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Efficacy of lactic acid and acetic acid against multidrug resistant Staphylococcus aureus, E. coli and klebsiella sp.

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ABSTRACT

In the phase of emerging antibiotic resistance, the efficacy of organic acids including lactic acid and acetic acid proved worth applicable. The rational use of antibiotics has always been challenging. The overuse and under-use of antimicrobials provokes the development of resistance, through which multidrug Resistant (MDR) strains have emerged. In this experiment we trialed MDR strains of *Staphylococcus aureus*, *Escherichia. coli* and *Klebsiella* sp. recovered from clinical cases of mastitis through broth dilution method followed by disc diffusion technique to demonstrate the minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) for lactic and acetic acids. For lactic acid the MIC for *Staphylococcus aureus*, *E. coli* and *Klebsiella sp.* was 0.78µl, 0.39µl and 0.39µl respectively at the 0.5 McFarland turbidity standard. The zone of inhibition (ZOI) was ranging from 18mm±1 to 20mm±1 in diameter. However, the acetic acid MIC of 0.78µl was found equally effective against all test bacterial spp. with ZOI of 18mm±1 to 19mm±1 in diameter. Moreover, the MBC for lactic acid was 1.56ul against *Staphylococcus*, 0.78ul for *E. coli* and *Klebsiella spp.* exhibiting the ZOI ranging from 20mm±1 to 22mm ±1 in diameter. Acetic acid revealed the MBC of 1.56ul against all test bacterial spp. having ZOI ranging from 19mm±1 to 22mm±1 in diameter.

Contribution/Originality: This paper provides data on the efficacy of lactic acid and acetic acid against multidrug resistant (MDR) strains of Staphylococcus aureus and Escherichia coli. The study aims to contribute valuable insights into antimicrobial resistance management of S. aureus and E. coli, and the development of alternative treatment options against them.

1. INTRODUCTION

Antibiotics are the drugs which are being produce by microbes that hampers the growth or kill other germs while being safe to host cell [1]. Sensible and wise use of antimicrobial has limited the emergence of refusal to acceptance of antibiotic and may be able to decrease effect of resistance that has already being develop, that can increase the durability of antimicrobials [2, 3].

Use of antibiotics option has been decreased due to MDR strains. Most common way to treat the resistance is the combined use of antibiotics therapy, having different mode of action to prevent the resistance mechanism against antimicrobial [4]. This method of synergism plays a very important role in decreasing the complexity to

treat MDR strains. Likewise, use of organic product with antimicrobial enhances the effect of antimicrobials and decreases the challenges of resistance [5, 6].

The evolution of multidrug antibiotic resistance in commensal bacteria is an important public health concern. Commensal bacteria such as *Escherichia coli*, *Streptococcus pneumoniae* or *Staphylococcus aureus* are also opportunistic pathogens causing a large fraction of the community-acquired and hospital-acquired bacterial infections [7]. MDR makes these infections harder to treat with antibiotics and may thus cause substantial additional morbidity and mortality.

Considering MDR, in light of these issues, there is a rising interest in the exploration of non-antibiotic antimicrobial agents.in contrast to antimicrobial, which can act according to a single biochemical mechanism, these antimicrobial agent generally attack more than one site on bacteria. These non-antibiotic antimicrobial agents, *viz*., antimicrobial proteins and silver nanoparticles, can work by attaching to and disrupt the thiol group, inhibit Deoxyribose Nucleic Acid (DNA) replication, causing changes in protein expression, induce reactive oxygen species (ROS), denaturalize enzyme, or breakage in bacterial cell membrane [8]. By binding to multiple molecular targets, chances to develop resistance against antibiotics should be very much less [9].

Silver, Zinc Oxide and Titanium dioxide are another group of nanoparticles that act as non-antibiotic antimicrobial agents. These nanoparticles can develop stronger antimicrobial effects on a large number of bacteria [10]. Through insects and bacteria, several peptides, protein and enzyme are obtained working as a non-antibiotic antimicrobial agent. Because of their antibacterial effect they are very important for food industry and for biomedical application [11, 12]. To treat the Nosocomial infection particularly pseudomonas, different antiseptics and disinfectants acting as NAAB such as chlorhexidine, dettol, povidone-iodine are commonly used superficially [13, 14]. Different naturally obtained acids such as acetic acid, ascorbic acid, salicylic acid, citric acid, boric acid and lactic acid are use topically having efficient results in treating the wound infection on skin [15, 16].

