

Evaluation of Antimicrobial Activities of Commercial Product (RH⁵⁺) and its Competitor Products against Clinically Isolated Bacterial Strains and Bacterial Population Present in Poultry Bed

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ABSTRACT

The gradual increase of antibiotic resistance in microbes has become the greatest health threat for human beings and animals. Disinfectants which are generally used in the farming industry effected on animal health and reduced productivity. Therefore, the aim of the present study was to evaluate antimicrobial activity of newer marketed disinfectant product RH⁵⁺ and its three competitor products (D1, D2 and D3) against clinically isolated multidrug resistant bacteria *Escherichia coli* (EC-1), *Pseudomonas aeruginosa* (PA-1), *Klebsiella oxytoca* (KO-1), *Proteus vulgaris* (PV-1), and *Staphylococcus aureus* (SA-1) and also investigate their efficacy against the bacteria found in Poultry bed. RH⁵⁺, D1, and D3 showed excellent antimicrobial efficacy against clinical isolates as well as against the bacterial populations found in the soil of poultry bed. The MIC, MBC values were noted at very low concentration (1µl/mL) of the tested compounds and inhibition zones were also significant as observed from agar diffusion assay. The RH⁵⁺, D1 and D3 also reduced bacterial colony formation from poultry bed soil. The D2 did not show any antimicrobial property against clinical isolates up to 100µl/mL dose. The quaternary ammonium, glutaraldehyde and other active ingredients of the commercial compounds present in those formulations were noted as potent bacteriostatic against multidrug resistant bacteria and against the bacterial populations found in the poultry bed soil. Thus, it was concluded that the commercial products i.e. RH⁵⁺ and competitor products could be used as an effective disinfectant in poultry farm to eliminate the pathogenic infections of the birds.

Keywords: Commercial antimicrobial, RH⁵⁺, minimum inhibitory concentration, minimum bactericidal concentration, poultry bed

INTRODUCTION

In recent time, multidrug resistant bacteria are a great threat to the world. Now-a-days, it has been found that abundant use of antibiotics without maintaining the real prescription policies develops massive emergence of multidrug-resistant bacterial strains. [1] This is not only the biggest risk for human health but also producing a remarkable threat to animals.

According to the research reports, severe crisis of new antibiotics for the treatment of those superbugs is the most shocking issue in this time. [2] Abundant use of antimicrobial drugs results a selective amplification of resistant bacteria, having capability to endure and replace the sensitive bacteria as well as greatly affect normal microbial flora. [3] From the last 30 years, alarming spreads of zoonotic diseases

produce a great challenge to global health. Zoonotic diseases are not the latest conception, they have an extensive periphery in human disease and most communicable diseases e.g. influenza, tuberculosis, yellow fever, anthrax etc., spreads from the domestic animals, poultry and farm animals. [4,5] Over the world, poultry industry is one of the most pervasive food industries, and on the other hand chicken is commonly consumed food item. Approximately, 90 billion tons of chicken meat produced in every year. [6] Various antibiotic resistant bacteria isolated from the poultry soil, and these enteric bacteria draw attention because of its direct exposure on public health, through uplifting the morbidity, mortality of infection caused by cross resistant to drugs applied in human medicine. [7] The most frequent causative microbes of wound infection, urinary tract infection, nosocomial and various other types of infections are *Staphylococcus* family, *Enterobacteriaceae* family and other bacterial species, [8] which have a prominent connection with the poultry industry. Many kind of opportunistic bacteria have been reported from the poultry, including *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Klebsiella oxytoca*. In the *Enterobacteriaceae* group *Escherichia coli* is a facultative anaerobe bacterium and thin layer of peptidoglycan indicate the gram negative phenomena of bacteria. [9] Importantly, *Escherichia coli* are present in intestinal tract of animals and widely used in the research field to examine antimicrobial resistant in food products found from animal sources. [10] Some *Escherichia coli* strains hosted by poultry farms are potential source of antimicrobial resistant genes that may transmit to humans. [11] *Staphylococci* family's *Staphylococcus aureus* is a gram positive coccus, non-motile and non-spore producer. A strain of *Staphylococcus* i.e. *Staphylococcus aureus* is one of the typical inhabitants of skin and nares (man and animals) which act as pathogen under various conditions. [12] In

the *Pseudomonas* genus, *Pseudomonas aeruginosa* has high significance in poultry industry, is an opportunistic bacterium capable of infecting all tissues in poultry. [13] *Proteus vulgaris* is a zoonotic human pathogen and have a great importance in public health. It is a member of enterobacteriaceae group found in intestinal tract of mammals, soil, water and they have been identified or isolated from the poultry. [14]

