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Impact of Non-Antibiotic Antibacterial Substances against Multidrug Resistant Staphylococcus aureus, E. coli and Klebsiella

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Article

Impact of Non-Antibiotic Antibacterial Substances against Multidrug Resistant *Staphylococcus aureus, E. coli* and *Klebsiella*

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Abstract: Conventionally, antibiotics have been regularly practiced as a cure of specific infections caused by specific bacteria. The rational use of antibiotics has always been challenging. Antibiotic residual time period is different for different antibiotics. The overuse and under-use of antimicrobials provokes the development of resistance, through which MDR strains have emerged. In this research, we intend to use Non-Antibiotic Antibacterial Agents (NAAB) that have no side effect and no residual time, in order to treat these MDR strains. Microorganisms do not develop resistance against NAAB. This study revolves around the use of Lactic Acid (LA) and Acetic Acid (AA) as NAAB against MDR strains of Staphylococcus aureus, E. coli and Klebsiella. Through broth dilution method, we identified the Minimum Inhibitory Concentration (MIC). For LA, MIC for Staphylococcus aureus, E. coli and Klebsiella is 0.78µl, 0.39µl and 0.39µl respectively and OD600 values at MIC is 0.041 for all microorganisms. Similarly, for AA the MIC was recorded at 0.78µl for Staphylococcus aureus, E. coli and Klebsiella, with OD600 value 0.041.Through Disc Diffusion Method, we got zone of inhibition (ZOI) 25mm ±1 to 18mm±1 at NAAB concentrations above MIC, indicating concentrations sufficient enough to kill bacteria while we got ZOI below 10mm at concentrations below MIC indicating that bacteria are resistant at these concentrations. We concluded that NAAB agents possess strong potential as disinfectants and antiseptics and can be ployed for the treatment of mastitis as their MIC concentrations are totally harmless for living tissue even if we inject directly into it.

Keywords: acetic acid; minimum inhibitory concentration,; multidrug resistance,; lactic acid,; zone of inhibition

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 (Chikezie et al., 2017). Sensible and wise use of antimicrobial has limit 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 (Nwobodo et al., 2022; English and Gaur, 2010).

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 (Kapoor et al., 2017). 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 (Qadri et al., 2022; Jenkins and Cooper, 2012).

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

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hospital-acquired bacterial infections (Eliott et al., 2020). 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 DNA replication, causing changes in protein expression, induce reactive oxygen species (ROS), denaturalize enzyme, or breakage in bacterial cell membrane (Naseri-Nosar and Ziora, 2018). By binding to multiple molecular targets, chances to develop resistance against antibiotics should be very much less (Cooper and Kirketerp-Moller, 2018)

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. (Mishra *et al., 2017).*Through insects and bacteria, several peptides, protein and enzyme are obtained working as a non-antibiotic antimicrobial agents. Because of their antibacterial effect they are very important for food industry and for biomedical application. (Yoon *et al., 2012; Singh et al., 2022)*.

To treat the Nosocomial infection particularly pseudomonas, different antiseptics and disinfectants acting as NAAB such as chlorhexidine, dettol, povidone-iodine are commonly used superficially (Tawre et al., 2021; Agrawal et al., 2017). 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 (Ji et al., 2023; Kramer et al., 2018).

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 COL-R *P. 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 (Feng et al., 2022).

Lactic acid is another organic agent used as food preservative and shows antimicrobial action in case of foodborne microorganism (Beuchat and Colden, 1989). Previously, many properties of lactic acid has been enlighten for decontamination of meat, fruits and vegetables (Alvarado-Casillas et al., 2007; Park et al., 2011). In addition to antimicrobial activity, LA is used as an artificial additive and flavonoid, inhibiting lipid oxidation by reducing the pro-oxidative effect of NaCl (Paelinck and Szczepaniak, 2005).

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 (Montinari et al., 2019; Ellen et al., 2024)

2. Material and Methods

Study plan:

In this study we use different organic acid *viz.*, Acetic Acid (AA) and Lactic Acid (LA) as Non-antimicrobial antibiotic substances (NAAB) against Multidrug Resistant (MDR) bacteria from pure cultures, and determine their Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) at which minimum concentration these 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

2.1. Phase I: Procurement and Evaluation of MDR Microorganisms

2.1.1. Procurement of Microorganisms:

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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.

2.1.2. Identification of Microorganisms:

Cultural identification:

For cultural identification of microorganisms, a single colony from pure culture was taken and grown on LB broth and incubated at 37ºC 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 37ºC, pure growths of *E. coli*, *Klebsiella* and *Staphylococcus* were obtained on their respective cultures and were characterized on the basis of their colony morphology.

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.

2.1.3. 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 CLSI guidelines were reported as described in Table 2.1.

Table 2. 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

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

2.2.1. 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.

 doi:10.20944/preprints202409.2261.v1

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.

2.2.2. Microdilution Assay:

To perform microdilution assay we follow the protocol as describe in this (Garza-Cervantes et al., 2023)

- 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 37ºC

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 MIC.