Acetic acid has served as anti-biofilm, antimicrobial and nontoxic qualities that can affect the pathogens cell wall and changes the membrane permeability. Presently topical use of acetic acid is considered as worthwhile in treatment of wound infection. The lower concentrations of acetic acid (0.00975% – 0.039% v/v) can be used as an anti-virulent agent for the medication of Colistin-resistant *Pseudomonas aeruginosa*, similarly its higher concentration (>0.156% v/v) can be used to disinfect biofilm-prone surgical instruments, as hospital shelf antiseptic agent and for treatment of external wound [17]. Lactic acid is another organic agent used as food preservative and shows antimicrobial action in case of foodborne microorganism [18]. Previously, many properties of lactic acid has been enlighten for decontamination of meat, fruits and vegetables [19, 20]. In addition to antimicrobial activity, LA is used as an artificial additive and flavonoid, inhibiting lipid oxidation by reducing the pro-oxidative effect of sodium chloride (NaCl) [21]. Salicylic acids is another very important non antibacterial antimicrobial agent that has been used in human and veterinary medicine because of its anti-inflammatory, anti-pyretic and pain reducing features for decades. Most important function of salicylic acid is immune system modulator in response to bacterial infections [22, 23].

2. MATERIALS AND METHODS

2.1. Study Plan

In this study we use different organic acid *viz*., Acetic Acid (AA) and Lactic Acid (LA) as non-antibiotic antibacterial (NAAB) substances against Multidrug Resistant (MDR) bacteria from pure cultures, and determine their Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) at which minimum concentration this acid can inhibit and kill the bacteria. Then determine the ZOI to determine the resistant and sensitive concentration of these acid against bacteria.

The whole research work was divided into two distinct phases.

Phase I: Procurement and Evaluation of MDR Microorganisms.

Phase II: In-Vitro evaluation of Acetic Acid & Lactic Acid against MDR.

2.2. Phase I: Procurement and Evaluation of MDR Microorganisms

2.2.1. Procurement of Microorganisms

Pure cultures of *Staphylococcus aureus*, *Escherichia coli* (*E. coli*) and *Klebsiella* already procured from skin samples were obtained from National Veterinary Laboratory (NVL), Islamabad, Pakistan.

2.2.2. Identification of Microorganisms

2.2.2.1. Cultural Identification

For cultural identification of microorganisms, a single colony from pure culture was taken and grown on LB broth and incubated at 37°C for 24 hours. After incubation, loop full cultures of *E. coli and Klebsiella* were taken and streaked on MacConkey agar. Similarly, loop full culture of *Staphylococcus aureus* was streaked on Blood agar.

After incubation for 24 hours at 37°C, pure growths of *E. coli*, *Klebsiella* and *Staphylococcus* were obtained on their respective cultures and were characterized on the basis of their colony morphology.

2.2.2.2. Biochemical Identification

After identification on the basis of colony morphology, the test cultures *E. coli* and *Klebsiella* were subjected to biochemical analysis by Api-20E® (*bioMérieux*, *France*) and *Staphylococcus* is confirmed by MALDI-TOF.

2.2.3. MDR Evaluation of Pure Cultures

Collect colonies from pure culture with help of swab and transfer the pure culture onto freshly prepared Muller Hinton (MH) Agar plates.

Disc of Cefoxitin (OFX), Ampicillin (AMP), Levofloxacin (LEVO), Amikacin (AMK), Trimethoprim/sulfamethoxazole (SXT), Augmentin (AUG), Ciprofloxacin (CIP), Chloramphenicol (C), Tetracycline (TET), Tezobactum (TZP), Cefepime (FEP), Clarithromycin (CLR), Gentamicin (CN), (CRO), Cefotaxime (CTX), Imipenem (IMP), Meropenem (MERO) and Ertapenem (ETP) were placed in these pure cultures in order to assess their Antibiotic Sensitivity Profile (AST).

The results of Antibiotic Sensitivity Test according to Clinical and Laboratory Standards Institute (CLSI) guidelines were reported as described in Table 1.

Table 1. Antibiotic sensitivity test (AST) results according to CLSI guidelines.