As a protective agent, antibiotics played a great role at sub therapeutic level in farm animals at the time of their discovery. [15] Extensive and unidirectional use of antibiotics lead to the rush in widespread and uncontrolled disease causes, related to antibiotic resistant bacterial infection. [16] The use of antibiotics as a growth enhancer was subsequently prohibited soon after the increase in prophylactic usage of therapeutic antibiotics against bacterial infections in animals. [17] Researcher found the alternative way out for the control of bacterial disease in poultry in a post-antibiotic era. A harmless sterilizer used continually in poultry production where a diminution in bacterial loads has been recorded. [18] Previous reports strongly suggested that for the management and elimination of pathogenic organisms, very little progress has been for finding of alternatives to the use of chemical disinfectants, which are generally applied, such as quaternary ammonium based compounds, glutaraldehyde, sodium hypochlorite, and a variety of organic acids. Those agents have multiple cellular targets such as the cell wall (or the outer membrane), the cytoplasmic membrane, functional and structural proteins, DNA, RNA and other cytosolic components. [19,20] Quaternary ammonium compound, which are USDA (United States Department of Agriculture) permitted and EPA (The Environmental Protection Agency) approved non-toxic sanitizers for food processing utensils, are exceedingly used in the poultry industry due to the flourishing efficacy. [21] Quaternary ammonium

compounds (QACs) served as antimicrobials agent with a cationic surfactants and an extensive spectrum of activities. [22] Predominant action of QACs is disruption of the bacterial cell membrane. [23] The length of the N-alkyl chain of QACs made it a potent antimicrobial agent. In case of gram-positive bacteria the chain contain 14 carbon whereas 16 carbons for gram-negative bacteria. [24] A quaternary ammonium blend alkyl dimethyl benzyl ammonium chloride (ADBAC), and a dialkyl agent, didecyldimethylammonium chloride (DDAC) performed as bactericidal, contributed to cell death of *S. aureus*. These agents caused immediate leakage of intracellular constituents by interacting with the cytoplasmic membrane of gram positive *S. aureus*. [25] Sublethal dose of DDAC showed a synergistic bactericidal effect against *S. aureus*. [26] Glutaraldehyde served as a biocidal agent against glutaraldehyde (GTA)-sensitive and -resistant strains of *Mycobacterium chelonae*. [27] Glutaraldehyde-didecyldimethylammonium bromide (GA-DDAB) a newly discovered bactericide components damaged the *E. coli* cell membrane by leakage of intracellular components and inactivation of H⁺ - ATPase. [28] Benzalkonium chloride and QAC was tested against 77 resistant bacterial strains and QAC performed more efficiently rather than the other two disinfectants. [20]

In the application field, disinfectants are regularly mixed with water in a container and exposed out to the severe environmental situation before their definite appliance. Disinfectants may have a small lifespan after their initial dilution and it is possible that heat, sunlight, time, organic matter and adulterants may reduce their efficacy. In this study we used Quaternary ammonium and other compounds (like glutaraldehyde, didecyl dimethyl ammonium chloride, benzalkonium chloride are) against gram positive and gram negative both bacterial stain, which was found in the poultry soil.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Culture Media and chemicals

Nutrient agar, Mueller-Hinton agar, Nutrient broth, Luria broth and peptone broth were used for antibacterial activity respectively. All the culture media were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India) and Merck Ltd. (India).

2.1.2. The antimicrobial Compounds

The experimental compounds are composed of antimicrobial components i.e., Di-decyl di-methyl ammonium chloride, Di-octyl di-methyl ammonium chloride, Octyl-Decyl di-methyl ammonium chloride, Benzalkonium chloride, Glutaraldehyde, Pine oil, and Terpeneol oil, cumulatively designated as RH⁵⁺ which is used as disinfectant in market, whereas, D2 and D3 were the competitor products. All the test compounds (in liquid state) were supplied by R.R Animal Health Care Ltd, Hyderabad, Telangana 500068 and were stored at room temperature until further use.