To determine the MBC (Tapouk et al., 2020), we streak a loop full of all the concentration from the microdilution plate on Muller Hilton Agar (MHA), incubate the MHA plates of 24hrs. After 24hrs check the colonies on agar.

2.2.3. 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 (Ye-won et al., 2013). 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 37ºC and record results in the form Zone of Inhibition (ZOI) around each disc and interpret results accordingly.

3. Results and Discussion:

In Table 3.1, OD_{600} value 0.041 is considered as the MIC value at concentration of 0.78µl, 0.39µl and 0.39µl for *Staphylococcus aureus*, *E. coli* and *Klebsiella* respectively in case of LA. While in case of AA, we got OD_{600} 0.041 as MIC value at concentration 0.78µl, 0.78µl and 0.78µl for *Staphylococcus aureus*, *E. coli* and *Klebsiella* respectively. These MIC values are directly related to negative control OD_{600} value *i.e* 0.041. The well before MIC is considered as MBC *i.e* minimum concentration at which bacteria were killed, which was 1.56µl, 0.78µl and 0.78µl for *Staphylococcus aureus*, *E. coli* and *Klebsiella* in case of LA. While in case of AA, MBC is 1.56µl, 1.56µl and 1.56µl for *Staphylococcus aureus*, *E. coli* and *Klebsiella* respectively.

Table 3.1 OD_{600} of microdilution plate.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
		100µL	**50µL**	**25µL**	**12.5µL**	**6.25µL**	**3.12µL**	**1.56µL**	**0.78µl**	**0.39µl**	**0.19µl**	**-ve**	**+ve**
LA *Staph*	A	0.0409	0.0435	0.042	0.0416	0.0414	0.0431	0.0413	**0.041**	0.669	0.562	0.038	0.453
E. coli	B	0.042	0.0433	0.0451	0.0438	0.0428	0.0435	0.0418	0.0393	**0.041**	0.486	0.044	0.743
Kleb.	C	0.0444	0.0476	0.0455	0.0425	0.041	0.0413	0.0404	0.0416	**0.04**	0.382	0.039	0.530
AA	D												
Staph	E	0.0395	0.0415	0.0398	0.0407	0.0399	0.039	0.0395	**0.041**	0.270	0.449	0.038	0.512
E.coli	F	0.0391	0.0429	0.0419	0.0405	0.0417	0.039	0.04	**0.0403**	0.295	0.523	0.039	0.599
Kleb	G	0.0402	0.0413	0.0402	0.0419	0.0404	0.0401	0.04	**0.041**	0.381	0.533	0.041	0.586

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Table 3.2 interprets the ZOI of *Staphylococcus aureus*, *E. coli* and *Klebsiella*, which is 18mm to 29mm±1 indicating that at these concentrations bacteria are sensitive. ZOI below 10mm indicate that at these concentrations, bacteria are resistant.

Table 3. 2: 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	20	19	**19**	5	6	8
E.coli	28	27	23	22	21	20	18	**18**	6	8
KP	29	28	25	22	19	19	18	**18**	6	8

Table 3.3 interprets the ZOI of *Staphylococcus aureus*, *E. coli* and *Klebsiella*, which is 18mm to 29mm±1 indicate that at these concentrations bacteria are sensitive. ZOI below 10mm indicate that at these concentrations, bacteria are resistant.

Table 3. 3: 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	0.39 µL	0.19µL
Staph	28	27	23	22	23	22	20	**18**	7	7
E.coli	29	27	24	22	21	20	19	**19**	5	7
KP	28	26	25	22	21	21	20	**19**	6	8

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 agent. In this case, we used acetic acid and lactic acid as non-antibiotic antimicrobial agents and studied their effects on gram positive and gram negative bacteria. We have taken 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 LA 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. After 24hrs incubation, we checked turbidity and OD_{600} value of wells as shown in Table 1. We concluded that the last clear well concentration has OD_{600} value 0.041 which is directly related to negative control OD_{600} value. In case of LA Concentration 0.78µl, 0.39µland 0.39µl shown this OD_{600} value while in case of AA concentration shown this OD_{600} value is 0.78µl, 0.78µl and 0.78µl for *Staphylococcus aureus*, *E. coli* and *Klebsiella* respectively, these concentration are considered as MIC. This MIC concentration of LA and AA are used for feed fortification, food preservation in tin, as an antiseptic and disinfectant on living and nonliving surfaces. It has been reported that LA is totally painless, nonirritant, don't cause any redness and tissue damage.

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 NAAB 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 NAAB agents is that the microorganism don't develop resistance upon there repeated usage and they have no residues in milk. Main origin of LA is milk, if the milk have residue of LA then it is not harmful as

 doi:10.20944/preprints202409.2261.v1

compare to antibiotic residues. While AA 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 NAAB agent. These MIC concentration are applied directly as a source of antiseptic on wound and we dip wound in the NAAB agent bath, as these organic acids don't cause any skin burn or irritation.

4. Conclusion

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

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