.3 ± 1.1	19.6 ± 0.5	18 ± 1	21 ± 1	13.3 ± 2.5	0 ± 0
Composition codes (Clindamycin + Pharmaceutical substance)							
S No. 4	**AA***	**AA* + B***	**AA* + G**	**AA* + G***	**G***	**AA* + H**	**B***
Average ZI	22.3 ± 2.3	25.6 ± 0.5	20.6 ± 1.1	18.3 ± 0.5	16.3 ± 0.5	19.3 ± 0.5	20.3 ± 0.5

Notes: Quantity of testing composition in their corresponding cup: A = clindamycin phosphate (2 µg); AA = clindamycin phosphate (5 µg); A* = clindamycin phosphate (4 µg); AA* clindamycin phosphate (10 µg); B = fusidic acid (0.05 µg); B* = fusidic acid (0.1 µg); C = sorbic acid (25 µg); D = citric acid (0.5 mg); E = sodium chloride (5 mg); F = sodium hydroxide (0.5 mg); G = SLS (1 mg); G* = SLS (0.5 mg); H = Tween-80 (1 mg).

Clindamycin phosphate showed significant zone of inhibition against *S. epidermidis*. Zone of inhibition of the compositions given in Table 1 against *S. epidermidis* are presented in Table 3. The zone of inhibition was concentration dependent and increased with the increase in clindamycin concentration (see Table 3; A, AA, A*, AA*). The combinations of fusidic acid and clindamycin (A + B, AA + B, A* + B* and AA* + B*) showed remarkable activity against *S. epidermidis*. At the lower as well as higher concentrations the zone of inhibition was increased when clindamycin and fusidic acid were used in combination. Fusidic acid (0.1 µg, B*) alone produced a zone of inhibition of about 20.3 ± 0.5 mm. Fusidic acid and clindamycin produced a zone of inhibition of about 23.3 ± 1.1 mm (A* + B*) and 25.3 ± 0.5 mm (AA* + B*). The sorbic acid (A + C, AA + C) and sodium chloride (A + E, AA + E) did not show any remarkable effect on the *in-vitro* efficacy of clindamycin against *S. epidermidis*. Citric acid did not show the activity against *S. epidermidis* (see D, Table 3). Sodium hydroxide slightly improves the *in-vitro* activity of clindamycin phosphate (A + F & AA + F) against *S. epidermidis*. In the presence of sodium hydroxide, the zone of inhibition by clindamycin was increased about 1.7 fold at lower concentration (A + F) and about 1.3 fold at higher concentration of clindamycin (AA + F). Tween-80 (H) does not have any inhibitory effect on the growth of *S. epidermidis*, and its combinations (A* + H, AA* + H) also did not influence the efficacy of clindamycin. SLS showed significant activity against *S. epidermidis* and produced distinct zone of inhibition at lower as well as at higher concentrations. The zone of inhibition at lower concentration of SLS (G*) was 16.3 ± 0.5 mm, while at higher concentration (G) the zone of inhibition was 21 ± 1 mm. No

additive or synergistic effect was observed in the combined activity of SLS and clindamycin phosphate (A* + G, AA* + G, A* + G*, AA* + G*) against *S. epidermidis*.

DISCUSSION

The pharmaceutical excipients were considered as pharmacologically and therapeutically inactive. Some recent investigations have revealed that pharmaceutical excipients may interfere in some of pharmacological activities. The excipients have been reported to inhibits P-glycoprotein efflux transporter in intestine, and improve the oral bioavailability of P-glycoprotein substrates (Tariq *et al*. 2016; Yan *et al.* 2008; Huang *et al*. 2010). The mechanism responsible for MDR includes: detoxification or cleavage of drug molecules by enzymes, genetic alteration of the intra- or extracellular targets, reduced permeability of cell membrane and drug efflux by multidrug transporters (Godsey, Zheleznova Heldwein and Brennan 2002; Tanwar *et al.* 2014). Hypothesis for present studies was that pharmaceutical excipients may improves the susceptibility of resistant microbes, either by increasing the permeability of cell membrane by altering its fluidity, or by interfering in some of the critical cell cycle, or by altering the drug receptor selectivity or by interfering with P-glycoprotein efflux in cell membrane (Stringaro *et al*. 2002). Surprisingly, the current investigation reveals that SLS (surfactant, solubiliser) showed inhibitory action against MRSA as well as *S. epidermidis.* Inhibitory action of SLS was better against *S. epidermidis* than MRSA. On contrary another excipient Tween-80, which also acts as surfactant, solubiliser; was ineffective against MRSA as well as *S. epidermidis*. The SLS is an anionic surfactant, while Tween-80 is non-ionic; and this anionic segment of SLS molecule may be responsible for its activity. The fusidic acid was also effective against MRSA as well as *S. epidermidis.* The *in-vitro* efficacy of fusidic acid against these microbes was better than clindamycin phosphate. Sodium hydroxide also improves the *in-vitro* efficacy of clindamycin against *S. epidermidis*, but there was no effect on MRSA. The other tested excipients sorbic acid, citric acid and sodium chloride did not show any noticeable effect against any of these tested microbes.

CONCLUSION

The present study investigated the antimicrobial effect of therapeutic and non-therapeutic pharmaceutical substances on MRSA and *S. epidermidis*. The study highlighted the importance of testing numerous other non-therapeutic pharmaceutical substances against other susceptible and resistant pathogenic micro-organisms. Observations from such investigations will help to select suitable pharmaceutical excipient to include in topical antimicrobial formulations.

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CONFLICT OF INTEREST

All authors (Mohd Aftab Alam, Fahad I. Al-Janoobi, Khaled A. Alzahrani, Mohammad H. Al-Agamy and Abdullah M. Al-Mohizea) declared that they do not have any conflict of interest.

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