2.1.3. Test microorganisms

Five clinically isolated multidrug resistant bacterial strains including four gram negative bacteria *Escherichia coli* (EC-1), *Pseudomonas aeruginosa* (PA-1), *Klebsiella oxytoca* (KO-1), *Proteus vulgaris* (PV-1), and one-gram positive bacteria *Staphylococcus aureus* (SA-1) were collected from department of Microbiology, Malda Medical college and Hospital, Malda, West Bengal, India. Species specific confirmation and antibiotic susceptibility pattern of those strains were confirmed by VITEK[®] 2 system, BioMérieux India. All the above mentioned strains were multidrug resistant strains and were subcultured accordingly and used throughout the study.

2.2. Methods

2.2.1. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) was determined by micro-dilution method according to the National Committee for Clinical Laboratory Standards (currently known as CLSI) with some modifications. Briefly, in 1ml Luria

broth, 10 µl bacterial suspension was added containing 2.5×10^5 CFU/mL strains. Different concentrations (1, 5, 10, 25, 50 and 100 µl/mL) of test antimicrobial compounds (RH⁵⁺, D1, D2 and D3) were added to the test tubes containing the bacterial strains and incubated for 24 h at 37 °C. The MIC value of each compound against each bacterial strain was noted at the lowest concentration inhibiting visible growth of bacteria after 24 h of incubation. [29]

The minimum bactericidal concentration (MBC) of the RH⁵⁺, D1, D2 and D3 were determined according to the method described elsewhere. [29] MBC is an addition part of the MIC experiment. In case of MBC, all MIC dilutions were sub-cultured on the sterile nutrient agar plates incubated at 37°C for 24 hours. The minimum concentration of the tested compounds (RH⁵⁺, D1, D2 and D3) required for completely killing of the multidrug resistant bacterial strains were noted as MBC value of the compound. The MBC value was shown as 100% bacterial killing, compared with the positive control (without treatment) and negative control (without bacterial inoculums).

2.2.2. Determination of Tolerance

The tolerance levels of the bacterial strain against RH⁵⁺, D1, D2 and D3 were determined according to the standard method using the following formula. [30]

Tolerance = MBC/MIC.

2.2.3. Agar diffusion assay

The antimicrobial susceptibility test using agar diffusion of four test compounds (RH⁵⁺, D1, D2 and D3) against five multidrug resistant bacterial strains was also determined according to the technique. [31] Muller Hinton agar (MHA) plates were prepared into sterile Petri plates by pouring 15 ml of molten media. The bacterial inoculums were taken from freshly grown culture (5 h) having 10^6 CFU/ml cells (compared with MacFarland's standard solution). By using sterile swab sticks (HiMedia, India) a bacterial lawn was prepared with on Muller Hinton agar media plate and was allowed to dry at 37°C incubator. Using 1ml micropipette tips, five holes on the MHA agar were done. Then 40 µl of different concentrations of the test compounds (crude, 1:2, 1:4, 1:8 and 1:16) were poured in each hole. Then the plates were kept for incubation at 37°C for 24 hours. The diameter of zone of bacterial growth inhibition was measured using zone scale (HiMedia, India). [29]

2.2.4. The onsite antibacterial activities of compounds RH⁵⁺, D1, and D3

At first 50 gm of poultry soil (collected from poultry bed of nearby firm) was mixed with 200 ml autoclaved distilled water and waited until all the solid precipitates deposited at the bottom of the container and clear supernatant found at the upper part. Then, 100 µl of that supernatant was mixed with peptone broth and incubated for 4 hrs in shaking condition for bacterial growth. For each set 100 µl of the fresh supernatant was mixed in peptone broth according to the following Table 1.

Table 1: Experimental setup of onsite antimicrobial study of compounds D1, D2, and D4

Drug dilution	(+) control	(+) control (Diluted) (200 times)	(-) control	1:1000 dilution	1:2000 dilution
Bacteria	100 µl	20 µl (+ control)	-	100 µl	100 µl
Antimicrobial compounds (RH ⁵⁺ , D1 and D3)	-	-	-	7 µl	3.5 µl
Peptone broth	6900 µl	1980 µl	7000 µl	6893 µl	6896 µl
Total	7000 µl	2000 µl	7000 µl	7000 µl	7000 µl

After the specified incubation period, the antimicrobial compounds (RH⁵⁺, D1, and D3) were added to each test tube maintaining dilution of 1:1000 and 1:2000

for each compounds and allowed to incubate for 30 min and 60 min. After that treatment 100 µl of bacterial culture were taken from each of the test tube including positive

control (diluted 200 times) and were spread on nutrient agar and incubated overnight at 37° C incubator. After overnight incubation period, the colonies were counted from each plate. The antimicrobial compound D2 was not used for the study as it showed no antimicrobial activity against clinical isolates.