Antibiotic	Staphylococcus	E. coli	Klebsiella
	Zone of inhibition in mm	Zone of inhibition in mm	Zone of inhibition in mm
OFX	--	6	6
AMP	0	6	6
LEV	17	6	6
AMK	13	27	14
SXT	11	6	12
AUG	6	6	6
CIP	19	6	6
C	15	6	6
TET	14	6	6
TZP	--	28	22
FEP	23	22	6
CLR	17	6	6
CN	8	24	12
CRO	14	10	6
CTX	17	10	6
IMP	21	22	17
MERO	--	24	22
ETP	--	20	14

2.3. Phase II: In-Vitro Evaluation of Acetic Acid and Lactic Acid against MDR

2.3.1. Standardization of Bacterial Cultures

In order to standardize bacterial cultures, a single colony from each pure culture was taken and suspended into 4.5 ml of Normal Saline separately and checked for 0.5 McFarland turbidity standards.

After standardization, the obtained bacterial suspensions were subjected to in-vitro testing against Acetic acid® *(Sigma-Aldrich, USA)* and Lactic acid® *(Sigma-Aldrich, USA)* by Microdilution assay and Disk Diffusion Assay.

2.3.2. Microdilution Assay

To perform microdilution assay, we follow the protocol as describe in this Garza-Cervantes, et al. [24].

- Add 100µl Peptone water from well 1-12 in microtitration plate.
- Add 100µl Lactic acid in well 1 and perform 2-fold serial dilution from well 1-11. Well 12 was kept as positive control.
- Add 25µl of 0.5 McFarland *Staphylococcus* suspension from well 1-10 and 12 in row A. Well 11 was kept as negative control.
- Similar protocol was repeated for *E. coli* and *Klebsiella* in row B and C respectively. Incubate the microtitration plate for 24 hours at 37°C

Results were recorded in the form Minimum Inhibitory Concentration (the least concentration of Lactic acid showing no bacterial growth) by checking for turbidity.

The same protocol was repeated for Acetic Acid in row E, F and G by using *Staphylococcus aureus*, *E. coli* and *Klebsiella* respectively and the results were recorded in form of minimum inhibitory concentration (MIC).

To determine the MBC, we streak a loop full of all the concentration from the microdilution plate on Muller Hilton Agar (MHA), incubate the MHA plates of 24hrs [25]. After 24hrs check the colonies on agar.

2.3.3. Disk Diffusion Method

Take a sterile cotton swab and soak it thoroughly in 0.5 McFarland standard suspensions of each bacteria and swab them on MH agar plates uniformly [26]. Now prepare discs of Lactic acid and Acetic acid by dipping 6mm thickness sterile filter paper discs into its different concentrations (100µl, 50µl, 25µl, 12.5µl, 6.25µl, 3.125µl, 1.56µl, 0.78µl, 0.39µl and 0.195µl). Air dry these discs in sterile environment and apply them on prepared agar plates containing bacterial cultures.

Incubate for 24 hours at 37°C and record results in the form Zone of Inhibition (ZOI) around each disc and interpret results accordingly.

3. RESULTS

The minimum inhibitory concentrations were determined from the optical density (OD) values at 600nm wavelength for lactic acid and acetic acid separately. The lowest concentrations at which OD600 values were comparable to OD600 values of negative control well, were terms as minimum inhibitory concentrations. At concentrations lower than MIC, significant turbidity was present in the wells, causing much higher OD600 values indicating the presence of bacterial. For lactic acid, MIC was recorded at 0.78ul against *Staphylococcus* with OD600 value of 0.041. Whereas, for *E. coli* and *Klebsiella* the MIC of lactic acid was found at 0.39ul for both exhibiting OD600 values of 0.041 and 0.04 respectively as shown in Table 2.

On the other hand, MIC of acetic acid was found effective at 0.78ul against all bacterial spp. At MIC, the OD600 values of *Staphylococcus*, *E. coli* and *Klebsiella* were 0.041, 0.0403 and 0.041 respectively as shown in Table 2.

Table 2. OD_{600} of microdilution plate.