3. RESULT

In the present study, commercial disinfectant compound RH⁵⁺, and competitor products D2 and D3 were examined against five clinical isolates of multidrug resistant bacterial strains viz. *E. coli*, *S. aureus*, *K. oxytoca*, *P. vulgaris*, and *P. aeruginosa* and certain parameters of antimicrobial activities were determined.

3.1. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

As presented in Table 1, RH⁵⁺ and competitor product were tested displayed against multidrug resistance bacteria at different concentrations, revealed a strong dose-dependent antimicrobial activity. Among the four compounds, D1 was the most active as it showed the highest inhibitory effects against all five clinical isolates of multidrug resistant bacterial strains (MIC value of 1µl/mL). Although RH⁵⁺ and D3 also displayed same MIC value i.e. 1µl/ml but there had a moderate result for *K. oxytoca*, *P. aeruginosa* and *S. aureus*. MIC values of *K. oxytoca*, *P. aeruginosa* were 5µl/ml against RH⁵⁺ whereas *S. aureus* exhibited 5µl/ml MIC value against D3. D2 did not show any significant inhibition of microbial growth (Table 2). The negative controls were used to compare with the inoculated tubes where no growths were present (Fig.1A, Fig.1B).

Table-2: Determination of MIC, MBC and Tolerance values

Bacteria	Commercial compounds	MIC value (µl/ml)	MBC value (µl/ml)	Tolerance level
<i>E. coli</i>	RH ⁵⁺	1	1	1
	D1	1	1	1
	D2	>100	>100	≥1
	D3	1	1	1
<i>S. aureus</i>	RH ⁵⁺	1	1	1
	D1	1	1	1
	D2	>100	>100	≥1
	D3	5	5	1
<i>K. oxytoca</i>	RH ⁵⁺	5	5	1
	D1	1	1	1
	D2	>100	>100	≥1
	D3	1	1	1
<i>P. vulgaris</i>	RH ⁵⁺	1	1	1
	D1	1	1	1
	D2	>100	>100	≥1
	D3	1	1	1
<i>P. aeruginosa</i>	RH ⁵⁺	5	5	1
	D1	1	1	1
	D2	>100	>100	≥1
	D3	1	1	1

The MBC values were also found at the same dose of MIC value of the respective compounds. Against all clinical isolated bacteria, D2 was showed no growth inhibition upto 100 µl/ml dose. (Table 2).

3.2. Determination of Tolerance

The Tolerance level of each multidrug resistance bacterial strain against the compounds RH⁵⁺, D1, D2 and D3 was calculated from the respective MIC and MBC value. In most of the multidrug

resistance bacterial stains *E. coli*, *S. aureus*, *K. oxytoca*, *P.vulgaris*, and *P. aeruginosa* the tolerance level is 1. The MBC/MIC ratio is a parameter that reflects the bactericidal capacity of the test compound. As seen in Table 1, there is no difference between the bactericidal effects of RH⁵⁺, D1, D2, and D3 on all drug-resistant microorganisms (Table 2).



Figure 1A



Figure 1B

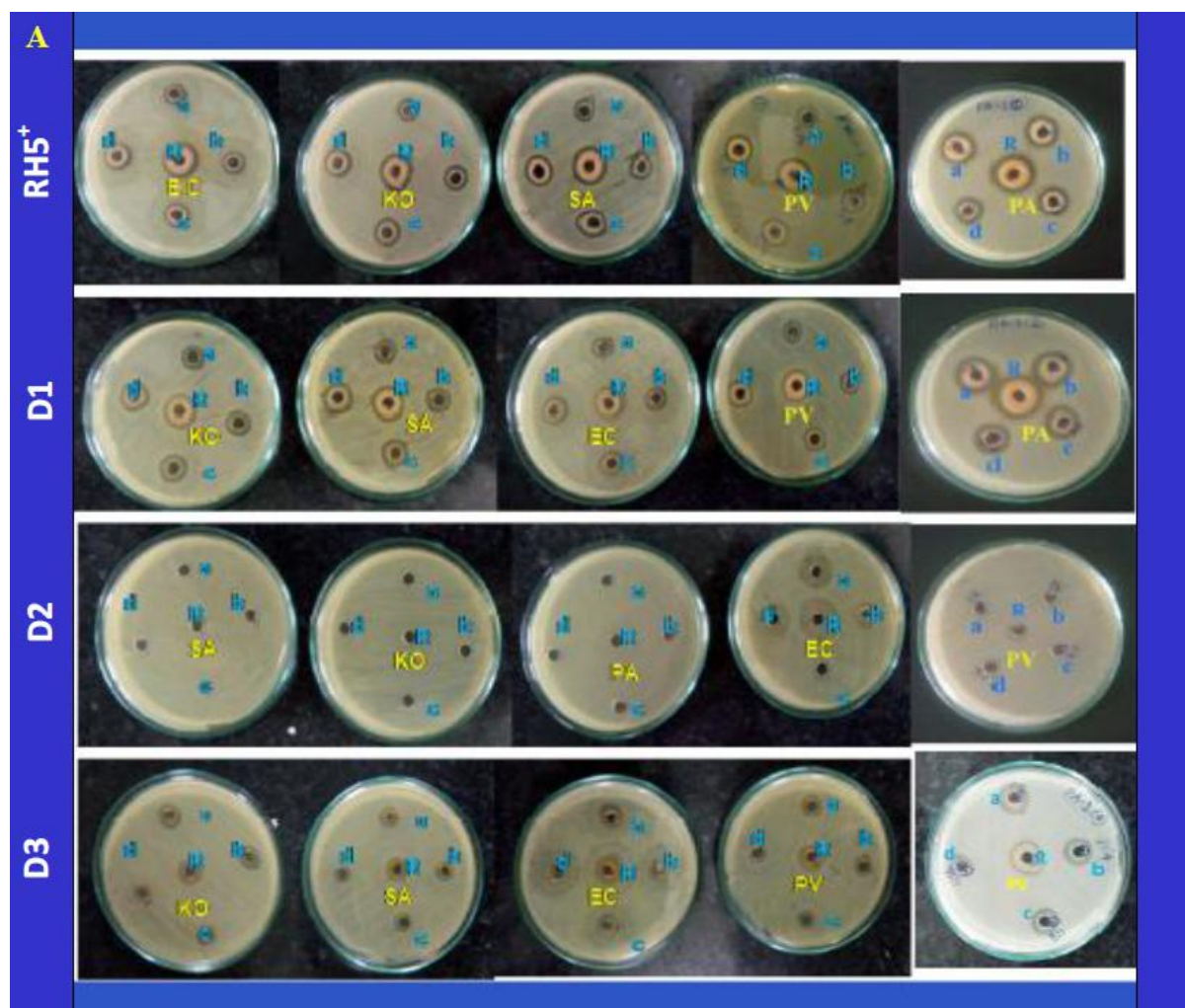


Figure 2 A

3.3. Determination of zone of inhibition by agar diffusion assay

The antibiotic resistance profile of the pathogenic gram positive and gram negative bacteria was determined by the agar diffusion test. During the assessment of antibacterial activity the diameter of zone of inhibition obtained as significant value against those selective tested compounds. The zone of inhibition of bacterial strain against the RH⁵⁺ and competitor product D1, and D3 within the range of 6 mm to 22 mm at concentration of undiluted (raw) were, 1:2, 1:4, 11:8, and 1:16 respectively, whereas the drug D2 showed the partial zone of inhibition against *E. coli*. The compounds RH⁵⁺, D1, and D3 showed better antibacterial activity than D2 against all multidrug resistant bacterial strains (Fig.2).

3.4. Onsite antibacterial activities using poultry soil bed

Zoonotic disease can be caused by bacteria present in poultry industries. So antibacterial activity of RH⁵⁺, D1, D3 against those zoonotic bacterial strains were determined using poultry bed soil. The different dilutions of the sample were spread on nutrient agar plate and the colonies of the bacteria were counted after 24 hrs of incubation at 37°C. The counted colonies are tabulated in Table 3. Due to low antimicrobial efficacy, D2 was not considered for the onsite antimicrobial activity study. The remaining three antimicrobial compounds were highly active against the all bacterial populations even at very low concentration (1:2000) and very short period of treatment time (30 mins and 60 mins). (Table-3, Fig.3 (A-F)).

Table-3: Time and dose dependent antimicrobial activity using colony count assay of D1, D2 and D4 against the bacterial populations found in poultry bed soil.