LA	1 100µL	2 50µL	3 25µL	4 12.5µL	5 6.25µL	6 3.12µL	7 1.56µL	8 0.78µl	9 0.39µl	10 0.19µl	11 -ve	12 +ve
Staph	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.67	0.56	0.04	0.45
E. coli	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.48	0.04	0.74
Kleb.	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.38	0.04	0.53
AA												
Staph	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.27	0.44	0.04	0.51
E.coli	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.29	0.52	0.04	0.60
Kleb	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.38	0.53	0.04	0.58

Minimum Bactericidal Concentration (MBC) is the concentration at which all bacteria are killed. For lactic acid, the MBC was recorded at 1.56µl, 0.78µl and 0.78µl for *Staphylococcus aureus*, *E. coli* and *Klebsiella* respectively. Whereas, in case of acetic acid, MBC of 1.56 ul was found equally effective for *Staphylococcus aureus*, *E. coli* and *Klebsiella* sp.

In second part of trial, we determined zone of inhibition (ZOI) for lactic acid and acetic acid. For lactic acid, ZOI at 0.78ul concentration (MIC) was measured at 19mm±1 in diameter. Whereas, ZOI of lactic acid against *E. coli* and *Klebsiella sp.* was measured at 18mm±1 for both at 0.39ul concentrations. The ZOI at MBC were also measured to be 19mm±1, 18mm±1 and 18mm±1 for lactic acid against *Staphylococcus*, *E. coli* and *Klebsiella sp.* respectively as shown in Table 3.

Table 3. Zone of inhibition of lactic acid on Muller Hilton agar.

LA conc.	100µL	50µL	25µL	12.5µL	6.25µL	3.12µL	1.56µL	0.78µL	0.39µL	0.19µL
Staph	29	27	22	24	22	21	20	18	6	8
E. coli	28	27	23	22	21	21	20	20	18	8
KP	29	28	25	22	19	19	19	20	19	8

ZOI of acetic acid against *Staphylococcus*, *E. coli* and *Klebsiella sp.* was measured at 18mm±1, 19mm±1 and 19mm±1 respectively at concentration 0.78ul (MIC). The ZOI at MBC were also measured to be 20mm±1, 19mm±1 and 20mm±1 for lactic acid against *Staphylococcus*, *E. coli* and *Klebsiella sp.* respectively as shown in Table 4.

Table 4. Zone of inhibition of acetic acid on Muller Hilton agar.

AA conc.	100µL	50µL	25µL	12.5µL	6.25µL	3.12µL	1.56µL	0.78µL	0.39µL	0.19µL
Staph	28	27	23	22	23	22	21	18	7	7
E. coli	29	27	24	22	21	20	19	18	5	7
KP	28	26	25	22	21	21	20	18	6	8

4. DISCUSSION

Bovine mastitis has been amongst the most economically significant diseases for the concern of dairy industry. It leads to marked reduction in both the quantity as well as quality of milk produce along with additional expenses of veterinary services, extra labor and other medicinal costs [27]. In order to control this notorious disease and prevent its economic losses, a mastitis control programme consisting of "5 point agenda" was surfaced by National Mastitis Council. One of the most important points of that agenda was to introduce some novel antimicrobial agents into teats as post-milking dips. It was also emphasized that the teat dip agents must bear no harm to the living tissue of teat and / or udder [28]. In past few decades, conventional antibiotic options have significantly decreased owing to the ever-increasing menace of antimicrobial resistance, not only making antibiotics ineffective against infectious pathogens, but also causing massive financial losses incurred on these ineffective antimicrobials for the farmers [4]. In such unusual situation, there is a special need to search for some natural product / s beyond the scope of antimicrobial resistance as well as having no harm on the living tissue. Considering all these circumstances, this study was planned to use organic acids like lactic acid and acetic acid as non-antibiotic antibacterial agents to treat multidrug resistant strains of common mastitis causing pathogens in *Staphylococcus aureus*, *E. coli* and *Klebsiella sp.* These natural organic acids possess strong antibacterial activity, pose no harmful effect to the diseased animal tissue and impart no negative impact on the consumer as well.