Incubation time	Untreated		D 1		D 2		D 4	
	Undiluted (uncountable)	Diluted (200 times)	1:1000	1:2000	1:1000	1:2000	1:1000	1:2000
30 minutes	3,249	(63X200) =12,600	6	7	0	5	2	8
60 minutes	4,142	(76X200) =15,200	0	2	2	6	4	7

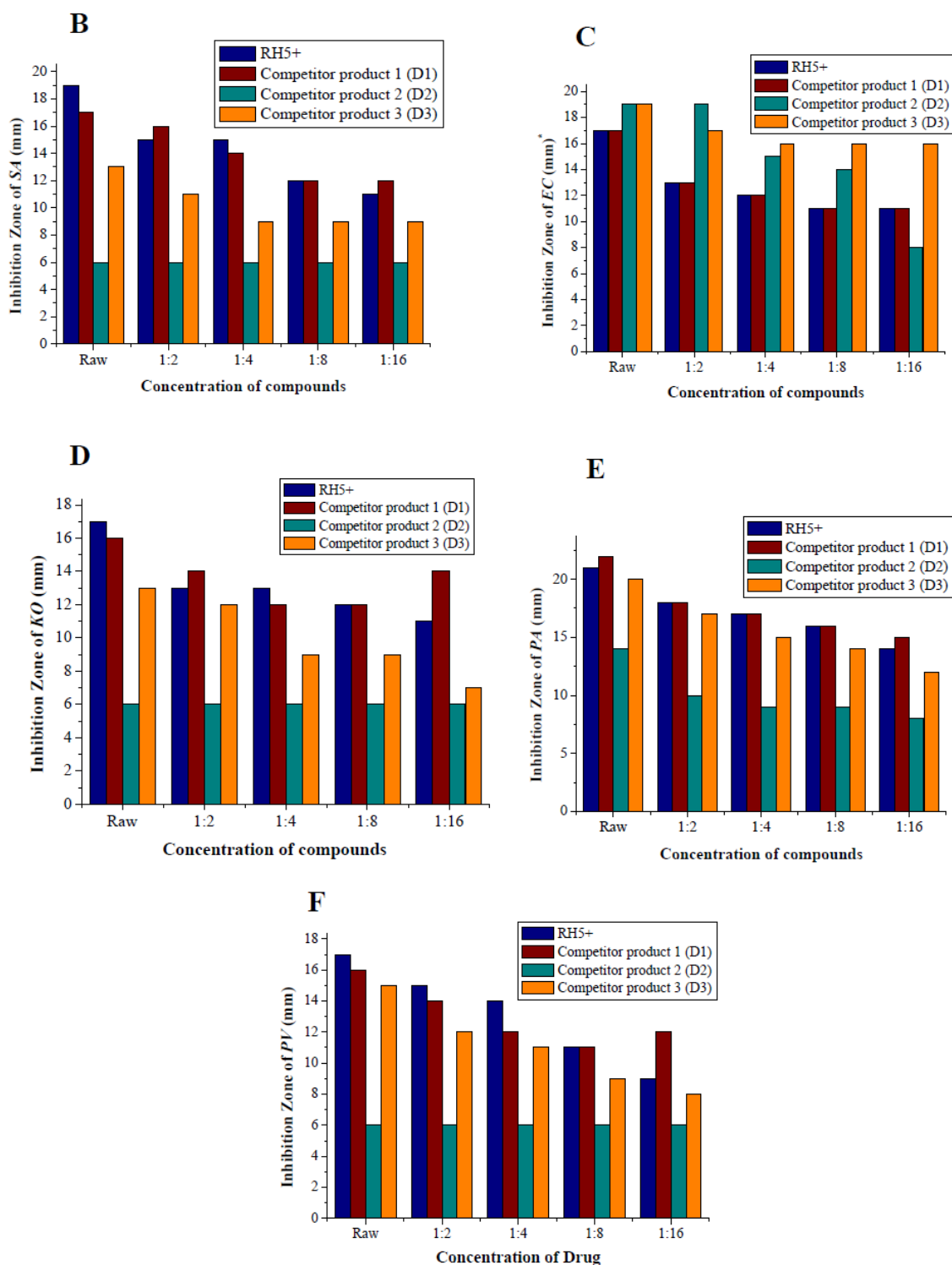


Figure 2

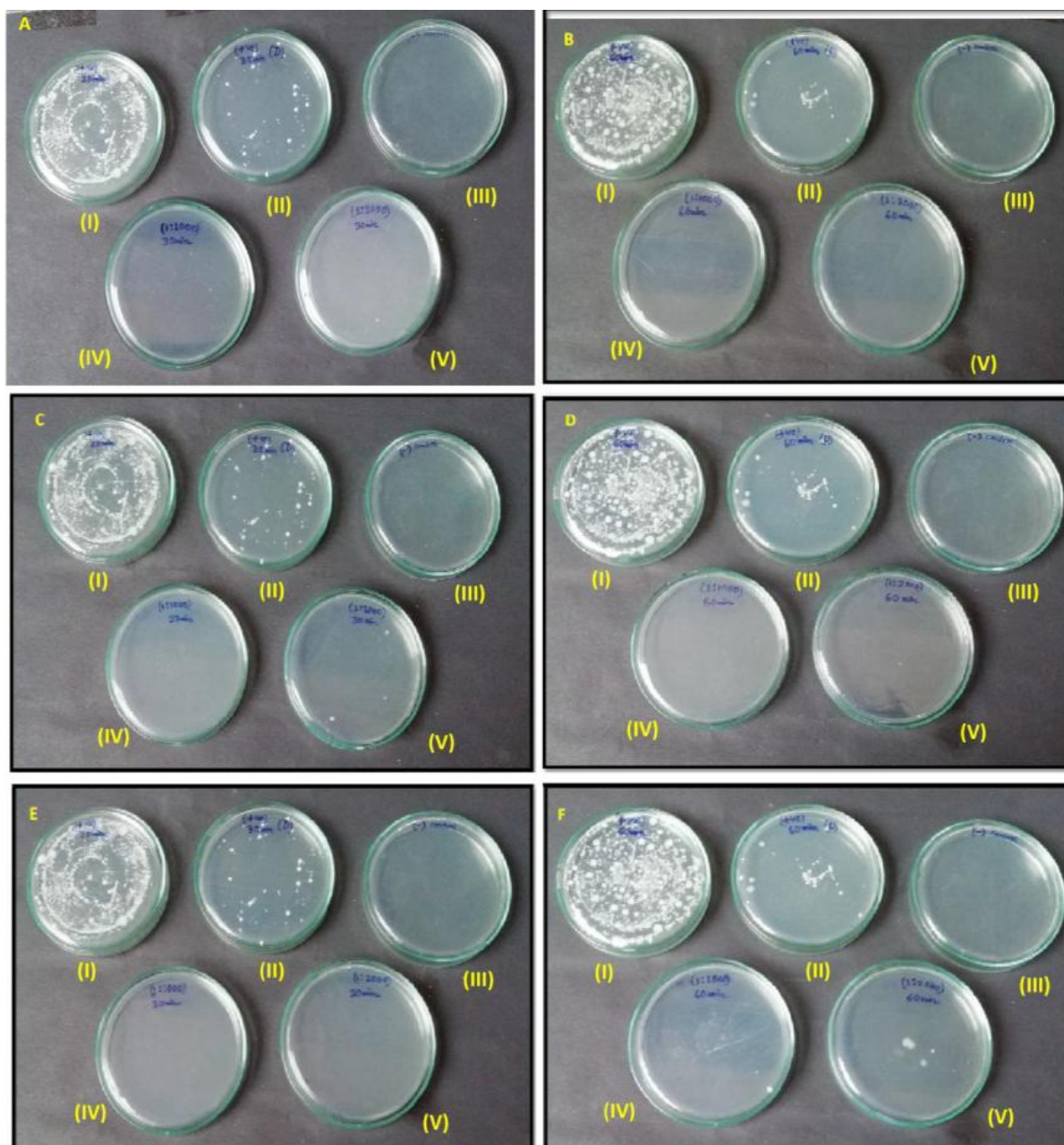


Figure 3

4. DISCUSSION

In the last year U.S. Geological Survey (USGS) investigated wildlife diseases, including zoonoses, at places where wildlife, domestic animals, humans, and the environment interacted with each other. [32] Along with the animal, food products such as eggs, meat, and milk are considered as vehicles for one or more of pathogens, causing food borne illnesses. In last few years, increasing number of organisms has been becoming resistant to several antibiotics and patients normally did not respond to high doses of antimicrobial

agents even if administered for prolonged periods. The poultry farms leading to a proliferation of resistant bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Klebsiella oxytoca* etc. which are now spreading in the environment because of insecure disposal of poultry litter and waste in agricultural fields, has a potential to infect human beings. [33, 34] Workers in poultry farm and farmers in agricultural field suffer most as they directly come in contact with most hazardous pathogen. Disinfectants are commonly used in poultry

farm to prevent spreading of pathological diseases caused by bacteria. Chemical disinfectants are rich in quaternary ammonium compounds, glutaraldehyde, sodium hypochlorite, and organic acids. QAC was showed bactericidal activity against *S. mutans* and other microorganisms present in dental material. [35] In a recent study QAC immobilized onto silica-decorated membrane surface which exhibited long-lasting antibiofouling and antibacterial efficacy against *Escherichia coli*, *Staphylococcus aureus* [36] An in vitro antimicrobial assays demonstrated that pine needle essential oil exhibits remarkable inhibitory and sterilizing activity against typical food-borne microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Penicillium*. [37] Condensation of glycine metal complexes with glutaraldehyde were screened for their antimicrobial activities against selected microorganisms like *Streptococcus aureus*, *Escherichia coli*, *Bacillus sphaericus*, and *Salmonella sp.* (Bacteria) and also *Fusarium oryzae*, *Candida albicans*, and *Aspergillus niger* (Yeast). [38] In this experiment the commercial disinfectants were mainly contained of a class of QACs. So in our study the marketed antimicrobial compounds RH⁵⁺, and three competitor products (D1, D2 and D3) which were composed of mainly Di-decyl di-methyl ammonium chloride, Di-octyl di-methyl ammonium chloride, Octyl-Decyl di-methyl ammonium chloride, Benzal konium chloride, Glutaraldehyde, Pine oil, and Terpeneol oil were used against five multidrug resistant bacteria (*Escherichia coli* (EC-1), *Pseudomonas aeruginosa* (PA-1), *Klebsiella oxytoca* (KO-1), *Proteus vulgaris* (PV-1), *Staphylococcus aureus* (SA-1) to determined antibacterial activities. Onsite antibacterial potency also was examined by using poultry bed soil. From the observed MIC value and MBC values (Table-1), it can be stated that the RH⁵⁺, D1, and D3 are highly active at low

concentration while D2 did not show any significant antibacterial activity. Most of the research reviewed here shows that whole plant extracts or combinations of compounds are more effective antimicrobials than isolated constituents. [39-43]

Combinations of non-specific mechanisms of action might create a more effective antimicrobial activity than a traditional antibiotic. [43]

The MBC/MIC ratio is a parameter that reflects the bactericidal and bacteriostatic capacity of the test compound. The high tolerance value indicates that the test compounds is less effective and have chances to get resistance. But here, it was observed that the tolerance values of RH⁵⁺, D1, and D3 were 1, against all multi drug resistant bacteria which indicates low chances for obtaining resistance property by the microorganisms. In disc agar diffusion study, the measurements of the diameter of zone of inhibition of bacterial strain against RH⁵⁺, D1, and D3 (Table-2), exhibiting diameters of zones of inhibition greater than 10 mm each (>10 mm) which was considered as active antimicrobial activity. [40] D2 showed the partial zone of inhibition against *E. coli*. and displayed zone of inhibition against other bacterial strain was <10 mm. Thus, it is assumed that the remaining compounds were highly effective towards the multidrug resistant bacterial strains. In the onsite antimicrobial study, shows high sensitivity towards the bacteria present in poultry soil bed. In onsite study it also demonstrated that minimum amount of drugs showed significantly high efficacy towards zoonotic bacteria present in poultry soil. It was also found that the antimicrobial components of RH⁵⁺ and competitor products were versatile disinfectant with a wide spectrum of antimicrobial action and safe for animals.

5. CONCLUSION

Gradual unresponsiveness of commercially available antibiotics becomes a major threat for not only the human health but also for the animal farming industries.

Moreover, unregulated use of antibiotics increases the chances of resistance property and also minimizes the quality of animal products. Considering the present alarming situation, the present study was carried out to investigate the antibacterial property of some unique formulations (RH⁵⁺ and competitor products) containing quaternary ammonium, Glutaraldehyde and other active ingredients. It was noted that RH⁵⁺ and its other two competitor products i.e. D1, and D3 were found as potent bacteriostatic agents against multidrug resistant bacteria and also against the bacterial populations found in the poultry bed soil. The D2 competitor product was observed to be non-effective against multidrug resistant bacterial strains and thus it was not used for further studies. So, it may be concluded that, the commercial products i.e. RH⁵⁺ and its variants could be used as an effective disinfectant in poultry farm to eliminate the pathogenic infections of the birds as well as for industrial and hospital areas to minimize the spreads of pathogenic bacterial strains from environment to human.

Conflict of Interests:

All authors contributed in the article declare no potential conflicts of interests.

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