Use of non-antibiotic agents against specific bacteria is considered as effective way of treatment, as in this strategy of treatment bacteria do not develop resistance against these non-antibacterial agents. In this research, we trialed Multidrug resistant (MDR) strains of *Staphylococcus aureus*, *E. coli* and *Klebsiella* sp. recovered from clinical cases of mastitis and determined their minimum inhibitory concentrations and maximum bactericidal

concentrations through broth microdilution assay against acetic acid and lactic acid as non-antibiotic antimicrobial agents. Trial was conducted on MDR strain of *Staphylococcus aureus*, *E. coli* and *Klebsiella*, in which *Staphylococcus aureus* is gram positive and other 2 bacteria are gram negative. From this study we concluded that the higher concentration of lactic acid is needed to kill gram positive bacteria as compare to gram negative, because of its outer and inner membrane composition as gram positive lack outer membrane but surrounded by layers of peptidoglycan many time thicker than gram negative imparting a need of higher concentration of acid to kill gram positive.

In order to check antimicrobial activity we performed Broth Dilution method and Disc Diffusion method. Through Broth Dilution method, we determined the MIC (Minimum inhibitory concentration) and MBC (Minimum bactericidal concentration) as MIC is the minimum concentration of acid used to inhibit the growth of bacteria, while MBC is the minimum concentration of agent at which all bacteria are killed.

From the results shown in Table 3 and Table 4, we can interpret that both the lactic acid and acetic acid have strong potential as antibacterial agents. MIC and MBC concentration of lactic acid is higher (0.78ul) against Staphylococcus aureus, whereas the same is lower (0.39ul) against E. coli and Klebsiella sp. This higher MIC against S. aureus can be correlated to its Gram +ve nature having a much thicker cell wall than other two bacterial spp. (Gram –ve). With the similar protocol, we also determined MIC and MBC of acetic acid against all bacterial spp. MIC and MBC values of acetic acid were 0.78ul and 1.56ul respectively for all bacterial spp.

Comparing MIC and MBC of both lactic acid and acetic acid against all bacterial spp. it can be interpreted that lactic acid has lower MIC and MBC values than MIC and MBC values of acetic acid for E. coli and Klebsiella sp. But comparing MIC and MBC of both the lactic acid and acetic acid, we surprisingly found them equal against S. aureus. Hence, we can conclude that lactic acid is more effective than acetic acid against MDR strains of E. coli and Klebsiella sp. whereas both lactic acid and acetic acid are equally potent against MDR strain of S. aureus.

Staphylococcus aureus, *E. coli* and *Klebsiella* are the skin inhabitants and opportunistic bacteria, for example whenever they get a favorable environment they will invade through the skin and cause infection. Mastitis is the most important disease caused by *Staphylococcus aureus* and cause much economical loses. In order to treat mastitis different precautionary measures as well as different antibiotics has been used, but due to their resistance and residues in milk there use has been limited. From this study we conclude that these non-antibiotic antibacterial agents are being used for the treatment of mastitis. The MIC concentration of these organic acid are being used to disinfect the teats before and after milking and can also be injected directly into the teats. The main advantage of these non-antibiotic antibacterial agents is that the microorganism don't develop resistance upon there repeated usage and they have no residues in milk. Main origin of lactic acid is milk, if the milk have residue of lactic acid then it is not harmful as compare to antibiotic residues. While acetic acid may cause a little bit irritation while infusing in teat but overall it is harmless, painless and don't cause a tissue injury.

E. coli and *Klebsiella* are also commensal bacteria of skin. They are mostly present in skin wounds. In order to treat these skin wounds that are infected with MDR strains, we can use MIC concentration of non-antibiotic antibacterial agent. These MIC concentrations are applied directly as a source of antiseptic on wound and we dip wound in the non-antibiotic antibacterial agent bath, as these organic acids don't cause any skin burn or irritation.

5. STUDY LIMITATIONS

Even though the use of non-antibiotic antibacterial agents has many advantages over the other conventional methods to combat bacterial infections, still work on these non-antibiotic antibacterial substances have some limitations. The regulatory pathways for non-antibiotic antibacterial agents are less well-defined as compared to the same for antibiotics. This ultimately leads to delay in drug approval chain and limits its availability as an effective biocide to be used in clinical settings. Also, there is a lack of investment on non-antibiotic antibacterial agents' development, which is very negligible as compared to antibiotics, which in turns limits the funding and pace of innovation.

6. CONCLUSION

From our this research, we concluded that we can use non-antibiotic antibacterial agents as an alternative to antibiotic against MDR strains because microorganism don't develop resistance against these non-antibiotic antibacterial agents and have no residual time period. In addition they are totally harmless for the living tissue as well.

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